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Amelioration of CFA induced arthritis in rats by buchnania lanzan

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***Abstract*---**Buchanania lanzan spreng commonly known as Char, Charoli is a tree 12-15 mt high, with straight trunk, belonging to family from Anacardiaceae. An herb known as priyal is a drug of the ayurveda and the unani system of medicine. It is known as chironji (or charoli). Buchanania lanzan plant has well-known traditional uses in ayurveda and unani system of medicine; almost all parts of the plant i.e. roots, rhizome, leaves, fruits, seeds and gum are used for the treatment of various disorders. The gum oozed from the cut-bark is used internally for treatment of intercostals pain and diarrhoea. The gum is mixed with goat`s milk for effective and curative results in intercostals pains, being analgesic. Some tribal communities of Andhra Pradesh consume a blend of the gum dissolved in cow`s milk for treating rheumatic pains. The ethnomedical use of plant in arthritis is evaluated using in vitro and in vivo (CFA induced) models. Among the pet ether, ethyl acetate, ethanol and aqueous extracts, ethanolic and aqueous extract found to exert better effect against arthritis in in-vitro models and hence only these 2 extracts at their high and low dose were evaluated for in vivo models of arthritis induced by CFA. The paw volume displacement, changes in body weight, arthritic index, alteration in serum biomarker, alteration in haematological parameters, secondary lesions changes as noticed from the radiograph and histopathology study were measured for both the extracts as a mark of antiarthritic activity. The results indicate that B.lanzan protects rats remarkably against all parameter induced

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by CFA in dose dependent manner. However the effect of ethanolic extract at its high dose was more significant, superior and pronounced than aqueous extract and also it is comparable to the standard drug.

***Keywords*---**CFA, buchnania lanzan, rheumatoid arthritis, radiology, histopathology.

Introduction

Buchanania lanzan spreng commonly known as Char, Charoli is a tree 12-15 mt high, with straight trunk, belonging to family from Anacardiaceae. [1] An herb known as priyal is a drug of the ayurveda and the Unani system of medicine. It is known as chironji (or charoli). [2] *Buchanania lanzan* is dicot plant of deciduous nature which produces seeds that are edible to humans. *Buchanania lanzan* plant has well-known traditional uses in ayurveda and Unani system of medicine; almost all parts of the plant i.e. roots, rhizome, leaves, fruits, seeds and gum are used for the treatment of various disorders. [3] *Buchanania lanzan*, being a vulnerable medicinal plant, is included in the Red Data Book published by International Union for Conservation of Nature and Natural Resources (IUCN). [4] The root is acrid; removes biliousness; cures blood diseases. The fruit is sour, sweet, fattening, laxative, binding cooling, and aphrodisiac; cures biliousness, fevers, thirst, ulcers, blood diseases. The oil is sweet; indigestible. The juice of the leaves is digestive, expectorant, aphrodisiac, purgative; purifies the blood; alleviates thirst; lessens biliousness. The seed have a slightly bitter pleasant taste; expectorant, tonic to the body and the brain, stomachic; remove bad humors; useful in gleet and urinary concretions; good in fevers; cause headache.[5] The gum oozed from the cut-bark is soluble in water and used internally for treatment of intercostals pain and diarrhoea. The gum is mixed with goat`s milk for effective and curative results in intercostals pains, being analgesic. Some tribal communities of Andhra Pradesh consume a blend of the gum dissolved in cow`s milk for treating rheumatic pains. [6]

Rheumatoid arthritis (RA) is a most prevalent chronic inflammatory, autoimmune, progressive, disabling and incurable disease that leads to painful inflammation, often irreversible joint damage, and eventually to functional loss. [7] Presently conventional treatments like non-steroidal, steroidal and immunosuppressive drugs are used to control inflammatory symptoms and pain; they are associated with certain undesirable adverse effects. [8] With regards to the limitations of synthetic drugs, many herbal drugs are seen as promising therapy with less adverse effects and better efficiency.

In Indian traditional medicines, Ayurvedic literature describes portions containing parts of certain plants for treating pain and inflammatory conditions like arthritis. [9] A large number of medicinal plants have been tested and found to contain active principles with curative properties against arthritis.[10] The present work attempts to expand the herbal treatment approach for arthritis by validating the potential of traditionally claimed plants for their use in arthritis.

Methods

Collection of plant material: The fresh bark of *Buchnania lanzan* was obtained from Toranmal region of Nandurbar district, Maharashtra. Authentication, Drying and Processing of selected plants materials: The fresh bark of *Buchnania lanzan was* authenticated at K.L.E Society's Raja Lakhamagouda Science Institute, Belgaum, India. The bark was cleaned and dried at room temperature. The size reduction of plant materials was done to get a coarse powder. The powdered drug was oven dried at 110°C for an hour and packed in air tight bottles until further use.

Extraction

The extraction of bark of *Buchnania lanzan* was carried out using continuous successive solvent extraction scheme. The powder of the air dried drugs was loaded in thimble of soxhlet apparatus and was extracted with petroleum ether, ethyl acetate, ethanol and water using successive method. The extract was filtered and concentrated extract was air-dried. [11]

Pharmacological Screening

In vitro anti arthritic study: [12]

A) Inhibition of Bovine serum albumin (BSA) denaturation:

Test solution: 0.5 ml of test solution consists of 0.45 mL of BSA (5% w/v) and 0.05 mL of extracts in various concentrations (100, 250, 500, 750 and 1000 µg/mL).
Test control solution: 0.5 mL of test control solution consists of 0.45 mL of BSA (5% w/v) and 0.05 mL of distilled water.
Standard solution: 0.5 mL of standard solution consists of 0.45 mL of BSA (5% w/v) and 0.05 mL of Diclofenac sodium solution (100 µg/mL).
The pH of the above solutions was adjusted to 6.3 using a small amount of 1N HCL. The samples were incubated at 37°C for 20 min and heated at 57°C for 3 min which were cooled and 2.5 mL of phosphate buffer (pH 6.3) was added to it. Control represents 100% proteins. After cooling, their absorbance was measured at 660 nm. The absorbance of Diclofenac sodium which was used as reference drug is measured. The percentage inhibition of protein denaturation was calculated as follows:
Percentage inhibition= OD control - OD sample /OD control.

B) Inhibition of egg albumin denaturation:

Test solution: 5 ml of test solution consists of 0.2 mL of egg albumin and 2.8 mL of phosphate buffer saline and 2 mL of in various concentrations of extracts. (100, 250, 500, 750 and 1000 µg/mL).
Test control solution: 5 ml of test control solution consists of 0.2 ml of egg albumin and 2.8 ml of phosphate buffered saline and 2 ml of distilled water.
Standard solution: 5 ml of standard solution consists of 0.2 mL of egg albumin and 2.8 mL of phosphate buffer saline and diclofenac 100 µg/mL.

The pH of the above solutions was adjusted to 6.4 using a small amount of 1N HCl. The samples were incubated at 37°C for 20 min and heated at 70°C for 5 min denaturations and the results were compared with standard diclofenac sodium.
After cooling, their absorbance was measured at 660 nm. The absorbance of Diclofenac sodium which was used as reference drug is measured. The percentage inhibition of protein denaturation was calculated as follows:
Percentage inhibition= OD control− OD sample /OD control
Acute Toxicity Study: The acute toxicity studies were carried out as per the Organization for Economic Co-operation and Development guideline (OECD) no. 423 (Acute Toxic Class Method).The limit dose of 5000 mg/kg (p.o.) was administered and they were observed for behaviour and other signs of toxicity such as twitches, respiratory changes, righting reflex and motor coordination for 4hrs and monitored up to 14 days. [13]

In vivo anti arthritic study

Experiment animals

Sprague-Dawley rats of either sex weighing from 200-300 g were used. The rats were housed under standard conditions of temperature (23 to 25°C), relative humidity (55%) with 12 hours light and 12 hours dark cycle. The study has got the approval from the Institutional Animal Ethical Committee (IAEC) of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) and the approval No. is SIPS/IAEC/2020/10.

Complete Freund's Adjuvant induced arthritis

Complete Freund's adjuvant (CFA)-induced arthritis is a scientifically justified standard experimental procedure for the induction of chronic immune-pathological RA in laboratory animals with similar cellular immunity response and pathological mechanism as in human. [14] Animals and experimental design: The animals were randomly assigned into different groups of six animals per group (n = 6): group as shown in Table 1.

