In-vivo Anti-arthritic Evaluation of Gugguluthikthakam kashayam in FCA induced arthritis in Rats

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ABSTRACT

AIM – The chief aim of this study was to evaluate the in-vivo anti-arthritic evaluation of Gugguluthikthakam kashayam in FCA Induced Arthritis in Rats. **MATERIALS & METHODS-** Adult female and male Wistar rats of weight 150- 200 g, which were bred and reared in the department animal house, were used for this study. In a study of Polyherbal formulations in collagen induced arthritis, the dose for the Gugguluthiktakam kashaya was reported to be 2 ml/kg body weight. So by consideration of that research article, in our study we have considered 2 ml/kg body weight as a safe dose for the further in-vivo investigation. The Wistar albino rats were divided into 5 groups of six animals in each. For the generation of chronic inflammatory response, FCA (0.1 ml) was injected through intra-articular injection in left ankle joint of rats on 0^{th} day. Body weight of all groups was measured, at 0^{th} day, before immunization and at 21^{st} day after treatments over by using a single pan weighing balance. At the end of the experimental period, rats were fasted overnight and the blood sample were collected by retro orbital blood sampling techniques from anaesthetized rats. On the last day of study, samples of plasma were send to the Pathology Lab, for estimation of TNF-alpha and Interleukin levels. The Estimation was done with the help of Elisa Reader in Pathology Lab. **RESULTS** - The assessment made on the 21^{st} day showed that the Gugguluthikthakam Kashayam treatments at both doses (low and high) had moderately significant and highly significant effect and reduced ($p< 0.01$& $p<0.001$) the adjuvant-induced lesions in the respective treatment groups as compared with the arthritis control group. MDA levels were observed to increase in Group II when compared with Group I. However, GSH levels and SOD activities were observed to decrease in Group II when compared with Group I. Administration of Gugguluthiktakam kashaya showed moderate

decrease (p <0.01) in MDA level and increase in GSH and SOD activities. The treatment of Gugguluthikthakam Kashayam was initiated at the onset stage of Polyarthritic development i.e., day 14th. During the initial phase of treatment, the articular indexes of the treated groups showed moderately significant (p<0.01) difference with those of arthritic control group. Treatment with Gugguluthikthakam Kashayam showed a highly significant decrease at both the doses (p<0.001) as compared to the arthritic rats. Prednisolone (10 mg/kg) treated rats also showed significant decrease in TNF-α and Interleukin levels. **CONCLUSION-** It can be stated that the Gugguluthikthakam Kashayam have beneficial effects in long lasting reduction in rat paw edema, arthritic index and various lysosomal enzymes.

KEYWORDS - *In-vivo* Models, Anti-arthritic Evaluation, Gugguluthikthakam Kashayam, FCA Induced Arthritis, Oxidative stress parameters.

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INTRODUCTION:

Ayurvedic Science has evolved on the facts which were available on the ancient time. Constant Researches and evaluations according to the current techniques are required for the advancement of this great system and for the benefit of the society. Rheumatoid Arthritis (RA) is a chronic, inflammatory condition, that primarily attacks peripheral joints and the surrounding muscles, tendons, ligaments, and blood vessels. Rheumatoid Arthritis is characterized by disturbed innate immunity that comprises of immune complexes mediated complement activation, adaptive immune response against self-antigens, disorganized cytokines, osteoclast chondrocytes activation, imprinting of stromal cells that in succession develop semi-autonomous features that support disease development. The environmental factors plays a major role in genetically predisposed individuals, in the pathogenesis and advancement of RA (Bellucci E *et al*, 2016; Picerno V *et al*, 2015).

In Ayurveda medicine, several compositions are advocated as shothaharam and for sandhi-asthi-majja gata conditions. Gugguluthikthakam kashaya (Ashtanga Hridaya, 2018, Vatavyadhi prakarana) is such a medication which can be used successfully for this condition, which can be proven with the support of modern diagnostic and assessment tools. But there is scarcity of scientific studies to prove the efficacy of these medicinal systems, although the medicines are cheap, more effective, with less or no side effects, easily available and affordable (Subramani Parasuraman*et al*,2014). Gugguluthiktakam kashayam (Ashtanga Hridayam, 2018) is indicated for sopha, sandhi-asthi-majja gata vyadhi and is

having relevance with the disease Rheumatoid Arthritis. Unpredictable exacerbations and remissions in this chronic disease makes confusions in the treatments implicated in patients. Hence experimental studies are valuable in Rheumatoid Arthritis. In this research work, in-vivo study has been carried out to prove the anti-inflammatory as well as anti-arthritic action of Gugguluthikthakam kashaya.

