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Pharmacological evaluation of a freshwater mollusc, *Bellamya bengalensis* lamarck on adjuvant induced arthritis in wistar rats

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Key words: *Bellamya bengalensis* Lamarck, Freund's Complete Adjuvant, TNF-α, X-ray, Histopathology.

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Abstract

Arthritis is a painful muscoskeletal disorder of unknown etiology affecting a large population globally. Non-steroidal antiinflammatory drugs have been used for long but have side effects like gastric ulcer, gastric bleeding, leucopenia etc. *Bellamya bengalensis* is a freshwater mollusc found abundantly of Viviparidae family was used to treat various ailments like conjunctivitis, arthritis etc. The present study aimed to show the attenuation of inflammation by phosphate buffer saline extract of *Bellamya bengalensis* Lam (PBB) in Freund's Complete adjuvant (FCA) induced arthritis model. The arthritis was induced in Wistar rats using FCA. Changes in body weight, paw volume, ankle diameter, arthritic score were studied as parameters of chronic inflammation. Serum cytokine profile and tissue antioxidant profile of the paw was studied at the end of the study. Radiology of the hindlegs and the histopathology of the ankle joints were also studied. The PBB treated group of rats showed normal gain in body weight with reduction in paw volume by 90.23% and 96.27% ($p<0.01$) at 200mg/kg and 400mg/kg when compared with FCA induced control. The proinflammatory cytokine was reduced by 56.79% in serum in PBB 400 mg/kg and the altered antioxidant parameters in inflamed rat paw were restored back to normal when treated with PBB extracts. Histopathology results revealed fewer inflammatory lesions in PBB treated rats when compared to adjuvant induced control rats. In conclusion, PBB at a dose of 200mg/kg and 400mg/kg, orally for 28 days showed significant reduction in chronic inflammation which may be through TNF-α inhibitory pathway.

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Introduction

Arthritis is an umbrella term that encompasses hundred distinct arthritic situations which are a persistent, painful, and debilitating institution of illnesses. Arthritis is ranked as the second most cause of disability worldwide [Briggs AM *et al.*, 2015] It accounts for one of the major reasons of disability worldwide. In United States alone, 22.7% people annually are diagnosed with some form of arthritis, fibromyalgia, gout, lupus. [Barbour KE *et al.*, 2017] A recent study reported that in low to middle income countries, prevalence of arthritis was more among those with least education, and in separated/divorced/widowed women. [Brennan-Olsen SL *et al.,* 2017] Pathophysiology of arthritis includes wear and tear of cartilage, bone destruction, painful swelling and morphological changes of the tissue, pannus formation and inflammation of the synovium. [Wang Q *et al.,* 2013] Freund's Complete Adjuvant (FCA) induces polyarthritis in rats by prolonging the life of autoantigen assuming a Th1 (T helper cells 1) profile resulting in strong delayed type hypersensitivity. [Billiau A and Matthys P, 2001].

Arthritis is widely treated by the intervention of steroidal and Non-steroidal Anti-inflammatory Drugs (NSAIDs). [Billiau A and Matthys P,2001] But the use of steroidal and Non-steroidal Anti-inflammatory Drugs (NSAIDs) is often associated with life threatening side effects which includes gastrointestinal ulcers, heartburn, leukopenia etc. [Kunnumakkara *et al.,*2018; Gor AP and Saksena M,2011] So, there is a continuous search for a safer drug from natural sources. The aquatic biosphere is one of the largest biospheres that nurses a variety of organisms. It is the secondary metabolites that gets produced during their adaptation to the aquatic environment which provides various pharmacological properties.30,000 compounds have been already discovered from marine origin and are identified by their diversity, structural novelty and complexity.[Lindequist U,2016] *Bellamya bengalensis* Lam. is a freshwater mollusc and is predominantly found in South East Asia and is commonly used in Indian traditional medicine for the treatment of various pathological states like conjunctivitis, arthritis etc.[Prabhakar AK and Roy SP,2009]. With relevance to the ethnopharmacological importance of this mollusc, the present study was designed to investigate the potential therapeutic effect of Phosphate buffer saline extract of *Bellamya bengalensis* Lam. in adjuvant induced arthritic model in rats.