Table 1: Treatment of different groups of animals in CFA induced anti-arthritic study

Sr. No	**Groups**	**Treatment**
1.	Normal Control	No CFA injection.
2.	Disease Control	Only vehicle after CFA
3	Standard/Positive control	Prednisolone 10 (day 8 to 28 day by oral route)
4	BL Ethanol High	*BL* ethanolic extract at 400mg/kg (day 8 till 28 day orally)
5	BL Ethanol Low	BL ethanolic extract at 200mg/kg (day 8 till 28 day orally)
6	BL Aqueous High	*BL* ethanolic extract at 400mg/kg (day 8 till 28 day orally)
7	BL Aqueous Low	*BL* ethanolic extract at 200mg/kg (day 8 till 28 day orally)

Induction of arthritis

All groups, except normal group, were made arthritic by injecting 0.1 mL Complete Freund's adjuvant intradermally into the subplantar region of left hind paw on day '0'. This adjuvant consists of dead mycobacterium tuberculosis bacteria suspended in heavy paraffin oil to give final concentration of 0.5 mg/mL. Saline or extracts or prednisolone were administered orally once daily from the 8th day of arthritis induction and continued till 28th day. Following parameters were evaluated. [15-16]

Hind paw volume measurement: [14]

The hind paw volume (HPV) of all animal groups was measured by plethysmometer on 0, 7, 14, 21 and 28th day after the injection of CFA emulsion. The percentage inhibition of paw edema in treated groups was then calculated.

Body weight examination: [14]

Animal's body weight of all groups was measured on day 0, 7, 14, 21 and 28 day after immunization. The percent weight change on day 28 was calculated.

Arthritic index [17]

Primary lesions and Secondary lesions are measured and arthritic index calculated as the sum of scores according to Table 2.

Table 2: Nature of lesions and its associated scores in Arthritic index calculation

Lesion Site	Nature of lesion	Score
Ear	a. Absence of nodules and redness	0
	b. Presence of nodules and redness	1
Nose	a. No swelling of connective tissue	0
	b. Intense swelling of connective tissue	1
Tail	a. Absence of nodules	0
	b. Presence of nodules	1
Forepaw	a. Absence of inflammation	0
	b. Inflammation of at least one joint	1
Hind paw	a. Absence of inflammation	0
	b. Slight inflammation	1
	c. Moderate inflammation	2
	d. Marked inflammation	3

Arthritic score was evaluated on 28th day using macroscopic scoring which is carried out by independent observers using scale from 0 (no sign of arthritis), 1 (mild swelling and redness of the paw), 2 (moderate swelling and redness of the paw) and 3 (severe swelling and redness of the paw). The arthritic index was calculated by adding the scores for each individual paw. [18]

Haematological parameters:
On day 28, haematological parameters like Red blood cell (RBC) count, Haemoglobin (Hb) are estimated. [18]
Erythrocyte Sedimentation Rate (ESR) Estimation:
ESR was measured on blood sample obtained from retro orbital plexus using Westergren's method. [19]

Serum Rheumatoid Factor (RF) [20] and C - reactive protein (CRP) Estimation [21]:
Serum RF factor estimation and C - reactive protein (CRP) was done by turbidimetry method.

Biochemical parameters:
On day 28, blood of the rats was withdrawn by retro-orbital puncture and serum was used for the estimation of serum AST, ALT and ALP. [22]

Radiological analysis:
For radiological studies, the affected paws of experimental rats were radiographed and checked for the soft swelling, bony erosions and narrowing of the spaces between joints. The animals x-rays were taken at the joints of the hind paw of the animals for evaluating the bone damage using x-ray apparatus. [23]

Histopathological analysis of ankle joints:
On day 28, ankle joints were separated from the hind paw and immersed in 10% buffered formalin for 24 h followed by decalcification in 5% formic acid, processed for paraffin embedding sectioned at 5 µm thickness. The sections were stained with haematoxylin-eosin and evaluated under light microscope with 10× magnifications for the presence of inflammatory cells, hyperplasia of synovium, pannus formation and destruction of joint space. [24]

Statistical analysis: The data were expressed as mean ± SEM (Standard error mean). The significance of the difference was evaluated by one way and two-way ANOVA followed by Bonferroni and Dunnet's test.

Result

Extraction: The colour, odour, consistency (nature) and percent yield of various extracts of *Buchnania lanzan* bark are calculated and the results are listed in Table No.3.

Table 3: Percentage yield and characterization of different extracts of *Buchnania lanzan* bark

Sr. No	**Extract**	**Colour**	**Odour**	**Nature**	**Yield (% w/w)**
1	Pet Ether	Light brown	Characteristic	Solid (powder)	6.63
2	Ethyl Acetate	Red	Characteristic	Lumps	11.26
3	Ethanol	Brown- red	Characteristic	Lumps	21.58
4	Aqueous	Light brown	Characteristic	Solid (flakes)	24.00

In Vitro Anti- Arthritic Assay

Assay: Inhibition of egg albumin

Table 4: The percentage Inhibition of egg albumin denaturation by different extracts of *Buchnania lanzan.*

Sr. No	**Concentration (µg/ml)**	**% Inhibition of egg albumin denaturation by different extracts of *Buchnania lanzan***				
		100	250	500	750	1000
1	Pet ether	38.48±0.24	57.26±0.59	59.36±1.25	74.25±1.69	85.54±1.87
2	Ethyl acetate	49.87±0.18	63.49±0.28	73.68±1.47	84.50±1.56	88.88±2.15
3	Ethanol	67.52±0.57	74.93±0.68	83.52±1.19	91.25±1.68	93.28±2.39
4	Aqueous	64.63±0.67	70.80±0.98	75.68±1.26	87.38±1.54	90.44±1.72
5	Std (Diclofenac-100)	91.39±0.56	93.44±0.27	94.62±0.91	95.27±0.26	97.62±0.82

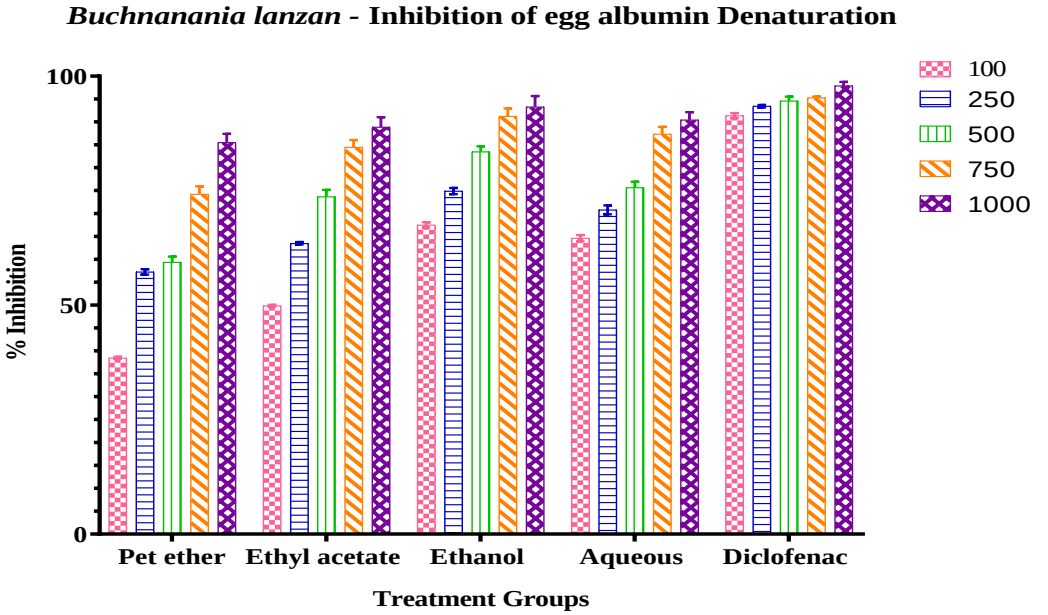

Fig 1 The percentage Inhibition of egg albumin denaturation by different extracts of *Buchnania lanzan*

Values are expressed as mean ± SEM for n=3 and analysis by two-way ANOVA followed by Bonferroni's multiple comparison test using Graph pad Software; *P < 0.0001 compared to Standard Diclofenac.