MATERIALS & METHODS

Formulation of Gugguluthikthakam Kashayam

The drugs used in the clinical study is Gugguluthikthakam Kashaya. The Ingredients and Quantity of Gugguluthikthakam Kashaya are given in the Table no.1.

Table No.1 Ingredients and Quantity of Gugguluthikthakam Kashaya

(Pharmacopiea, Kerala Govt. Publication, Dr.K Lalithamma, Revised 1996, pg.no.71-72).

1) Nimba – 12 g	16) *Chavya* – 1 g
2) Amrita – 12 g	17) *Kushtha* - 1 g
3) Vrisha – 12 g	18) *Tejovati* – 1 g
4) Patola - 12 g	19) *Maricha* – 1 g
5) Nidigdhika – 12 g	20) *Vatsaka* – 1 g
6) Shuddha Guggulu – 12 g	21) *Dipyaka* – 1 g
7) Patha – 1 g	22) *Agni* – 1 g
8) Vidanga – 1 g	23) *Rohini* – 1 g
9) Suradaru – 1 g	24) *Arushkara* – 1 g
10) Gajopakulya – 1 g	25) *Vacha* – 1 g
11) Yavakshara – 1 g	26) *Kanamula* – 1 g
12) Souvarchala – 1 g	27) Yuktha – 1 g
13) Nagara – 1 g	28) *Manjishtha* – 1 g
14) Nisha – 1 g	29) *Ativisha* – 1 g
15) Misi – 1 g	30) *Visha* – 1 g
	31) *Yavani* – 1 g

Method of Preparation:

Gugguluthikthakam Kashayam is prepared by soaking one part of coarse kashaya powder with 16 parts water, boiled till its volume reduced to 1/8 which was filtered and used. (Bhavaprakasha, Vol.1)

Experimental Animals:

Adult female and male Wistar rats of weight 150- 200 g, which were bred and reared in the department animal house, were used for this study. The animals were provided with laboratory chow (Hindustan Lever Lab diet) and water ad libitum throughout the experimental period. The rats were housed in polypropylene cages in a room with the temperature maintained at 26 ± 10C and a 12-hour light dark cycle. All experiments were conducted as per the guidelines of the Animal Ethics Committee CPCSEA (Registration No. IAEC/BRNCOP/2021/004). In a study of Polyherbal formulations in collagen induced arthritis, the dose for the Gugguluthiktakam kashaya was reported to be 2 ml/kg body weight. So by consideration of that research article, in our study we have considered 2 ml/kg body weight as a safe dose for the further in-vivo investigation (Aswathi *et al*, 2021).

In-Vivo Evaluation of anti-arthritic activity

Evaluation of anti-arthritic activity of Gugguluthiktakam kashaya

The Wistar albino rats were divided into 5 groups of six animals in each. FCA (0.1 ml) was injected for the induction of chronic inflammatory response, through intra-articular injection. It was done in the left ankle joint of rats on 0^{th} day. Prior to the injection of Freund's Complete Adjuvant (FCA), pre-induction baseline was taken by measuring the left paw volume of each animal at 0^{th} day for the induction of arthritis in Wistar rats. The treatments with all plant extracts were given once daily from day of injection to 21^{st} day. A suspension of the test extracts were prepared in 1% Tween 80. The animals were grouped as following. (Arulmozhi *et al*, 2011).

Group-I: Normal control, treated with 1% Tween 80 on zero day

Group-II: Arthritic control, treated with 0.1 mL of FCA on 0^{th} day.

Group-III: Standard control: treated with prednisolone (10 mg/kg, p.o.) + FCA

Group-IV: Treated with Gugguluthiktakam kashaya (2 ml/kg, p.o.) + FCA

Group-V: Treated with Gugguluthiktakam kashaya (4 ml/kg, p.o.) + FCA

Measurements of paw volume

The percentage inhibition of the rat paw volume was measured by following formula (Arulmozhi *et al*., 2011; Ignacimuthu *et al*., 2011).

$$\text{Percentage inhibition} = \frac{Vc - Vt}{Vt} \times 100$$

Where,

Vc-Paw volume of control animals

Vt-Paw volume of treated animals

Measurements of body weight

Body weight was measured of all groups at zero days before immunization and at 21st day after treatments over by using a single pan weighing balance (Jalalpure *et al*., 2011).

