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Research Article

ANTI-ARTHRITICACTIVITY OF *ANNONA MURICATA* LEAVES METHANOLIC EXTRACT ON ADJUVANT INDUCED ARTHRITIS IN RATS

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ABSTRACT

The current research has aimed to evaluate the anti-arthritic effect of *Annona muricata* methanolic leaves extract (AMME) on the progression of adjuvant induced arthritis in Wistar rats. AMME was obtained by bioactivity guided fraction by 5-Lipooxygenase inhibitory activity. The AMME was administrated orally at 100 and 200 mg/kg body weight for 35 days to the experimental animals. Arthritis was induced on day 8 by a single intra-plantar injection of 0.1 ml suspension of heat killed *Mycobacterium tuberculosis* (100 µg/animal) in incomplete Freund's adjuvant of left foot pads of female Wistar rats. The anti-arthritic activity extract was screened by measuring hind limb paw volume, biochemical and haematological parameters, pathological and radiography changes. The levels of pro-inflammatory cytokines TNF-α, IL-1β and inflammatory mediators PGE2, LTB_4 was measured in serum samples on day 35. The stabilizing ability of lipid peroxide and activities of enzymatic antioxidants catalase, superoxide dismutase (SOD) and non-enzymatic antioxidants reduced glutathione (GSH) levels were measured in liver. Treated groups AMME 100 and 200 mg/kg and prednisolone 10 mg/kg significantly decreased hind paw volume, diminished serum levels of TNF-α, IL-1β, PGE_2 and LTB_4, reduced MDA levels and increased levels of catalase, SOD and GSH levels in liver and promising results of serum biochemistr y, haematology, histopathology and radiographic changes suggests the administration of AMME was effective in modulating the inflammatory response and conquer the advancement of arthritis in experimental animal model. The safety of AMME was established (LD50 >2000 mg/kg) by acute oral toxicity limit test according to OECD guideline 423. These findings may help to improve the treatment of rheumatoid arthritis.

INTRODUCTION

Inflammation is body's self-protective mechanism, which is an action against microbes, toxins, foreign materials at injury site to prevent their spread further to other tissues or an attempt to restore tissue homeostasis at site of tissue repair (Charles *et al,* 2008). The prolonged acute inflammation may lead to chronic inflammatory disorders like rheumatoid arthritis. Rheumatoid arthritis (RA) is an autoimmune disease, where the body's own immune system attacks its own tissues like articular cartilage and joint linings. RA is characterized by joint swelling with pain and loss of function. Synovial membrane inflammation is the primary symptom of this disease and arthritis is usually occurs bilaterally. If disease untreated, the synovial membrane thickens and fluid accumulates and results in pain and tenderness. Then the membrane produces pannus is an abnormal granulation tissue, which adheres to articular cartilage and destroys cartilage tissue completely (Weyand, 2000). When cartilage completely destroys, the fibrous tissue exposes to bone ends and this tissue fuses the joints. So that the

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joints will become immovable and the granulation tissue growth causes the distortion of bones that characterizes the Rheumatoid arthritis. The prevalence of RA is higher in females as compared to males and the chances of RA occurrence is higher at age group above 50 (Kvien *et al*, 2006). Arachidonic Acid (AA) metabolism plays a vital role in inflammatory process and associated diseases. Most of the anti-inflammatory drugs inhibit the lipoxygenase (LOX) and cyclooxygenase (COX) pathways and these two pathways can be used for potential interventions against inflammation. Regrettablymaximumnumber of the anti-inflammatory drugs, predominantly steroids and COX inhibitors are frequently accompanying with adverse side effects including, gastrointestinal irritation, hypertension, ulcers and cardiac abnormalities (William, 1989; Wolfe *et al*, 1999). PGE_2 and LTB_4 are the key inflammatory mediators derived from the arachidonic acid metabolism through COX and LOX pathways of mammal's cell membrane (Khanapure *et al*, 2007). PGE_2 and LTB_4 are involved in modulating the intensity of inflammatory responses, and also have number of pro-inflammatory effects, including increasing vascular permeability, vasodilation, enhances local blood flow and local pyrexia and potentiation of pain caused by other agents. PGE_2promotes the production of MMPs and stimulates bone resorption (Lewis *et al*, 1990). LTB_4 is a potent chemotactic agent for leucocytes, induces release of lysosomal enzymes, and enhances release reactive oxygen species and inflammatory cytokines, such as TNF-α, IL-1β and IL-6 (Tilley *et al*, 2001). TNF-αand IL-1β are the key pro-inflammatory cytokines produced by immune system.

