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Anti-osteoarthritis, anti-nociception, anti-inflammatory activities of isolated fraction of flesh extract Viviparous bengalensis in experimental model

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A B S T R A C T

Study was to isolate, characterize and evaluate the anti-osteoarthritic, anti-nociception, anti-inflammatory activities of VB-P4/VB-P5 (flesh extract of fresh water snail *Viviparous bengalensis*) in experimental model. Live adult fresh edible water snail (*Viviparous bengalensis)* were collected commercially from the market and authenticated by the Zoological Survey of India, Kolkata. Foot pad portion was dissected out and 10% aqueous flesh homogenate (VBE) was prepared in PBS (0.01M, pH 7.2) by homogenization using an Elvenjan homogenizer fitted with a Teflon piston (Remi Motors Ltd., India). The homogenate was subjected to centrifuge at 8000 rpm for 30 mins (4^0C). The concentration of *Viviparous bengalensis* extract (VBE) was expressed in terms of protein content and purified by Ion exchange chromatography (Mitchell *et al.*, 1970). RP-HPLC was performed with two isolated fractions VB-P4/VB-P5 having higher protein content and biological activities. RP-HPLC purified fractions were analyzed through CD spectra, UV spectra analysis, SDS-PAGE for molecular weight determination. OA was induced in male rats- Gr.1: Sham control, Gr.2:OA control, Gr.3:Standard, Gr.4:VB-P4 ($200\mu g.100g^{-1}$), Gr.5:VB-P4 ($400\mu g.100g^{-1}$), Gr.6:VB-P5 ($200\mu g.100g^{-1}$), Gr.7:VB-P5 ($400\mu g.100g^{-1}$). Anti-osteoarthritic activities of VB-P4/ VB-P5 were examined through physical/ urinary/ serum/ synovial/ blood parameters/ histopathological examination. Anti-nociception/ anti-inflammatory models were assessed in male mice. Data expressed in Mean±SEM (n=6). ANOVA was performed, $p < 0.05$. IEC fractions of VBE, VB-P4/VB-P5 showed sharp peak in RP-HPLC. UV-spectra confirmed protein nature of these fractions. CD-spectra analysis showed strong α-helical structure followed by β-sheet and random coil. SDS-PAGE of VB-P4/VB-P5 showed 140kD, 150kD molecular weight respectively. In biological studies, ankle and knee diameters, urinary markers, serum markers and cytokines, synovial marker levels, blood mAbs were changed significantly and restored after VB-P4/ VB-P5 treatment. BrdU conjugation data also clarified the same result. VB-P4/VB-P5 also restored the nociceptic, inflammatory activity. Thus it can be assumed that VB-P4/ VB-P5 treatment showed significant anti-osteoarthritis, anti-nociception, anti-inflammatory activity in animal models. Isolated fractions VB-P4/VB-P5 from the fresh water snail flesh extract possess anti-osteoarthritic, anti-nociception and anti-inflammatory activity in experimental animal models.

Introduction

Arthritis means joint in pain, which is very common now a day. Three types of arthritis generally well known to us – rheumatoid arthritis, osteoarthritis and psoriatic arthritis. Among them osteoarthritis is a major type seen all over the world. Osteoarthritis is derive from the Greek word ***osteo*** meaning the bone *artho* meaning joint and ***itis*** meaning inflammation although many suffers many have little or no inflammation. OA is the most common form of arthritis and one of the leading causes of chronic disability in the elderly person. Approximately 25% of persons of 55 yrs of age or older have knee pain and almost half of them having symptomatic osteoarthritis, that leads to physical disabilities or loss of functional capacity and reduce quality of life (Reva *et al.,* 1994). OA is classified into primary or idiopathic, when there is no obvious pre disposing cause and secondary when there is clearly defined pre disposing cause. Idiopathic osteoarthritis is the most common form of arthritis.

The factors which include the changes in the expression of collagen and other matrix molecules in OA had not been yet identified, but may include changes of the chondrocyte environment, mechanical loading, cytokines, growth factors and perhaps molecular fragments produced by the matrix metabolism (Goldring, 1999). The degradation of cartilage and proteoglyacans ion OA is due to the imbalance in the proteinases and the inhibitors synthesised by the chondrocyte (Garnero, 2000). Matrix metalloproteinases (MMPs) have been detected in the synovial fluids and cartilage of OA patients. It has been found that the increased levels of inhibitors of metalloproteinases in the synovial fluid of OA reflect an adaptive response to the increased levels of active MMPs (Nakamura *et al.,* 2007). Genetic and hormonal factors also play an important role and established that the mutations in collagen genes (types II, IX, X) appear to contribute to the development of premature idiopathic OA (Novack, 2011).

