



Anti-inflammatory, antinociceptive and antiarthritic potential of apis cerana indica bee venom by reducing pain and degeneration of articular cartilage in adjuvant and collagen induced rat models of arthritis

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Introduction

Rheumatoid Arthritis (RA) is a chronic, relapsing inflammatory and autoimmune multisystem illness that affects the joints and characterized by inflammation of the synovial membrane, pain and restricted joint movement [1]. Bee venom has traditionally been used in oriental medicine to relieve pain and to treat Rheumatoid arthritis [2]. Bee venom consists of a variety of different peptides like Melittin, Adolapin, Apamin and Mast Cell Degranulating Peptide (MCDP) [3] (Eiseman et al, 1982). It

contains enzymes, such as phospholipase A₂, biologically active amines and non-peptide components [4]. The efficacy of bee venom in the treatment of arthritis and rheumatism is initiated after it is known to stimulate the production of cortisol in the adrenal glands, which in turn has anti-inflammatory activity [5]. Recent studies have reported several of mechanisms for the anti-arthritis and anti-inflammatory effects of bee venom. The decrease in cyclooxygenase (COX-2) and phospholipase (PLA₂)



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expression and the decline in the levels of tumor necrosis factor alpha (TNF- α), interleukin IL-1, IL-6, Nitric oxide (NO) and reactive oxygen species (ROS) are suggested to be associated with the anti-arthritis effect of Melittin [6,7,8]. Adolapin has anti-inflammatory activity most probably due to its ability to inhibit the prostaglandin synthesis through COX inhibitory properties [9].

It was reported in our earlier study that *Apis dorsata* bee venom therapy produced anti-nociceptive and anti-inflammatory effects on adjuvant and collagen induced arthritis model [10]. In present study, we have evaluated *Apis cerena indica* for its effectiveness in the carrageenan and cotton pellet induced inflammation model, hot plate test and tail immersion experimental model for analgesia. Further we have evaluated its anti-arthritic activity in FCA and CII induced experimental model for RA for acquisition supplementary vision into the mode of action and to explore the anti-inflammatory, anti-nociceptive and anti-arthritic activity of *Apis cerena indica* bee venom.

Material and methods

Bee Venom

The bee venom was obtained by electrical stimulation of the bees from bee keeper Shivsagar Madh Udyog, Pune, Maharashtra, India. The apparatus used in this procedure consists of a pulse generator and 10 glass-collecting plates. The apparatus was installed at the hive entrance, in such a way that the bees were induced (an electrical stimulus voltage was 415–420 V) to sting the plate, thus releasing the venom over its surface. Obtained *Apis Cerena Indica* bee Venom (ACIV) was weighed and dissolved in phosphate buffer until use.

Animals

Experiments were performed on healthy albino Wistar rats (180-200 g) and Swiss albino mice (20-25 g) of either sex purchased from the National Institute of Biosciences, Pune, (MS), India and acclimatized in our own animal house prior to experimental study. The animals were housed at $22 \pm 1^\circ\text{C}$, under a 12 h light/dark cycle, with free access to commercial diet and water ad libitum throughout the study. The care, use and experimental procedures were conducted in accordance with guidelines and procedures for animal experimentation as prescribed by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India. Experimental protocol was approved by Institutional Animal Ethics Committee (IAEC) (Ref: MCP/IAEC/29/2011). Each group containing six animals was used for the study.

Chemicals

Carrageenan was procured from Analab Fine Chemicals (Mumbai, India). Indomethacin, Diclofenac sodium, Freund's complete adjuvant and Bovine sternal type-2 collagen was purchased from Sigma Aldrich pvt.ltd. U.S.A. All other chemicals and reagents used were of analytical grade procured from SRL (Mumbai, india), E.Merck (India).

Anti-inflammatory study of ACIV

Acute inflammatory study using Carrageenan induced paw oedema in rats

The carrageenan- induced hind paw oedema in rats was performed according to Guilhon- Simplicio et al [11]. Inflammation was induced by subplantar injection of 0.1 ml of (1% w/v) of

Carrageenan suspension in normal saline in left hind paw of animals after 1h of administration of ACIV (2 mg and 4 mg/200g rat i.p.) in test group, Indomethacin (10 mg/kg p.o.) in standard group and control group received vehicle. The % inhibition in paw volume was calculated by using following formula,

$$\% \text{ inhibition in paw volume} = 100 \times (1 - V_t / V_c)$$

Where, V_c = Mean paw volume in control group, V_t = Mean paw volume in the drug treated group.

Cotton pellet induced granuloma in rats.

Sterile cotton pellet weighing 25 ± 1 mg was implanted subcutaneously in the groin region of rat under thiopental sodium (25 mg/kg) anesthesia.

Group I: The control group received vehicle

Group II: The standard group received Indomethacin (10 mg/kg p.o.)

Group III: Test group received (ACIV 4 mg/200g rat i.p.)

The animals received the respective treatments for 7 consecutive days starting from the day of cotton pellet implantation. The rats were sacrificed on eighth day, blood was collected by cardiac puncture and the pellets surrounded by granuloma tissue were dissected out carefully and dried at 60°C to a constant weight. The granuloma tissue formation and exudates formation was calculated using following formula.

Measure of granuloma tissue formation = Constant dry weight – Initial weight of pellet.

Measure of exudate formation = Wet weight – Constant dry weight of pellet [12].

Analgesic study of ACIV

Eddy's hot plate method

Animals were divided in 3 groups of 6 mice each.

Group I: Control (Vehicle treated)

Group II: Standard Diclofenac sodium (9 mg/kg p.o.)

Group III: Test group ACIV (1 mg/20g mice i.p.)