These two cytokines play a major role in potentiating the inflammatory response (Feldmann *et al*, 1996). The process of rheumatoid arthritis produced reactive oxygen species (ROS), these increases the cell membrane permeability by formation of lipid peroxides. Antioxidants like catalase, SOD, glutathione peroxidase and reduced glutathione etc. effectively detoxifies ROS. The herbal medicines with antioxidant properties reduce ROS production and renovate the endogenous antioxidants activity and reverts joint destruction. As antioxidant activity appears significances, these became promising agents for RAtreatment. In recent years, use of herbal origin medicines for the treatment arthritis has been gaining momentum (Chrubasik *et al*, 2007). Therapeutic use of COX inhibitors has some concern due to their adverse side effects; especially some COX inhibitor mechanism based products were either withdrawn or made to carry warning by the US FDA (Naesdal *et al*, 2006; Solomon 2006). Herbal origin products based on 5-LOX inhibitors on other hand are reported for significant amelioration of inflammation without adverse effects. Thus 5-LOX inhibitors are becoming first source of treatment for chronic inflammatory disease like arthritis (Oliver Werz, 2007). *Annona muricata* leaves extracts were previously tested for antioxidant activity and anti-hypertensive, anti-microbial activity, anti-diabetic activity, Inflammatory and antinociceptive activity. However the leaf extract was not tested for chronic inflammatory or arthritis models to prove efficacy. Hence, we try to research the 5-lipoxygenase inhibitory and anti-arthritic properties of *Annona muricata* leaves methonalic extract.

MATERIALS AND METHODS

Plant Material and Preparation of Extract

The leaves of *Annona muricata* were collected from Kondapalli reserve forest area, Andhra Pradesh, India, during the month of January. The authentication of the above plant was done by Prof. Dr. S. M. Khasim and deposited in Department of Botany, Acharya Nagarjuna University. The Leaves of *Annona muricata*was shade dried and cut into small pieces. The pieces were milled to a coarse powder. The powdered material was successively extracted with Hexane, Ethyl acetate, Methanol and water by Soxhlet extraction method. The powdered raw material of *Annonamuricata* leaves was extracted successively using solvents of non-polar to polar grade with hexane, ethyl acetate, methanol, aqueous methanol and water using a soxhlet apparatus. The extracts were concentrated independently and dried under reduced pressure using rotary evaporator. A potent 5-Lipoxygenase inhibitory fraction obtained through bioactivity guided fractionation was used for *in vivo* efficacy and toxicity studies. Methanol extract (AMME) was found to be the most potent 5-LOX inhibitor.

Experimental Animals

Female *Wistar* rats, weighing 180g to 220g was housed (3 animals per cage) in polypropylene cages with stainless steel with paddy husk bedding and fed with Nutrilab standard rodent diet manufactured (Provimi. Pvt. Ltd.,) and UV treated filtered water was supplied *ad libitum* for all the animals. The animal room was maintained at a controlled temperature (22 ± 3°C), humidity (30 to 70%) and light (12 h light and dark cycles). All the experiments involving animals were performed according to the standard protocol and guidelines of Animal Ethical Committee, Government of India **Reg.No. 1629/PO/a/12/CPCSEA,** aftergetting proper approval.

In vitro 5-Lipoxygenase enzyme inhibitory assay

5-Lipoxygenase (5-LOX) enzyme inhibitory activity of AMME was measured using the method of Reddenna *et al,* 1990; Ulusu *et al,* 2002. The assay mixture contained 80 µM linoleic acid and 10 µL potato 5-lipoxygenase enzyme in 50 mM phosphate buffer (pH 6.3). The reaction was initiated by the addition of enzyme buffer mix to the substrate (linoleic acid) and the enzyme activity was monitored as an increase in absorbance at 234 nm. The reaction was monitored for 120 seconds using UV-Kinetic mode on Varian Cary-50 UV-Vis spectrophotometer. The inhibitory potentials of different test substances were measured by incubating various concentrations of test substances with enzyme buffer mix for 2 minutes before addition of the substrate. All assays were performed in triplicate and mean values were used for the calculation. Percentage inhibition was calculated by comparing increase in absorbance of test substances with that of control enzyme activity by using percentage inhibition formula. The activity of plantextracts was compared with the standard positive control Nordihydroguaiaretic acid (NDGA).