Materials and methods

Collection of the specimen and preparation of the test drug

The living univalve molluscs were collected from the ponds near Kolkata. The authenticity of the specimen was validated by Zoological Survey of India, Kolkata (Specimen No :1242/Lot No-63). Deionized water is used for cleaning up of mud and dirt from the molluscs. The molluscs were kept for 24 hours in laboratory conditions for acclimatization. On the next day, the shell was removed carefully and the whole mass was submersed in Phosphate Buffer Saline (PBS, 1M, pH 7.4) overnight at 4°C.The mass was grounded and made into a fine paste and was centrifuged for 10 minutes at 5000 rpm at 4°C.The supernatant was filtered through Whatmann filter paper (Ø125 mm).The extract was lyophilized to powdered form and stored at -20°C. The test drug was abbreviated as PBB.

Animals used

Wistar albino rats (150 ± 10g) were used for the study. The animals were procured from CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) registered breeders and were kept in polypropylene cages, maintained at a temperature of 22 ± 1°C, 50 ± 10% humidity and 12 hours light and 12 hours dark cycle. The animals were fed with food pellets (fortified with minerals and vitamins) and water *ad libitum*. The study was approved by the Institutional Animal Ethics Committee and the experiments were done following the accepted principles of Committee for the Purpose of Control and Supervision of Experiments on Animals. (Ethical No.-RKC/IAEC/13/18 dated 15.03.2016).

Experimental Induction of arthritis

Arthritis was induced by injecting 0.1ml of Freund's Complete Adjuvant (Sigma, St. Louis, USA) in the sub plantar region of left hind paw of the animals. [Liu Y*et al.*, 2009] Thirty-six healthy Wister rats were then divided into six groups (n=6, per group). Diclofenac sodium was used as the standard drug. The group division along with the treatment schedules are as follows:

Group I: Normal control treated with normal saline,2ml/kg

Group II: FCA induced rats treated with normal saline, 2ml/kg

Group III: FCA induced rats treated with diclofenac sodium, 10mg/kg

Group IV: FCA induced rats treated with the test drug PBB 100mg/kg

Group V: FCA induced rats treated with the test drug PBB 200mg/kg

Group VI: FCA induced rats treated with the test drug PBB 400mg/kg

Experimental design

The oral administration of the drugs was carried out for a period of 28 days. The doses of the test drug were selected on the basis of pilot study. Body weight, paw volume, ankle diameter and motility score of the rats were measured on 0^{th} ,7^{th}, 14^{th}, 21^{st} and the 28^{th}day. The paw volume was measured using a digital plethysmometre (Orchid Scientific, India) and the ankle diameter was measured by Vernier Caliper.

The locomotory activity was estimated by digital actophotometer (Labman, India) and motility score was measured by wire maze. Radiological imaging of the hind paw was done, blood sample was collected from each rat by retro-orbital puncture. The rats were then sacrificed by administering overdose of thiopentone sodium. [CPCSEA,2003] Paw tissues were collected for *in-vivo* antioxidant assay and the ankle joints were taken for histopathological study.

Motility test: The motility test was estimated by a standardized scoring system. The score is as defined under [Adhikari A *et al.*,2017]

Rats avoiding touch with the ground-0

Rats touching the ground with difficulty-0.5

Rats with normal gait-1

Biochemical analysis

Determination of serum TNF-α level: TNF-α concentration in serum was done by ELISA kits following manufacture's protocol (Ray Biotech, USA). Optical density (OD) was measured at 450nm (Bio-Rad ELISA reader).

Determination of tissue antioxidant parameters: Assessment of the anti-oxidant activity was carried out using rat hind paw tissue homogenate.10% of the tissue was homogenized dissolved in 0.1M phosphate buffer (pH 7.4). The tissue samples were then centrifuged at 5000rpm for 10mins at 4°C.The supernatant was used for the evaluation of lipid peroxidation, nitric oxide (NO), reduced glutathione (GSH) and superoxide dismutase (SOD) in liver and kidney tissues. Protein concentration in the tissue homogenate was determined by Lowry methods [Lowry OH *et al.*, 1951].

Lipid Peroxidation Assay: 0.5ml of tissue homogenate was mixed with 1ml of TBARS solution and heated at 90° C for 30 mins for estimation of lipid peroxidation in the sample. The reaction mixture was cooled and centrifuged at 5,000 rpm for 10 mins. The optical density of the supernatant was measured spectrophotometrically at 532 nm. [Buege JA and Aust SD, 1978] Concentration of Malonaldehyde (MDA) was determined by plotting a standard curve against malonic acid in respect to the amount of protein present in tissue.