The mice were placed on the hot plate maintained at 55°C . The response time was noted as the time at which animals reacted to the pain stimulus either by paw licking or jump response, whichever appeared first. The response latency was measured at 0, 1, 2, 3, 4, 5 and 6 h [13].

2.5.2. Tail immersion method

Animals were divided in 3 groups of 6 mice each.

Group I: Control (Vehicle treated)

Group II: Standard Diclofenac sodium (9 mg/kg p.o.)

Group III: Test group administered with ACIV (1 mg/20g mice i.p.)

The tip of tail was dipped up to 5 cm in hot water maintained at 58°C . The response time was noted as a sudden withdrawal of the tail from the hot water. The latency response was measured at 0, 1, 2, 3, 4, 5 and 6 h [14].

Immunosuppressant activity

Animals were divided in three groups of six mice each:

Group I (Control): Vehicle treated

Group II (Standard): administered with methotrexate (0.5 mg/kg, i.p.)

Group III (Test): administered with ACIV (1mg/20 g mice i.p.)

The animals were immunized by injecting 1×10^8 sheep red blood cells (SRBC's) intraperitoneally (0 day). ACIV and Methotrexate were administered to all the animals from day 0 to day 7 as shown above. Blood samples were collected from individual animals of all the groups by retro orbital bleeding on day 7 and serum was separated. Antibody levels were determined by the hemagglutination technique [15].

Assessment of Anti-arthritic activity

Effect of ACIV for anti arthritic activity using Freund's complete adjuvant induced arthritis.

Freund's complete adjuvant (FCA) was prepared by suspending in liquid paraffin. The arthritis was induced by a single intradermal injection of 0.1 ml of FCA into the left hind metatarsal footpad of rat (Wei W et al 1986). FCA produced definite oedema within 24 h with progressive arthritis by day 9 after inoculation. In prophylactic model animals were treated from day 0 to 28 days, while in therapeutic model animals were treated from day 14 to 28 days. Animals were divided as

Group I: Control (Vehicle treated)

Group II: Arthritic control (Disease control)

Group III: Standard (Indomethacin (i.p.) (Prophylactic treatment)

Group IV and V: ACIV (2 and 4mg/ 200 g rat, i. p.) (Prophylactic treatment)

Group VI: Standard (Indomethacin i. p.) (Therapeutic treatment)

Groups VII and VIII: ACIV (2 and 4mg/ 200 g rat, i. p.) (Therapeutic treatment)

Drug treatment to prophylactic groups was started from the initial day i.e. from the day of adjuvant injection (0 day), 30 minutes before adjuvant injection and continued till 28th day and drug treatment for therapeutic groups was started from 14th day after chronic disease induction and continued till 28th day.

Paw oedema and Joint thickness (perimeter) was measured on 7th, 14th, 21st and 28th day by using digital vernier calliper. Paw volume and joint thickness, were calculated on respective days and % inhibition of paw oedema and joint thickness with respect to untreated group was calculated using following formula.

$$\% \text{ inhibition in paw oedema volume/ Joint thickness} = 100 \times (1 - V_t / V_c)$$

Where,

V_c = Mean paw oedema volume/ joint thickness in control group.

V_t = Mean paw oedema volume/ joint thickness in the drug treated group [16].

Assessment of Arthritic index

Assessment of Arthritic index is done by visual observation

of level of inflammation found on left hind paw in terms of oedema and redness [17].

0- No sign of oedema or redness

1- Redness without oedema

2- Redness with mild oedema

3- Redness with severe oedema

4- Redness, severe oedema and stiffness in movement

Arthritic dorsal flexion pain test

The ankle joint was gently flexed dorsally until the toes touched the front of the leg for 5 times with an inter-test interval of 5 seconds. Pain was scored zero when the animal showed neither squeaking nor quick leg-withdrawal. The scores was 1 when either reaction appeared and scored 2 when both reactions appeared. All the groups were evaluated in this manner on day 7th, 14th, 21st and 28th [18].

Stair Climbing Activity Test

Overnight fasting animals were trained for one week to climb a staircase with steps at 5, 10, and 15 cm having water at the second and food at the third step. Climbing ability of the rats in above groups was scored 0 if the rats did not climb; 1, if the rats climbed onto step-1; 2, if the rats climbed onto step-2 and 3, if the rat could climb all the three steps. All the groups were evaluated in this manner on day 7th, 14th, 21st and 28th day [19].

Motility Test

The motility pattern of the rats was observed for a period of 5 minutes on a plane surface and scored 0, if the rat walked easily, scored 1, if rat walked with little difficulty and scored 2, if rat walked with more difficulty and avoided touching the toes of the inflamed paw to the floor. All the groups were evaluated in this manner on day 7th, 14th, 21st and 28th day [18].

Hematological determinations in arthritic rats

On 28th day blood was withdrawn through retro-orbital vein puncture and the biochemical parameters like haemoglobin content, total WBC count, HCT (Hematocrit value), Hb (Hemoglobin) count and RBC count were analyzed [20].

Histopathological analysis of arthritic rats

On 28th day all animals were sacrificed and their hind limbs were removed surgically and fixed in 10% buffered formalin. Limbs were decalcified in 15% formic acid, processed for paraffin embedding and were sectioned to 5 μ m thickness. These sections were stained with haematoxyline and eosin for histological examination in all the above groups under light microscope [21].

Radiological analysis of arthritic rats

Before sacrificing the animals, X-rays were taken on 14th and 28th day at the joints of the hind paw of the animals. Radiographs were taken using X-ray apparatus (Siemens- 60MA, Germany) and industrial X-ray film (Fuji photo film, Japan). The X-ray apparatus was operated at 220 V with a 40 V peak, 0.2 second exposure time, and a 60 cm tube-to film distance for anterior-posterior projection [21].