Acute toxicity study

The acute toxicity of AMME was determined as per the OECD 423 guideline (Acute oral toxicity class method). Six animals (Step I and II) were used for the screening of acute oral toxicity. After single oral dose administration of AMME at

2000 mg/kg, animals observed over a period of 14 days for clinical symptoms.

Adjuvant Induced Arthritis

The potent bioactive guided fraction AMM Eobtained through *in vitro* screening of *Annonamuricata* leaves was tested for *in vivo* efficacy for chronic inflammatory model using Freund's Complete Adjuvant [FCA] Induced Arthritis in Wistar rats. Wistar rats were divided into 4 groups (6 animals each group). Group I animals served as arthritic control and received 0.5 % CMC (Carboxy Methyl Cellulose). The required quantity of test item was weighed using an analytical balance and transferred into a mortar and pestle. The desired volume of CMC added and triturated to obtain the final concentration of 10 and 20mg/mL of test item. The standard drug Prednisolone 10 mg/kg in 0.5% CMC was used as positive control. Animals were treated daily from day 0 to 35 by oral route to the respective groups. The dose volume was calculated and adjusted according to individual animal's body weight recorded on a weekly basis. Following dose administration, the rats were transferred immediately to their respective cages. The treatment regimen as follows; Group I was arthritic animals without drug treatment, Group II was arthritic animals treated with AMME (100 mg/kg), Group III was arthritic animals treated with AMME (200 mg/kg) and Group II was arthritic animals treated with Prednisolone (10 mg/kg).On day 8, arthritis was induced in Wistar rats of all groups by a single intra-plantar injection of 0.1 mL suspension of heat killed *M. tuberculosis* (100 µg/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 1 mg/mL of Mycobacterium tuberculosis H37Ra].

Animals were observed for general clinical signs once daily on all days throughout the study duration. Body weights of animals were recorded prior to initiation of treatment and thereafter weekly once during course of the study and prior to termination of the study. Paw volume was measured by a plethysmometer (UGO basile) on days 0 (prior to treatment), 7 (prior to AIA induction), 20, 24, 28 and 35.The difference between the final and initial paw volume was considered as oedema volume. The percent inhibition of oedema volume was calculated by using the formula;

$$\{(V_c - V_t / V_c)\} \times 100$$

Where,

V_0 - Oedema volume of normal control group

V_c- Oedema volume of AIA control group

V_t - Oedema volume of treatment group

Blood Analysis

Blood was collected by retro-orbital plexus puncture on day 35 under isoflurane anaesthesia. The serum was separated by centrifugation of blood at 1500 *rpm* for 15 minutes and the samples was analysed by using Humastar 600automated biochemistry analyser for AST/SGOT, ALT/SGPT, ALP, total bilirubin, total protein, albumin, cholestrol, triglycerides, creatinine, urea and CRP. The rats were sacrificed on day 35 and blood samples were collected into 1% EDTA sample tubes for hematological analysis (RBC, WBC, Hb, Platelets, Lymphocytes and PCV) using the Automated Hematology Analyzer (Humacount 60ts, HUMAN and Germany).The Erythrocyte Sedimentation Rate (ESR) is a nonspecific assay used to screen for the presence or absence of active disease and is measured in mm/hr using the modified Westergren Method.

Cytokines and inflammatory mediators

Blood was collected by retro-orbital plexus puncture on day 35 under isoflurane anaesthesia. The serum was separated by centrifugation of blood at 1500 *rpm* for 15 minutes and the samples were analysed for pro-inflammatory cytokines TNF-α and IL-1β, inflammatory mediators PGE_2 and LTB_4levels in serum was estimated using ELISA assay kit (R&D systems) according to manufacturer's protocol. Finally, the specific chromophore reactions were read at 540 nm in a micro well plate ELISA reader (BioRad, USA).

Antioxidant Studies

On termination of study, all the experimental animals were euthanized by over dose of ketamine anesthesia and necropsy was performed. The liver was collected and washed with ice cold saline, preserved in -20°C until the antioxidant analysis. The protein levels of liver samples different group animals were estimated by Braadford method form 20% of liver homogenate. The lipid peroxide levels of liver samples estimated by Nichans *et al*., 1968 and Ohkawa *et al*., 1979 method. The enzymatic antioxidant activities like catalase estimated by Sinha 1972, superoxide dismutase (SOD) estimated by Kakkar *et al*., 1984 and the non-enzymatic antioxidant activities like reduced glutathione (GSH) estimated by Ellman 1959.

