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In vivo evaluation of anti-inflammatory activities of ethanolic extract of *Buchanania lanzan* Spreng bark

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Abstract
The present study was carried out to evaluate the *in-vivo* anti-inflammatory potential of *Buchanania lanzan* Spreng used in Indian traditional medication. The sequentially extracted plant sample in ethanol was evaluated for *in-vivo* anti-inflammatory and antioxidant activities. The *in-vivo* anti-inflammatory activity of selected samples showing promising inflammatory markers was assessed using the carrageenan (CIP) and Cotton wool granuloma (CWG) method in mice animal models. The results obtained reveal that the ethanolic extract was found carrageenan induced animals treated with EEBL showed a significant reduction in the paw volume at 2nd and 3rd hour after carrageenan induction when compared to the control group. The ethanolic extract of the plant demonstrated an effective total number of leukocytes in the blood of all the cotton wool inserted animals that were recorded on the 0^{th} and 8^{th} day. The data showed that on the 8^{th} day, there should be a highly significant reduction in leukocytes migration on blood analysis. It shows a significant impact on inhibition of edema formation.

Keywords: EEBL Ethanolic extract of *Buchanania lanzan* Spreng, CIP carrageenan induced Paw oedema CWG cotton wool granuloma

1. Introduction
India is a country with a vast reserve of natural resources and a rich history of traditional medicine. Medicinal plants contain numerous biologically active compounds that help improve the life and treatment of disease. Compounds such as carbohydrates, proteins, enzymes, fats, oils, terpenoids, flavonoids, sterols pure phenolic compounds, etc. Natural products are the source of synthetic and traditional herbal medicine and are still the primary health care system. The presence of many life-sustaining constituents in plants made scientists investigate these plants for their use in treating certain infective diseases and the management of chronic wounds. The traditional medicine literature describes the potential role as a source of many vitamins and a domestic remedy for many disorders like diabetes, cancer, arthritis, and many others. There is a proportional increase in demand for herbal products, both locally and internationally. The demand for herbal products is caused by population increase, poverty, increasing awareness of herbal products, high cost of modern medicine. Recent research has focused on natural plant products alternatively for disease control and cure. Medicinal plants are cheaper, more accessible to most of the population in the world. Thus, there is a need to encourage the use of medicinal plants as potential sources of new drugs. There has been as highly increased interest in herbal remedies in several parts of the world. With the chemically synthesised drugs for several diseases, natural products of plant origin have their importance and have maintained the essential resource for developing new drugs to treat various diseases. (Handral *et al*., 2012)

2. Materials and Methods
2.1. *In vivo* studies
Selection and preparation of dose of *Buchanania lanzan* Spreng. Ethanolic bark extract for pharmacological screening
The extract was suspended in water with the help of suspending agents Carboxymethylcellulose (CMC) and performed the Acute Toxicity study according to OECD guidelines 423, Acute Toxic Class Method to find out the safe and effective dose level in rodents.

2.2. Acute oral toxicity studies

Acute oral toxicity studies were carried out according to OECDNo.423 guidelines (Acute Toxic Class Method). Three female *Wistar* albino rats weighing 150 to 200g were selected for the studies were fasted overnight for food with free access for water before test extract. The ethanolic extract of *Buchanania lanzan* bark was suspended in CMC and administered with a higher dose of 2000mg/Kg (p.o). Individual rats were observed after dosing at least once during the first 30min., periodically during the first 24 hours, with particular attention given during the first 4h, and daily after that, for a total of 14 days. The rats were observed for systemic and behavioural toxicity patterns, as described in OECD Test guidelines. At the end of the study, all surviving animals were sacrificed.