Analysis of serum transaminases

The blood was collected after sacrificing the animals of all

groups on 28th day and serum was then analysed for lysosomal enzymes such as SGPT, SGOT and ALP by using analysis kits (Pathozyne laboratories Ltd.) [22].

Effect of ACIV for anti arthritic activity using Collagen type-II induced arthritis.

CII was dissolved in 0.1M acetic acid (0.4 mg/ml) by stirring overnight at 4°C and emulsified with an equal volume of FCA to a final concentration of 0.1 mg/ml. Rats were injected intradermally twice with 1ml of the emulsion (containing 200 mg of CII). First injection was made in the left hind paw with 0.1 ml and the tail and other 3–5 sites on the back with 0.9 ml; second immunization was done for 7 d after with a similar method. The day of the first immunization was defined as day 0. Arthritis symptoms appear by day 7 and became severe by 17th day. Animals were treated therapeutically from day 18 to day 36 [23].

Animals were divided into 4 groups of 6 rats each.

Group I: control group (Vehicle treated)

Group II: The standard (Indomethacin (10 mg/kg p.o.)

Group III: Test group ACIV (4 mg/200g rat i.p.)

Drug treatment to standard and test groups was started from the 18th day after chronic induction of disease and continued till 36th day. All the parameters were evaluated as like FCA model of arthritis.

Statistical data analysis

Data obtained were subjected to one-way analysis of variance (ANOVA) followed by Dunnett’s test. *p < 0.05, **p < 0.01 was considered significant.

Results

Anti-inflammatory activity

Carrageenan induced paw oedema model in rats

In the carrageenan-induced paw oedema, there was a gradual increase in the oedema paw volume in the control group during the experiment (6 h). ACIV showed significant decrease (p < 0.01) in carrageenan induced paw oedema when compared to control (Figure 1). Atam ius nenis. Sat nordit; num occididet re, se

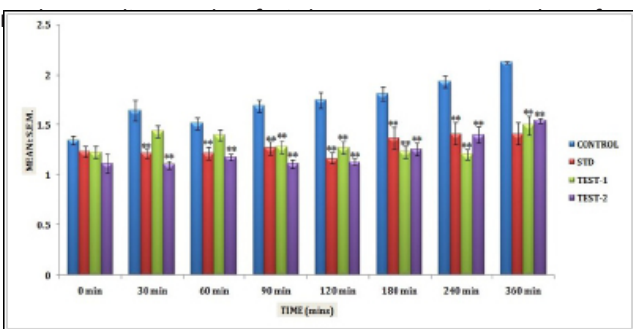


Figure 1: Effect of ACIV on carrageenan induced paw oedema

Values are expressed in mean ± SEM (n=6); *p<0.05, **p<0.01 vs. Control group, Data analysed by One-way ANOVA test followed by Dunnett’s multiple test for comparison. Treatment of various group are as follows, C: Control, STD: Indomethacin, Test: ACIV.

Cotton pellet induced granuloma model in rats

Administration of ACIV inhibited the formation of granulomatous tissue (p<0.01) and inhibition of the transudative granuloma weights in comparison to the control group (Figure 2).

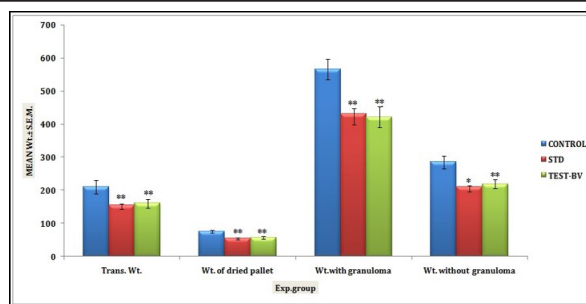


Figure 2: Effect of ACIV on cotton pellet induced granuloma model

Values are expressed in mean ± SEM (n=6); *p<0.05, **p<0.01 vs. Control group, Data analysed by One-way ANOVA test followed by Dunnett’s multiple test for comparison. Treatment of various group are as follows, Control: Vehicle treated, STD: Indomethacin, Test: ACIV treated.

Analgesic activity

Eddy’s hot plate method.

ACIV produced a significant analgesic activity as it significantly increased the withdrawal response latency period in Eddy’s hot plate model between 30m to 360 m (Figure 3).

Figure- 3: Effect of ACIV on Eddy’s hot plate method in mice

Values are expressed in mean ± SEM (n=6); *p<0.05, **p<0.01 vs. Control group, Data analysed by One-way ANOVA test followed by Dunnett’s multiple test for comparison. Treatment of various group are as follows, Control: Vehicle treated, STD: Indomethacin, Test: ACIV treated.

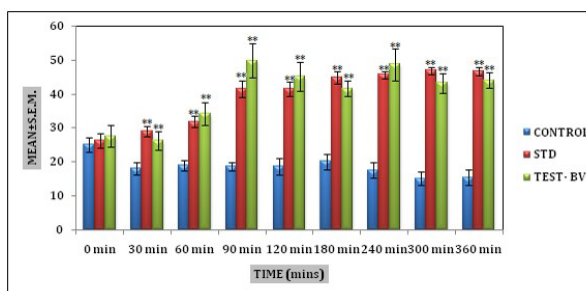


Figure 3: Effect of ACIV on Eddy’s hot plate method in mice

Values are expressed in mean ± SEM (n=6); *p<0.05, **p<0.01 vs. Control group, Data analysed by One-way ANOVA test followed by Dunnett’s multiple test for comparison. Treatment of various group are as follows, Control: Vehicle treated, STD: Indomethacin, Test: ACIV treated

Tail immersion method

ACIV showed significant analgesic activity after 1 h (p<0.01) with increased in withdrawal response (Figure 4).

