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Anti-Inflammatory Activities of Indian Fresh Water Edible Mollusca

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Abstract: *Animal derived natural products contributed a major part in traditional medicine to prevent inflammation related diseases. The proposed work was aimed to study on the extrapallial fluid of the freshwater mollusca, Bellamya bengalensis (L), family Viviparidae in experimental regimes. The extrapallial fluid of Bellamya bengalensis was inspirited out, partially purified with centrifugation (3500 rpm for 15 min at 4°C) and standardized using protein calibration (BBE). The bioactivities of BBE were examined for erythrocytes membrane stabilizing, protein denaturation inhibiting and proteinase inhibiting actions in test tubes. Further, carrageenan induced acute and Freund's adjuvant induced sub-acute paw inflammations were conducted in rats. Finally, central analgesic activities were also done using Eddy's hot plate and tail immersion tests. BBE contained ~0.1% proteins in slightly acidic medium (pH 6.6) and exhibited significant proteinase inhibitory actions and membrane stabilizing properties. BBE showed dose dependent inhibition in rat paw inflammation measured plethysmographically and also histopathologically. It also showed significant analgesic properties in mice. The results indicated the analgesic and anti-inflammatory actions of extrapallial fluid of Bellamya bengalensis.*

Keywords: *Bellamya bengalensis, mollusca, inflammation, analgesia*

I. Introduction

Searching of foods and therapeutic remedies from animal origin invertebrates have long been practice, since the time of human civilization. Modern systemic efforts established several therapeutically active animal derived compounds such as, bryostatin 1, didemnin B, halichondrin B, ectenascidin 743 etc. Most of these compounds showed their anti-inflammatory, immunosuppressant, anti-tumor, anticancer activities.[1-3] In India and other south Asian countries animal derived medicines are very popular, particularly in chronic disorders.[4] It has been reported that fresh water mollusca (gastropod) *Bellamya bengalensis* L (family Viviparidae) is widely used in Indian traditional medicine to treat conjunctivitis, visional dilemma, joint pains, enlarged spleen, gastritis, jaundice, asthma, and healing of wounds.[5-9] The species is ovoviviparous and its operculum is a thin teardrop-shaped disk of flexible protein that forms a door close the aperture and two long cephalic tentacles are located dorsally beside the base of the snout.[10] The extrapallial fluid fills the cavity between the most outer visceral organ (mantle) and the external shell. By considering the essential role of extrapallial fluid in mineralization/demineralization process *in vivo*, it was decided to investigate the characteristics of extrapallial fluids of *Bellamya bengalensis* on inflammations in laboratory animals.

II. Materials & Methods

2.1 Test Drug Preparation

The identically matured fresh water mollusca, *Bellamya bengalensis* Lamarck (weight 6 to 9 g with shell) were collected from the marshland in Kolkata and authenticated by Zoological Survey of India, Kolkata (Specimen 1242/Lot No-63, November 11, 2013). The living mollusca were cleaned in tap water and kept in sufficient deionized water for 24 h for their acclimatization. Thereafter, the extrapallial fluid was inspirited out with a sterile 1 ml syringe, placing bevel down on the shell margin and slid beneath the mantle at the pallial line into extrapallial space without disturb the mantle, shell attachment.[11] The fluid was then centrifuged at 3500 rpm for 15 min at 4°C to precipitate the residual debris. The clear supernatant was collected (BBE). Thereafter, pH, specific gravity, suspended matter and protein concentration were analyzed. [12-14]

2.2 In vitro Anti-inflammatory Studies

2.2.1 Inhibition of Protein Denaturation

The protein denaturation inhibition of BBE was estimated using the method of Mizushima *et al.*, 1968.[15] The reaction mixture consisted of 0.4 ml of 5% bovine aqueous serum albumin and 0.5 ml of BBE

(at serial dilutions) in glass tubes and incubated at 37^{o}C for 20 min. Thereafter, the tubes were further incubated at 57°C for 3 min and diluted with phosphate buffer saline (4.5 ml, pH 6.3). Distilled water was used in blank control and diclofenac sodium solution was used as standard, while Bovine Serum Albumin was missing in all product control tubes. Finally the turbidity was measured at 660 nm and IC_{50} of BBE was determined.

