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Anti-arthritic activity of *Citrullus colocynthis* against Complete Freund's adjuvant (CFA) induced arthritis in experimental model rats

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Abstract

Aim The present study was mainly aimed to evaluate the anti-arthritic activity of ethanolic extract of *Citrullus colocynthis*.

Methods Plant extract was tested for its effect on Arthritis at two dose levels i.e., 200 and 400 mg/kg respectively. Prior to that the animals were induced with arthritis by giving Complete Freund's adjuvant (CFA) (0.1 ml/rat, s.p.). And, the rats were observed for the changes in Body weights, Paw edema (inflammation), hematological parameters and histological analysis.

Results The data represented in this study clearly demonstrates the anti-arthritic activity of ethanolic extract of *Citrullus colocynthis*.

Conclusion Hence, the ethanolic plant extract of *Citrullus colocynthis* has an effective anti-arthritic activity.

Keywords Arthritis, Anti-arthritic activity, *Citrullus colocynthis*, Complete Freund's adjuvant.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, inflammatory, systemic autoimmune disease that affects about 1% of the general population in Western countries and is two to three times more common in women than in men. It is characterized by both local and systemic inflammation with elevated plasma concentration of pro-inflammatory cytokines, such as interleukins-6 (IL-6), interleukin 1β (IL- 1β), tumor necrosis factor-α (TNF-α) and acute phase proteins [1].

Conventional treatments for RA, including Non-steroidal Anti-inflammatory Drugs (NSAID's), disease modifying anti-rheumatoid drugs (DMARD's) and corticosteroids, aim to reduce the patient's pain, joint inflammation; minimize loss of function and decrease the progression of joint damage. However, such treatments are rarely totally effective and some pharmacological therapies have the potential to cause side effects. All anti inflammatory drugs are not anti-arthritic because, it does not suppress T-cell and B-cell mediated response. RA is associated with poor nutritional status in relation to various nutrients due to not only because of increased requirements and reduction in their absorption but also due to NSAID's, DMARD's and corticosteroids prescribed to alleviate symptoms of this disease [2].

Citrullus colocynthis Schard belongs to the family Cucurbiataceae. It is reported to possess many phytoconstituents like alkaloids, flavonoids, tannins, saponins, triterpenes and glycosides and it is used in the treatment of tumors, as antipyretic [3], anti-inflammatory [4], antioxidant [5] and also against hepatic diseases [6], hyperglycemia, Anti diabetic, ulcers, urinary diseases and rheumatism.

MATERIALS AND METHODS

Collection of plant material

Citrullus colocynthis roots were obtained from the local area of Kadapa district, Andhra Pradesh, India. The plant was authenticated by Dr. Madhava chetty, Taxonomist, S.V. University, Tirupathi, India. The

collected roots were washed immediately and dried at room temperature for one month, powdered mechanically, sieved (10/44) and stored in air-tight containers.

Preparation of Ethanolic extract

About 2000 g of the powdered material was subjected to soxhlation and exhaustively extracted with 80% ethanol for 48 h. The solvent was distilled off at low temperature under reduced pressure using rotary flash evaporator. The semisolid mass obtained was dried in an oven at 40^0C, powdered, labeled as EECC (Ethanolic extract of *Citrullus colocynthisis)* and stored in desiccator.

Experimental animals

Healthy male adult wistar albino rats weighing about (150 – 200 g, b.wt) were used They were acclimatized for 7 days under standard husbandry conditions, i.e., room temperature 22±2^{o}C, relative humidity 45-55% and light dark cycle 12:12 hours. The animals were fed with commercial pellet rat feed (Raghavendra enterprises Bangalore) and water was given ad libitum. The Institutional Animals Ethics Committee (IAEC) approved the experimental protocol and care of animals was taken as per guidelines of CPCSEA (Ref NO: 1423/PO/a/11/CPCSEA).

Chemicals

CFA (Complete Freund's adjuvant) was procured from Sigma-Aldrich Pvt. Ltd, Bangalore. Ibuprofen and all other chemicals and reagents used were of analytical grade, procured from SD fine chemicals Ltd. India.