2.2.1. Animals

Healthy adult Albino *Wistar* male rats between 4-8 weeks of age and weighing 150-250g and healthy adult *Sprague Dawley* rats between 4 to 8 weeks of age and weighing 145-200g were used for the study. Animals were procured from the animal house of University College of Pharmacy, Cheruvandoor CPCSCEA Reg no: 499/CPCSEA and College of Veterinary and Animal Sciences, Mannuthy P.O., Thrissur-680651. CPCSEA Reg No:328/CPCSEA. Animals were housed in polypropylene cages at a temperature of 25-30 °C and relative humidity 35-45%, in light and dark cycles of 12 and 12 hours respectively, for one week before and during the experiments. Animals were provided with a standard rodent pellet diet (Dayal Industries, Banglore) and water. All studies were performed in accordance with the guides for care and use of laboratory animals and approved by the Institutional Animal Ethical Committee of UCP, Cheruvandoor, Kottayam, India (003/MPH/UCP/CVR/13).

2.3. Acute model

2.3.1. Carrageenan induced paw oedema method

Carrageenin, from the Irish word “carraigin” meaning Irish moss discovered by the British pharmacist Stanford in 1862. The name was later changed to carrageenan to comply with the “an” suffix for polysaccharides. Structurally, the carrageenans are a heterogeneous group of polysaccharides made up of repeating related galactose monomers and are of three main types; lambda, kappa, and iota. The lambda form does not require gel strongly at room temperature and is injectable to induce an inflammatory response. Inflammation-induced by carrageenan, originally described by Winter, is acute, nonimmune, well-researched, and highly reproducible. Cardinal signs of inflammation, edema, hyperalgesia, and erythema, develop immediately following subcutaneous injection, resulting from the action of proinflammatory agents like bradykinin, histamine, tachykinins, complement and reactive oxygen, and nitrogen species. Such agents can be generated *in situ* at the site of insult or by infiltrating cells. Neutrophils readily migrate to sites of inflammation and can generate proinflammatory reactive oxygen and other species producing inflammation. The inflammatory response is usually quantified by an increase in paw size (edema). (Winyard *et al.*) Experimental details:

Experimental design

Group I: Control: received 5% carboxymethyl cellulose; p.o (30 min before carrageenan injection)

Group II: Standard: received Diclofenac sodium; p.o (received 30 min before carrageenan injection)

Group III: Test: received ethanolic extract of *Buchanania lanzan* bark; p.o (200mg/Kg) (received 30 min before carrageenan injection)

Procedure

Male Albino *Wistar* rats with bodyweight between 150-200g were used. The animals were starved overnight, water given adlibitum. Group I receives a vehicle (CMC), Group II receives Diclofenac Na 10mg/Kg, and group III receives 200mg/Kg of ethanolic extract of *Buchanania lanzan* Spreng. Bark. Thirty minutes later, the rats were challenged by subcutaneous injection of 0.1ml of 1% solution of carrageenan in saline into the plantar region of the left hind paw. The paw was marked with ink at the level of the lateral malleolus. The readings were measured by immersing the paw in mercury up to the mark. The paw volume was measured plethismographically immediately after the injection and again 1,2 and 3hour after challenge **(**Winter *et al*., 1962; Rahman *et al*., 2012**)** [9, 5].

Evaluation

The difference in the paw volume was calculated as a percentage and compared with the paw volume measured immediately after injection of the irritant for each animal. The percentage inhibition was calculated as,

$$\text{Percentage inhibition of paw edema} = \frac{(V_t\text{-}V_0)_{\text{control}} - (V_t\text{-}V_0)_{\text{test}}}{(V_t\text{-}V_0)_{\text{control}}} \; 100$$

The difference in the average values between treated animals and control groups was calculated for each time interval and statistically evaluated.

Statistical evaluation is done by One Way ANOVA followed by Dunnet’s multiple comparison test using GraphPad Prism 6 version computer software.

2.3.2 Cotton wool granuloma method

The cotton wool granuloma method is an animal screening model of the subacute phase of inflammation. The subcutaneous implantation of a sterile cotton pellet in a rat evokes a short-lasting phase of increased capillary permeability lasting for some 20 min after implantation followed by a more sustained phase, which occurs after 2.5-3h. After implantation of sterile cotton pellets, there will be changes in the differential cell count, DNA levels, cellular proliferation, and hydrolytic enzyme release into the inflammatory exudates. At two days, the predominant cell type was the polymorphonuclear leucocytes (PMNL), but on day5, there will be an increasing number of mononuclear cells (macrophage and lymphocytes). (Tsurumi *et al*., 1989) [7].