2.2.2 Erythrocytes Membrane Stabilization

The membrane stabilization of BBE was determined using method of Sur *et al.* (2002).[16] The reaction volume was made up to 4.5 ml by adding 2 ml of 0.25% hypotonic saline, 1 ml 0.15 M phosphate buffer saline (pH 7.4) and 1 ml of BBE (at serial dilutions). Thereafter, 0.5 ml of 1% ($2X10^5$) fresh rabbit erythrocytes in normal saline was added. Isotonic saline was used in blank control and diclofenac sodium in standard, while, product control tubes lacked with red cells. All tubes were incubated for 30 min at 56°C. After centrifugation the absorbance of the supernatants was read at 560 nm and IC_{50} of BBE was determined.

2.3 Proteinase Inhibitory Action

The reaction mixture in proteinase enzyme inhibitory assay consisted with 2.0 ml containing 0.06 mg of trypsin (EC 3.4.21.4), 1.0 ml of 25 mM tris-HCl buffer (pH 7.4) and 1.0 ml BBE (at serial dilutions). Diclofenac sodium was used as standard. Incubation was made at 37°C for 5 min followed by adding 1 ml of 0.08% (w/v) casein. Thereafter, the tubes were further incubated for 20 min and finally the reaction was terminated by adding 2 ml of 70% perchloric acid. The cloudy mixture was centrifuged and read at 280 nm against buffer as blank. [16] The proteinase inhibitory action of BBE was expressed as IC_{50}.

2.3 Animal Protocol

Experiments were carried out on male Wistar rats (150-160 g body weight) and male Swiss mice (25-30 g body weight). The animals were kept in polypropylene cages and received food pellets and water *ad libitum*. Institutional animal ethics committee approved the experimental protocol (RKC/IAEC/13/18 dated 10.05.2013).

2.3.1 Acute Toxicity Study

The acute oral toxicity studies of BBE were performed as per revised Organization for Economic Cooperation and Development (OECD) guidelines 423.[17] BBE was administered orally to overnight fasted rats at the doses of 0.25, 0.5, 1.0 and 2.0 ml/100 g of body weight. After BBE administration the animals were closely observed for any toxic manifestation. Thereafter, observations were made at regular intervals for 48 hours. Further, the animals were under investigation up to a period of 2 weeks for mortality and general behavior.

2.3.2 Acute in vivo Anti-inflammatory Test

2.3.2.1 Carrageenan –induced Paw Edema in Rats

Wistar rats were divided into five groups (N=6) as follows: Group I: 2 ml/kg body weight normal saline; Group II: diclofenac sodium 10 mg/kg body weight; Group III: BBE 1.66 µl/g (~1.8 µg protein/g) body weight; Group IV: BBE 3.33 µl/g (~3.6 µg protein/g) body weight; Group V: BBE 6.66 µl/g (~7.2 µg protein/g) body weight. All test drugs were given orally 30 mins prior to carrageenan (0.1 ml of 1% in normal saline) injection (s.c.) into the sub plantar surface of the left hind paw.[18-19] The paw volume up to ankle joint was measured plethysmographically at 1 h, 2 h, 3 h and 4 h after injection.

2.3.3 Sub-acute in vivo Anti-inflammatory Test

2.3.3.1 Freund's adjuvant-induced Poly-arthritis

Wistar rats were divided into five groups (N=6) as describe before. At the volume of 0.1 ml of Freund's complete adjuvant (FCA) was injected into the sub plantar pad of each rat. All test drugs were given orally for 21 consecutive days. [20] The paw volume was recorded plethysmographically at day 1, 7, 14 and 21 after injection. The animals were autopsied and the injected paw was processed for histopathological examinations.