Treatment schedule

Induction of arthritis

On the 1st day they were injected with 0.1 ml of CFA into the sub plantar region of the left hind paw. This consists of killed Mycobacterium butyricum suspended in heavy liquid paraffin oil. Administration of test compounds and standard drug was started on the next day and continued for 28 days.

Experimental setup

The animals were randomly selected into five groups, each group comprising of 6 animals and treated for 28 days, as following:

Group I: Normal control receives distilled water.

Group II: Positive Control receives CFA (0.1 ml/rat, s.p.)

Group III: Standard Control receives CFA (0.1 ml/rat, s.p.) + Ibuprofen (15 mg/kg, p.o.)

Group IV: EECC (low dose) receives CFA (0.1 ml/rat, s.p.) + EECC (200 mg/kg, p.o.)

Group V: EECC (High dose) receives CFA (0.1 ml/rat, s.p.) + EECC (200 mg/kg, p.o.)

Parameters monitored

Measurement of paw volume

Paw volumes were measured at 0, 7th, 14th, 21st and 28th day using Plethysmometer for paw volumes were noted [7].

% Inhibition = Vc - Vt / Vc ×100. Where,

Vc is mean changes in paw volume of control group and Vt is mean changes in paw volume of test group.

Measurement of paw thickness

Paw thickness was carried out by using micrometer screw gauge till the pain elicited as indicated by squeaking or leg withdrawal at 0, 7th, 14th, 21st and 28th day [8].

The percentage inhibition of the growth of paw thickness was calculated by using the following formula:

% Inhibition = Vc - Vt / Vc ×100. Where,

Vc is mean changes in paw thickness of control group and Vt is mean changes in paw thickness of test group.

Body and organ weights

Initial and terminal body weights were measured along with weight of isolated organs such as kidney, liver and spleen on the 28th day.

Hematological Parameters

After completion of 28th day blood was collected from the retro-orbital Venus plexus and rats were sacrificed using cervical dislocation and serum was separated. Parameters like hemoglobin (Hg), RBC and WBC counts were measured using Semi auto analyzer (Maxlyzer, Avecon model no: NB-201).

Radiographic analysis

The rats were anaesthetized using Ketamine (50 mg/kg, i.p.) and radio graph was recorded on a digital system and seimen's X-ray machine.

Statistical analysis

Results were analyzed using one way analysis of variance (ANOVA) followed by the Dennett's t test by using statistical software package, Graph Pad Prism; version 5.03. Values were expressed as mean ± SEM and the $p<0.05$ were considered as statistically significant.

RESULTS

Anti-arthritic activity of EECC

Effect on inflammatory parameters

Effect on paw volume & paw thickness

Rats treated with CFA (0.1ml) shown significant ($p<0.001$) increase in paw volume & thickness in arthritis control (G-II) on day 7 and same was observed throughout the studied period as compared with normal control (G-II). And, rats treated with standard control (G-III) shown significant prevention in the paw volume on 28th ($p<0.001$) day. Rats treated with EECC at low dose (200mg/kg p.o.) shown significant prevention of the paw volume on 28th ($p<0.01$) and also, high dose (400mg/kg) showed significant ($p<0.001$)

Table 1: Effect of EECC on CFA Induced Rat Paw volume & Percentage inhibition of paw volume in CFA induced arthritis rat paw volume

SI. No	Group	Paw volume (ml) (Mean ± SEM) on					Percentage inhibition of paw volume				
		0th day	7th day	14th day	21st day	28th day	0th day	7th day	14th day	21st day	28th day
1	I	0.20±0.04	0.20±0.04	0.20±0.04	0.20±0.04	0.20±0.04	-	-	-	-	-
2	II	0.22±0.02	1.95±0.09###	2.27±0.08###	2.35±0.10###	2.32±0.06###	-	-	-	-	-
3	III	0.27±0.02	2.12±0.07	1.82±0.06**	1.22±0.06***	0.52±0.04***	0	0	19.82	48.08	77.58
4	IV	0.25±0.02	2.22±0.06	2.15±0.06	1.87±0.04**	1.85±0.11**	0	0	5.28	20.42	20.25
5	V	0.27±0.02	1.97±0.14	1.97±0.08*	1.32±0.04***	0.6±0.07***	0	0	13.21	43.82	74.13