Nitric Oxide Estimation in Tissues: For estimation of nitric oxide in the sample, 100µL of 10% tissue homogenate and 500µL of Griess reagent was incubated at room temperature for 30 mins.

Absorbance was measured at 540nm. Concentration of nitric acid in the sample was estimated from the protein concentration in the sample and the nitric oxide standard curve. [Moshage H *et al.*, 1995].

Reduced Glutathione Estimation: Reduction of 5,5 dithiobis (2-nitrobenzoic acid) (DTNB) with reduced glutathione (GSH) yields a yellow coloured compound. Absorbance of this yellow compound (reduced chromogen) is directly proportional to GSH concentration in the tissue homogenate.100 µl of the tissue homogenate was dissolved in 600 µl of 20 mM EDTA (Ethylenediaminetetraacetic acid) and incubated in ice for 10 mins. To this, 500 µl of water and 250 µl of Tricholoroacetic acid (10% w/v) was added and incubated at room temperature for 5 mins.500 µl of water, 250 µl of Tricholoroacetic acid (10% w/v) was added and incubated at room temperature for 5 mins. The reaction mixture was then centrifuged at 5000 rpm for 10mins. 1ml of the supernatant, 2ml of 0.4M Tris buffer and 100µl of 0.1 M DTNB was mixed and incubated at room temperature for 3 minutes.

The absorbance was measured at 412nm. [Lawrence R and Burk R, 1976]

Superoxide Dismutase Assay: Presence of Superoxide dismutase activity in the tissue homogenate is estimated by formation of purple coloured formazan complex. Initiation of the reaction starts with the addition of 100µl of 186µM phenazine methosulphate (PMS),1.2ml of 0.052mM sodium pyrophosphate buffer (pH 8.3), 0.2ml of nicotinamide adenine dinucleotide (NADH, 780µmol),0.2ml of tissue homogenate in dark at room temperature. PMS and NADH reacts to form a complex to generate superoxide radicals. To this, 300µl nitroblue tetrazolium was added that forms the purple coloured formazan. Reaction is stopped immediately after 90 secs by addition of 1ml of glacial acetic acid. The amount of chromogen formed was measured by recording color intensity at 560 nm. [Patro G, 2016] Sodium dismutase activity was calculated from the standard curve and the amount of protein present in tissue homogenate.

Radiological study of the hind paw

Each rat was anaesthetized by injecting thiopentone sodium intraperitoneally at a dose of 40mg/kg. Radiographs were done with X-ray equipment (PHILIPS Diagnose X-ray) operated at a voltage of 55kV against 3.2mA/s with a tube-to-film distance of 110cm for lateral projection. [Harith JM *et al.*, 2018] The photographs were then examined for the soft tissue swelling, bony erosions and narrowing of the spaces between joints.

Histological study

Ankle joint tissues were taken for histopathological study. Initially, the joints were washed in normal saline and stored in 10% formalin. The tissues were processed, cut into thin sections using cryotome (3-5µm thickness), paraffinized and deparaffinized following standardized methods. The tissues were stained with hematoxylin and eosin (H&E) and were evaluated under light microscope for any morphological changes compared to the normal. [Desai SD *et al.*, 2015]

Statistical analysis

All the data were expressed as the mean ± SEM (Standard Error of Mean). In the present study, analysis of variance (ANOVA) followed by post-hoc Dunnett test was performed for statistical data evaluation. SPSS version 20.0 was used for analyzing the data. Differences were considered statistically significant at $p < 0.05$ and $p < 0.01$.

Results

Body weight profile of rats

The body weight profile (Figure 1) of the FCA treated arthritic control showed a gradual decrease in body weight during the period of 28days ($p<0.01$) when compared with normal healthy control (Group I).

The rats treated with Diclofenac Sodium (Group III) did not show significant changes in body weight; whereas the PBB treated rats at a dose of 200mg/kg and 400mg/kg showed marked increase in body weight ($p<0.01$) when compared to FCA induced arthritic control (Group II) rats.

Measurement of paw volume and ankle diameter

The severity of inflammation was estimated by measurement of paw volume and ankle diameter on

7th, 14th, 21st and 28th day of the study period. Figure 2 revealed the percentage inhibition of paw volume and ankle diameter. The PBB treated group of rats at 200mg/kg and 400mg/kg reduced paw volume by 90.23% and 96.27% ($p<0.01$) when compared to arthritic control group. The ankle diameter was also reduced by of PBB 79% and 84% ($p<0.01$) in PBB 200mg/kg and PBB 400mg/kg when compared to arthritic control group. Diclofenac Sodium decreased the paw volume by 72.55% and ankle diameter by 60%. (Figure 2).