The aim of the management of osteoarthritis is to reduce pain and to minimize the change occurs during arthritis development. Physiotherapy, physical exercise and analgesics are often prescribed by the rheumatologists. Non-steroidal anti-inflammatory drugs (NSAIDS) like accelophenac, dichlophenac are the first line of defense against arthritis (Halliwell *et al.,* 1998). But NSAIDS work through cyclo-oxygenase (COX-1 and COX-2) inhibition that inhibit prostaglandins. COX-1 inhibition causes side effects like G.I tract irritation, platelet aggregation etc. Sometimes patients treated with COX-1b shows cardiovascular Problems. The COX inhibition helps to protect against arthritic inflammation, pain and rheumatic fever. Glucocortiocoid therapy also shows certain side effects. For this reason disease modifying anti rheumatic drugs (DMARDS) are better advised. But DMARDS like methotrexate, cyclosporine A, anti-cytokine therapies all have certain side effects like sepsis, pulmonary and extra pulmonary tuberculosis etc.

For all these reasons the past decades or two have seen a dramatic increase and growing interest in the use of alternative therapies by osteoarthritis patients.

Natural products such as honey, mussel, snake venom, ginger, black tea (Datta *et al.,* 2012) are used for the treatment for arthritis. Treatment of osteoarthritis using natural products is an old age practice (Buckwalter *et al.,* 2005; Chrubasik *et al.,* 2007). In India, snails are consumed by the villagers of low income group as local belief says that

snail increases the strength of bone and prevents joint disorders. Recently Gomes *et al.,* 2011 showed that snail flesh extract is useful for liver problems (Gomes *et al.,* 2011). In the North Bihar, India, the flesh of *Viviparous (Bellamya) bengalensis* is used as a traditional medicine against arthritis (Prabhakar and Roy, 2009). Recently from this laboratory it has been proved that, the crude flesh extract of the fresh water snail *Viviparous bengalensis* showed antiosteoporotic and antiarthritic activity (Sarkar *et al.,* 2013).

Use of snail in the past history proves from the Tarots card. Golden snail is important evidence from the past history which increases strength of bone and joint, liver problem. It also showed positive result against cancer to some extent. In past evidence showed that molluscs were worshiped as God, e.g. "Snail God". Snails and mussels were also evident in Hindu mythology. This is because of the usefulness of these two things. Cone snail venom was useful in eye treatment like conjunctivitis, eye irritations etc. In the north Bihar, the flesh of *Bellamia* (*Vi- viparous*) *bengalensis* is used as a traditional medicine against arthritis (Prabhakar and Roy, 2009). Indian local people said that flesh of snail and mussel increase the strength of bone and prevent joint disorders. There is also evidence in Chinese folk medicine that Shell of vivipardae used in acupuncture treatment. Snail moxibustion is used in the treatment of eye disease. In the folk-traditional medicine, snail and mussel purify blood, boost immune system, snail water prevents conjunctivitis, mussels and snail flesh for liver problems (Gomes *et al.,* 2011). In Ayurveda, snail dust is used in hypoacidity, hyperacidity, anorexia, piles, any type of inflammation, anemia, leprosy, pain, diabetes, high fever, Spru syndrome, cough, chronic pulmonary syndrome, emaciation, tuberculosis, arthritis, acidity etc.

In Ayerveda, snail moxibustion is termed as "Shakha Vasma" which is very useful in pthisis and all sorts of apasmara-neurological disorder. Moxibustion of snail, clam, octopuse, squid, oyster, and mussel are used as medicine to cure waste, indigestion. They also cure weakness of digestion heat, temporary indigestion, fever, anaemia, leprosy, colic, spermatorrea, piles.