Experimental design

Group I: Control: received 5% carboxy methyl cellulose; p.o

Group II: Standard: received Prednisolone 10 mg/Kg p.o

Group III: Test: received ethanolic extract of *Buchanania lanzan* bark; p.o (200mg/Kg)

Procedure

The rats were anesthetised with diethyl ether, and a sterilized cotton pellet weighing approximately 20 mg was inserted in the subcutaneous layer of the groin. The incised skin was sutured correctly, and an antiseptic was applied to prevent the infection. The control group receives a vehicle (CMC); the Standard group receives prednisolone (10 mg/Kg) p.o; Test

group animals were treated with ethanolic extract of *Buchanania lanzan* bark (200mg/Kg p.o) once a day for seven consecutive days. On 8th day animals were sacrificed by cervical dislocation, and the pellets along with granuloma mass were removed, washed, and taken the wet weight. Then the granuloma mass was dried at 60 °C for 18 h in an oven and taken the dry weight (Kaneria *et al*., 2006; Phanse *et al*., 2012). On the 0th day and 8th day (before sacrificing), blood was withdrawn from each animal by a retro-orbital puncture for evaluating the leucocyte count (Mishra *et al*., 2010) [3].

Evaluation

Weight of the granuloma mass was calculated as a percentage and compared the weight of the treated group with the control group. Differences in the leucocytes count in three groups were compared and statistically evaluated.

Statistical evaluation was done by One Way ANOVA followed by Dunnet's multiple comparison test using GraphPad Prism 6 version computer software.

1. Paw volume

The inflammatory reaction was measured using mercury plethysmometer on 1, 14, 21, 28, 35, and 40 days from the day of CIA injection.

2. Body Weight

The body weight was monitored using weighing balance from 1to 40 days from the day of

2.4. Histopathological examination

Paws from rats were fixed in Bouin's fluid; subsequently, the specimens were decalcified with 10% EDTA for seven days, dehydrated and embedded in paraffin blocks. Sections of ankle joints (5µm thick) were cut mounted on slides and stained using haematoxylin and eosin. Grading of cellular infiltration, synovial hyperplasia, pannus formation, joint space narrowing, and cartilage and bone erosion of the ankle joints was blindly investigated by a pathologist using a semi quantitative scale from 0 (normal), 1 (mild changes), 2 (moderate changes), 3 (severe changes). (Salvemini, *et al*., 2001; Lin *et al*., 2007) [6, 2].

2.5. Statistical analysis

Data were statistically evaluated by one-way ANOVA, followed by Dunnet's multiple comparisons using Graph Pad Prism 6.

3 Results of *in vivo* Studies

3.1: Effect of ethanolic extract of Buchanania lanzan bark on carrageenan-induced paw oedema method

Paw volumes of animals in all groups were recorded at 0th, 1st, 2nd, and 3rd hour. Data obtained were tabulated in Table 1.

Table 1: Effect of ethanolic extract of *Buchanania lanzan bark* on paw volume of Carrageenan induced animals.

Groups	Animal(n=6)	0th hour	1st hour	2nd hour	3rd hour
GroupI Control (CMC)	1	0.7	0.9	1.3	1.0
	2	0.8	1.1	1.6	1.2
	3	0.7	1.0	1.4	1.1
	4	0.6	0.9	1.2	1.0
	5	0.8	1.2	1.4	1.3
	6	0.7	1.3	1.8	1.4
Mean±SEM		0.72±0.03	1.07±0.07	1.45±0.09	1.17±0.07
GroupII Diclofenac Treated	1	0.7	0.9	0.8	0.8
	2	0.8	1.0	0.8	0.9
	3	0.8	1.0	09	0.9
	4	0.7	1.1	1.0	1.0
	5	0.6	0.9	0.8	0.7
	6	0.7	1.0	0.9	0.8
Mean±SEM		0.72±0.03	0.98±0.03	0.87±0.03 ***	0.85±0.03 **
Group III EEBL treated	1	0.8	1.1	1.0	0.9
	2	0.7	1.0	0.9	0.8
	3	0.6	1.0	1.0	0.7
	4	0.7	0.9	0.8	0.9
	5	0.8	1.1	1.0	0.8
	6	0.7	1.0	0.9	0.9
Mean±SEM		0.72±0.03	1.01±0.03	0.93±0.03 **	0.82±0.02 ***

