

In vitro and *In vivo* Anti-Arthritic and Anti-Inflammatory Activity of *Bungarus Fasciatus* Venom

Keywords: *Bungarus fasciatus*; Banded krait; Snake venom; Anti-arthritis; Anti-inflammatory

Abstract

In the Indian traditional medicinal system Ayurveda and Unani, the use of snake venom has been mentioned to treat various diseases including arthritis. This prompted us to investigate the anti-arthritic and anti-inflammatory activity of banded krait venom (BFV). In the present study, *in vitro* anti-arthritic activity of BFV was evaluated by bovine serum protein denaturation and egg albumin denaturation and *in vivo* anti-arthritic activity was evaluated by collagenase induced arthritis in male albino Wistar rats. *In vitro* anti-inflammatory activity was evaluated by RBC membrane stabilization and *in vivo* anti-inflammatory activity was evaluated by histamine/ formalin induced paw edema in male albino Swiss mice. BFV treatment significantly inhibited bovine serum protein and egg albumin denaturation. In collagenase induced arthritis model, BFV treatment significantly reduced serum proinflammatory cytokines TNF- α , IL-1 β and IL-17, significantly increased percentage stabilization of RBC membrane and significantly decreased histamine/ formalin induced paw edema. The findings of the present study showed that *Bungarus fasciatus* venom possesses significant *in vitro* and *in vivo* anti-arthritic and anti-inflammatory activities and may serve as a natural biomedical remedial agent against inflammatory conditions.

Introduction

Inflammation, a body defence mechanism is triggered by several conditions including injury. Macrophages and neutrophils take part in acute inflammation while T-cells and plasma cells are leading candidates in chronic inflammation [1]. Osteoarthritis, the most common type of arthritis [2], is most common in the knee joint (6%) and hip joint (3%) [3]. Clinical symptoms include joint pain, swelling and stiffness which may worsen after activity or inactivity [4]. Many studies have indicated that inflammation of the synovium may play an important role in the pathogenesis of OA [5,6]. Activated synoviocytes, macrophages and articular cartilage itself produce a variety of proinflammatory cytokines like TNF- α and IL-1 β contributing to tissue destruction [7,8]. Rheumatoid arthritis, another form of inflammatory arthritis, involves chronic inflammation of the synovial membrane in the joints of hands and feet with subsequent cartilage and bone erosion and destruction [9]. Nearly 1% of the world population is affected by rheumatoid arthritis [10]. Interplay between pro- and anti-inflammatory cytokines contribute to the pathogenesis in RA. There are many anti-inflammatory drugs (NSAIDs) and anti-arthritic drugs (DMARDs) that have wide applications in clinical conditions [11] and are associated with several side effects like G.I tract complications, ulcers, cardiovascular problems [12,13]. Therefore, alternative therapies from natural resources are ventured throughout the world.

Therapeutic use of snake venom to treat various diseases including arthritis has been mentioned in age old medicinal systems like Ayurveda, Unani and folk medicine [14]. Snake venom

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protein toxins like NKCT-1, NN-32 showed anti-arthritic activity in experimental animal models [15]. *Bungarus fasciatus*, one of the member of elapidae snake family, is distributed throughout eastern India including West Bengal. Though its venom contains many lethal cardiotoxic, neurotoxic compounds, several research works has been carried out with its venom which revealed its potential to treat various diseases. *Bungarus fasciatus* venom possesses an anticancer compound that could induce apoptosis of cancer cells and arrest cell-cycle progression in the check points [16]. An antibiotic compound named Cathelicidin BF was isolated from *Bungarus fasciatus* venom [17]. But there is no scientific evidence of using *Bungarus fasciatus* venom to treat arthritis. The present research work was aimed to evaluate the *in vitro* and *in vivo* anti-arthritic and anti-inflammatory activity of *Bungarus fasciatus* venom in experimental models.

Materials and Methods

Chemicals and drugs

Diclofenac sodium used under the brand name Voveran (Novartis, India). Other chemicals and reagents used are- BSA, Histamine, Collagenase (Sigma-aldrich, USA); Formaldehyde and Indomethacin (SRL, India); TNF- α , IL-1 β and IL-17 ELISA kits (R & D Systems, USA). All other chemicals and reagents used were of analytical grade.