Assay: Inhibition of BSA

Table 5: The percentage Inhibition of BSA denaturation by different extracts of *Buchnania lanzan*

Sr. No	Concentration (µg/ml)	% Inhibition of BSA denaturation by different extracts of *Buchnania lanzan*				
		100	250	500	750	1000
1	Pet ether	23.69±2.36	29.95±1.56	54.74±0.83	61.64±0.96	73.11±2.77
2	Ethyl acetate	41.37±1.89	53.68±1.63	60.19±2.47	70.19±2.16	75.68±3.14
3	Ethanol	68.78±2.25	76.67±2.56	87.05±1.84	90.52±2.59	94.83±1.57
4	Aqueous	47.68±2.68	64.16±1.89	76.12±2.57	83.33±2.18	91.32±1.67
5	Std (Diclofenac-100)	92.11±1.16	93.49±1.56	94.06±2.72	94.98±1.89	96.28±2.26

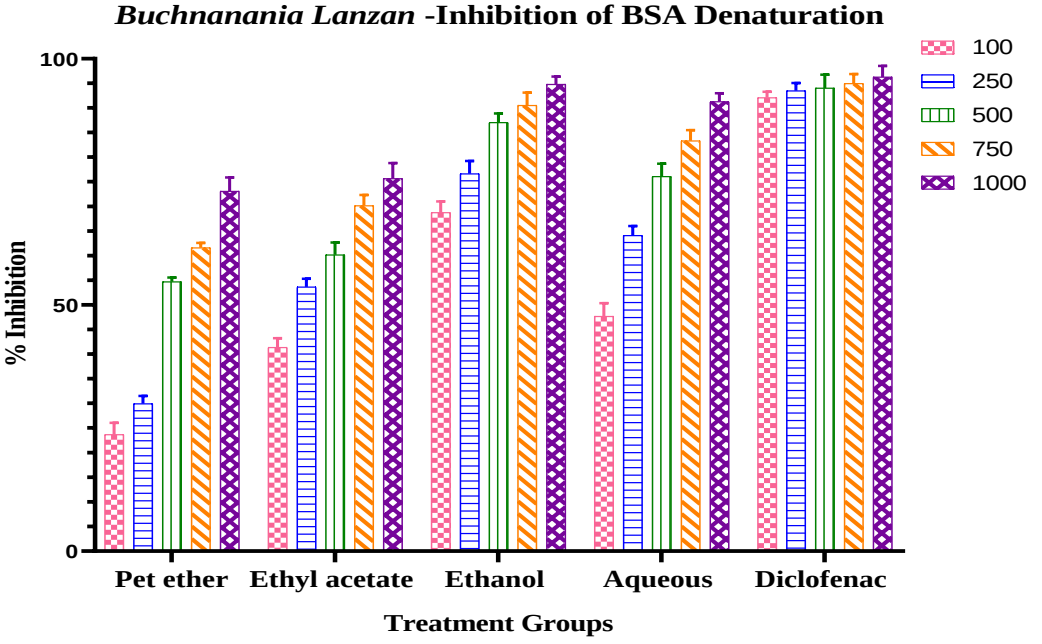

Fig 2 The percentage Inhibition of bovine serum albumin denaturation by different extracts of *Buchnania lanzan.*

Values are expressed as mean ± SEM for n=3 and analysis by two-way ANOVA followed by Bonferroni's multiple comparison test using Graph pad Software; *P < 0.0001 compared to Standard Diclofenac.

Acute Toxicity Studies

The acute toxicity of *Buchnania lanzan* was determined as per the OECD guidelines no. 425. The non-toxic nature of extracts of *BL* is evident even at the

highest dose of 5000 mg/kg. Hence 5000 mg/kg is considered as cut off LD50 and 200 mg/kg and 400 mg/kg were selected for the further study.

In vivo Anti-arthritic studies

Based on the results of in vitro anti-arthritic protein denaturation assay, only those extracts showing significant and promising outcome were evaluated further for in vivo anti-arthritic activity. Thus aqueous and ethanolic extracts at their high (400 mg/kg) and low dose (200 mg/kg) were selected for evaluation of in vivo anti-arthritic activity of *Buchnania lanzan.*

Paw volume: - Subplantar administration of CFA intradermally in the rat paw resulted in the progressive increase in the volume of the injected paw as well as non-injected paw which indicates primary and secondary arthritic lesions. Maximum Paw volume was observed on day 14, after which there was a gradual decrease except in the disease controlled group. It is found that the animals treated with ethanolic and aqueous extract of *Buchnania lanzan* (200 and 400 mg/kg) caused a significant reduction in paw edema after day 14 in comparison to the control animals. The aqueous as well as ethanolic extract at their high dose showed comparable effect with that of standard drug Prednisolone. The maximum reduction in paw volume was seen in animal group which was treated with ethanolic extract at a dose of 400 mg/kg as shown in table no.6 and fig 3.

Table 6: Effect of ethanolic and aqueous extract of *Buchnania lanzan* at high (400 mg/kg) and low dose (200 mg/kg) on paw volume of CFA-induced arthritic rats

Groups	**Day 0**	**Day 7**	**Day 14**	**Day 21**	**Day 28**	**PV % Inhibition**
	(Mean+SEM)					
Normal	0.22±0.012	0.22±0.02	0.22±0.013	0.22±0.014	0.22±0.015	72.5
Control	0.23±0.022	0.31±0.013	0.49±0.016	0.61±0.017	0.80±0.024	NA
Prednisolone 10	0.21±0.023	0.3±0.013	0.34±0.015	0.3±0.024	0.28±0.026	65
BL-Eth-High	0.24±0.028	0.37±0.032	0.42±0.012	0.39±0.013	0.37±0.023	53.75
BL-Eth-Low	0.26±0.021	0.38±0.016	0.49±0.014	0.47±0.011	0.46±0.013	42.5
BL-Aq- High	0.25±0.024	0.37±0.021	0.46±0.013	0.43±0.013	0.41±0.012	48.75
BL-Aq- Low	0.31±0.011	0.42±0.012	0.53±0.015	0.49±0.015	0.48±0.018	40

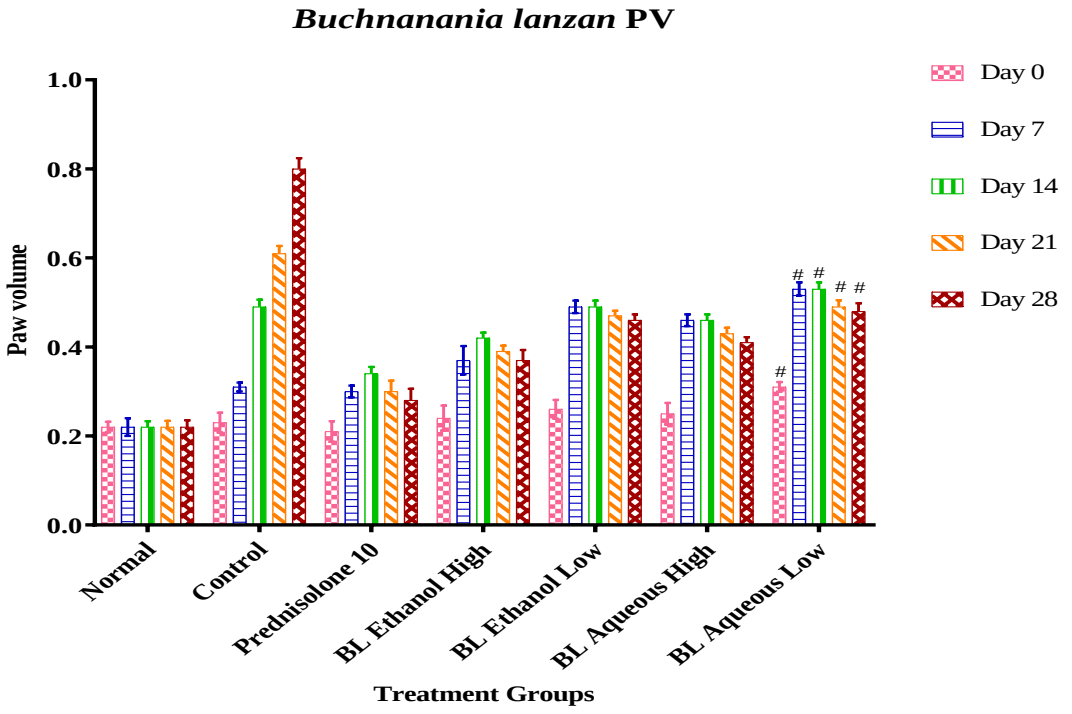

Fig 3. Effect of ethanolic and aqueous extract of *Buchnania lanzan* at their high (400 mg/kg) and low dose (200 mg/kg) on paw volume of CFA-induced arthritic rats.