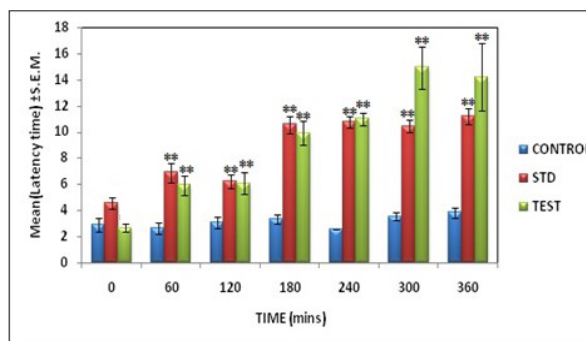


Figure 4: Effect of ACIV on Tail immersion method in mice

Values are expressed in mean \pm SEM (n=6); *p<0.05, **p<0.01 vs. Control group, Data analysed by One-way ANOVA test followed by Dunnett's multiple test for comparison. Treatment of various group are as follows, Control: vehicle treated, STD: Indomethacin, Test-ACIV Treated.

Immunosuppressant activity

In present study it was observed that on 7th day ACIV treatment effectively inhibited the agglutination reaction between test serum antibodies and antigens (mesh formation) present in SRBC's in ACIV and methotrexate treated group in comparison with agglutination of control group (Table 1).

Effect of ACIV on FCA and CII induced arthritis

Effect of ACIV on paw oedema and joint thickness in FCA and CII induced arthritis

In FCA and CII induced arthritis, there was a gradual increased in the paw oedema volume and joint thickness. Standard (Indomethacin), and ACIV treated groups showed significant decreased in paw oedema volume and joint thickness (Table 2 &3).

Effect of ACIV on pain perception parameters in FCA and CII induced arthritis

In FCA and CII induced arthritis, there was a gradual increased in arthritic index score, Motility test score and dorsal flexion test

score in the disease control group. Standard (Indomethacin), and ACIV treated groups showed significant decreased arthritic index score Motility test score and dorsal flexion test score were reduced significantly and stair climb ability test score were increased significantly (p<0.05) in standard (Indomethacin) and ACIV treated groups in comparison with disease control groups. (Table 4 &5).

Effect of ACIV on Serum transaminases and hematological parameters in FCA induced arthritis

In present study it has been observed that prophylactic (28 days) and therapeutic (14 days) treatment of ACIV significantly (p<0.01) decreased the raised level of serum transaminases like SGPT, SGOT, ALP and decreased the raised WBC count to normal level prophylactically and therapeutically along with significant increase in RBC, HB, HCT count on 28th day by in comparison with disease control group respectively (Table 6).

Effect of ACIV on Serum transaminases and hematological parameters in CII induced arthritis

In present study it has been observed that therapeutic (18 days) treatment of ACIV significantly (p< 0.01) reduced the increased level of SGPT, SGOT and ALP by on day 36th in comparison with disease control group. Also ACIV significantly (p< 0.01) restored the raised WBC count to normal level along with significant increase in RBC, HB, HCT count in comparison to disease control group (Table 7).

Table 1: Inhibition of Serum hemagglutination antibody titre by ACIV.

EXPERIMENTAL ANIMAL	% IMMUNE SUPPRESSION	
	STD	TEST-BV
1	25	37.5
2	22.22	66.66
3	50	60
4	70	80
5	33.33	66.66
6	50	50

Values of % immunesuppression of STD: standard and Test-BV group were expressed

Table 2: Effect of ACIV on Arthritic parameters in FCA induced arthritis.

Exp. Grps.	PAW OEDEMA(mm)				JOINT THICKNESS	
	7	14	21	28	21	28
DC	8.23 \pm 2.204	8.47 \pm 0.128	8.34 \pm 0.112	7.82 \pm 0.064	10.50 \pm 0.0685	9.33 \pm 0.351
BV-11	7.93 \pm 0.158	7.77 \pm 0.092**	6.82 \pm 0.155**	6.41 \pm 0.209**	7.74 \pm 0.194**	7.05 \pm 0.136**
BV-12	7.29 \pm 0.195*	7.18 \pm 0.112**	6.70 \pm 0.191*	6.13 \pm 0.112**	8.62 \pm 0.194**	7.60 \pm 0.140**
BV-21	7.30 \pm 0.167*	7.79 \pm 0.128**	7.59 \pm 0.116*	6.61 \pm 0.128**	9.76 \pm 0.454	8.14 \pm 0.386**
BV-22	7.08 \pm 0.271	7.19 \pm 0.172**	6.63 \pm 0.169**	5.531 \pm 0.201**	9.06 \pm 0.366*	8.35 \pm 0.228*
STD-P	6.62 \pm 0.243*	7.37 \pm 0.182**	7.19 \pm 0.181**	6.37 \pm 0.124**	8.72 \pm 0.387*	8.06 \pm 0.313**
STD-T	7.36 \pm 0.157*	7.81 \pm 0.170**	6.46 \pm 0.32**	6.18 \pm 0.158**	8.561 \pm 0.374**	7.49 \pm 0.156**

Values are expressed in mean \pm S.E.M. (n=6); *p<0.05, **p<0.01 vs. Control group, Data analysed by One-way ANOVA test followed by Dunnett's multiple test for comparison. Treatment of various group are as follows, DC: Disease Control, STD: Indomethacin, BV: ACIV Prophylactic (BV-11: 2mg/kg, BV-12: 4mg/kg), Therapeutic (BV-21: 2mg/kg, BV-22: 4mg/kg)

Table 3: Effect of ACIV on Arthritic parameters in CII induced arthritis.