All values are shown as mean ± SEM and n=6.
indicate p<0.001 when compared to normal group.
* indicate p<0.05, ** indicate p<0.01, *** indicate p<0.001 when compared to control group

Table 2: Effect of EECCT on CFA Induced Paw thickness & Percentage inhibition of paw thickness in CFA induced arthritis rat paw thickness

SI. No	Group	Paw thickness (cm) (Mean ± SEM)					Percentage inhibition of Paw thickness				
		0th day	7th day	14th day	21st day	28th day	0th day	7th day	14th day	21st day	28th day
1	I	0.275±0.04	0.275±0.04	0.275±0.04	0.275±0.04	0.275±0.04	0	0	0	0	0
2	II	0.3±0.04	1.125±0.04###	1.225±0.08###	1.3±0.04###	1.275±0.04###	0	0	0	0	0
3	III	0.3±0.05	0.8±0.04	0.775±0.04**	0.7±0.04***	0.45±0.08***	0	28.57	36.73	46.15	64.705
4	IV	0.325±0.02	1.2±0.07	1.2±0.07	1.1±0.07	0.925±0.04**	0	0	2.04	15.38	27.45
5	V	0.275±0.04	1.125±0.04	0.975±0.04	0.9±0.07**	0.55±0.06***	0	0	20.408	30.76	56.86

All values are shown as mean ± SEM and n=6.
indicate *p*<0.001 when compared to normal group.
* indicate *p*<0.05, ** indicate *p*<0.01, *** indicate *p*<0.001 when compared to control group

Protection. Percentage inhibition of CFA induced hind paw volume on 28th day; ibuprofen treated rats were 77.58 % inhibition and the rats treated with EECC 400 mg/kg showed 74.13% inhibition. Moreover, Percentage inhibition of CFA induced hind paw thickness in ibuprofen treated rats showed 64.70 and EECC 400 mg/kg high dose showed 56.86 (Table 1, 2).

Effect on body weight & organ weights

The arthritis control (G-II) animals exhibited a remarkable decrease in body weight gain when compared to the normal group (G-I) rats. At the same rats treated with EECC (400mg/kg) showed remarkable increase in body weights. CFA (0.1ml) induced arthritis rats showed significant increase in the liver & spleen weight ($p<0.001$) and EECC (400 mg/kg) treated rats inhibited it significantly ($p< 0.05$) (Table. 3).

Effect on hematological parameters

All CFA treated rats showed decrease in Hb level ($p<0.001$) and RBC count ($p<0.001$) when compared to the normal rats. The EECC at low dose (200 mg/kg) (G-IV) treated rats showed significant protection in the Hb level ($p<0.05$) and RBC count ($p<0.01$) when compared to the control group (G-II) rats. Whereas, the EECC at high dose (400 mg/kg) was showed significant protection in Hb level ($p<0.01$) and RBC count ($p<0.001$) when compared to control rats (G-II) (Table. 4).

Radiographic analysis

The results were observed from X- ray was the normal group animals showed no soft tissue swelling and bony destruction. The arthritis control group animals showed soft tissue swelling along with narrowing of joint spaces and sign of bony destruction.

Table 3: Effect of EECCT on CFA Induced body weight & Organ weights

SI. No	Groups	Mean body weight in g at initial day	Mean body weight in g on day 28	Mean difference body weight in g (Mean±SEM)	Organ weigh (mg/100gm) (Mean ± SEM)		
					Liver	Spleen	Kidney
1	I	177.5±3.22	211±3.14	33.75±3.14	3.62±0.103	0.33±0.004	0.81±0.01
2	II	176.3±2.39	185±2.88	8.75±1.25	4.55±0.15###	0.402±0.002###	0.812±0.004###
3	III	176.3±3.75	200±3.53	23.75±4.2	3.97±0.04**	0.34±0.002***	0.85±0.008**
4	IV	180±4.08	193.8±2.39	13.77±2.39	4.22±0.062	0.39±0.004	0.817±0.008
5	V	178.8±1.25	201.3±3.14	22.5±3.22	4.15±0.02*	0.37±0.002**	0.812±0.007

All values are shown as Mean ± SEM and n=6
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###indicate $p<0.001$ when compared to normal group.
* indicate $p<0.05$, ** indicate $p<0.01$,
*** indicate $p<0.001$ when compared to control group.