2.4 Analgesic Activity Test

2.4.1 Eddy's Hot Plate Method

Swiss mice (25-30 g body weight) were divided into six groups (N=6) as follows: Group I: 2 ml/kg body weight normal saline; Group II: Diclofenac sodium 10 mg/kg body weight; Group III: 1.66 µl/g (~1.8 µg protein/g) body weight; Group IV: BBE 3.33 µl/g (~3.6 µg protein/g) body weight; Group V: BBE 6.66 µl/g (~7.2 µg protein/g) body weight; and Group 6: BBE 13.32 µl/g (~14.4 µg protein/g) body weight. All drugs

were given orally. The animals were placed on the Eddy's hot plate maintained at 55±1°C and the reaction time in sec was recorded at 0, 30 and 60 min after the treatment, with a cut-off time of 90 sec. [21]

2.4.2 Tail Immersion Method

Swiss mice (25-30 g body weight) were divided into six groups (N=6) as mentioned before. The test drug was given orally 1 h prior to examination. The lower 5 cm portion of the tail was immersed in a beaker of water maintained at 55±1°C; with a cut-off time of immersion at 30 sec. [21] The time in sec for tail withdrawal from the hot water was recorded.

2.5 Statistical Analysis

Results were expressed as Mean ± standard error of mean. The data were statistically analyzed by one way analysis of variance (ANOVA) and Duncan post-hoc test. p values less than 0.05 were considered as significant.

III. Results

The gross weight of Bellamya bengalensis (N=50) including outer shell was estimated 8.82 g (range between 6 to 9 g weight) and the volume of extrapallial fluid inspirited from each gastropod was maximum 0.6 to 1.0 ml in accordance with their size. The pH of BBE was slightly acidic in nature (6.63). The specific gravity was estimated to 1.0068, while the suspended matter was calculated nearly 0.021%. The protein concentration on BBE was 11.47 (range between 10 to 12 mg/ml or ~0.1%). The effective doses for in vivo experiments were calculated on the basis of protein concentration in BBE [Table 1].

In vitro assay revealed 50% inhibitory concentration (IC_{50}) of BBE on protein denaturation was 87.56 µl/ml, rabbit erythrocyte membrane lysis was 58.82 µl/ml and proteinases inhibitory action was 87.56 µl/ml, while diclofenac sodium exhibited 5.33 µg/ml, 3.15 µg/ml and 8.52 µg/ml on respective tests [Table 2].

BBE also showed efficacy in anti-inflammatory actions in rats by lowering paw volume − both in acute and sub-acute models similar to standard diclofenac sodium. BBE significantly and dose dependently inhibited paw inflammation in carrageenan-induced rats up to 54% [Table 3] and also Freund's adjuvant-induced rats nearly 56% [Table 4] in respect to negative control.

Histopathological studies showed BBE reduced the number of vacuoles in subcutaneous tissue and infiltration of inflammatory cells than control rats [Figure 1].

BBE also exhibited significant and dose dependant analgesic effects as evidenced by Eddy's hot plate method (110.18%) and tail immersion (63.30%) in mice [Table 5].

IV. Discussion

Inflammation is a response of the body towards immune system that are likely to be mediated either through macrophages, inflammatory leukocytes such as neutrophils, lymphocytes and monocytes and or by group of inflammatory cytokines.[9,22-23] It is now well known that the generation of free radicals particularly during joint inflammation denatures the proteins in cartilages by activating lysosomal protease enzyme.[24] Further, prostaglandins, a regulatory inflammatory mediator facilitate in all phases of inflammatory reactions and elicit pain either by direct stimulation of sensory nerve endings or to other pain provoking stimuli.[24-25] Hence, prostaglandin synthesis inhibitors or non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used in the treatment of inflammations, arthritis and pain, but serious adverse events have already alerted against repeated use of NSAIDs.[25-26] Hence, it may an urgent need to develop new safer and effective anti-inflammatory drugs, perhaps from natural origin.