Histopathology and Radiology of knee joint

At the termination of the study, tibio-tarsal joint of all animals were dissected out and fixed in 10 % buffered formalin and subsequently decalcified in 1:1 formic acid and distilled water for one week. Tissue samples were dehydrated in series of isopropyl alcohol (70, 80, 90 and 100 %) each for 2 hours and processed for paraffin embedding and then sectioned at 3-5 µm thickness. The tissue sections were stained with Hematoxylin & Eosin (H&E) and Toluidine Blue (TB). The slides were examined under light microscope for histopathological lesions. The ankle joints were fixed in 10% buffered formalin and subjected to radiography on Siemens triodors (100 MA at 100 cm focal distance, 40 KV and 2 mAs exposure). The lateral radiographs were taken and the exposure was recorded.

Statistical Analysis

Data was statistically analyzed using one-way ANOVA as primary test followed by Dunnett's test, using graph pad prism software. All the results were expressed as Mean ± S.D and considered significant when $P \leq 0.05$.

RESULTS

5-lipoxygenase inhibitory Activity

5-Lipoxygenase enzyme inhibitory activity of *Annona muricata* extracts AMHEX, AMET, AMME, AMAM and AMW exhibited inhibitory activity 5-Lipoxygenase enzyme by showing IC_{50} values as 358.99, 85.35, 33.94, 62.96 and 155.51 µg/mL respectively, Standard NDGA showed an IC_{50} value of

5.11 µg/mL.5-Lipoxygenase enzyme inhibitory action of *Annona muricata* was depicted **Figure 1**.

Acute oral toxicity

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

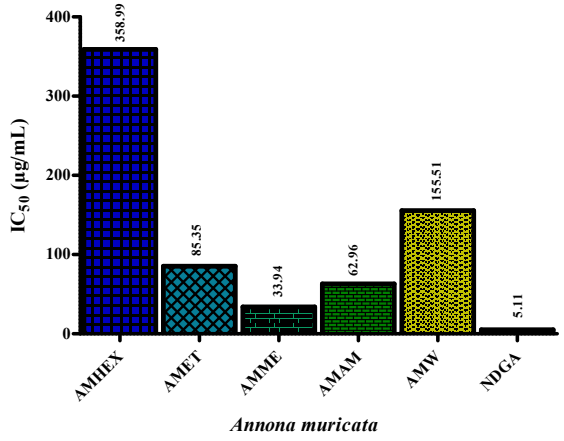

Figure 1 5-Lipoxygenase Inhibitory Activity

Adjuvant Induced Arthritis

Administration of FCA (100 µg/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 **(Figure3)**.

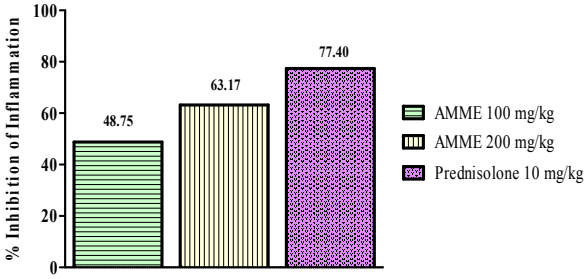

Figure 3 FCA induced Arthritis – Paw Swelling

Oral administration of AMME (100 & 200 mg/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 ($P \leq 0.001$) reduction in *Annona muricata* methanolic extract animals (AMME 100 and 200 mg/kg).

Percent inhibition of paw oedema observed in AMME 100 mg/kg treated animals was 2.38%, 25.88%, 38.71% and 48.75%, whereas in AMME 200 mg/kg treated inhibition observed as 5.85, 31.53, 47.31 and 63.17 on day 14, 21, 28 and 35 respectively as compared to adjuvant induced arthritis (AIA) control.**Table2** 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 **(Table1)**. 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.