Table 1. Effect of phosphate buffer saline extract of *Bellamya bengalensis* Lam. on locomotory activity and motility score in FCA induced rats.

	Score									
	Actophotometer					Wire maze				
	0th Day	7th Day	14th Day	21st Day	28th Day	0th Day	7th Day	14th Day	21st Day	28th Day
Group I	200 ±14.9	146±9.6	152±5.9	170±4.1	200±1.6	1±0	1±0	1±0	1±0	1±0
Group II	104.5±11.6	88±13.2	84.75±13.2	82.5 ± 14.3	71.25±11.4	1±0	0	0	0.2±0.1	0.4±0.1
Group III	110.6±11.6	77±15.1	88.4±9.7	101.2±12.4	115.8±12.3	1±0	0.2±0.1	0.4±0	0.5±0	0.8±0.1
Group IV	121±2.4	51.3±4.5	81 ± 9.5	112±4.5	118±2.3	1±0	0.3±0.1	0.5±0	0.5±0	0.75±0.1
Group V	185.8±17.1	89.5±22.7	114±7	124.3±6.3	169±6.84	1±0	0.5±0	0.7±0.1	0.9±0.1	0.9±0.1
Group VI	218.3±7.1	90±16.5	122.6±13.9	174.3±14.2	186.3±13.4	1±0	0.38±0.1	0.7± 0.1	1±0	1±0

Values are expressed as mean ± SEM, n=6.

Measurement of locomotory activity and motility score

It was observed that after induction with FCA, the locomotory activity and motility score decreased considerably on 7th day (Table 1). PBB treated rats (200mg/kg and 400mg/kg) showed steady increase in locomotory movement and improved motility score on 14th, 21st and 28th day. Diclofenac Sodium also exhibited improved locomotory activity and the motility scores as compared to FCA induced arthritic control.

Table 2. Estimation of antioxidant enzymes in the left hind paw induced with arthritis by FCA (n=6).

Group no.	Nitric Oxide (mM/mg protein/mg tissue)	Superoxide Dismutase (mM/mg protein/mg tissue)	Reduced glutathione (mM/mg protein/mg tissue)	Malonaldehyde (mM/mg protein/mg tissue)
Group I	0.093±0.009	1.58±0.2	5.18±0.12	0.012±0.003
Group II	0.168±0.003	0.43±0.13	0.17±0.51	0.028±0.003
Group III	0.127±0.054	0.84±0.26	2.44±0.07	0.054±0.013
Group IV	0.092±0.078	1.52±2.59	1.58±1.29	0.023±0.003
Group V	0.079±0.049	1.61±1.15*	2.36±0.85	0.011±0.008
Group VI	0.077±0.015*	1.97±0.54**	4.29±0.11**	0.008±0.002*

Values are expressed as mean ± SEM, n=6. Data were analyzed by One-way ANOVA followed by Dunnett post-hoc test, *p < 0.05, **p < 0.01 when compared to when compared to FCA induced arthritic control (Group II).

Estimation of serum cytokine level

Proinflammatory cytokines like TNF-α play a major role in pathogenesis of inflammation. A significant increase in the concentration of TNF-α was observed in the serum of arthritic animals after 28 days of FCA induction. Post-treatment with PBB at a dose of 200mg/kg and 400mg/kg showed marked reduction in TNF-α concentration ($p<0.01$) that was induced by Freund's adjuvant (Figure 3).

Evaluation of paw erethyma, radiological analysis and histopathological study

Oral administration of PBB extract at 400mg/kg dose significantly reduced paw erythema and ankle

diameter as depicted in Figure 4A. The radiological analysis in FCA induced arthritic control showed swelling of soft tissue, narrowing of joint spaces and sign of bone destruction. However, the effect was much reduced in PBB treated 400mg/kg dose and Diclofenac Sodium 10mg/kg where they provided protection against narrowing of joint spaces, destruction of bones, inflammation of the soft tissue (Figure 4B).