Exp. Groups	Paw edema(mm)				Joint thickness(mm)			
	9	18	27 th	36 th	9 th	18 th	27 th	36 th
DC	9.42 ± 0.598	9.74 ± 0.324	9.23 ± 0.303	9.73 ± 0.227	11.06 ± 0.087	11.85 ± 0.48	10.55 ± 0.34	10.53 ± 0.345
STD	8.02 ± 0.316	8.54 ± .381*	7.53 ± .425**	6.93 ± .222**	9.71 ± 0.48	9.78 ± 0.57*	8.34 ± .325**	7.83 ± 0.285**
TEST	8.55 ± 0.403	8.49 ± 0.275*	7.01 ± .279**	6.81 ± 0.468**	9.90 ± 0.51	9.95 ± 0.472	8.63 ± 0.455**	7.98 ± 0.503**

Values are expressed in mean ± S.E.M. (n=6); *p<0.05, **p<0.01 vs. Disease Control group, Data analysed by One-way ANOVA test followed by Dunnett’s multiple test for comparison. Treatment of various group are as follows, DC: Disease Control, STD: Indomethacin, Test: ACIV.

Table 4: Effect of ACIV on Pain perception parameters in FCA induced arthritis

Exp. groups	PAIN PERCEPTION PARAMETER														
	DORSAL FLEXION SCORE				MOTILITY SCORE				ARTHRITIC INDEX				STAIR CLIMB SCORE		
	7 th	14 th	21 st	28 th	7 th	14 th	21 st	28 th	7 th	14 th	21 st	28 th	14 th	21 st	28 th
DC	1.03± 0.120	1.1± 0.100	1.16± 0.095	1.33± 0.066	1.5± 0.223	1.66± 0.210	1.66± 0.210	1.5± 0.223	3.33± 0.2108	3.5± 0.223	3.5± 0.223	3.5± 0.223	1.33± 0.211	1.16± 0.166	0.83± 0.307
BV-11	0.3± 0.044**	0.33± 0.042**	0.33± 0.042**	0.26± 0.042**	0.16± 0.166**	0.16± 0.166**	0.5± 0.223**	0.16± 0.166**	2.166± 0.210**	2.00± 0.001**	1.6± 0.210**	1.66± 0.220**	2.66± 0.212**	2.83± 0.177**	2.83± 0.166**
BV-12	0.23± 0.033*	0.23± 0.033**	0.26± 0.042**	0.2± 0.001**	0.17± 0.167**	0.33± 0.211**	0.51± 0.223**	0.17± 0.167**	1.666± 0.166*	1.5± 0.223**	1.3± 0.210**	1.33± 0.210**	2.83± 0.166**	3.00± 0.001**	3.00± 0.333**
BV-21	0.73± 0.042*	0.73± 0.042*	0.73± 0.040**	0.63± 0.061**	1.00± 0.012	1.00± 0.001	0.83± 0.212*	1.00± 0.012	2.666± 0.210	2.833± 0.307	2.33± 0.210*	2.33± 0.220**	1.16± 0.307	2.33± 0.333*	2.33± 0.333*
BV-22	0.5± 0.44*	0.5± 0.044**	0.53± 0.041**	0.4± 0.073**	0.50± 0.223**	0.66± 0.212**	0.66± 0.210*	0.50± 0.223**	2.666± 0.210**	1.66± 0.210**	2.00± 0.001**	2.00± 0.500**	1.5± 0.223	2.5± 0.341**	2.50± 0.341**
STD-P	0.3± 0.068**	0.3± 0.167**	0.26± 0.043**	0.23± 0.033**	0.16± 0.170**	0.16± 0.166**	0.50± 0.223**	0.16± 0.170**	1.333± 0.210**	2.66± 0.210**	1.5± 0.223*	1.6± 0.213**	2.66± 0.210**	2.83± 0.167**	2.83± 0.166**
STD-T	0.63± 0.061	0.63± 0.061**	0.56± 0.061**	0.4± 0.051**	0.83± 0.307	1.00± 0.258*	0.66± 0.213*	0.83± 0.307	2.666± 0.210**	2.66± 0.228*	2.33± 0.210**	2.33± 0.223**	1.33± 0.220	2.00± 0.258	2.16± 0.167**

Table 5: Effect of ACIV on Pain perception parameters in CII induced arthritis

Exp. group	PAIN PERCEPTION PARAMETER															
	Arthritic index				Dorsal flexion score				Motility score				Stair climb score			
	9	18	27	36	9 th	18 th	27 th	36 th	9 th	18 th	27 th	36 th	9 th	18 th	27 th	36 th
DC	3.5± 0.223	3.51± 0.225	3.33± 0.212	2.83± 0.307	1.56± 0.061	1.46± 0.066	1.63± 0.061	1.63± 0.095	1.5± 0.223	2.00± 0.002	2.00± 0.003	1.5± 0.223	1.5± 0.428	0.66± 0.211	0.68± 0.333	0.83± 0.307
STD	2.66± 0.333	2.5± 0.233*	2.16± 0.166**	1.33± 0.211**	1.36± 0.158	1.16± 0.095*	0.96± 0.061**	0.76± 0.12**	1.33± 0.210	0.66± 0.210*	0.69± 0.215**	0.5± 0.22**	1.33± 0.494	1.5± 0.223*	2.16± 0.307**	2.16± 0.167**
TEST	2.5± 0.428	2.33± 0.210*	1.83± 0.307**	1.16± 0.167**	1.23± 0.140	1.1± 0.085*	0.8± 0.115**	0.46± 0.084**	1.00± 0.001	0.68± 0.22*	0.67± 0.220**	0.33± 0.23**	1.66± 0.3073	1.66± 0.23	2.5± 0.225**	2.68± 0.212**

Values are expressed in mean ± S.E.M. (n=6); *p<0.05, **p<0.01 vs. Disease Control group, Data analysed by One-way ANOVA test followed by Dunnett’s multiple test for comparison. Treatment of various group are as follows, DC: Disease Control; STD: Indomethacin; Test: ACIV.