*Values are plotted as the mean ± SEM, n = 6 in each group; significant reduction in paw volume was analysed by Two-way analysis of variance followed by Dunnett's multiple comparison test using Graph pad Software; *P < 0.0001 compared to CFA control.*

Body weight

The incidence and severity of arthritis increased the changes in the body weight of the rats induced with CFA models. [25] The changes in the body weight were monitored as apparent indicator of arthritic symptoms and the loss of body weight usually began to appear at the onset stage of arthritis. [26] As shown in table no.7 and fig. 4, arthritic control animals showed a marked weight loss (weight change is -4.78%) after 7 days of CFA injection till the end of study. Rats of normal group showed progressive weight gain (weight change is 7.69 %) from 0 day to 28 days as they were not administered CFA injection. In standard group, Prednisolone 10 treated rats significantly improved the body weight (weight change is 6.97 %). In *BL* treated groups, it is found that the groups which received ethanolic extract 400 mg/kg showed highest improvement in weight gain (weight change is 4.95 %) which shows its superior efficacy compared to other groups.

Table 7: Effect of ethanolic and aqueous extract of *Buchnania lanzan* at high (400 mg/kg) and low dose (200 mg/kg) on body weight of CFA-induced arthritic rats

Groups	**Day 0**	**Day 7**	**Day 14**	**Day 21**	**Day 28**	**% change in body weight**
	(Mean+SEM)					
Normal	208±0.36	212±0.25	215±1.06	220±1.29	224±1.59	7.69
Control	230±0.63	232±0.93	225±0.36	222±0.44	219±0.57	-4.78
Prednisolone 10	244±0.93	248±0.73	251±0.63	255±1.23	261±1.09	6.97
BL-Eth-High	202±0.52	203±0.23	206±0.84	209±0.62	212±0.13	4.95
BL-Eth-Low	211±0.25	212±0.42	214±0.81	216±0.45	218±0.94	3.32
BL-Aq- High	213±0.82	215±0.64	217±0.23	220±0.46	222±0.62	4.23
BL-Aq- Low	212±0.52	213±0.97	215±0.35	218±0.65	220±0.32	3.77

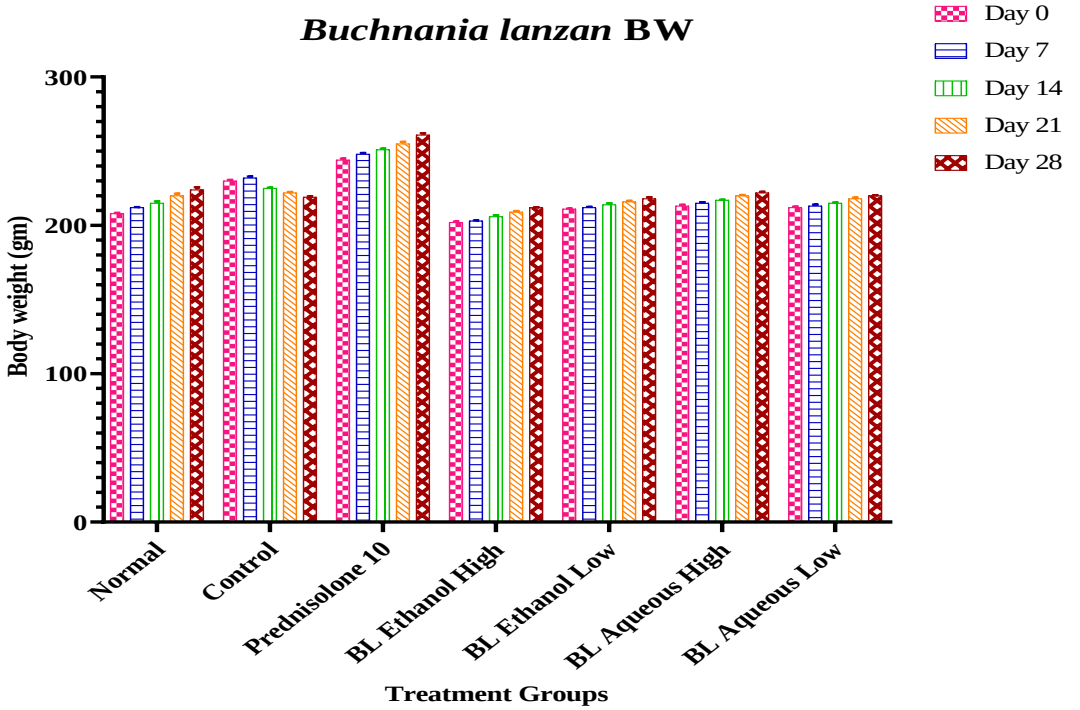

Fig 4. Effect of ethanolic and aqueous extracts of *Buchnania lanzan* at high (400 mg/kg) and low dose (200 mg/kg) on body weight of CFA-induced arthritic rats.

Values are plotted as the mean ± SEM, n = 6 in each group; significant reduction in paw volume was analysed by Two-way analysis of variance followed by Dunnett's multiple comparison test using Graph pad Software; *P < 0.0001 compared to CFA control.

Arthritic Index

CFA induced arthritis model is associated with destruction of the joints. Paw swelling and arthritic index are indicative measures to determine antiarthritic activity of any drug.[27] Arthritic scoring is a benchmark of puffiness of joints succeeding to introduction in a CFA-induced arthritic study.[28] Arthritic index is not evident in animals from normal groups as they were not administered CFA injection. Arthritic control groups are more vulnerable to develop the signs of inflammation, swelling, redness after CFA injection and no reduction in arthritic index was observed until the 28 Days. As shown in table no.8 and fig.5, animals treated with standard drug prednisolone showed a marked decrease in arthritic index on day 28. Also, the rats treated with ethanolic and aqueous extracts of BL caused significant ($P < 0.0001$) and dose dependent reduction in arthritic index on day 28 as compared to CFA control animals. However, ethanolic extracts of *B.lanzan* at 400 mg/kg demonstrated suppressive effect (1.53) which is closer to standard (1.41).

Table 8: Effect of ethanolic and aqueous extract of *Buchnania lanzan* at high (400 mg/kg) and low dose (200 mg/kg) on body weight of CFA-induced arthritic rats.

Arthritic index (AI) on Day 28		
Sr. No	**Treatment Group**	**AI (Mean ±SEM)**
1	Normal	0±0
2	Control	5.12±0.26
3	Prednisolone 10	1.41±0.18
4	BL ethanol High	1.53±0.13
5	BL ethanol Low	1.63±0.24
6	BL Aqueous High	1.58±0.22
7	BL Aqueous Low	1.63±0.14

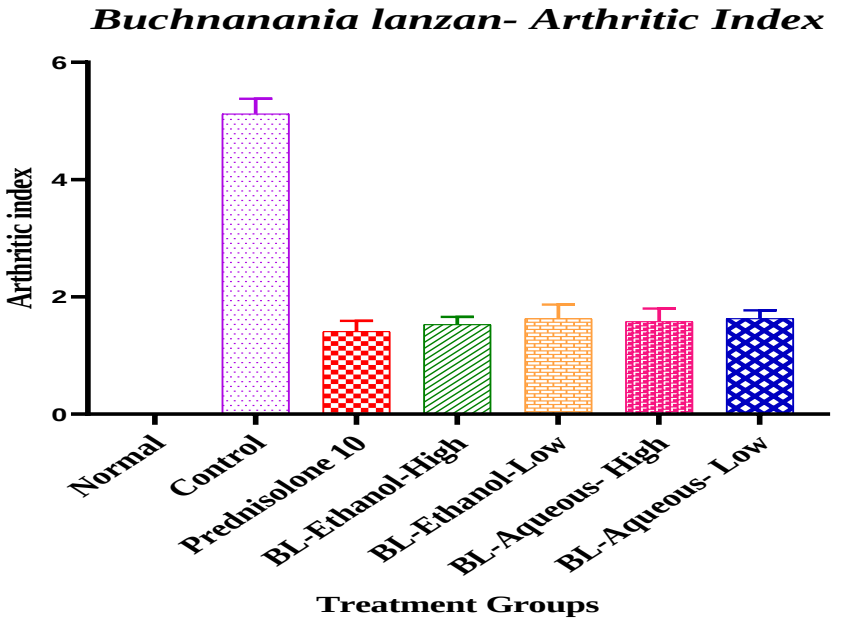

Fig 5. Effect of ethanolic and aqueous extracts of *Buchnania lanzan* at high (400 mg/kg) and low dose (200 mg/kg) on arthritic index of CFA-induced arthritic rats.