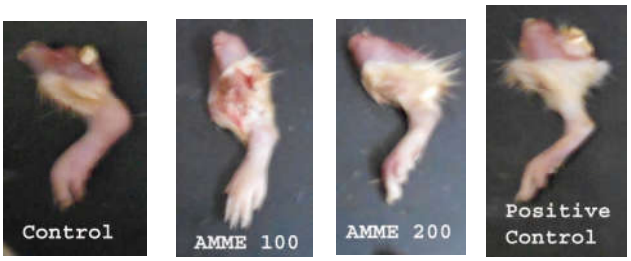

Figure 3 FCA induced Arthritis – Paw Swelling

Biochemical Studies

Blood was collected by retro-orbital plexus puncture on day 35 and centrifuged for serum at 1500 rpm 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 AMME (100 and 200 mg/kg) and prednisolone (10 mg/kg) caused a highly significant ($P \leq 0.001$) reduction in AST, ALT, ALP, total bilirubin, total protein, albumin, creatinine, urea, triglycerides and CRPlevels 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 AMME (100 mg/kg) and prednisolone (10 mg/kg) did not caused a significant reduction in cholesterol level when compared to AIA control. All the serum biochemistry results were depicted in **(Table3).**

Hematological analysis

Blood was collected by retro-orbital plexus puncture in EDTA tubes from each animalon 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 AMME (100 and 200 mg/kg) and prednisolone (10 mg/kg) treated groups it was significantly recovered to normal levels.

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	211.33 ± 9.91
AMME	100	166.17 ± 8.64	186.17 ± 8.93	199.33 ± 9.16	213.17 ± 8.95	224.50 ± 9.35	237.00 ± 8.46
	200	167.33 ± 6.12	185.83 ± 5.71	199.17 ± 6.43	213.83 ± 7.05	226.83 ± 7.70	239.00 ± 8.74
Prednisolone	10	166.17 ± 6.82	181.33 ± 7.28	190.83 ± 7.91	198.83 ± 9.35	202.00 ± 9.94	195.17± 10.40

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

The reduced levels of RBC, Haemoglobin and percentage packed cell volume was observed in AIA control group, whereas in AMME (100 and 200 mg/kg) and prednisolone (10 mg/kg) treated groups it was significantly increased. All the hematology results were depicted in **(Table4)**.

Inflammatory Biomarkers

Serum TNF-α, IL-1β, PGE_2 and LTB_4 Profile

There was an increase in serum pro-inflammatory cytokines such as TNF-α and IL-β levels observed in AIA control group

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***
AMME	100	1.78 ± 0.12	2.01 ± 0.10***	1.88 ± 0.17***	1.71 ± 0.14***
	200	1.72 ± 0.10	1.86 ± 0.11***	1.62 ± 0.11***	1.23 ± 0.05***
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≤0.001 versus control, S.D: Standard deviation

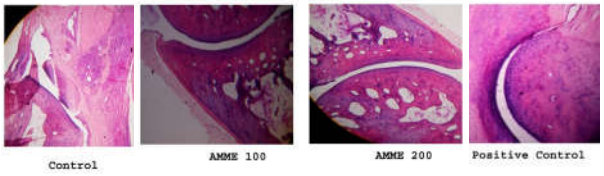

Figure 4 FCA induced Arthritis – Histopathology

Whereas treatment with AMME (100 and 200 mg/kg) and prednisolone (10 mg/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 $PEG_2$and LTB_4levels observed in AIA control group when compared to normal control on day 35.

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
AMME 100 mg/kg	130.17 ± 6.37***	68.17 ± 6.52***	164.67 ± 14.83***	0.35 ± 0.05***	6.90 ± 0.99***	3.59 ± 0.32***	84.33 ± 3.88	79.33 ± 4.97***	0.83 ± 0.07***	28.90 ± 3.15***	3.59 ± 0.28***
AMME 200 mg/kg	124.00 ± 4.60***	66.67 ± 5.92***	162.83 ± 13.54***	0.32 ± 0.07***	7.17 ± 0.44***	3.46 ± 0.31***	79.50 ± 5.61*	80.00 ± 0.89***	0.87 ± 0.07***	27.88 ± 2.84***	3.22 ± 0.37***
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≤0.05, ** P≤0.01 and, *** P≤0.001 versus control, S.D: Standard deviation

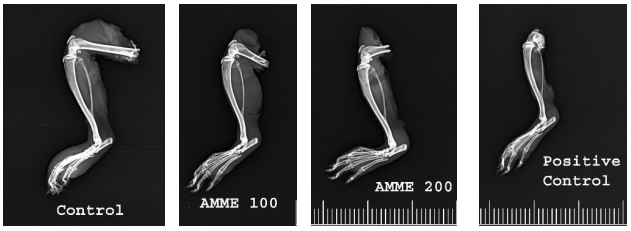

Figure 5 FCA induced Arthritis – Radiography

However treatment with AMME (100 and 200 mg/kg) and prednisolone (10 mg/kg) caused a significant (p≤0.001) reduction in $PEG_2$and LTB_4level when compared to AIA control on day-35 **(Table 5)**.