Collection and preparation of venom

Lyophilized *Bungarus fasciatus* venom (BFV) was commercially purchased from Calcutta snake park, Kolkata, India. A concentration of 1mg/ ml was achieved by dissolving lyophilized BFV in 0.9% NaCl.

Animals

Adult Wistar albino male rats (120 \pm 10 g) and Swiss albino male mice (20 \pm 2 g) were purchased from approved animal breeders. They were kept in polypropylene cages, temperature maintained at 25 \pm 2 °C, relative humidity 65% and given standard pellet diet and water *ad libitum*. Animal ethical clearance was availed before all experiments (IAEC-III/ Proposal/ AG-02/2012 dated 07.06.2012).

In vitro anti-arthritic activity

In vitro anti-arthritic activity of BFV was evaluated using

bovine serum protein denaturation method [18] and egg albumin denaturation method [18].

Bovine serum albumin (BSA) denaturation method: Test solution (0.5 ml): 0.5% w/v aqueous solution of BSA (0.45 ml) and test solution (0.05 ml) of different concentrations were used.

Test control solution (0.5 ml): 0.5% w/v aqueous solution of BSA (0.45 ml) and distilled water (0.05 ml) were used.

Product control (0.5 ml): 0.45 ml distilled water and test solution (0.05 ml) of different concentrations were used.

Standard solution (0.5 ml): 0.5% w/v aqueous solution of BSA (0.45 ml) and diclofenac sodium (0.05 ml) of different concentrations were used.

Test solution (0.05 ml) of different concentrations (2, 5, 10, 20, 30 and 60 µg/ml) and standard drug diclofenac sodium (0.05 ml) of different concentrations (2, 5, 10, 20, 30 and 60 µg/ml) were mixed with 0.5% w/v aqueous solution of BSA (0.45 ml). Then the samples were incubated at 37 °C for 20 min followed by incubation at 57 °C for 3 min. 2.5 ml of phosphate buffer (pH 6.3) was added to all the above samples after cooling. UV-Visible spectrophotometer (Analab) was used to measure the absorbance at 255 nm. The control represents 100% protein denaturation. The percentage inhibition of protein denaturation was calculated by the following formula:

Percentage inhibition = $100 - \left[\frac{\text{optical density of test solution} - \text{optical density of product control}}{\text{optical density of test control}} \right] \times 100$.

Egg albumin denaturation method: 0.2 ml of fresh hen's egg albumin, 2.8 ml of phosphate buffered saline (pH 6.4) and test solution of different concentrations (2, 5, 10, 20, 30 and 60 µg/ml) or standard drug diclofenac sodium (0.05 ml) of different concentrations (2, 5, 10, 20, 30 and 60 µg/ml) were mixed to form a reaction mixture of 5 ml. Double distilled water of same volume served as control. The samples were incubated at 37±2 °C in a BOD incubator for 15 min followed by heating at 70 °C for 5 min. UV-Visible spectrophotometer (Analab) was used to measure the absorbance at 255 nm. The percentage inhibition of protein denaturation was calculated by the following formula:

Percentage inhibition = $100 \times \left[\frac{\text{absorbance of test sample}}{\text{absorbance of control} - 1} \right]$

In vivo anti-arthritic activity

In vivo anti-arthritic activity of BFV was evaluated using collagenase induced osteoarthritis model [19].

Collagenase induced arthritis model: Induction of osteoarthritis was done in adult Wistar male albino rats (120±10 gm) by intra-articular injection of 20 µl bacterial collagenase (5 CDU) solution in the right knee joint. Same amount of saline was injected in the left knee joint. After treatment of 14 days, animals were sacrificed and serum pro inflammatory cytokines were measured.

Treatment schedule: Animals were randomised in 4 groups (n=6). Group 1: Sham control, Group 2: Osteoarthritic control, Group 3: Standard drug, Indomethacin treated (0.25 mg.kg⁻¹, p.o. × 5 days, alternatively), Group 4: BFV treated (0.3 mg.kg⁻¹, i.p. × 14 days)

Measurement of serum cytokines: Serum pro inflammatory cytokines TNF-α, IL-1β and IL-17 were measured using ELISA kits (R & D Systems, USA).

In vitro anti-inflammatory activity

In vitro anti-inflammatory activity of BFV was evaluated using RBC membrane stabilization method [20].