Values are mean ± SEM, n=6. ANOVA followed by multiple comparisons of Dunnet's test. **p*<0.05, ***p*<0.01, ****p*<0.001, as compared to positive control

From the above tabulated data, it is clear that, carrageenan induced animals treated with EEBL showed a significant reduction in the paw volume at 2nd and 3rd hour after carrageenan induction when compared to the control group.

Table 2: Statistical summary of Carrageenan induced paw edema method

ANOVA	0hr	1hr	2hr	3hr
P value	0.391	0.453	< 0.0001	0.0002
F value	1.000	0.833	30.41	15.50
DF value	17 (2,15)	17(2,15)	17 (2,15)	17(2,15)

Table 2 represented the percentage inhibition of paw oedema on the Diclofenac and EEBL treated animals.

Table 3: Effect of ethanolic extract of Buchanania lanzan on %inhibition of paw oedema in carrageenan-induced animals.

Percentage Inhibition of Paw Edema			
Hour	1	2	3
Diclofenac	25.71	79.45	71.11
Eebl	17.14	71.23	77.0

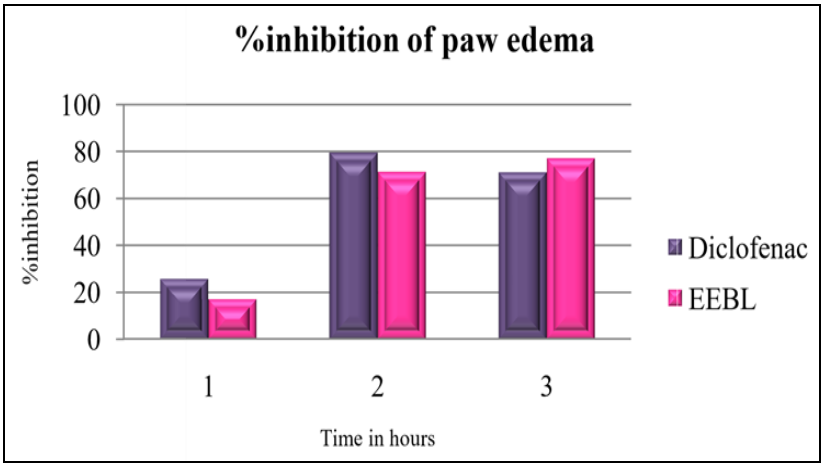

Fig 1: Effect of ethanolic extract of *Buchanania lanzan* on %inhibition of paw oedema in carrageenan-induced animals

EEBL treated animals showed a highly significant reduction in the percentage inhibition of paw oedema during the 2nd and 3rd hours.

3.4: Effect of ethanolic extract of Buchanania lanzan bark on cotton wool granuloma method

3.2.1.a: Effect on Granuloma weight.

The study demonstrated that there was a granuloma formed around the inserted cotton pellet of all the animals. Table 4 depicted the weight of granuloma of each group of animals. The tabulated data indicated that the weights of the granuloma were significantly reduced in the EEBL and Prednisolone treated animals.

Table 5: Effect of ethanolic extract of Buchanania lanzan on the weight of granuloma in cotton wool inserted animals

	Wet weight (g)	Dry weight (g)
Control	0.354 ± 0.01	0.264 ± 0.02
Diclofenac treated	0.281 ± 0.05 ***	0.208 ± 0.02 *
EEBL treated	0.237 ± 0.05 ***	0.198 ± 0.06 **

ANOVA	wet weight	Dry weight
F value	42.13	6.648
P value	<0.0001	0.009
DF value	17 (2,15)	17 (2,15)

Values are mean ± SEM, n=6. ANOVA followed by multiple comparisons of Dunnet's test. *p<0.05, **p<0.01, ***p<0.001, as compared to positive control.