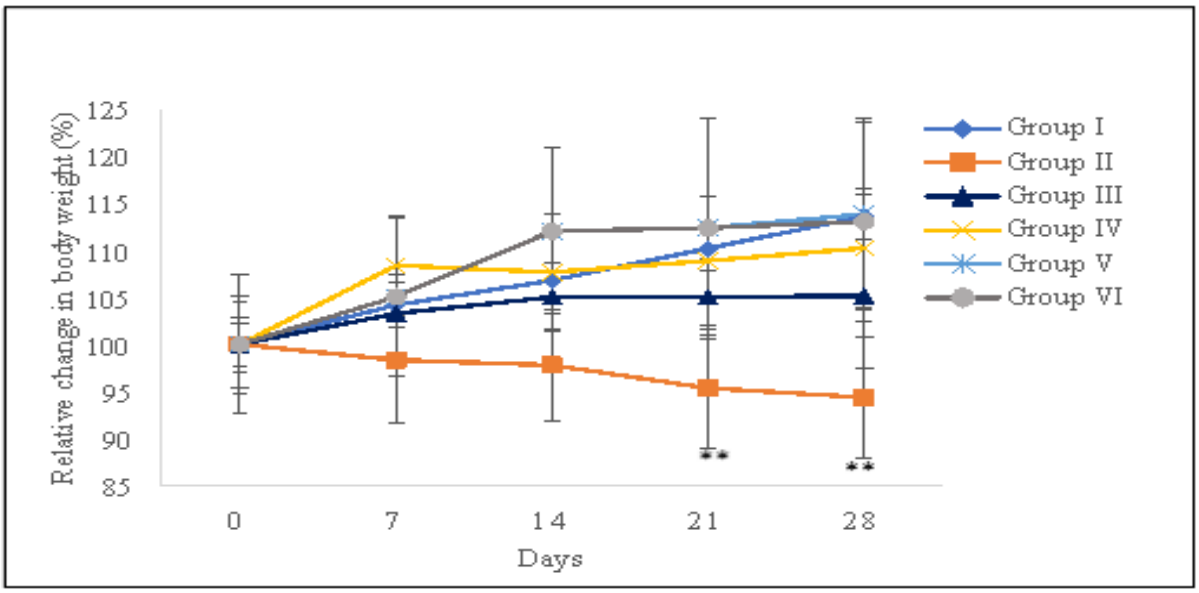

Fig. 1. Effect of phosphate buffer saline extract of *Bellamya bengalensis* Lam. on body weight of FCA induced rats. Values are expressed as mean ± SEM, n=6. Data were analyzed by One-way ANOVA followed by Dunnett post-hoc test, $^{*}p < 0.05$, $^{**}p < 0.01$ when compared to FCA induced arthritic control (Group II).

Figure 4C depicts histological analysis of the ankle joints. The arthritic control showed numerous edematous vacuoles, inflammatory cells, cartilage destruction and synovial hyperplasia; The PBB treated rats at 400mg/kg dose reduced edematous vacuoles, inflammatory cells and showed smooth articulation of cartilage surface. The standard drug, Diclofenac sodium also lowered the number of inflammatory cells but presence of edematous vacuole was well observed.

Antioxidant analysis of the paw

Free radicals play a major role in induction of inflammation which are released in high concentration in the surrounding tissue of the inflamed area. [Abotsi WM *et al.*,2010] Decreased concentration of Nitric oxide, Malonaldehyde and increased concentration Superoxide dismutase, Glutathione reductase affirms that the PBB extract played a protective role in prevention of free radical generation (Table 2).

Discussion

Body weight profile

Preclinical testing of numerous antiarthritic and chronic inflammatory diseases uses Freund's adjuvant induced model in experimental rat model [Tuncel J *et al.*,2016]. In the present study, phosphate buffer saline extract of whole mass of *Bellamya bengalensis* reduced significantly all the inflammatory parameters. Chronic arthritis is associated weight loss in living beings. Weight loss in arthritis signifies muscle loss, low appetite and metabolic burden of inflammatory response. [Mbiantcha M *et al.*, 2017] Arthritis stimulates high concentration of proinflammatory cytokines like TNF-α, IL-1β which are thought to stimulate protein catabolism. This leads to weight loss and reduced lean body mass which is also known as "rheumatoid cachexia. [Ben IO *et al.*, 2017] The study revealed that after a period of 28 days there was significant decrease in body weight in FCA induced arthritic control. The PBB treated rats at 200mg/kg and

400mg/kg showed increase in body weight by 14.02% and 12.78% respectively while the Diclofenac Sodium showed increase in body weight by 5.22%.