Values are plotted as the mean ± SEM, n = 6 in each group; significant reduction in paw volume was analysed by One-way analysis of variance followed by Dunnett's multiple comparison test using Graph pad Software; *P < 0.0001 compared to CFA control.

Haematological Parameters (Haemoglobin [Hb], RBC and ESR) Estimation

The CFA-induced haematological perturbations, such as an increase in the WBC count, a decreased RBC count, a decreased hemoglobin (Hb) count and an increased erythrocyte sedimentation rate. [29] Haemoglobin and RBCs decreases in RA due to reduced bone marrow erythropoietin response and demolition of premature RBCs. [30] The ESR is an indirect method for the measurement of inflammation in the body and it is key biomarkers that is regulated during inflammation, stress and cell necrosis. [31-32] Increase in ESR reflects the chronicity of the disease. [33]

As shown in Table no.9 induction of arthritis by CFA resulted in significant decrease in RBC count, haemoglobin and increase in ESR compared to Normal rats. The effects of ethanolic and aqueous extracts of BL are promising in exerting their anti anaemic action by stabilising the altered haematological changes effectively. Among all the groups the group which is treated with ethanolic extract of BL at a dose of 400 mg/kg prevented alterations in haematological parameters to a greater extent than any other group and it is comparable to standard (Prednisolone). The ethanolic extracts of *BL* significantly restored ESR, blood Hb content and hence it supports the involvement of antioxidant and anti-anaemic properties in maintenance.

Effect of ethanolic and aqueous extract of *BL* at high (400 mg/kg) and low dose (200 mg/kg) on haematological parameters (Hb, RBC and ESR) of CFA-induced arthritic rats is shown in Figure 6-a, 6-b, 6-c respectively.

Table 9: Effect of ethanolic and aqueous extract of *Buchnania lanzan* at high (400 mg/kg) and low dose (200 mg/kg) on Haematological parameters of CFA-induced arthritic rats.

Haematological parameters				
Sr. No	**Treatment Group**	**Haemoglobin (Hb) (gm%) (Mean ±SEM)**	**RBC count (million/cmm) (Mean ±SEM)**	**ESR (mm/hr) (Mean ±SEM)**
1	Normal	15.23±0.15	6.15±0.4	0.69±0.14
2	Control	12.54±0.16	5.1±0.12	4.92±0.24
3	Prednisolone 10	14.81±0.11	5.95±0.18	1.15±0.07
4	BL Ethanol High	14.87±0.15	5.93±0.24	1.39±0.12
5	BL Ethanol Low	14.13±0.24	5.68±0.19	1.58±0.13
6	BL Aqueous High	14.56±0.17	5.81±0.23	1.43±0.04
7	BL Aqueous Low	14.38±0.16	5.87±0.31	1.63±0.21

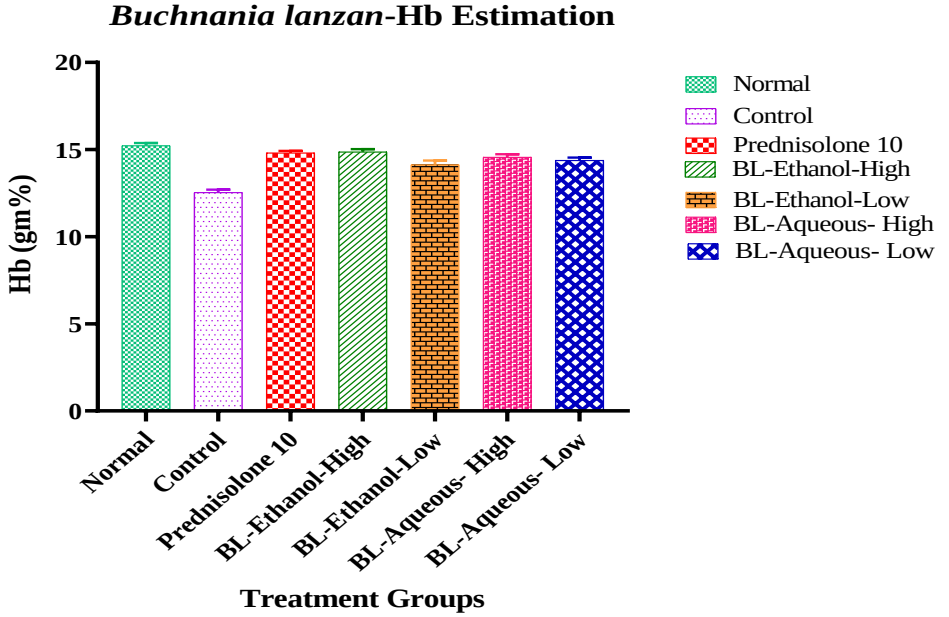

Fig 6-a. Effect of ethanolic and aqueous extracts of *Buchnania lanzan* at high (400 mg/kg) and low dose (200 mg/kg) on Hb of CFA-induced arthritic rats.

Values are expressed as mean ± SEM for six animals and analysis by One-way ANOVA followed by Dunnett's multiple comparison test using Graph pad Software; *P < 0.0001 compared to CFA control**.**

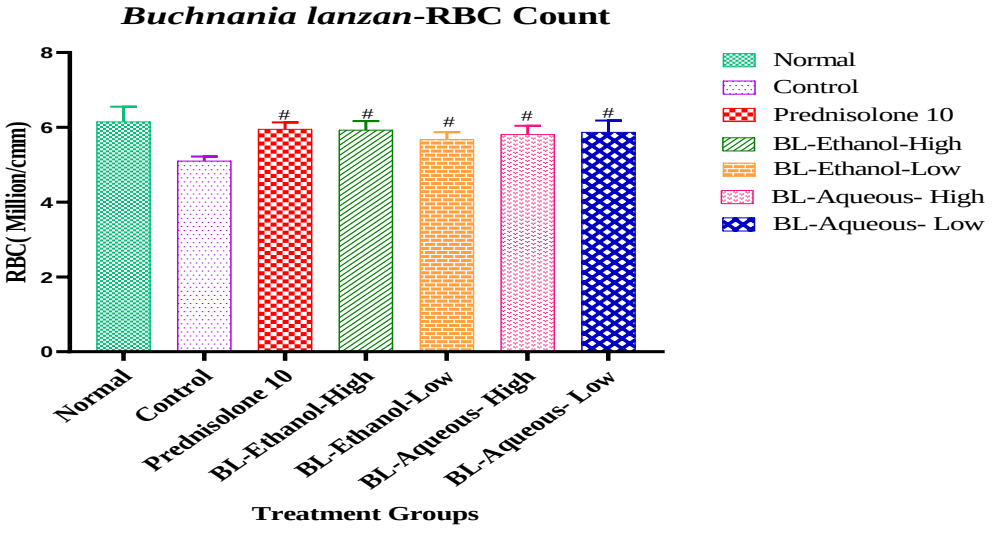

Fig 6-b. Effect of ethanolic and aqueous extracts of *Buchnania lanzan* at their (400 mg/kg) and low dose (200 mg/kg) on RBC of CFA-induced arthritic rats.

Values are expressed as mean ± SEM for six animals and analysis by One-way ANOVA followed by Dunnett's multiple comparison test using Graph pad Software; *P < 0.0001 compared to CFA control.