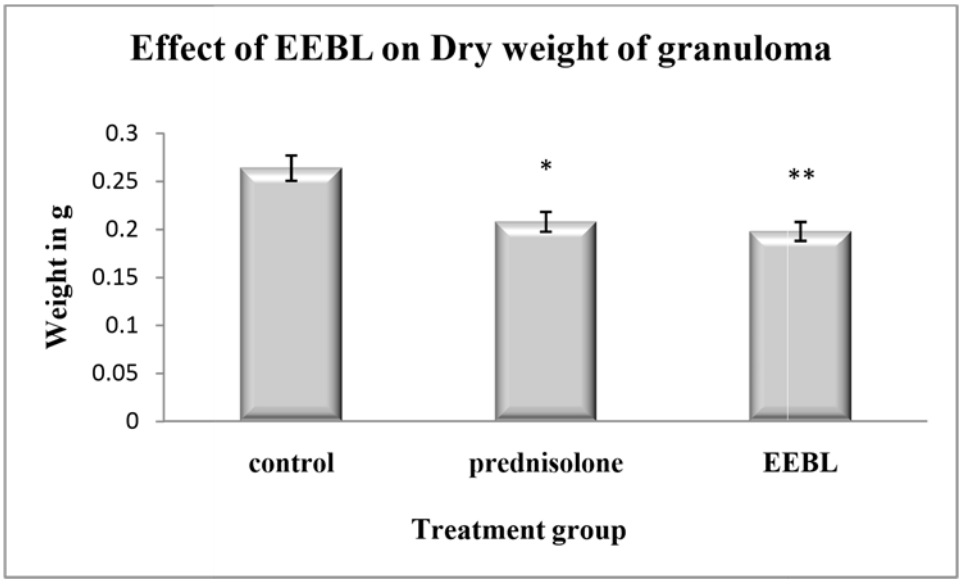

Fig 2: Effect of EEBL on Dry weight of granuloma

3.2.1.b: Effect on Leucocyte migration:

The total number of leukocytes in the blood of all the cotton wool inserted animals was recorded on the 0th and 8th day and tabulated in Table 6. The data showed that on 8th day there should be a highly significant reduction in leukocytes on blood analysis. The statistical summary was tabulated in Table 7

Table 6: Effect of EEBL on Leucocyte migration

Groups	DAY 0	DAY 8
Control	13842 ± 822	26158 ± 1248
Diclofenac	13880 ± 771	9214 ± 900 ***
EEBL	13978 ± 728	8947 ± 3275 ***

Values are mean±SEM n=6 ANOVA followed by multiple comparison Dunnet's test

* *P*<0.05, ***P*<0.01, *P*<0.001 as compared to a positive control

Table 7: Effect of EEBL on leucocyte migration

ANOVA	day 0	Day 8
P value	0.99	<0.0001
F value	0.008	117.3
DF value	17 (2,15)	17 (2,15)