Estimation of various biochemical parameters

At the end of the experimental period, rats were fasted overnight and the blood sample were collected by retro orbital blood sampling techniques from anaesthetized rats.

Estimation of Malonaldehyde (MDA)

In the articular cartilage, the thiobarbituric acid-reactive substance was measured as a marker of lipid peroxidation. The homogenized tissue was added with 1.5 ml of 8.1% sodium dodecyl sulphate, 1.5 ml of 20% acetate buffer (pH 3.5) and 1.5 ml of 0.8% TBA (thiobarbituric acid) solution. The mixture was heated for 1 hour, at 95 ◦C and after cooling, 5ml of n-butanol pyridine (14:1) was added for extraction. For the determination of TBA reactive substance, the absorbance of n-butanol–pyridine layer at 532 nm (Shimadzu UV Vis 1700) has been measured. (Kumar *et al*, 2009; Arulmozhi *et al*, 2011).

Estimation of glutathione (GSH)

An aliquot of articular tissue homogenate supernatant (0.4 ml) was added to dark polyethylene tube containing 1.6 ml of 0.4M Tris–EDTA buffer, pH 8.9. After vortex-mixing, 40µl of 10mM dithiobisnitrobenzoic acid in methanol was added. Again the samples were vortex-mixed and the absorbance was read at 412 nm after 5 min (Shimadzu UV-Vis 1700). The values of unknown samples were drawn from a standard curve plotted by assaying different known concentrations of glutathione (GSH). The amount of GSH was expressed as µmol/g of protein (Kumar *et al*., 2009; Arulmozhi *et al*, 2011).

Estimation of superoxide dismutase (SOD)

Total SOD activity was measured by determining the capability to inhibit the auto-oxidation of pyrogallol. The rate of auto-oxidation was determined by measuring increases in the absorbance at 420 nm. The reaction mixture which contained, 0.2mM pyrogallol in 50mM Tris–cacodylic acid buffer (pH 8.5) and 1mM diethylene triamine pentaacetic acid was incubated for 90s at 25^0C. One unit of SOD activity is defined as the amount of the enzyme required to inhibit the rate of pyrogallol auto oxidation by 50% (Kumar *et al*, 2009; Arulmozhi *et al*, 2011).

Estimation of TNF-alpha & Interleukin: On the last day of study, samples of plasma were sending to the Pathology Lab, for estimation of TNF-alpha and Interleukin levels. The Estimation was done with the help of Elisa Reader in Pathology Lab.

STATISTICAL ANALYSIS

The values are expressed in mean ± SEM. The results were analyzed by using one way analysis of variance (ANOVA) followed by Dunnet's "t" test to determine the statistical significance. $P < 0.05$ has been taken as the level of significance.

RESULTS & DISCUSSION

ANTI-ARTHRITIC ACTIVITY

Freund's complete adjuvant (FCA) induced rat paw edema

Observations of paw volume were recorded on 4^{th}, 8^{th}, 12^{th}, 16^{th}, 21^{st} day after adjuvant injection. The FCA-induced arthritic control group showed signs of arthritis development, as seen by the increase in the paw volume and other indications, such as decreased body weight, also showed induction of arthritis in the FCA-treated control group rats. The assessment made on the 21^{st} day showed that the Gugguluthiktakam kashaya treatment at both doses (low and high) had moderately significant and highly significant effect and reduced ($p< 0.01$& $p<0.001$) the adjuvant-induced lesions in the respective treatment groups as compared with the arthritis control group.