RBC membrane stabilization method: Goat red blood cells were used for the study of membrane stability. Goat blood was collected and mixed with 3.8% tri-sodium citrate solution and centrifuged at 3000 rpm. After washing the packed cells in isosaline, 10% suspension was made. 4.5 ml reaction mixture was prepared with 0.5 ml of 10% RBC suspension, 1 ml of phosphate buffer (pH 7.4), 2 ml of hyposaline (0.25% NaCl) and 1 ml of test solution of different concentrations (2, 5, 10, 20, 30 and 60 µg/ml). The samples were then incubated at 37 °C for 30 min followed by centrifugation for 20 min at 3000 rpm. Supernatant solution was tested for haemoglobin content at 560 nm using a UV-Visible spectrophotometer (Analab).

In vivo anti-inflammatory activity

In vivo anti-inflammatory activity of BFV was evaluated using histamine induced paw edema model [21] and formalin induced paw edema model [22].

Histamine induced paw edema model: Histamine induced paw edema was analyzed in mice model. Mice were randomised into three groups (n=6). Gr.1: Histamine control, Gr.2: Standard drug, Indomethacin treated (10 mg.kg⁻¹, i.p.), Gr.3: BFV treated (1.25 mg.kg⁻¹, i.p.). The initial volume of right hind paw of mice was measured using digital caliper (Mitutoyo, Japan). Then, 0.02 ml of 1% histamine was injected subcutaneously into the subplantar region of right hind paw. The volume of right hind paw was measured at 1, 2, 3 hour after histamine injection for determining the volume of paw edema. Standard drug, Indomethacin and BFV were given one hour before histamine injection.

Formalin induced paw edema model: Formalin induced paw edema was analyzed in mice model. Mice were randomised into three groups (n=6). Gr.1: Formalin control, Gr.2: Standard drug, Indomethacin treated (10 mg.kg⁻¹, i.p.), Gr.3: BFV treated (1.25 mg.kg⁻¹, i.p.). The initial volume of right hind paw of mice was measured using digital caliper (Mitutoyo, Japan). Then, 0.02 ml of 2% formalin was injected subcutaneously into the subplantar region of right hind paw. The volume of right hind paw was measured at 1, 2, 3 hour after formalin injection for determining the volume of paw edema. Standard drug, Indomethacin and BFV were given one hour before formalin injection.

Statistical analysis

Data expressed as mean ± SEM (n=6). One way ANOVA was carried out for determination of significant differences between groups. *p<0.05 was considered to be statistically significant (Gr.1 vs Gr.2) and **p<0.05 was considered to be statistically significant (Gr.2 vs Gr.3/Gr.4).

Results

In vitro anti-arthritic activity

Effect of BFV on bovine serum albumin (BSA) denaturation:

BFV at a concentration of 2, 5, 10, 20 and 30 µg/ml showed inhibition of BSA denaturation by 3.06%, 4.36%, 4.97%, 9.39% and 20.86% respectively whereas standard drug, diclofenac sodium at a concentration of 2, 5, 10, 20 and 30 µg/ml showed inhibition of BSA denaturation by 2.92%, 5.69%, 9.79%, 13.05% and 20.29% respectively (Figure 1).

Effect of BFV on egg albumin denaturation: BFV at a concentration of 2, 5, 10, 20 and 30 µg/ml showed inhibition of egg albumin denaturation by 12.06%, 17.25%, 21.81%, 26.59% and 31.15% respectively whereas standard drug, diclofenac sodium at a concentration of 2, 5, 10, 20 and 30 µg/ml showed inhibition of egg albumin denaturation by 5.67%, 7.11%, 12.46%, 17.33% and 21.65% respectively (Table 1).

In vivo anti-arthritic activity

Effect of BFV on serum proinflammatory cytokines: BFV significantly reduced serum pro inflammatory cytokines TNF-α, IL-1β and IL-17 by 42.17%, 58.14% and 26.91% respectively; whereas standard drug, Indomethacin showed a reduction of TNF-α, IL-1β and IL-17 by 46.83%, 57.07% and 45.81% respectively when compared to arthritic control rats (Figure 2).

In vitro anti-inflammatory activity

Effect of BFV on RBC membrane stabilization: BFV at a concentration of 2, 5, 10, 20 and 30 µg/ml showed stabilization of RBC in hypotonic solution by 25.98%, 32.66%, 40.43%, 56.40 % and 60.29% respectively whereas standard drug, diclofenac sodium at a concentration of 2, 5, 10, 20 and 30 µg/ml showed stabilization of RBC in hypotonic solution by 22.67%, 29.84%, 45.52%, 52.59% and 56.66% respectively (Figure 3).