Table 6: Effect of ACIV on Serum and hematological parameter in FCA induced Arthritis

EXP. GROUP	SERUM PARAMETERS			HEMATOLOGICAL PARAMETERS			
	SGOT	SGPT	ALP	WBC	RBC	HB	HCT
DC	399.37± 14.32	185.06± 7.275	1023.28± 19.10	24.73± 0.68	4.60± 0.15	8.63± 0.061	26.5± 1.08

BV-11	204.99± 46.044**	122.71± 12.43**	592.81± 26.05**	5.83± 0.30*	5.50± 0.11**	10.45± 0.13**	33.36± 0.88*
BV-12	263.23± 20.73**	79.76± 8.49**	567.83± 39.52**	7.48± 0.75**	6.15± 0.21**	11.1± 0.42**	33.11± 1.25*
BV-21	298.56± 12.18*	96.21± 8.49*	611.75± 45.21**	19.71± 2.01**	7.08± 0.22*	12.43± 0.316**	35.85± 0.60**
BV-22	268.91± 17.70**	99.59± 1.90**	741.96± 32.36*	13.62± 2.01**	6.53± 0.44**	12.01± 0.67**	36.98± 1.87**
STD-P	273.36± 27.22**	116.25± 8.83**	926.5± 18.59	13.62± 0.91**	6.53± 0.17**	12.36± 0.23**	35± 0.49**
STD-T	264.2± 21.44*	78.72± 8.67**	565.76± 39.71**	16.25± 1.32**	5.86± 0.14**	10.56± 0.44**	35.18± 2.65*

Values are expressed in mean ± S.E.M. (n=6); *p<0.05, **p<0.01 vs. Control group, Data analysed by One-way ANOVA test followed by Dunnett's multiple test for comparison. Treatment of various group are as follows, DC: Disease Control; STD: Indomethacin; BV: ACIV. Prophylactic (BV-11: 2mg/kg, BV-12: 4mg/kg), Therapeutic (BV-21: 2mg/kg, BV-22: 4mg/kg).

Table 7: Effect of ACIV on Serum and Hematological parameters in C-II induced arthritis

EXP. GROUP	SERUM PARAMETERS			HEMATOLOGICAL PARAMETER			
	SGOT	SGPT	ALP	WBC	RBC	HB	HCT
DC	409.61±16.322	133.93±2.68	882.2±12.88	26.336±1.22	6.111±0.20	10.105±0.21	31.913±0.35
STD	169.95±8.88	67.313±2.68	650.5±51.88	19.325±1.53*	7.301±0.20**	13.033±0.48**	39.03±1.08**
TEST	260.46±16.95	95.15±5.11	632.08±61.86	14.483±1.94**	6.953±0.16*	13.9±0.28*	37.883±0.65**

Values are expressed in mean ± SEM (n=6); *p<0.05, **p<0.01 vs. Disease Control group, Data analysed by One-way ANOVA test followed by Dunnett's multiple test for comparison. Treatment of various group are as follows, DC: Disease Control; STD: Indomethacin; Test: ACIV.

Radiographic evaluation

Radiographic evaluation in FCA induced arthritis

On 14th and 28th day, radiological assessment of rat paw through X-rays showed significant reduction in various imaging parameters like spur formation, bone erosion, soft connective tissue swelling around joint, calcium deposition and increase in interspacing between the bones in case of ankle joint, in Standard Indomethacin group, Prophylactic test (Test-P) and Therapeutic test (Test-T) groups respectively in comparison with disease control (DC) group (Figure 5).

Radiographic evaluation in CII induced arthritis

On 18th and 36th day, radiological assessment of rat paw through X-rays showed significant reduction in various imaging parameters like spur formation, bone erosion, soft connective tissue swelling around joint, calcium deposition and increase in interspacing between the bones in case of ankle joint, in Indomethacin treated group, and therapeutic test (Test: ACIV-T) groups respectively in comparison with disease control (DC) group (Figure 6).

Histopathological evaluation

Histological evaluation in FCA induced arthritis

Histopathological studies of left hind paw joints in normal control rats shown intact articular cartilage and normal syn-

ovial lining. Distorted articular cartilage, bone erosion, pannus formation and inflamed cells infiltration like lymphocytes and eosinophils were abundant at synovial lining with disease control rats. It was found that Indomethacin and prophylactic ACIV treatment showed better retention of joint parameters like very less disruption of synovial cartilage, reduction in synovial hyperplasia, bone erosion, tissue infiltration with lymphocytes, eosinophils and pannus formation. While in case of therapeutic treatment group above parameters were found to have slightly more effect in comparison with prophylactic treatment group but less effect than disease control group (Figure 7).

Histological evaluation in CII induced arthritis

Histopathological studies of left hind paw joints in normal control rats shown intact articular cartilage and normal synovial lining. Distorted articular cartilage, bone erosion, pannus formation and inflamed cells infiltration like lymphocytes and eosinophils were abundant at synovial lining with disease control rats with diffused joints. It was found that standard (Indomethacin 10 mg/kg) and therapeutic ACIV treatment rat showed better retention of joint parameters like very less disruption of synovial cartilage, reduction in synovial hyperplasia, bone erosion, tissue infiltration with lymphocytes, eosinophils and pannus formation in comparison with disease control which has been also reported as more diffused in nature as found in histopathology (Figure 8).