Table 4: Effect of EECCT on CFA Induced hematological parameters on 28th day

Group	RBC (x 10^6 / mm^3)	WBC (x 10^3 / mm^3)	Hb (g/dl)	ESR (mm/hr)
I	5.91±0.36	5.35±0.07	14.38±0.175	2.95±0.17
II	3.31±0.08###	13.38±1.3###	10.73±0.33###	10.67±0.45###
III	5.50±0.12***	7.01±0.09***	13.94±0.17***	3.5±0.21***
IV	4.25±0.09**	10.4±0.52*	11.78±0.143*	8.9±0.23**
V	5.16±0.09***	7.33±0.14***	12.13±0.209**	4.47±0.12***

All values are shown as mean ± SEM and n=6.
indicate $p<0.001$ when compared to normal group.
* indicate $p<0.05$, ** indicate $p<0.01$, *** indicate $p<0.001$ when compared to control group

Standard group (G-III) showed no soft tissue swelling and no bony destruction. The EECC 200 mg/kg treated group shown less prevention of soft tissue swelling and narrowing of joint. The EECC 400 mg/kg treated group shown prevention against bony destruction and narrowing of joint spaces by showing less soft tissue, swelling (Fig. 1).

DISCUSSION

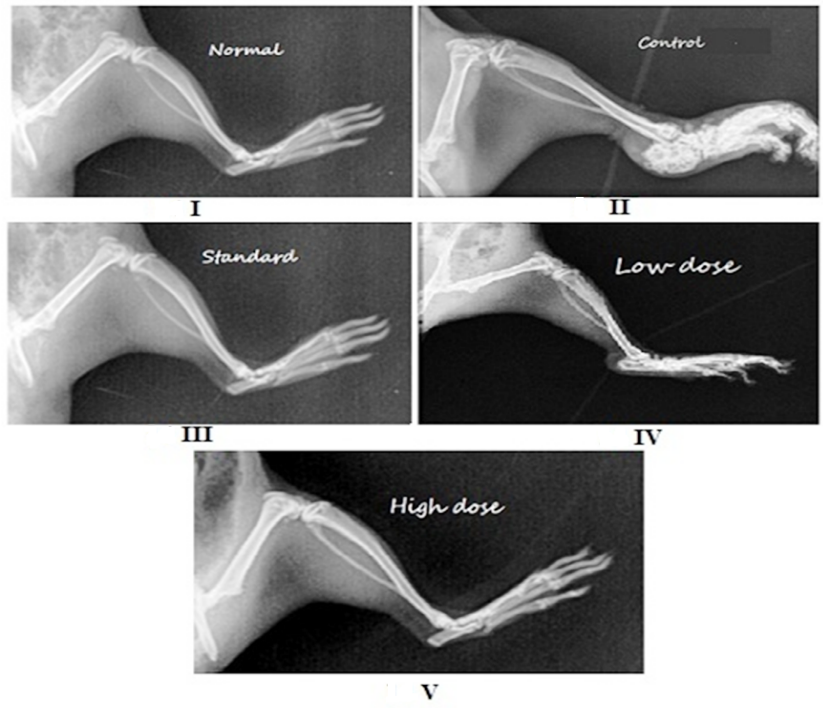
In the present study, standard drug ibuprofen significantly inhibited the paw volume, paw thickness and knee diameter in complete Freund's adjuvant induced arthritis rat due suppressed the swelling of the rat paws. The ethanolic extract of CC at doses of 200 and 400 mg/kg significantly and dose-dependently suppressed swelling of the paw volume, paw thickness and knee diameters in both acute and chronic phase, which may be due to the suppression of inflammatory mediators released due to induction of Freund's adjuvant. Though the actual mechanism of suppressing inflammation is substances such as flavonoids, which have been reported in EECC can be contributing to the anti-arthritic effect since flavonoids show anti-inflammatory [13,14], and anti-arthritic action [14,15]. Arthritis-characteristic events such as the IL-1 induced production of progelatinase B and prostaglandin E2, and synovial fibroblast proliferation have been suppressed by flavonoids [16].