The use of fresh water molluca as protein-rich food is very much in practice in number of south Asian countries namely India, Bangladesh, Taiwan, Philippines and Thailand. [27-28] Research reveals, molluscan shell growth occur throughout the life. It is assumed to be an organic matrix mediated process controlled by a network of macromolecules composed of protein, carbohydrates and glycoprotein. Interestingly, it should be consider that extrapallial fluid is mainly derived from the secretary organic materials of mantle and also is a dynamic physiological medium of the gastropod. [11-12] In the present study, the characteristic nature of extrapallial fluid of Bellamya bengalensis (BBE) involved the presence of acidic conjugated proteins/or lower peptides Mizushima et al., (1968) [15] has been described the role of NSAIDs in rheumatoid arthritis were mediated through the suppression of proteins denature in inflamed joints. Earlier it was reported that erythrocytes membrane are prone to damaged by free radicals through lipid peroxidation, similar to lysosomal membrane and hence, used as a tool for anti-inflammatory drug monitoring.[15-16] In this study, BBE showed promising erythrocytes membrane stabilizing activity. Similarly, neutral serine proteinases have reported to abundantly present in lysosomal granules and facilitate to initiate the release of pro-inflammatory cytokines during inflammation. [24] Nevertheless, BBE showed significant anti-proteinase action and thereby confirm its anti-inflammatory properties. Hence, present experiments exhibited BBE have efficacy on erythrocytes membrane lipid protection, safeguard for proteins from denature and defend protease enzyme activation.

Most of the investigators have reported inhibition of carrageenan induced inflammation is one of the acceptable procedure to identify anti-inflammatory agents.[18-19] The dose dependent inhibitory actions obtained after BBE treatment on carrageenan induced inflammation in rats may be mediated either through prostaglandins-cyclooxygenase mediatory pathways or by protease regulatory cytokines inhibitory pathways. Moreover, BBE attenuated nociceptive thermal stimulation induced pain activities, which supported the suggestion that BBE has possesses centrally acting analgesic activity via monoaminergic-neuronal system.[18] FCA induced arthritis has been used as a model of sub-acute inflammation in rats which is relevant to the pathophysiological control of inflammatory processes correlated to rheumatoid arthritis in human.[19-20] Histopathologically, cellular infiltration, joint space narrowing, synovial hyperplasia, pannas formation and damage of cartilage have been observed in FCA arthritis rats. In this study, BBE dose dependently and significantly negated FCA induced inflammatory symptoms like, paw edema and inflammation, secondary lesions in paw, tail, ear and nose and painful behavioral symptoms. Nevertheless, BBE also reduced cellular inflammatory cell infiltration in vacuoles, enlarged the joint space and lowered the synovial hyperplasia in rats paw. Since, this edible mollusca are available, cheap and used as regular diet in this subcontinent,[27] thus its additional analgesic and anti-inflammatory therapeutic potentiality would be a new lead of research.

V. Conclusion

On the basis of the data obtained in this study it may conclude that the extrapallial fluid of *Bellamya bengalensis* L contains low molecular conjugated protein molecule as bioactive constituents and its anti-inflammatory and analgesic actions could be mediated through its inhibitory actions on protease or prostaglandin biosynthetic pathways. Further studies may require for isolation of more potent compound(s) from extrapallial fluid of the mollusca.

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Table 1: Characterization Of Extrapallial Fluid Of *Bellamya Bengalensis* (BBE)

	Weight (g)	pH	Sp. gravity	Suspended matter (%)	Protein concentration (mg/ml)
BBE	8.32±0.094	6.63±0.082	1.0068±0.007	0.021±0.003	11.47±0.096

Results are expressed as Mean ± standard error of mean; average weight of 50 living species with intact shell (range 6 to 9 g); N=10 for each test; BBE means extrapallial fluid of *Bellamya bengalensis* L; protein concentration was determined using Lowry's method and it was ~0.1% of BBE.

Table 2: *In-vitro* Anti-Inflammatory Activity Of Extrapallial Fluid Of *Bellamya Bengalensis* (BBE)

	Membrane stabilizing activity (IC_{50})	Inhibition of protein denature (IC_{50})	Proteinase inhibitory activity (IC_{50})
BBE (µl/ml)	58.82±2.79	79.36±3.15	87.56±5.07
DC (µg/ml)	3.15±0.68	8.52±0.73	5.33±0.62

Results are expressed as Mean ± standard error of mean; N=6 for each test; BBE means extrapallial fluid of *Bellamya bengalensis* L, DC means Diclofenac sodium and IC_{50} mean inhibitory 50% concentration.