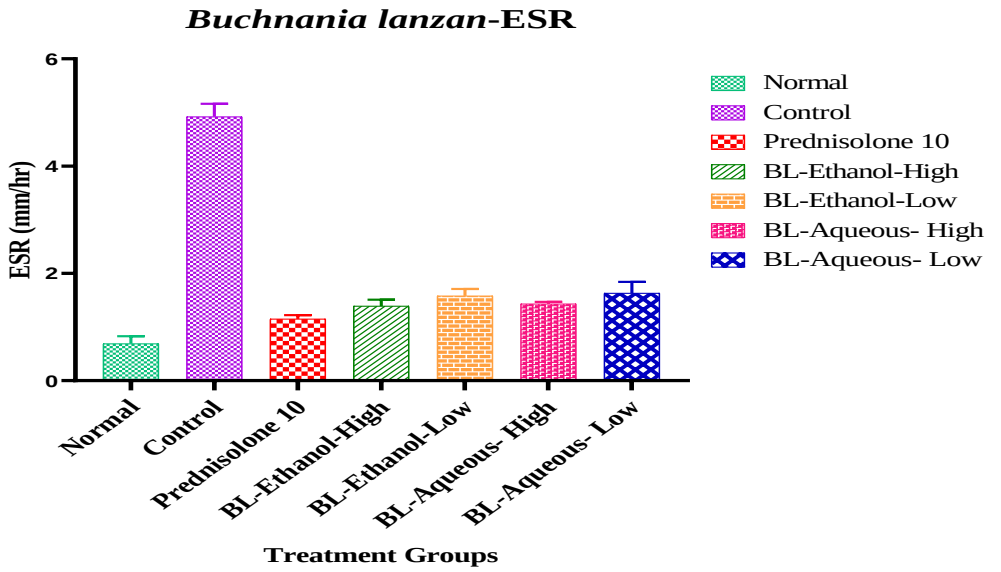

Fig 6-c. Effect of ethanolic and aqueous extracts of *Buchnania lanzan* at high (400 mg/kg) and low dose (200 mg/kg) on ESR of CFA-induced arthritic rats.

Values are expressed as mean ± SEM for six animals and analysis by One-way ANOVA followed by Dunnett's multiple comparison test using Graph pad Software; *P < 0.0001 compared to CFA control.

Estimation of Rheumatoid factor (RF) and C - reactive protein (CRP)

Rheumatoid factors contribute actively to disease severity and chronicity by enhancing immune complex formation and complement fixation; Therefore, RF-producing B cells and their activation mechanisms, including Toll-like receptor ligation may be important targets for RA treatment. [34] Predominant CRP check is followed as an excellent lab technique for scrutinizing RA and other inflammatory diseases arising from inflammation. [35] The increase in the level of CRP is due to the rise in the plasma concentration of IL-6, which is produced by the macrophages and the adipocytes.[36] In general, CRP plays an important role in host defence mechanisms against infectious agents and in the inflammatory response. [37]

As shown in Table no.10, the serum RF (49.44±0.35 IU/mL) and CRP (12.8±0.63 mg/L) which are markers of systemic inflammation and antibody production against the injected adjuvant were dramatically increased in CFA control group rats. The BL exerted its anti-arthritic effect by suppressing the elevated level of serum RF and CRP significantly (p 0.001) as compared to CFA controlled group. Nevertheless, the effect was found to be dose dependent in both ethanolic and aqueous extracts and ethanolic extract displayed maximum efficacy in reducing Serum RF (29.94±0.42 IU/mL) and CRP (4.5±0.38 mg/L) at its dose of 400 mg/kg which is shown in figure 7-a and 7-b.

Table 10: Effect of ethanolic and aqueous extract of *Buchnania lanzan* at high (400 mg/kg) and low dose (200 mg/kg) on Rheumatoid factors (RF) and C-reactive protein (CRP) of CFA-induced arthritic rats

Sr. No	**Treatment Group**	**RF (IU/ml) (Mean ±SEM)**	**CRP (mg/L) (Mean ±SEM)**
1	Normal	0±0	3.07±0.2
2	Control	49.44±0.35	12.8±0.63
3	Prednisolone 10	25.78±0.22	4.2±0.23
4	BL Ethanol High	29.94±0.42	4.5±0.38
5	BL Ethanol Low	33.24±0.15	5.9±0.34
6	BL Aqueous High	30.15±0.38	4.8±0.42
7	BL Aqueous Low	36.12±0.21	6.2±0.16

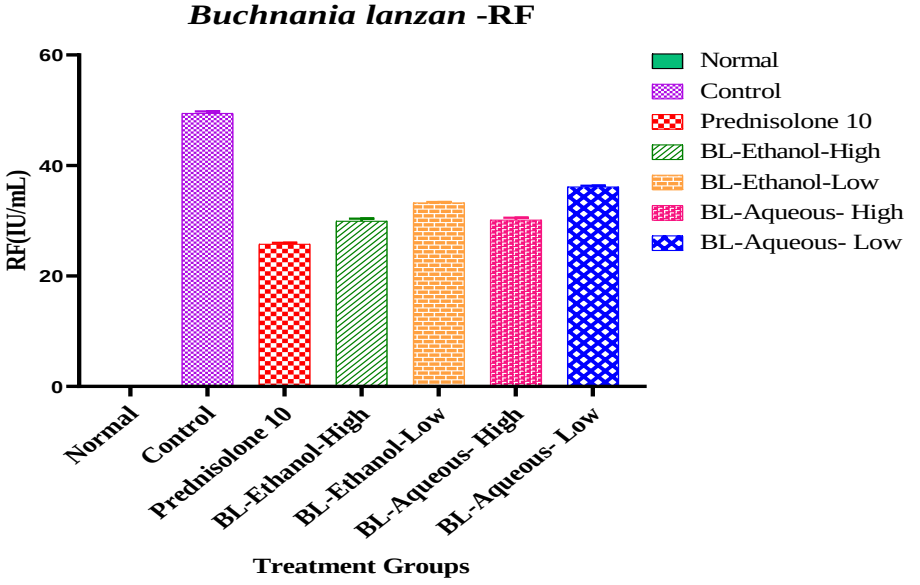

Fig 7-a. Effect of ethanolic and aqueous extracts of *Buchnania lanzan* at high (400 mg/kg) and low dose (200 mg/kg) on RF of CFA-induced arthritic rats**.**

Values are expressed as mean ± SEM for six animals and analysis by One-way ANOVA followed by Dunnett's multiple comparison test using Graph pad Software; *P < 0.0001 compared to CFA control.

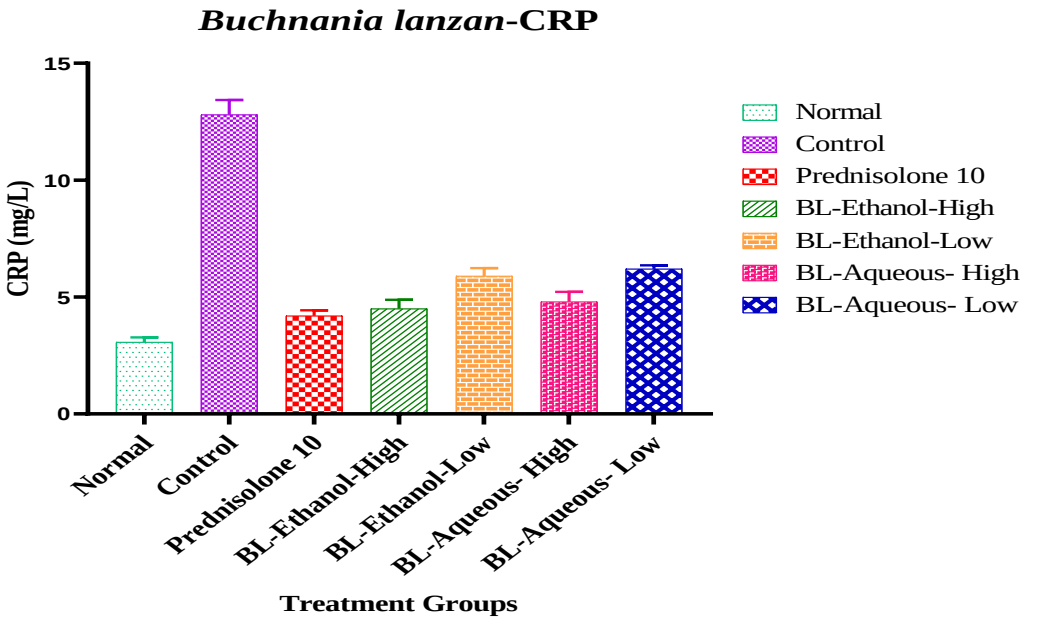

Fig 7-b. Effect of ethanolic and aqueous extracts of *Buchnania lanzan* at high (400 mg/kg) and low dose (200 mg/kg) on CRP of CFA-induced arthritic rats.