In vivo anti-inflammatory activity

Effect of BFV on histamine induced paw edema: BFV showed significant reduction of histamine induced paw edema by 65.85% whereas standard drug, Indomethacin showed significant reduction of histamine induced paw edema by 70.73% when compared to histamine control group (Table 2).

Effect of BFV on formalin induced paw edema: BFV showed significant reduction of formalin induced paw edema by 34.23% whereas standard drug, Indomethacin showed significant reduction of formalin induced paw edema by 44.97% when compared to formalin control group (Table 3).

Table 1: Effect of Bungarus fasciatus venom on egg albumin denaturation. Data expressed as mean ± SEM (n=4).

Treatment	Dose (µg/ml)	Absorbance	% Inhibition
Control	-	1.252±0.014	-
BFV	2	1.403±0.041	12.06
BFV	5	1.468±0.012	17.25
BFV	10	1.525±0.019	21.81
BFV	20	1.585±0.024	26.59
BFV	30	1.642±0.017	31.15
Diclofenac	2	1.323±0.029	5.67
Diclofenac	5	1.341±0.056	7.11
Diclofenac	10	1.408±0.014	12.46
Diclofenac	20	1.469±0.022	17.33
Diclofenac	30	1.523±0.031	21.65

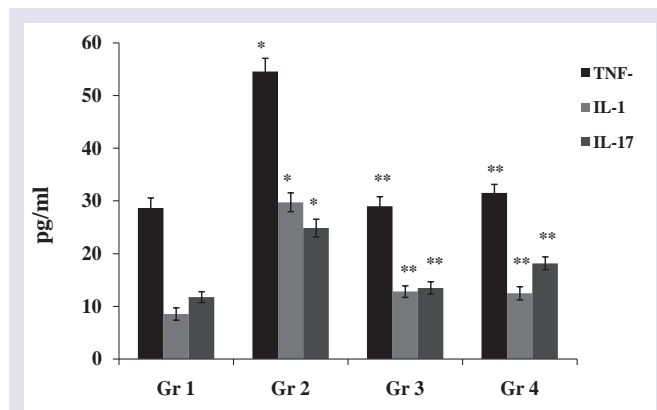


Figure 2: Effect of Bungarus fasciatus venom on serum pro-inflammatory cytokines TNF-α, IL-1β and IL-17 levels in FCA induced rats. Data expressed as mean ± SEM (n=6). One-way ANOVA was done for statistical analysis and *p<0.05 was considered as significant, (Gr.1 vs Gr.2) and **p<0.05 was considered as significant, (Gr.2 vs Gr.3/Gr.4) where Gr.1- Sham control, Gr.2- Arthritis control, Gr.3- Standard drug (Indomethacin) treated, Gr.4- BFV (0.3 mg.kg⁻¹) treated.

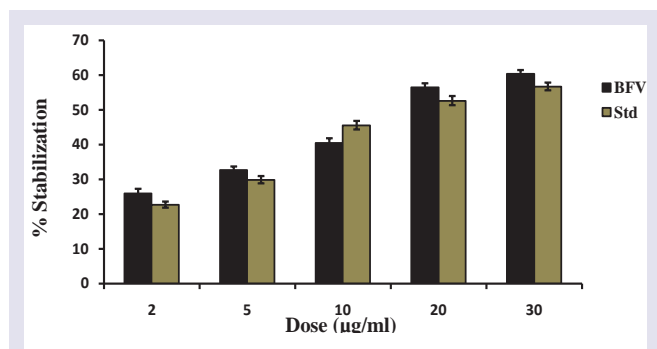


Figure 3: Effect of Bungarus fasciatus venom on RBC membrane stabilization. Data expressed as mean ± SEM (n=4).

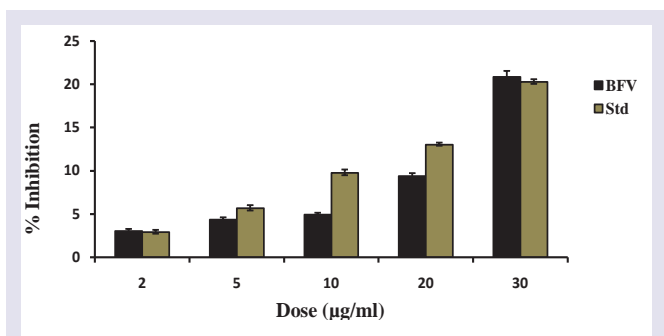


Figure 1: Effect of Bungarus fasciatus venom on bovine serum albumin denaturation. Data expressed as mean ± SEM (n=4).