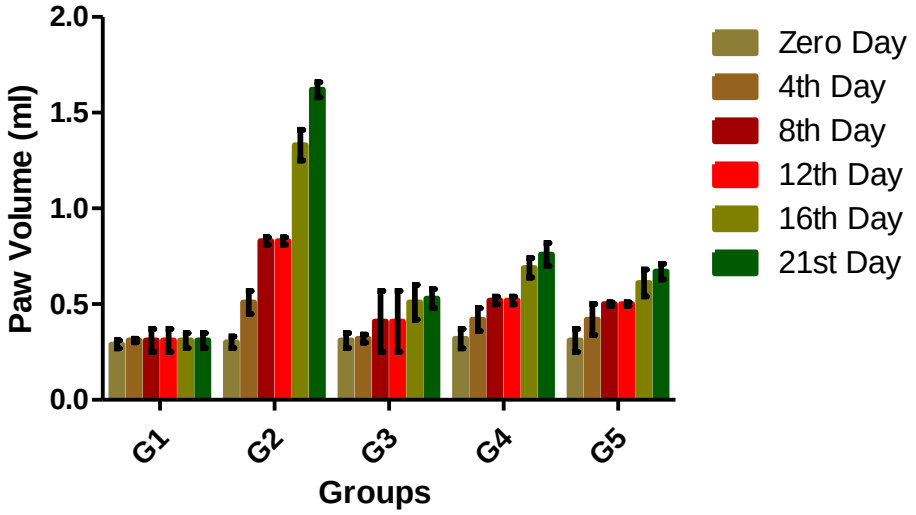

Figure No.1: Effects of Gugguluthiktam kashaya on paw volume in FCA induced arthritis in rat

Effects on body weight

Although the weights were almost identical in all group of animals at 0 to 7 days during the subsequent course of disease, the body weight always declined in arthritic control group from 14th day to 21st day. In arthritic group, decrease in body weight was observed on the subsequent days, whereas groups treated with standard, and the Gugguluthiktakam kashaya showed improvements in body weight.

Table No.2: Effects of Gugguluthiktakam kashaya on body weight in FCA induced arthritis in rat

S. No.	Groups & Treatments	Days	
		Zero	21st
1	Normal Control	190.20±0.78	192.45±0.28
2	Arthritic Control, 1% Tween 80, p.o.	191.40±0.18	162.45±0.34***
3	Prednisolone, 10 mg/kg, p.o.	191.80±0.20	205.33±0.11***
4	Gugguluthiktakam kashaya, 2 ml/kg, p.o.	190.18±0.12	202.31±0.55**
5	Gugguluthiktakam kashaya, 4 ml/kg, p.o.	192.25±0.40	205.62±0.42***

Values are expressed as mean±SEM, n=6 in each group; *p<0.05, compared to disease control ** p<0.01, compared to disease control. ***p<0.001, compared to disease control

Effects on arthritic assessment in arthritic rats

The treatment of Gugguluthikthakam Kashayam was initiated at the onset stage of Polyarthritic development i.e., day 14th. During the initial phase of treatment, the articular indexes of the treated groups showed moderately significant (p<0.01) difference with those of arthritic control group.

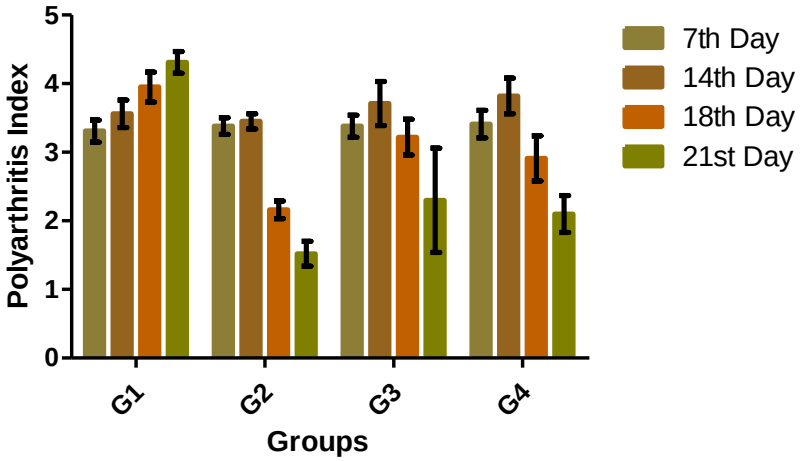

Figure No.2: Effects of Gugguluthiktakam kashaya on arthritic assessment in rats

Estimation of biochemical parameters

Oxidative Stress Parameters

As shown in Table 3, MDA levels were observed to increase in Group II when compared with Group I. However, GSH levels and SOD activities were observed to decrease in Group II when compared with Group I. Administration of Gugguluthiktakam kashaya showed moderate decrease (p <0.01) in MDA level and increase in GSH and SOD activities.