In santal medicine water of snail is often used in treating opthalmia, hazy vision and night blindness. Snail after cooking in master oil, used as an ointment over different types of sore and corn. Shell of forest snail is used to treat hydrosyl or epidydimititis. A mixture in which snail is an important ingredient is used in the treatment of cough. The extracts of some marine gastropode were evaluated in terms of antioxidant and composition properties and their healing properties were tested on skin burns in Wistar rats. Recently Conotoxin isolated from Australian cone snail *Conus victoriae* has anti-analgesic property. Cone snail also used in diabetic neuropathy and in different CNS disorders (Leipold *et al.,* 2007). But it also has a limitation. When administered it can increases blood pressure. ACV1 isolated from a cone shell does not show any side effects & is more powerful than conotoxin in treating chronic pain common in diseases such as cancer, AIDS and arthritis. Different sea snails are also used in cardio vascular and respiratory disorders. Snail extract cream can be useful in maintaining skin tone and help to maintain wrinkle free skin. Cone snail also used in diabetic neuropathy and in different CNS disorders. Different sea snails are also used in cardio vascular and respiratory disorders. There is very little scientific information available about Indian

fresh water edible snail. Though in villages it is belied that this species can help in preventing bone and joint disorders.

The present study was an effort to explore the effect of fresh water snail flesh protein fractions and to establish the osteoarthritis, nociception and inflammatory activity in experimental animals.

Materials and Methods

Chemicals & reagents

Chemicals, reagents and kits were purchased from Sigma, USA; SRL, India; Merck, India; BD Bioscience, USA; R & D, USA.

Purification and Characterization of anti-osteoarthritic factor of Viviparous bengalensis flesh extract (VBE)

Preparation of VBE and purification through Ion exchange chromatography (IEC),Reverse phase-HPLC (RP-HPLC)

Edible water snails (*Viviparous bengalensis)* were collected, identified from Zoological Survey of India and 10% aqueous flesh homogenate (VBE) was prepared in PBS (0.01M, pH 7.2) using homogenization using an Elvenjan homogenizer fitted with a Teflon piston (Remi Motors Ltd., India). The homogenate was subjected to centrifuge at 8000 rpm for 30 mins (4^0C). The concentration of *Viviparous bengalensis* extract (VBE) was expressed in terms of protein sing standard protein estimation by Lowry *et al.* (1951).

Crude VBE was subjected to ion exchange chromatography (IEC) using DEAE cellulose column with phosphate buffer. Fractions were collected using graded NaCl and proteins were estimated (Datta *et al.*, 2012). The 5 peaks were tested for biological activity through osteoarthritis rat model and also nociceptic and inflammatory model in mice. Among them only peak 4 (VB-P4) and 5 (VB-P5) showed the required biological activity in osteoarthritis (OA) rat model and inflammation and nociception model in mice. VB-P4 and VB-P5 were further analyzed through HPLC through C18 column (4.6×250 mm), using methanol and water mixture (70:30) with a flow rate 1 ml/min. Protein peak was detected at 280 nm and the retention time was calculated.

Determination of molecular weight

Molecular weight of the IEC purified proteins VB-P4 (981 $\mu g.ml^{-1}$ protein) and VB-P5 (760 $\mu g.ml^{-1}$ protein) were determined by SDS-PAGE using standard molecular weight marker proteins (10–170 kDa) after the method of Lammelli (Buckwalter *et al.,* 2005). Protein bands were visualized and photographed.

UV Scan and CD- Spectra analysis

UV-Scan of VB-P4 and VB-P5 at length of 200-400 nm were done. CD-Spectroscopic analysis was done and analyzed through CD spectra analysis software K2D3 online.

Experimental animals

Wistar male albino rats (120±10 g) and male Swiss mice (20±2 g) were procured (Animal Ethical Committee approval no. of CPCSEA: PHY/CU/IAEC/20/2008).

Development of Osteoarthritis model

Experimental osteoarthritis was developed in 9 to 12 weeks old Wister male albino rats (120±10gm) by intra-articular injection of 20 µl bacterial collagenase (5CDU) solution in the right knee joint. Same amount of

saline will be injected in the left knee joint (Chrubasik *et al.*, 2007).

Urinary parameters analysis for confirmation of OA development

On the day 2, 24 hr. urine was collected from the rats and hydroxy-proline (OH-P) (Gomes *et al.,* 2011), glucosamine (Prabhakar and Roy, 2009), calcium (Ca^{2+}), creatinine (CRE) and phosphate (PO_4^{3-}) were measured using biochemical kits.