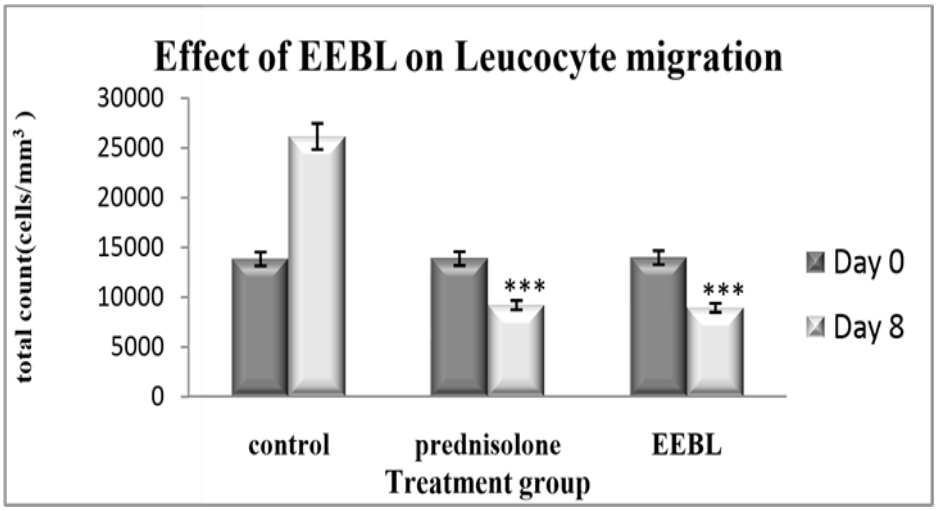

Fig 3: Effect of EEBL on leucocyte migration

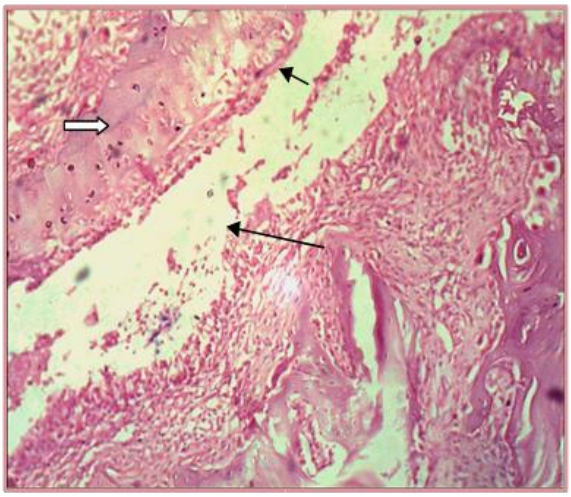

Fig 4: Synovial joint of CIA induced rat. (H&E, 5µm, 10X)

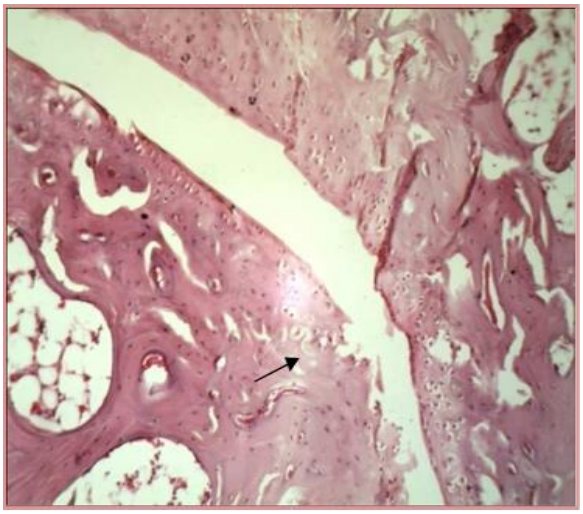

Fig 5: Synovial joint of CIA+ prednisolone treated rat. (H&E, 5µm, 10X)

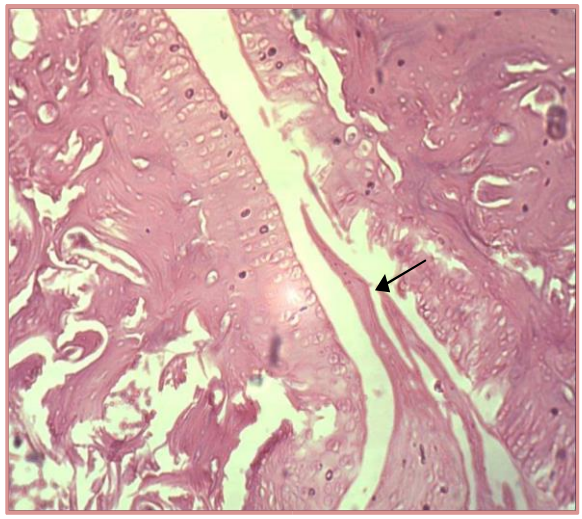

Fig 6: Synovial joint of CIA+EEBL treated rat. (H&E, 5µm, 10X

Figure 4 showed a severe chronic inflammatory change like cartilage erosion (black arrow), cellular infiltration (white arrow), pannus formation in the joint (short arrow). Figure5 illustrated that prednisolone reduced the cellular infiltration and showed a moderate synovial membrane degradation (black arrow). Figure 6 showed an EEBL treated synovial joint with only a mild distortion of joint architecture.