A change in body weight of rats was also measured as one of the parameter to assess the course of the disease and the response to therapy of anti-inflammatory and arthritic drugs [17]. As the incidence and severity of arthritis increased, a decrease in body weights of the rats also occurred during the course of the experimental period and this observation was supported by the findings of C.V. Winder [18] on alterations in the metabolic activities of diseased rats. In addition to the absorption of ^{14}C- glucose and ^{14}C- leucine in rat's intestine was reduced in the case of inflamed rats.

The increase in spleen weight in the adjuvant induced arthritic rats has been reported to be associated with splenomegaly, generalized lymphadenopathy and altered hepatic function [19]. Injection of the CFA significantly increased spleen weight in arthritis control and that was significantly decreased in the animals treated with EECC 400 mg/kg. Thus, these findings indicated the inhibition of lymphocytes and decreased immunological response that may be responsible for the anti-arthritic potential of EECC.

In present study, arthritic control rats showed a reduced RBC count, reduced Hb levels, and an increased erythrocyte sedimentation rate (ESR).

Figure 1: Effects of EECCT on radiographic evaluation in CFA induced arthritis in rats



I = (Normal = Vehicle treated); II = (Control = CFA (0.1ml/rat, s.p.); III (Standard = CFA (0.1 ml/rat, s.p) + Ibuprofen (15 mg/kg, p.o.); IV = (Low dose CFA (0.1 ml/rat, s.p) + EECCT (200 mg/kg, p.o.); V = (High dose CFA (0.1 ml/rat, s.p) + EECCT (400 mg/kg, p.o.)

All these symptoms indicate an anemic condition. The two most common reasons for anemia in arthritic patients are gastrointestinal blood loss from arthritis medications and bone marrow changes in patients with inflammatory arthritis, which prevent the release of iron for incorporation into RBCs.

The significant increase in release of IL-IB inflammatory response, IL-IB increases the production of both granulocyte and macrophages colony stimulating factors [20] in adjuvant-induced arthritic rats may be due to the stimulation of immune system against the invading antigens. In the present study, the migration of leucocytes into the inflamed area is significantly suppressed by ethanolic extract of CC treated groups showed its immune modulation effect when compared to control rats, as seen from the significant reduction in the total WBC count. Similar effect was observed with Ibuprofen also.

RA is an autoimmune disorder, where auto antibodies are produced against self antigen (IgG). These auto antibodies are termed as "rheumatoid factor", which are immunoglobulin's of predominantly IgM class, which combine with Fc portion of immunoglobulin IgG molecules. RF is present in sera of 80% cases of rheumatoid arthritis [21,22].

It was also reported that among the various phytoconstituent flavonoids have beneficial effects in the inflammatory conditions and that the anti-inflammatory activity is a common property of many triterpenoids [23]. Flavonoids are particularly reported for significant antioxidant, vasculoprotector, anti-hepatotoxic, anti-allergic, anti-inflammatory, anti ulcerogenic and an even anti-tumor activity [24]. The anti-inflammatory effects of triterpenes have been attributed to various mechanisms including inhibition of lipoxygenase and cyclooxygenase activities [25]. The antioxidant activity has been described for several triterpenes, such as α- and β- amyrins, oleanolic acid, ursolic acid, lupeol and glycirretinic acid [26].These results showed the significant anti-arthritic effect of *Citrullus colocynthis.*

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Conflict of interest

No potential conflict of interest relevant to this article was reported.

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