Treatment schedule

Osteoarthritis (OA) was developed in male albino rats (120±10g) divided into- Gr.1: Sham control (without OA induction and without any treatment); Gr.2: OA control (only OA induced), Gr.3: Standard (OA induced and treated with indomethacin-0.25mg.100g^{-1} i.p. x 5days alternately), Gr.4: VB-P4 (OA induced and treated with 200 μg.100g^{-1}; i.p. x 15 days), Gr.5: VB-P4 (OA induced and treated with 400 μg.100g^{-1}; i.p. x 15 days), Gr.6: VB-P5 (OA induced and treated with 200 μg.100g^{-1}; i.p. x 15 days), Gr.7: (OA induced and treated with 400 μg.100g^{-1}; i.p. x 15 days) in both cases. Urine and serum were collected on Day 14 and Day 16 respectively. Anti-osteoarthritic activity of IEC fractions VB-P4 and VB-P5 were examined through physical, urinary, serum and blood parameters.

Biochemical markers of urine and serum

24 hrs urine was collected from all groups of animals and urinary OH-P and glucosamine and minerals Ca^{2+}, PO_4^{3-} and CRE were measured on day 14. Serum enzymes alkaline and acid phosphatase (Sarkar *et al.*, 2013) and minerals Ca^{2+}, PO_4^{3-}, CRE were measured on day 16 after scarifying those animals. Serum pro and anti-oxidant LPO (Lipid peroxide), GSH (glutathione), SOD (superoxide dismutase), Catalase, serum and synovial fluid interleukins TNFα (Tumor necrosis factor α), IL-1β (Interleukin-1β), CINC-1 (Cytokine-induced neutrophil chemoattractant-1), IL-4 (Interleukin-4), IL-6 (Interleukin-6), IL-10 (Interleukin-10), IL-12 (Interleukin-12), pyridoline, deoxypyridoline, osteocalcin were measured using ELISA kit.

Blood AntiCD4 and CD25 analysis through Flow cytometry

To investigate the anti CD4 (cluster of differentiation-4) and anti CD25 (cluster of differentiation-25) activity in arthritis condition, flow cytometric analysis was done by dot plot assay. Blood was collected in a heparinized tube from the previously mentioned same groups of rats. Then mAb (monoclonal antibody) (eBioscience, USA) was added to it. Cells were pelleted down, centrifuged at 1000 rpm for 10 mins, washed with lysing solution. Again after centrifuging at 1000 rpm for 5 mins, cell pellets were dissolved in PBS. After 15 min incubation in dark at room temperature flow cytometric analysis was done. Flow-cytometric reading was taken using 488 nm excitation and band pass filters of 530 nm (for CD4-FITC detection and CD25-PE) and 585 nm (for CD3-PE detection) (Becton-Dickinson FACS Verse double laser cytometer). In this experiment, CD3-PE was plotted in X-axis to identify the amount of FITC untagged populations. Y-axis showed the antibody tagging with FITC, e.g., CD4 and CD25.

Bromodeoxyuridine labeling through Flow cytometry

After OA induction, on day1 rats were i.p. injected with 10 mg of bromodeoxyuridine (BrdU) in 1ml of PBS in 3groups of male rats for 5 alternative days. Treatment of VB-P4 and VB-P5 (400 μg.g^{-1}; i.p. x 10 days)

were given also in Gr. 2 and 3 of rats, respectively. Gr.1 rats (Sham Control) were given vehicle saline. Intracellular staining of lymphoid cells was performed using a commercial kit according to the manufacturer's recommendations (BrdU Flow Kit; BD Bioscience, USA).

Joint histology and X-ray

Ankle joints sections (6μm) were cut from all the groups of rats and stained with haematoxylin-eosin, observed and photographs were captured with Motic software (Motic Images Plus 2.0 software). X-ray studies of knee joints of those rats were done through Digital X-ray machine (film focus distance 60 inches and at 43kV peak, 2mA).