Figure 5: Radiological assessment of arthritic joints in FCA induced arthritis

NC: Normal Control; DC: Disease Control; STD: Standard (Indomethacin), and TEST-P: Prophylactic BV; TEST-T: Therapeutic BV

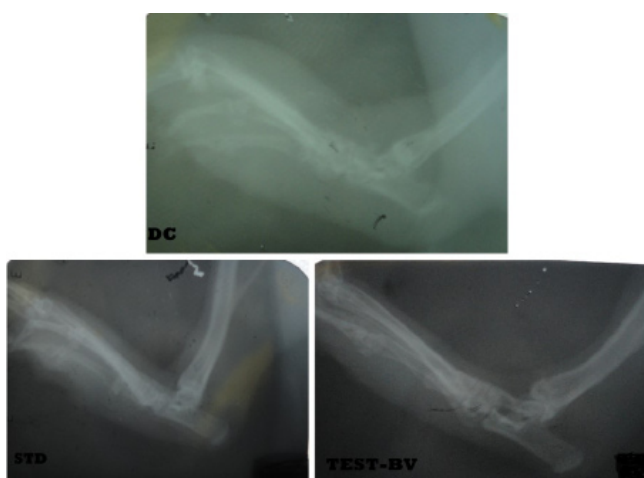


Figure 6: Radiological assessment of arthritic joints in Bovine collagen type-II induced arthritis

DC: Disease control; STD (Indomethacin) standard; TEST-BV: Bee venom

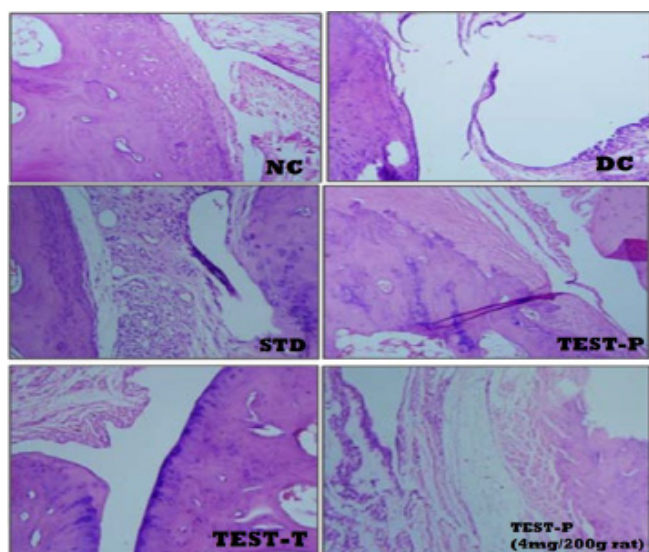


Figure 7: Histological assessment of arthritic joints in FCA induced arthritis

NC-Normal Control; DC-Disease Control; STD: Standard (Indomethacin); and TEST-P: Prophylactic BV; TEST-T: Therapeutic BV

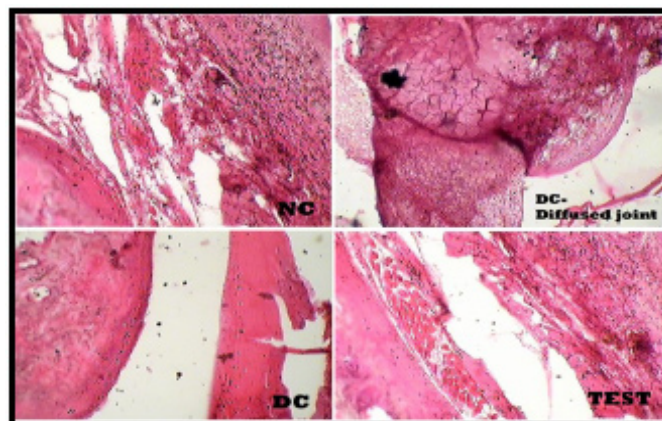


Figure 8: Histological assessment of arthritic joints in CII induced arthritis.

NC: Normal control; DC: Disease Control; STD (Indomethacin) standard, TEST-BV: Bee venom (4 gm/200g rat)

Discussion

In spite of the discovery of several newer agents, the search for better anti-inflammatory and anti-nociceptive drugs continues because of their existing side effects and none of them is suitable for prolonged use. Therefore, there is need of development of new and more powerful drugs with fewer side effects.

From various animal sources, different toxins were discovered and utilized as an effective drug therapy for arthritic pain and inflammation in RA. These venoms are released for the defense mechanism of animals for their protection and paralyze their prey [24].

The results of the present study indicate that ACIV showed significant ($p < 0.01$) suppressive activity in both phases of inflammation in carrageenan-induced paw edema and in cotton-pellet induced granuloma by inhibition of total granulomatous, transudative weight. This may reflect that ACIV might be involved in the inhibiting fibroblasts proliferation, synthesis of collagen fibers and mucopolysaccharides during granuloma tissue formation. Based on these results, it might be suggested that the suppression of inflammation is due to the presence of melittin in ACIV. In Eddy's hot plate-induced hyperalgesia and tail flick method, the nociceptive behavior of mice was inhibited by ACIV and showed antinociceptive activity. Probable anti-nociceptive effect of ACIV is mediated by the selective activation of spinal α_2 adrenergic and serotonergic receptors.