Table No.3: Effects of Gugguluthiktakam kashaya on oxidative parameters in rats

S. No.	Groups & Treatments	Oxidative stress parameters		
		MDA nmol/mg of protein	Glutathione µmol/g of protein	SOD U/mg of protein
1	Normal Control	4.52±0.32	7.47±0.28	7.82±0.31
2	Arthritic Control	14.24±0.37***	2.32±0.12***	3.13±0.18***
3	Prednisolone 10 mg/kg	7.52±0.29***	6.57±0.24***	5.87±0.27**
4	Gugguluthiktakam kashaya,2 ml/kg, p.o.	9.40±0.28*	5.45±0.23*	4.56±0.31*
5	Gugguluthiktakam kashaya,4 ml/kg, p.o.	8.28±0.24**	5.91±0.27**	5.78±0.96**

Values are expressed as mean±SEM, n=6 in each group; *p <0.05, compared to arthritic control, ** p <0.01, compared to arthritic control. *** p<0.001, compared to arthritic control

Estimation of TNF-α

Treatment with Gugguluthiktakam kashaya showed a highly significant decrease at both the doses (p<0.001) as compared to the arthritic rats. Prednisolone (10 mg/kg) treated rats also showed significant decrease in TNF-α levels.

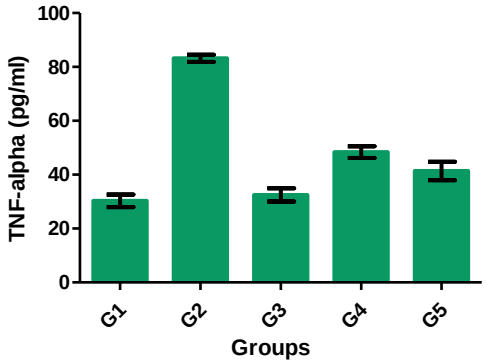

Figure No.3: Effects of Gugguluthikthakam Kashaya on TNF-α level in arthritic rats

Estimation of IL-1β

Treatment with Gugguluthiktakam kashaya showed a highly significant decrease at both the doses (p<0.001) as compared to the arthritic rats. Prednisolone (10 mg/kg) treated rats also showed significant decrease in IL-1β levels.

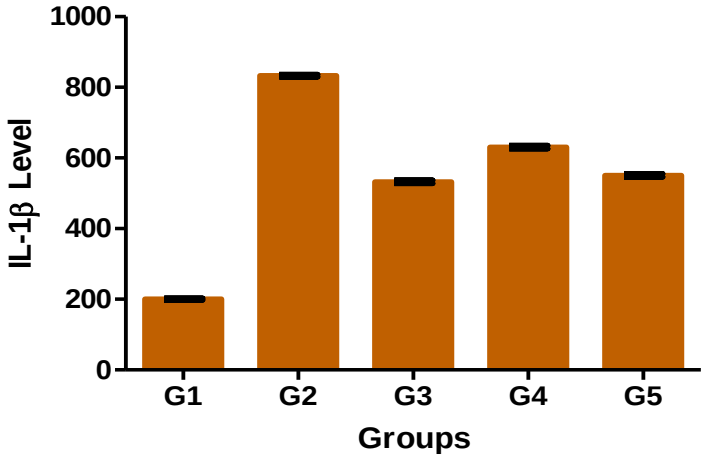

Figure No.4: Effect of Gugguluthiktakam kashaya on IL-1β levels

DISCUSSION

Rheumatoid arthritis (RA) is a chronic inflammatory disease which leads to the destruction of synovial membranes, cartilage and bone. Pro-inflammatory cytokines are considered to be one of the most vital mediators involved in the pathogenesis of RA. Mclnnes *et al*, compiled the recent investigations of this subject meticulously and discussed the role of cytokines in RA (Mclnnes & Schett, 2007). Tumor necrosis factor (TNF-α) plays a critical role in the pathogenic mechanisms of RA, and is now targeted in the standard management of patients with RA. Interleukin-1 (IL-1) is well recognized as another key pro-inflammatory cytokine involved in RA. Lately, macrophage migration inhibitory factor (MIF) has been considered to have pro-inflammatory effect in RA. MIF is implicated in leukocyte recruitment, activation, proliferation and survival, as well as the production of pro-inflammatory cytokines and mediators, and mechanisms of bone and cartilage injury, all of which contributes for the pathology of RA (Eric *et al*., 2006).

FCA-induced experimental model for arthritis is considered closest to simulating human rheumatoid arthritis and therefore it is the most widely used chronic test model in which the associated clinical and histopathological changes are similar to those seen in human form (Billingham & Davies, 1979). Humoral immune mechanisms appear not to add to the disease process. This unique rat disease model represents a systemic process that involves not only the joints but also the gastrointestinal and genitourinary tracts, the skin and the eyes.