Values are expressed as mean ± SEM for six animals and analysis by One-way ANOVA followed by Dunnett's multiple comparison test using Graph pad Software; *P < 0.0001 compared to CFA control.

Estimation of Biochemical parameters

Cellular enzymes, such as Aspartate transaminase (AST), Alanine transaminase (ALT), membrane bound indicator of type II cell secretary activity or the lysosomal enzyme glucuronidase, an indicator of phagocytic activity, can also be used as sensitive markers of cellular integrity and cellular toxicity induced by pathological conditions. [38] However, the alkaline phosphatase (ALP) function is associated with annihilation of the bone and is an assessment of lysosomal integrity. [39] Increased activities of AST, ALT and ALP were observed in arthritic rats, which may be attributed towards persistent inflammation and also useful in the evaluation of liver damage. [40]

As a result of inflammation induced by CFA, the levels of all the biochemical parameters were increased in all arthritis rats as compared to control rats. However, the treatment of animals with Prednisolone significantly reduced the elevated levels of serum enzymes as seen in Table 11. Treatment with ethanolic and aqueous extracts of *BL* found to be promising in stabilizing the altered biochemical changes effectively. Among all the groups, ethanolic extract of *BL* (400 mg/kg) treated group prevented alterations in serum biomarkers AST (72 ±2.67 U/L), ALT (86±2.25 U/L) and ALP (143±3.28 U/L) to a greater extent as shown in Figure 8.

Table 11: Effect of ethanolic and aqueous extract of *Buchnania lanzan* at high (400 mg/kg) and low dose (200 mg/kg) on Biochemical Parameters (AST, ALT and ALP) of CFA-induced arthritic rats

Biochemical Parameters				
Sr. No	**Treatment Group**	**AST(U/L)**	**ALT(U/L)**	**ALP (U/L)**
		(Mean ±SEM)		
1	Normal	42±2.3	42±1.7	73±3.3
2	Control	127±4.4	173±2.8	475±1.6
4	BL Ethanol High	72±2.67	86±2.25	143±3.28
5	BL Ethanol Low	86±4.02	95±1.37	165±3.16
6	BL Aqueous High	76±3.25	92±2.46	149±3.18
7	BL Aqueous Low	92±2.04	102±1.58	172±3.05
8	BL Ethanol High	72±2.67	86±2.25	143±3.28

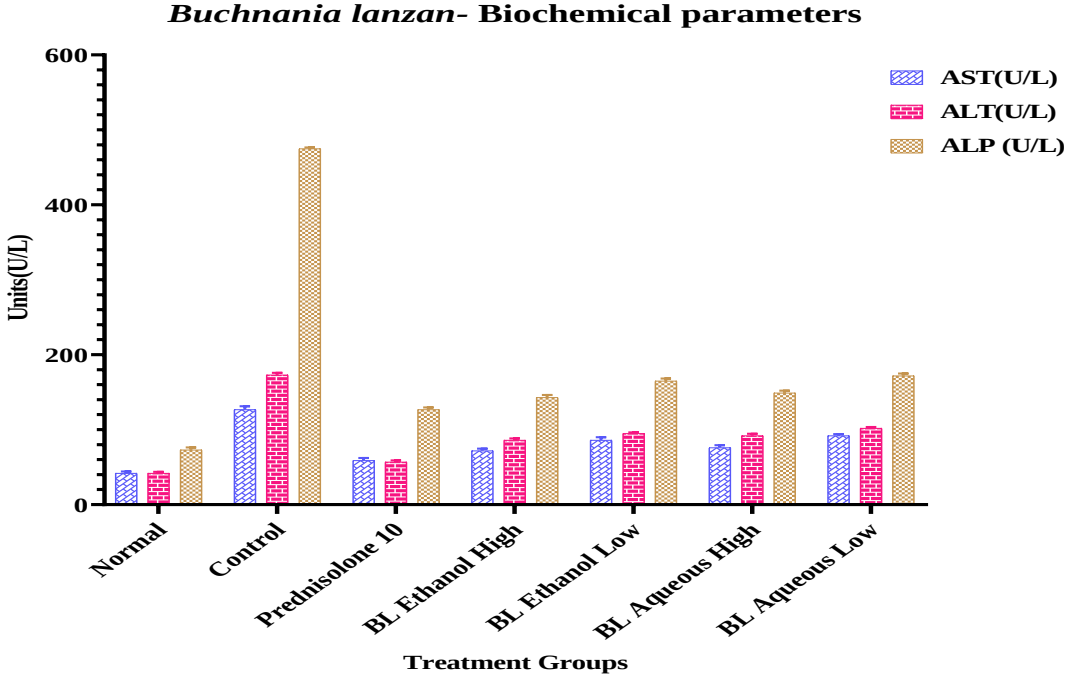

Fig 8. Effect of ethanolic and aqueous extracts of *Buchnania lanzan* at high (400 mg/kg) and low dose (200 mg/kg) on biochemical parameters of CFA-induced arthritic rats.

Values are expressed as mean ± SEM for six animals and analysis by Two-way ANOVA followed by Dunnet's multiple comparison test using Graph pad Software; *P < 0.0001 compared to CFA control.

Radiological study

The severity of rheumatoid arthritis is indicated by the radiographic changes and hence it is considered as useful diagnostic measures. Early phase RA is characterised by soft tissue swelling, whereas the prominent signs like bony erosions and narrowing of joint spaces can be observed only in the developed stages of rheumatoid arthritis. [36]

The arthritic control rats show the narrowing of joint space, severe soft tissue swelling, pronounced decrease in bone density, marked destruction of bones and abnormal ossification in the joints of interphalangeal regions. All these symptoms symbolises the presence of subchondral erosion in arthritic conditions. However it is found that the animals treated with the different extracts and standard drug prednisolone have contributed to noticeable reduction in joint erosion. The treatment also resulted in normalization of other changes. The standard prednisolone showed better effect compared to all other group. The radiographs of the rat joints in CFA-induced arthritic rat model shown in the Figure 9.

The ethanolic and aqueous extract of Buchnania lanzan given animals remarkably diminished all the signs and symptom associated with arthritic induced rats. Treatment with ethanolic and aqueous extract of Buchnania lanzan at their 200 mg/kg and 400 mg/kg revealed reduction in gap spaces, alteration in bone erosion, connective tissue inflammation comparable to standard prednisolone. The best dose of Buchnania lanzan that exhibited maximum rectification in radiographic changes was 400mg/kg of its ethanolic extract.

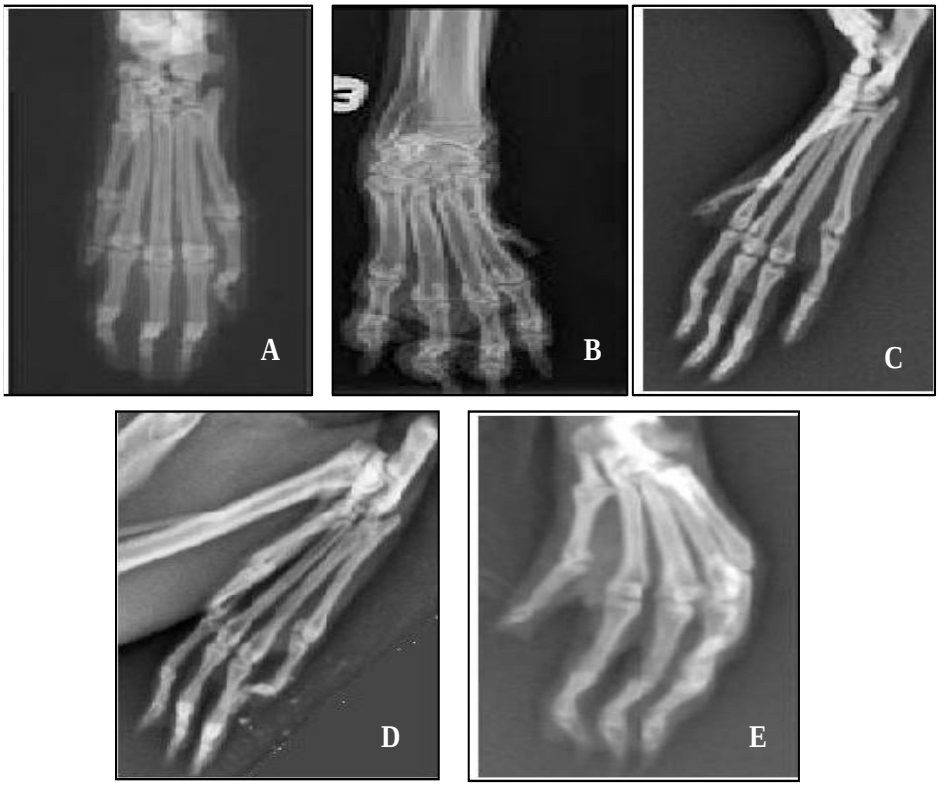

Fig.9 X-ray Radiographic assessment of hind paws of CFA-induced arthritis rats after treatment with standard and different extracts of *B. lanzan*, Where,