Major part of drug therapy of rheumatoid arthritis include the drugs like Methotrexate and Cyclophosphamide which are having immunosuppressant actions as it is an autoimmune disease, these drugs will be useful for the prevention of autoimmunity along with inhibition of the hazards associated with it. As immunosuppression is important parameter in prevention of RA, hence ACIV is assessed for immunosuppressant activity by serum hemagglutinin titre assay. ACIV treatment is analyzed for its immunosuppressant activity. It was observed that on 7th day ACIV treatment effectively inhibited the agglutination reaction between test serum antibodies and antigens (mesh formation) present in SRBC's in comparison with agglutination of control group wells in micro-titre plates. The probable mechanism might be due to inhibition of antigen uptake by mature dendritic cells of bone marrow cells of immune system as it has been seen in case of Apis melifera bee venom [25]. The evaluation of ACIV for anti-arthritic potential was seen with FCA model which has been used to induce an arthritic immunopathological con-

dition that displayed many pathological features of human RA [26]. Therefore it is used extensively to analyse the anti-arthritic effects of new drugs with potential therapeutic application to chronic arthritis [27]. (Chang Y.L et al, 1979). Immunization with CII is well known to be able to induce inflammatory polyarthritis in rats and susceptible strains of mice [28-29]. It is implicated that anti-CII antibodies are solely responsible for connective tissue damage in synovial cartilage. Since CII induced arthritis in rats and mice are well known to have both clinical and histological similarities to human RA, these models have been widely used to evaluate anti-arthritic drugs [30] (Paska et al, 1986). It was observed that administration of whole ACIV for 28 and 36 days in FCA and CII significantly inhibited the arthritic index score, paw oedema volume, joint area and significant inhibition of pain perception parameters like dorsal flexion and motility respectively along with significant rise in stair climb score in comparison with disease control. After 14th day in FCA and 18th day in CII prominent inhibition was observed in all parameters. Here, in agreement with other studies it was observed that, whole ACIV administration effectively inhibited the development of oedema, a characteristic sign of inflammation in arthritic hind paw of rat.

Lipid peroxidation results in damage and loss of functional integrity of cell membrane causing leakage of SGOT, SGPT, and ALP which are effective tool in assessing anti-inflammatory activity. At the end of treatment, significant reduction of increased levels of various serum transaminases like SGPT, SGOT and ALP was also observed in standard, prophylactic and therapeutic groups in comparison with disease control group in both FCA and CII models.

Radiographic changes in RA conditions are useful diagnostic measures which indicate the severity of disease. Soft tissue swelling is the earlier radiographic sign, whereas prominent radiographic changes like bone erosions and narrowing of joints spaces can be observed only in the developed stages of arthritis [31]. (Harris A. Et al, 1990). Radiological assessment of prophylactic, therapeutic and standard (Indomethacin) groups through X-rays showed significant reduction in various imaging parameters like spur formation, calcium deposition, interspacing between the joints, bone erosion and connective tissue swelling around joint in comparison with disease control in both FCA and CII models of arthritis.

In present study the histological assessment of joints showed less cellular infiltration and destruction of synovial cartilage in case of prophylactic, therapeutic (ACIV) and standard treatment groups in comparison with disease control group in both FCA and CII models. Joint space had also been increased in case of ACIV treated groups more than disease control group.

It was observed that administration of ACIV significantly restored the increased WBC count to normal in standard, prophylactic and therapeutic (ACIV) groups of FCA and in therapeutic (ACIV) group of CII models in comparison to disease control group respectively. This may be probably due to inhibition of matured bone marrow dendritic cell growth by ACIV which might be responsible for development of innate immunity.

Anaemia arises due to abnormal storage of iron in the reticuloendothelial system and synovial tissue due to which RBC count, Hb count and Hematocrit value decreases below normal levels [32]. It was observed that RBC, Hb count and hematocrit value significantly increased in prophylactic, therapeutic (ACIV) groups in FCA and therapeutic (ACIV) group in CII models re-

spectively, which might be due to ACIV activity by which matured bone marrow cells responds to anaemic condition by preventing the abnormal deposition of iron in reticuloendothelial system and synovial tissue.

It was already demonstrated that ACIV was able to suppress the arthritic inflammation in joints that was induced by adjuvant arthritis [33]. The anti-inflammatory effects of ACIV might be implicated to the inhibition of cyclooxygenase (COX₂) expression and subsequent production of proinflammatory cytokines (TNF- α , IL-b) and suppression of leukocyte migration that is induced by adrenal catecholamine release through beta-adrenoceptors [34-35]. In addition, it was reported that *Apis mellifera* bee venom regulates the free radical production by blocking the neutrophil superoxide production and/or induces the corticosteroid concentration that is correlated with their anti-arthritic effects [36-37]. Hence, from above reported data it can be concluded that ACIV possess same polypeptides with similar pharmacological properties as *Apis mellifera* bee venom so it has showed anti-arthritic activity. From above results it can be stated that as ACIV reduced inflammation and produced significant analgesia along with immunosuppression so that it might give symptomatic relief in arthritic condition.

As ACIV seen to be effective in FCA and CII induced arthritis as it reduced the joint inflammation (thickness), paw oedema, arthritic index along with reduction of pain perception parameters such as dorsal flexion and motility and rise in stair climb score. Hence it can be stated that ACIV was found to be effective in prophylactic and therapeutic treatment of RA.

Conclusion

From the present study, it can be concluded that ACIV showed potential antinociceptive, anti-inflammatory, immunosuppressant and antiarthritic activity due to presence of mellittin which possesses anti-inflammatory property, causes cytokine inhibition, PLA₂- T-cell proliferation prevention. Adolapin which is another peptide causes COX 2 inhibition and inhibition of matured dendritic cells in bone marrow which prevents antigen uptake responsible for autoimmunity.

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