Table 3: *In- vivo* acute anti-inflammatory activity of extrapallial fluid of *Bellamya bengalensis* (BBE)

	Carrageenan induced paw inflammation (Paw volume in ml)				
	0 h	1 h	2 h	3 h	4 h
Group I	1.22±0.22	2.78±0.81	3.12±0.94	3.25±0.75	3.10±0.64
Group II	1.28±0.28	1.75±0.54[a] (-37.05%)	2.08±0.65[a] (-33.33%)	1.71±0.66[a] (-47.38%)	1.46±0.42[a] (-52.9%)
Group III	1.24±0.29	2.36±0.56 (-15.10%)	2.49±0.82[b] (-20.19)	2.18±0.92[a] (-38.15%)	1.95±0.53[a] (-32.09%)
Group IV	1.29±0.35	2.08±0.61[a] (-25.17%)	2.27±0.71[a] (-27.24%)	2.01±0.85[a] (-39.92%)	1.79±0.74[a] (-42.26%)
Group V	1.25±0.23	1.95±0.75[a] (-29.85%)	2.16±0.58[a] (-30.76%)	1.84±0.67[a] (-43.38)	1.53±0.40[a] (-50.64%)

Results are expressed as Mean ± standard error of mean; N=6 for each test; The data were statistically analyzed by one way analysis of variance (ANOVA) and Duncan post-hoc test; [a:]significant different from negative control at P<0.01 and [a:]significant different from negative control at P<0.05; parenthesis indicated percent change in comparison to negative control

Table 4: *In-vivo* Sub-Acute Anti-Inflammatory Activity Of Extrapallial Fluid Of *Bellamya Bengalensis* (BBE)

	Freund's adjuvant induced paw inflammation (Paw volume in ml)			
	Day 0	Day 7	Day 14	Day 21
Group I	1.28±0.34	3.75±0.58	4.13±0.61	3.84±0.47
Group II	1.25±0.31	2.91±0.42[a] (-22.40%)	2.65±0.38[a] (-35.83%)	1.58±0.35[a] (-58.85%)
Group III	1.27±0.29	3.55±0.68 (-5.33%)	3.48±0.72 (-15.74%)	3.10±0.63[b] (-19.27%)
Group IV	1.30±0.26	3.21±0.5 (-14.40%)	3.05±0.61[a] (-26.15%)	2.56±0.51[a] (-33.34%)
Group V	1.26±0.23	2.78±0.39[a] (-25.86%)	2.54±0.34[a] (-38.49%)	1.74±0.32[a] (-54.68%)

Results are expressed as Mean ± standard error of mean; N=6 for each test; The data were statistically analyzed by one way analysis of variance (ANOVA) and Duncan post-hoc test; [a]:significant different from negative control at P<0.01 and [b]:significant different from negative control at P<0.05; parenthesis indicated percent change in comparison to negative control