Paw edema and ankle diameter

The Freund's Complete Adjuvant induce arthritis induces soft tissue swelling in joints, erosion of joint cartilage and bone destruction. [Rajaram C *et al.*, 2015] These changes result in loss of joint stability and slows down motility and locomotory activity in rats. Phosphate buffer saline extract of *Bellamya bengalensis* at a dose of 200mg/kg and 400mg/kg showed inhibition of paw edema by 90.23% and 96.27% and the ankle diameter was reduced by 79% and 84% respectively when compared with FCA induced arthritic controlled rats.

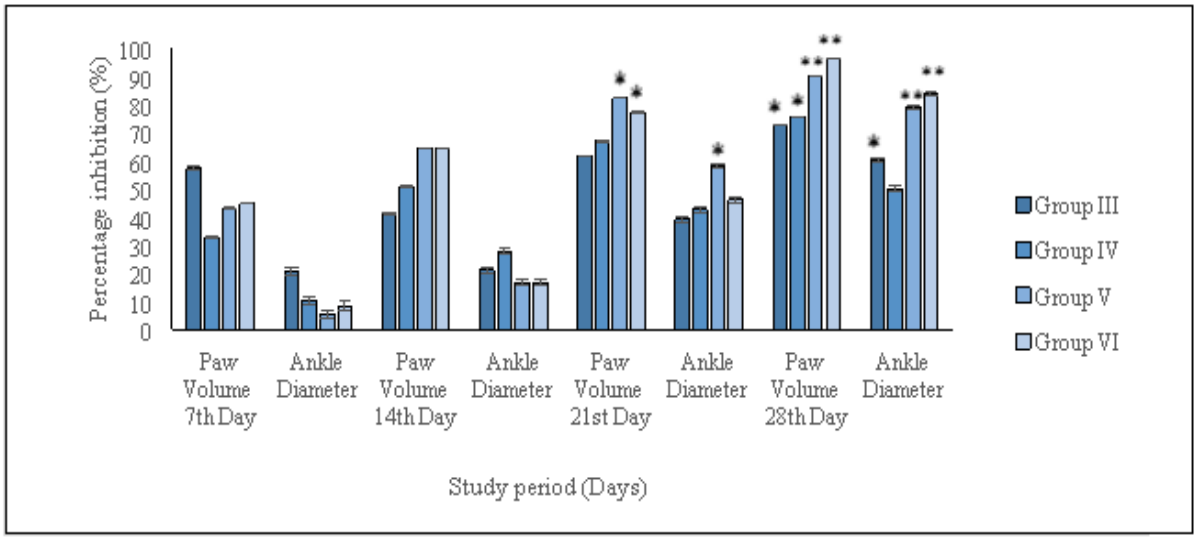

Fig. 2. Effect of phosphate buffer saline extract of *Bellamya bengalensis* Lam. on paw volume and ankle diameter in FCA induced rats. Values are expressed as mean ± SEM, n=6. Data were analyzed by One-way ANOVA followed by Dunnett post-hoc test, $^{*}p < 0.05$, $^{**}p < 0.01$ when compared to FCA induced arthritic control (Group II).