Table 4 FCA induced Arthritis - Hematology on day-35

Test item	Dose (mg/kg)	Hematology Analysis						
		ESR (mm/hr)	RBC count (x10^6 Cells/µL)	WBC count (x10^3 Cells/µL)	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
AMME	100	8.90 ± 1.16***	7.30 ± 0.53***	6.69 ± 0.29***	12.23 ± 0.99**	8.57 ± 0.36***	49.19 ± 0.53***	28.10 ± 0.59***
	200	7.63 ± 0.45***	7.22 ± 0.36***	6.17 ± 0.50***	12.78 ± 0.72***	8.42 ± 0.46***	47.12 ± 0.54***	28.62 ± 0.61***
Prednisolone	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
AMME	100	25.40 ± 0.37***	122.74 ± 0.89***	150.22 ± 0.96***	154.66 ± 0.72***
	200	23.81 ± 0.39***	104.63 ± 3.11***	120.54 ± 2.43***	116.41 ± 1.18***
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

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 AMME (100 and 200 mg/kg) and prednisolone (10 mg/kg) significantly ($p \leq 0.001$) 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 AMME (100 and 200 mg/kg) and prednisolone (10 mg/kg) significantly ($p \leq 0.001$) restored the catalase and SOD levels **(Table6)**.

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
AMME	100	0.68 ± 0.36***	86.34 ± 2.36***	3.38 ± 0.16***	3.02 ± 0.49**
	200	0.49 ± 0.27***	91.63 ± 7.68***	4.92 ± 0.13***	4.85 ± 0.44***
Prednisolone	10	0.29 ± 0.05***	77.95 ± 5.34*	3.89 ± 0.34***	3.51 ± 0.23***

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

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 AMME (100 and 200 mg/kg) and prednisolone (10 mg/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 AMME (100 and 200 mg/kg) and prednisolone (10 mg/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 muricata* methanol extract. Earlier studies have indicated that the different solvent extracts of the selected plant have shown a significant anti-inflammatory activity *Annona muricata*. 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.

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 (Agnihotri *et al,* 2010). 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 inflammation may be one of the reasons for many of the chronic inflammatory diseases like rheumatoid arthritis (Biswa *et al,* 2009).

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.

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 AMME at dose levels of 100 and 200 mg/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 (Durai *et al*, 2007). 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 AMME treated groups. 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. 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 AMME (100 and 200 mg/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 (Weiss and Goodnough 2005). These decreased levels of RBC, haemoglobin and PCV was significantly increased by AMME (100 and 200 mg/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 (Maria 1983). These increased levels of WBC and platelet count was significantly decreased by AMME (100 and 200 mg/Kg). The increased levels of lymphocytes indicates incidences of active inflammation, lymphocytes are the highly responsible to the initiation and maintenance of immune response. The concentration of lymphocytes in control group is high, whereas this concentration of lymphocytes in AMME (100 and 200 mg/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 (Brennan and Mclnnes, 2008).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 (Lewis *et al*, 1990). 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., (Tilley *et al*, 2001). The literature reveals high levels of PGE_2 and LTB_4 are found in serum and joint tissues in arthritis induced rat models (James and Gibson 2000). 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 AMME 100 and 200 mg/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 antioxidant 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 AMME (100 and 200 mg/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 (Crow and Beckman, 1995). The Superoxide dismutase and catalase enzymes are key enzymes in obstructing superoxide radical activity. The Superoxide 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 (Blake *et al.,* 1981). 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) (Bazzichi *et al.,* 2002). 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 AMME (100 and 200 mg/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 AMME treated groups at 100 and 200 mg/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 muricata*in 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 auto immune disease rheumatoid arthritis by inhibiting the both COX and 5 LOX pathways. This was proven by inhibition of PGE_2 (COX pathway) and LTB_4 (LOX pathway). The antioxidant activity exerted by AMME 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.

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