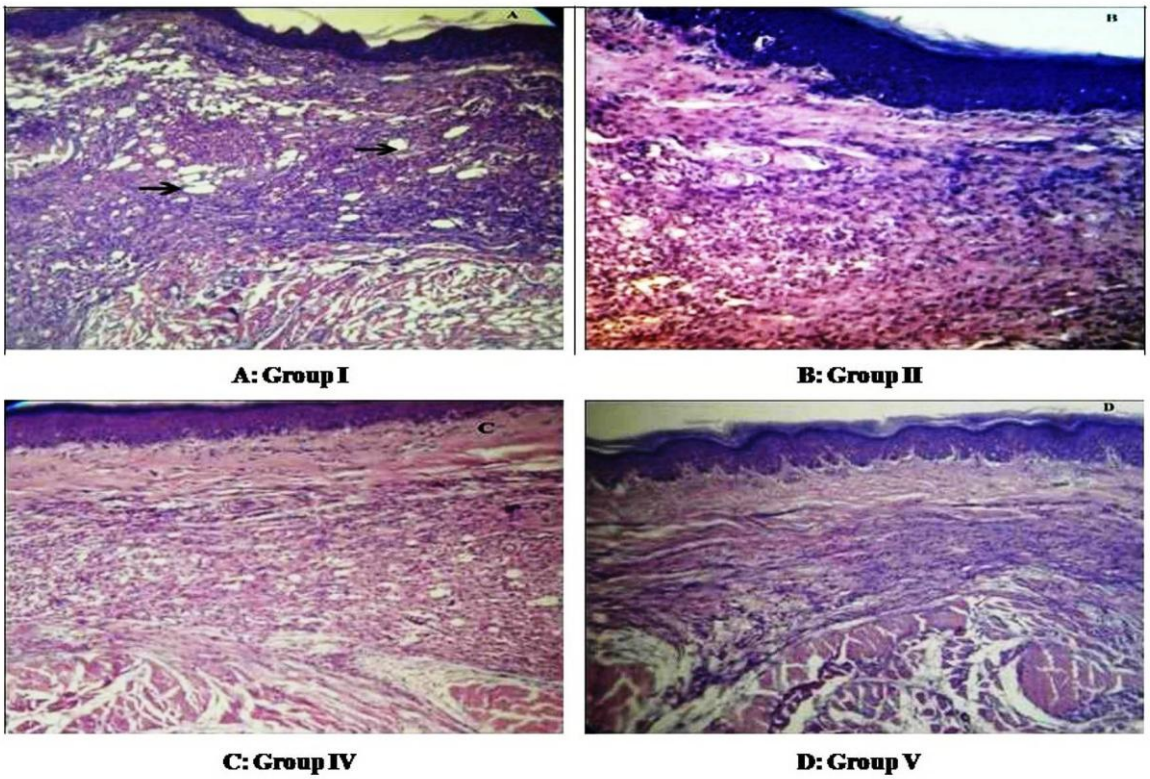
Table 5: *In-vivo* analgesic activity of extrapallial fluid of *Bellamya bengalensis* (BBE)

	Eddy's Hot Plate method			Tail immersion method	
	0 min	30 min	60 min	Initial	60 min
Group I	3.26±0.17	3.66±0.25	3.83±0.42	1.44±0.32	1.83±0.41
Group II	3.18±0.12	8.10±0.41[a] (121.31%)	8.25±0.68[a] (115.40%)	1.48±0.38	4.15±0.64[a] (126.77%)
Group III	3.15±0.19	5.63±0.89[a] (58.82%)	5.92±0.96[a] (54.56%)	1.51±0.29	2.22±0.59[b] (21.31%)
Group IV	3.21±0.13	5.19±0.92[a] (41.80%)	6.02±0.48[a] (57.10%)	1.42±0.33	2.47±0.51[a] (34.97%)
Group V	3.25±0.11	5.52±0.39[a] (50.82%)	6.68±1.08[a] (74.41%)	1.47±0.35	2.82±0.48[a] (54.09%)
Group VI	3.17±0.18	6.72±0.34[a] (83.60%)	8.05±0.51[a] (110.18%)	1.43±0.37	3.08±0.42[a] (63.30%)

Results are expressed as Mean ± standard error of mean; N=6 for each test; The data were statistically analyzed by one way analysis of variance (ANOVA) and Duncan post-hoc test; [a]:significant different from negative control at P<0.01 and [b]:significant different from negative control at P<0.05; parenthesis indicated percent change in comparison to negative control

Legends for Figure 1.

A: Group I. Large numbers of inflammatory cells in subcutaneous tissues (arrows mark vacuoles); B: Group II. Vacuoles showed less numbers than control; C: IV. Less number of vacuoles and inflammatory cells noted; D: V. Negligible number of vacuoles and cells. [H &E X 100]



A: Group I. Large numbers of inflammatory cells in subcutaneous tissues (arrows mark vacuoles); B: Group II. Vacuoles showed less numbers than control; C: IV. Less number of vacuoles and inflammatory cells noted; D: V. Negligible number of vacuoles and cells. [H &E X 100]