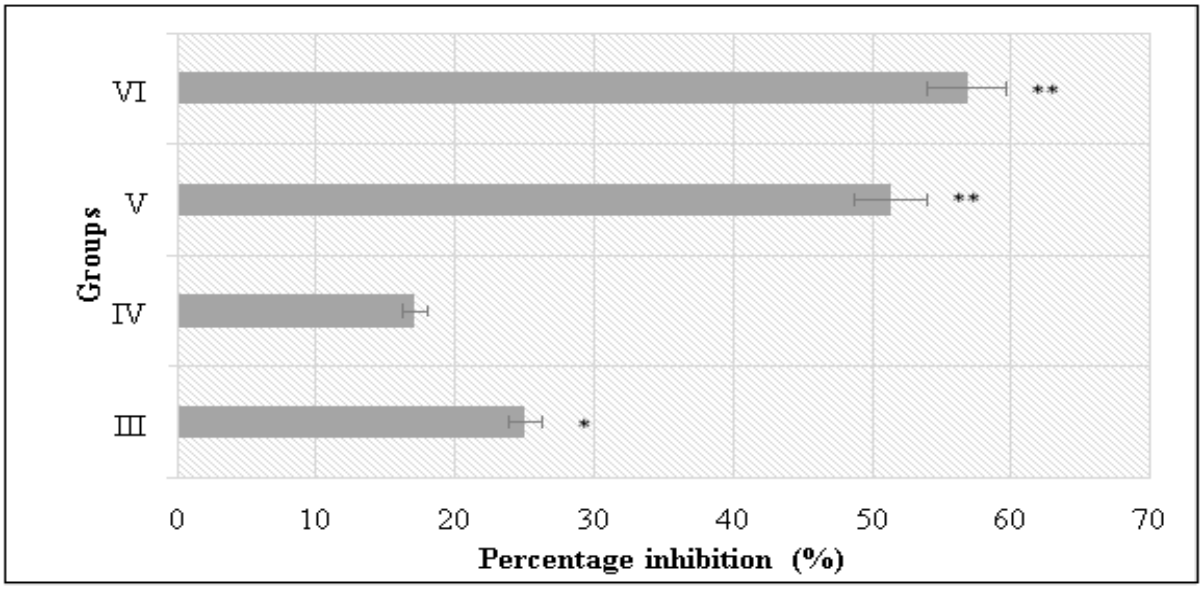

Fig. 3. Effect of phosphate buffer saline extract of *Bellamya bengalensis* Lam. on proinflammatory cytokine marker, TNF-α in FCA induced rats. Values are expressed as mean ± SEM, n=6. Data were analyzed by One-way ANOVA followed by Dunnett post-hoc test, $^{*}p < 0.05$, $^{**}p < 0.01$ when compared to FCA induced arthritic control (Group II).

This result can be compared with a similar study done on the lymph extracted from *Bellamya bengalensis* f. annandalei showed significant reduction in paw volume in Freund's induced arthritic model at 50mg/kg and 100 mg/kg of the extract. [Ray M *et al.*, 2017] The analgesic effect of the PBB extracts at a dose of 200mg/kg and 400mg/kg was evident as the locomotory activity and motility score increased considerably when arthritic control.