The results emanating from the present study demonstrated that the Gugguluthiktam kashaya dose dependently attenuated chronic inflammatory responses in adjuvant induced arthritis and also facilitated recovery as measured by the decreasing paw edema, body weight and various biochemical parameters. All the test extracts were found to effectively reduce the primary lesions in arthritic rats.

During the development of arthritic syndrome, the body weight of rats used as an indirect index in restoration of health. The body weight was significantly decreased in arthritic rat as compared to normal rat, but in the test extracts and standard drug treated groups, the body weights of the rats did not decline. The results of our study therefore indicated that there is a relationship between the extent of inflammation and loss of body weight. Polyarthritic index was associated with an immune system mediated inflammatory reaction and after FCA treatment, experimental animal developed Polyarthritic index (Cai *et al*, 2006). The Polyarthritic index was an initial reaction of edema and soft-tissue thickening at the site and the irritant effect of the adjuvant and the disease progression in the injected foot are supposed

to be immunologic events (Ward & Cloud, 1965). In our study, the average scores for each group of drug treated animals were compared with that of disease control animals. In disease control group, arthritic Index was significantly higher compared to normal control group while prednisolone and Gugguluthiktakam kashaya treated groups showed significantly less score as compared to model control group. This indicates the protective effect and immunosuppressant properties of test drugs against adjuvant-induced arthritis. In arthritic animals, damage to the synovial cavity has been correlated with the overproduction of ROS, dysregulation of anti-oxidant enzymes and free radical-scavenging molecules in the joint (Sen, 1988). MDA is a metabolic result of lipid peroxidation, and its level is raised in oxidative stress. Lipid peroxidation is a critical mechanism of the injury that occurs during rheumatoid arthritis, which is often measured by analysis of tissue Malonaldehyde. The large amount of Malonaldehyde in arthritic control group is consistent with the occurrence of damage mediated by free radicals. Gugguluthiktakam kashaya treated groups showed a significant attenuation of Malonaldehyde and having anti-lipoperoxidative activity, which might be the proposed mechanism of anti-inflammatory activity (Wills, 1987; Arulmozhi *et al*, 2011).

In arthritic control group, there was a significant decrease in glutathione levels in joints. Due to depletion of glutathione level, there is decreased protection against various invading pathogens (Tastekin *et al*, 2007). Treatment of Gugguluthiktakam kashaya treated groups causes a significant increase in glutathione level. Anti-inflammatory action of fractions probably may be due to scavenging property of free radicals which was generated due to oxidative stress. In arthritic animals, there was a significant decrease in SOD level and all fractions and standard treated groups showed a significant increase in this enzyme. The significant up regulation of antioxidant enzymes, SOD, GSH and MDA by Gugguluthiktakam kashaya supports it's role in anti-arthritic potential through effects on antioxidant parameters.

In Rheumatoid arthritis, several cytokines, e.g. interleukin (IL)-1, IL-6, IL-8, IL-12, IL-17, tumour necrosis factor-α (TNF-α), interferon-γ(IFN-γ) and granulocyte macrophage colony stimulating factor (GM-CSF), are concerned in almost all aspects of articular inflammation and destruction. TNF-α has been considered as an essential cytokine in the pathogenesis of RA. Previous researchers also reported that TNF-α blockade has become a main strategy for the therapy of RA. Tumor necrosis factor (TNF) has an enormously broad spectrum of

biological activities. It is one of the key cytokine molecules that causes inflammation in RA. (Fox 2000; Elliott & Maini *et al*, 1993).

CFA injection caused activation of immune system, resulting in abnormal leukocyte proliferation and differentiation. Dendritic cells react with adjuvant components; it enhanced phagocytosis, proliferation of CD4+ lymphocytes, and secretion of cytokines (TNF-α & IL-1β). Several studies have reported that IL-1β play crucial roles in inflammation and synovial tissue damage, and their levels were increased in patients with RA. Thus, IL-1β represents crucial targets for treating RA. Our results revealed that administration of Gugguluthiktakam kashaya significantly reduced TNF-alpha and Interleukin levels as compared to the arthritic rats.

CONCLUSION

In conclusion, it can be stated that the Gugguluthikthakam Kashayam have beneficial effects in long lasting reduction in rat paw oedema, arthritic index and various lysosomal enzymes. Further studies are required to elucidate the exact mechanism based on molecular and genetic level responsible for anti-arthritic activity.

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