4. Discussion

Oedema and inflammation induced by carrageenan are shown to be mediated by histamine and serotonin during the first hour. After which, increased vascular permeability is maintained by the release of kinins up to 2.30 hour and finally through the release of bradykinin and prostaglandins from 2 to 6 hours (Winter *et al*., 1962) [9]. The later phase is reported to be sensitive to most of the clinically useful antiinflammatory agents. The mediators appear to be prostaglandins. The Carrageenan induced paw oedema model

in rats is known to be sensitive to cyclooxygenase (COX) inhibitors (COX1 and COX2) and has been used to evaluate the effect of non-steroidal antiinflammatory agents (Rahman, H *et al*., 2012) [5].

In the present study, 200mg/Kg EEBL exhibited a significant reduction in paw volume during the 2nd and 3rd hours when compared to the control group animals. EEBL showed no significant difference in the percentage reduction of paw edema when compared with Diclofenac. These observations indicated the possibilities of EEBL being clinically useful in acute inflammation. The mechanism behind this use could probably due to the inhibition of prostaglandins via the inhibition of cyclooxygenase and lipoxygenase pathway.

Inflammatory granuloma is a typical feature of a subacute inflammatory reaction. In order to assess the efficacy of EEBL against proliferative phase of inflammation, selected cotton pellet granuloma animal model in which tissue regeneration and fibrosis occur. During the repair process of inflammation, there is a proliferation of macrophages, neutrophils, fibroblasts, and multiplication of small blood vessels (angiogenesis), which are the basic sources of forming a highly vascularised reddish mass, termed granulation tissue (Kaneria *et al*., 2006). The fluid adsorbed by the pellet greatly influences the wet weight of granuloma, whereas, the dry weight correlated well with the amount of granulomatous tissue. During the inflammatory process, there should be a huge amount of leucocyte infiltration. Most of the NSAIDs, like Diclofenac, possess only slight inhibition on the granuloma formation. The steroidal drugs, on the contrary, exhibit a profound reduction of granuloma and leucocyte migration (Ashok P.*et al*., 2010).

In the present study, EEBL elicited a significant inhibitory activity on the wet and dry weight of granuloma when compared with prednisolone (10mg/Kg). EEBL and prednisolone treated animals showed a significant reduction in the leukocytes migration as compared with control. The statistical evaluation showed that there was no significant difference in EEBL and pr The reduction in the granuloma weight may be due to the decreased production of P.G.s (either by the induction of lipocortin or by direct negative regulation of COX2) or negative regulation of genes for various cytokines like IL-1, IL-2, IL-6, TNF-α. Reduction in the leukocyte migration may be due to the downregulation of prostaglandins mediated ICAM-1 in the endothelial cells (Thripathi, 2008).

Collagen induced arthritis model is the gold standard approach for the screening of antiarthritic activity in rodents as it possesses many of the cellular and humoral immune responses associated with human rheumatoid arthritis. (Khan *et al*., 2012).

This study demonstrated that the curative oral treatment of Sprague Dawley rats. 200mg/Kg of EEBL suppressed the joint inflammation and ultimately reduced the destruction of bone and joint. Prednisolone. These findings also strengthen the anti inflammatory activity of EEBL.

5. Conclusion

Findings of the *in vivo* studies revealed that *Buchanania lanzan* Spreng. Bark at 200mg/Kg was produced beneficial effects on acute, sub-acute, and chronic phases of inflammation. Treatment of EEBL on carrageen induced animals showed a significant reduction in the paw volume during the 2nd and 3rd hours. Granuloma formation is a proliferative phase of inflammation. The study demonstrated that there is a significant reduction in granuloma tissue. From the *in vivo* antiarthritic study, EEBL offered a beneficial effect on behavioral parameters of EEBL treated collagen-induced arthritic animals.

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