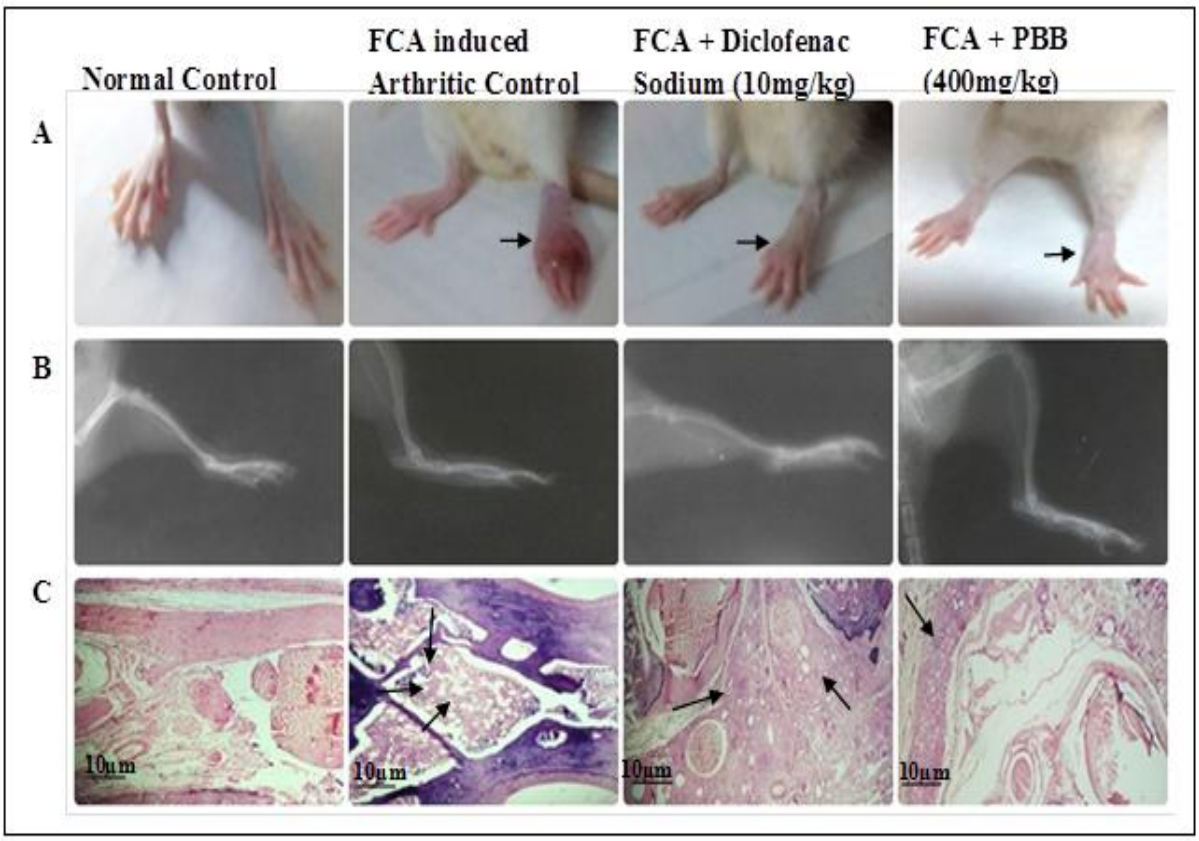

Fig. 4. (A)Photographs depicting inhibition of paw erythema after 28 days of oral administration of the drug. The right hind paw depicting the normal paw while the left hind paw was induced with FCA (indicated with arrows); (B) Radiological imaging of the left hind paw of normal rats, arthritic control induced with FCA, FCA induced rats treated with Sodium diclofenac 10mg/kg and PBB 400mg/kg;(C) Histological sections of paw tissues of normal rats, arthritic control rats, Diclofenac sodium 10mg/kg treated rats, PBB 400mg/kg dose treated rats [C] and PBB 200mg/kg dose treated rats. Sections were stained with Hematoxylin and Eosin, magnification=40x.FCA: Freund's complete adjuvant; PBB: Phosphate Buffer saline fraction of *Bellamya bengalensis* Lamarck.

Serum cytokine level

Pro-inflammatory cytokines play an important role in pathophysiology of chronic inflammatory disorders. TNF-α mediates the signaling through two transmembrane receptors TNFR1 and TNFR2 which regulates cell proliferation, survival, differentiation and apoptosis. [Parameswaran N and Patial S,2010] Pathogenesis of inflammatory diseases increases aberrant expression of TNF-α in serum. High concentration of the cytokine aggravates tissue degeneration. Researchers have shown that the presence of TNF-, IFN-Y, iNOS in the hemocytes of *Bellamya bengalensis*. [Ray M *et al.*,2016].

In the present study, PBB reduced the TNF-α expression in the serum of Freund's adjuvant induced rats by 56.79% at 400 mg/kg.

This can be compared to another study where treatment with water extract of *Bellamya bengalensis*, reduced TNF-alpha levels in arsenic induced toxicity in rats. [Ali S Sk and Maiti S, 2017].

Radiological imaging and histology

One of the important diagnostic tools for arthritis is radiological imaging which helps in determining the severity of the disease. Earlier signs of the disease include soft tissue swelling whereas later narrowing of joint spaces, bony erosions mark the developed stages of arthritis. [Ekambaram S *et al.*, 2010] In the present study, oral administration of PBB extract (400mg/kg) for 28 days have significantly reduced the soft tissue swelling, prevented bony erosion and narrowing of joint spaces. The standard drug Diclofenac Sodium, also considerably reduced the disease progression by abridging soft tissue swelling and narrowing of joint space. Another important biomarker is the histological examination of the inflamed joints. The PBB 400mg/kg lowered the influx of inflammatory cells, cartilage destruction and bone damage.

Antioxidant study of the paw tissue

FCA induced arthritis stimulates polymorphonuclear leucocytes and produce superoxide radicals and hydrogen peroxide, which in the presence of traces of iron salts found in synovial fluids have interaction to form the reactive hydroxyl radical. [Abotsi WM *et al.*, 2010] This could lead to cartilage destruction, impairment of articular constituents and synovial fluid. Superoxide radicals, in addition, is known to cause cellular disruption of membrane lipids. PBB extract (200mg/kg and 400mg/kg) significantly reduced nitric oxide concentration and lipid peroxidation in the paw tissue. PBB also confirms its antioxidant activity by increasing the concentration of glutathione reductase and superoxide dismutase.

Conclusion

In summary, *Bellamya bengalensis* Lamarck was effective in attenuating the Freund's chronic adjuvant induced arthritis in a dose dependent manner in rats. The positive effect of PBB extract in rats in adjuvant induced arthritis model is possibly due to the inhibition of proinflammatory cytokine and prevention of oxidative burst which prevents joint destruction. The results obtained from the present study affirm the use of PBB extract in traditional medicine for management of arthritic conditions.

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