A: Normal control, B: Disease control, C: Standard Prednisolone, D: 400 mg/kg of *B. lanzan* ethanolic extract, E: 400 mg/kg of *B. lanzan* aqueous extract,

Histopathological Analysis

The finding of joint histopathological study 28 days post CFA induction revealed noticeable changes in hind paw joints. This significant changes includes synovial hyperplasia, infiltration of leukocytes, erosion of bone and cartilage, joint space narrowing, pannus formation, infiltration of inflammatory cells, as seen in picture of disease control animals (B) compared to vehicle control animals (A). After treatment with the standard drug prednisolone the intensity of the changes were drastically reduced as seen in picture (C). The effect of ethanolic and aqueous extracts of BL at their dose of 400 mg/kg resulted in normalisation of changes to great extent and in many cases the outcome is comparable to standard prednisolone as seen in figure 10.

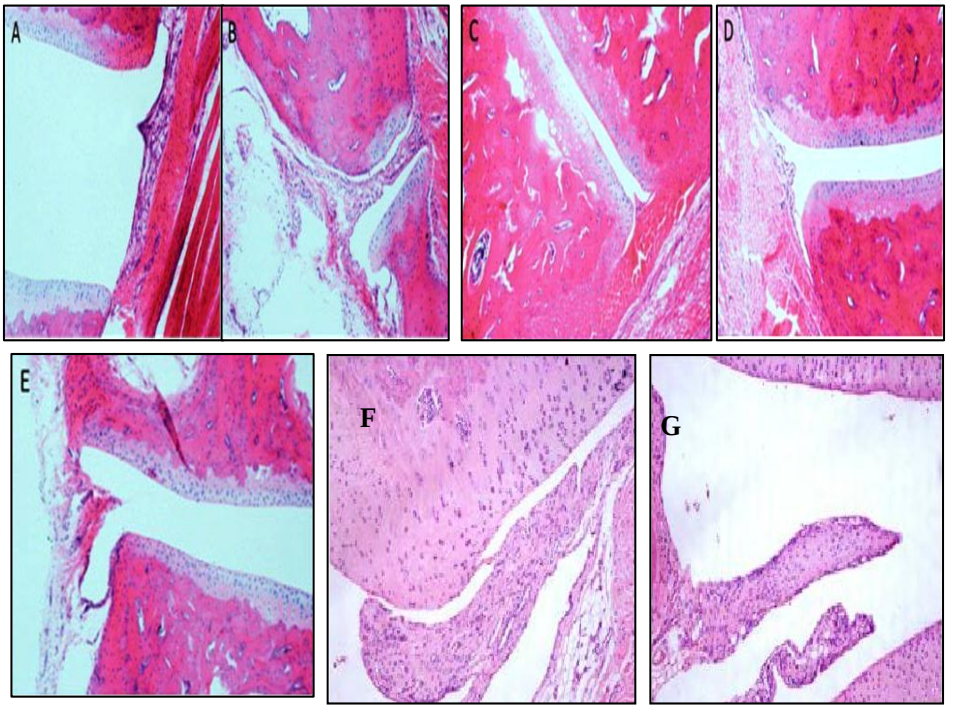

Fig.10 Histopathological assessment of hind paws of CFA-induced arthritis rats after treatment with standard and different extracts of *Buchnania lanzan* where,

A: Normal control, B: Disease control, C: Standard Prednisolone, D: 400 mg/kg of *B.lanzan* ethanolic extract, E: 400 mg/kg of *B.lanzan* aqueous extract, F: 200 mg/kg of *B.lanzan* ethanol extract, G: 200 mg/kg of *B.lanzan* aqueous extract respectively.

Histopathological studies of animals revealed effect of ethanol extracts of *BL* at 400 mg/kg as superior effect as it shows no evidence of leukocyte infiltration, synovial hyperplasia, bone and cartilage erosion and pannus formation.

Discussion

As the recent healthcare scenario is mainly focussed on use of synthetic drugs though having numerous side effects associated with them, the use of herbal drugs always remains in unique position because of less side effects, easy availability and affordability. In order to increase the widespread use of herbal drugs, efforts are taken to validate the ethno medical claim of various medicinal plants for treatment of various disorders.

The plant *Buchnania lanzan* has been traditionally claimed for use in Rheumatism. To explore the potential of *BL* for treatment of arthritis, it has been subjected for continuous soxhlet extraction using various solvents of increasing order of polarity. It has been found from the percentage yield of Pet. ether, ethyl acetate, ethanol and aqueous extracts of *BL*, maximum phytoconstituents are

present in ethanolic extract followed by aqueous extract. The amount of phytoconstituents that are soluble in solvents like pet ether and ethyl acetate is comparatively less. Initially, all the four extracts were screened for anti-arthritic activity using in vitro models of inhibition of protein i;e egg albumin and Bovine serum albumin (BSA) denaturation.

Denaturation of the protein involves the disruption of secondary, tertiary and quaternary structure of the molecules and finally leads to cell death, it occurs due to stress like a high level of salt, high temperature and high level of acidity. Denaturation of protein is one of the causes of RA due to the production of auto-antigens in certain rheumatic diseases. [41] It has been reported that inhibition of denaturation of BSA at pathological pH (6.2-6.5) was accountable for anti-inflammtory action of various NSAIDs. [42]

Agents that can prevent protein denaturation therefore, would be worthwhile for antiarthritic drug development. The anti arthritic activity was also shown in a concentration dependent manner and the activity was increased on increasing the concentration of extracts. The increments in absorbance of test samples with respect to control indicated stabilization of protein i.e. inhibition of heat induced protein denaturation by plant extract. Hence, maximum activity was reported at highest concentration (1000 µg/ml) of ethanolic extract of *BL.* The in vitro anti-arthritic protein denaturation assay showed that among all extracts, only ethanolic and aqueous extract are significant and promising and hence evaluated further for in vivo anti-arthritic activity.

Agents that can prevent protein denaturation therefore, would be worthwhile for antiarthritic drug development. The anti arthritic activity was also shown in a concentration dependent manner and the activity was increased on increasing the concentration of extracts. The increments in absorbance of test samples with respect to control indicated stabilization of protein i.e. inhibition of heat induced protein denaturation by plant extract. Hence, maximum activity was reported at highest concentration (1000 µg/ml) of ethanolic extract of B*L.* The in vitro anti-arthritic protein denaturation assay showed that among all extracts, only ethanolic and aqueous extract are significant and promising and hence evaluated further for in vivo anti-arthritic activity.

The purpose of preliminary acute toxicity studies is to determine the LD50 values to help us in evaluating the safe dose range at which the drug can be used without any harmful or lethal effect on the experimental animals. Accordingly 5000 mg/kg is fixed as cut off LD50. In vivo anti-arthritic activity of *BL* was validated using CFA induced arthritis model and different parameters were observed like changes in Paw volume, Body weight, arthritic index, haematological system (RBC, Hb, ESR), Biochemical marker (AST, ALT, ALP), CRP level, RF level, Radiological and histopathological study.

In Freund's Adjuvant Arthritic rat model, treatment with ethanolic and aqueous extract of *BL at 400mg/kg* showed significant inhibitory effect on injected hind paw edema, body weight changes, arthritic index, haematological changes, biochemical changes and maximum inhibition was observed on the group receiving ethanolic extract. The study also illustrates the superior effect of

ethanolic extract in other parameters like level of CRP and RF in serum, radiological changes and histopathological changes.

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