Discussion

Rheumatoid arthritis, an autoimmune disease, involves denaturation of proteins and hence production of auto-antigens. Heat induced denaturation of BSA involves presentation of antigens that are related to diseases such as rheumatoid arthritis [23]. Denaturation of BSA was inhibited by BFV which suggested that BFV might prevent

Table 2: Effect of Bungarus fasciatus venom and standard drug (Indomethacin) on histamine induced paw edema. Data expressed as mean ± SEM (n=6).

Treatment	Dose (mg/kg)	Paw diameter (Initial)	Paw diameter (After 3 hours)	Mean paw edema (mm)	% Inhibition
Histamine control	Vehicle	1.87 ± 0.03	3.10 ± 0.05	1.23 ± 0.09	-
BFV	1.25	2.06 ± 0.04	2.48 ± 0.02	0.42 ± 0.08	65.85
Indomethacin	10	2.09 ± 0.03	2.45 ± 0.04	0.36 ± 0.05	70.73

Table 3: Effect of Bungarus fasciatus venom and standard drug (Indomethacin) on formalin induced paw edema. Data expressed as mean ± SEM (n=6).

Treatment	Dose (mg/kg)	Paw diameter (Initial)	Paw diameter (After 3 hours)	Mean paw edema (mm)	% Inhibition
Formalin control	Vehicle	1.93 ± 0.02	3.42 ± 0.04	1.49 ± 0.05	-
BFV	1.25	2.01 ± 0.03	2.99 ± 0.02	0.98 ± 0.02	34.23
Indomethacin	10	1.96 ± 0.02	2.78 ± 0.03	0.82 ± 0.04	44.97

denaturation of protein in rheumatoid arthritis and hence a potential anti-arthritic agent. Heat induced denaturation of egg albumin was inhibited by BFV which again suggested that BFV might prevent protein denaturation in rheumatoid arthritis and hence a potential anti-arthritic agent.

Due to infiltration of T-lymphocytes and macrophages into the synovial joint as well as their presence in peripheral blood, high levels of pro-inflammatory cytokines like TNF-α, IL-1β and IL-17 were found in the serum of arthritic patients [24]. Activated macrophages release TNF-α which in turn induces the release of IL-1β. Activated T-cells release IL-17 which in turn act as a stimulator of the release of TNF-α and IL-1β from macrophages [25]. BFV significantly reduced the levels of pro-inflammatory cytokines TNF-α, IL-1β and IL-17, this indicated that BFV might restore the balance of cytokines and hence could act as a potential anti-arthritic agent.

It is known that membrane of RBC is structurally equivalent to the lysosomal membrane. So any substance protecting RBC membrane can be predicted as a stabilizer of lysosomal membrane. Extracellular release of lysosomal content from neutrophil causes tissue damage and inflammation in arthritis [26]. RBC membrane lyses due to different injuries and causes haemolysis and oxidation of haemoglobin. Such type of damage of RBC further causes secondary damage through free radical induced lipid peroxidation as well as release of inflammatory mediators like phospholipases [11]. According to our result BFV could actively protect RBC membrane lysis and hence could be predicted as a potential anti-inflammatory agent.

Histamine induced inflammation is a good model for studying paw edema as histamine is a potent vasodilator, increases vascular permeability and can attract neutrophils at the target site. This results in increased blood flow and plasma exudates leak into the inflamed area, ultimately leading to redness and swelling [27]. Histamine induced paw edema was significantly reduced by BFV which suggested that BFV might interfere with the release or synthesis of this inflammatory mediator. Formalin induced inflammation is due to cell damage that result in the release of other inflammatory mediators like histamine, serotonin, bradykinin and prostaglandins [22]. This ultimately leads to redness, swelling and edema. Formalin induced paw edema was significantly reduced by BFV which suggested that BFV might interfere with the release or synthesis of these inflammatory mediators.

Conclusion

It may be concluded that *Bungarus fasciatus* venom possesses significant *in vitro* and *in vivo* anti-arthritic and anti-inflammatory activities. Further studies are warranted on the active venom constituents involved in this process.

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