Experimental pain models-

a) Hot Plate and Tail Flick Model:

24 male mice (20±2 g) were taken for each model and divided into Gr.1: Sham control; Gr.2: Standard (Aspirin- 150 $mg.kg^{-1}$; i.p.), Gr.3: VB-P4 (400 $\mu g.100g^{-1}$; i.p.), Gr.4: VB-P5 (400 $\mu g.100g^{-1}$; i.p.). Hot plate model (Prabhakar and Roy, 2009) and analgesia (Gomes *et al.*, 2011) were assessed. In case of hot plate model, mice was placed over a hot plate machine (55 ± 1^0C) and the basal reaction time was monitored in which the mice produced licking or jumping response. Standard drug, VB-P4 ad VB-P5 was injected one hr. prior the experiment to the groups of animals and the same method was repeated after 1 hr.

In tail flick model, 3-4 cm area of the tail was marked and immersed in the water bath thermo-statically maintained at 51 ± 0.5^0C. The withdrawal time of the tail flick latency was noted. Standard drug, VB-P4 ad VB-P5 was injected one hr. prior the experiment to the groups of animals and the same method was repeated after 1 hr.

b) Acetic acid induced writhing reflex and Formalin induced pain model:

Acetic acid writhing (Leipold *et al.*, 2007) and formalin induced pain (Lowry *et al.*, 1951) were analyzed in 30 male mice (20±2 g). Animals were divided - Gr.1: Sham control; Gr.2: Acetic acid control/formalin control; Gr.3: Standard (Aspirin- 150 $mg.kg^{-1}$; i.p.), Gr.4: VB-P4 (400 $\mu g.100g^{-1}$; i.p.), Gr.5: VB-P5 (400 $\mu g.100g^{-1}$; i.p.).

In acetic acid writhing model, first acetic acid was injected intraperitonally in male mice at a dose of 10 $ml.kg^{-1}$ and then number of writhes during the following 15 mins time were observed. Standard drug, VB-P4 ad VB-P5 were injected one hr. prior the experiment to the groups of animals and the same method was repeated after 1 hr. Reduction in number of writhing response in treatment groups of animals were noted and compared to the control animals.

In formalin induced pain model, 20 μl of formalin (2.5%) was injected in the right hind paw of male mice and the time animal spent in licking of injected paw was determined during 0-5 mins (early phase), and during 15-30 mins (late phase) after the injection of formalin. 1 hr. prior to formalin injection aspirin (standard drug), VB-P4 and VB-P5 were injected to the groups of mice intraperitonally. Reduction in the number of licking response was also noted and compared with control groups.

Experimental Inflammation models-

a) Xylene induced inflammation:

30 male mice (20±2 g) were taken and into 5 groups- Gr.1: Sham control; Gr.2: Xylene control; Gr.3: Standard (Aspirin- 150 $mg.kg^{-}$

^{1}; i.p.), Gr.4: VB-P4 (400 μg.100g^{-1}; i.p.), Gr.5: VB-P5 (400 μg.100g^{-1}; i.p.). Xylene induced inflammation (Laemmli *et al.,* 1970) was done by giving a total of 30 μl of xylene on the anterior and posterior surfaces of the right ear lobe. The standard drug, VB-P4 and VB-P5 were injected 1 hr. prior to the experiments in those respective groups intraperitoneally and after 1 hr. the same experiment was repeated on those mice. The animals were scarified by cervical dislocation and right and left ears of each animal were removed using cork borer (diameter of 7 mm) and weighed. The left ear was considered as control.

b) Carrageenan induced inflammation:

30 male mice (20±2 g) were taken and divided into Gr.1: Sham control; Gr.2: Carrageenan control; Gr.3: Standard (Aspirin- 150 mg.kg^{-1}; i.p.), Gr.4: VB-P4 (400 μg.100g^{-1}; i.p.), Gr.5: VB-P5 (400 μg.100g^{-1}; i.p.). Carrageenan induced inflammation (Van der Kraan *et al.,* 1989) was done on those mice.

The initial right hind paw volume of the mice was measured using digital calipers and then 20 μl of 1% (w/v) carrageenan was subcutenously injected into the sub-planter region of the right hind paw. The volume of right hind paw was measured at 1 hr., 2 hr., 3 hr., 4 hr., 5 hr., after carrageenan injection and edema volume was determined. In case of standard drug, VB-P4 and VB-P5 groups, treatments were given 1 hr. prior to the experiment to the respective groups of mice.

Statistical analysis

Data were expressed as Mean±SEM (n=6). The repeated measure analysis of variance (ANOVA) was used to determine significant differences between groups. *p<0.05 was considered to be statistically significant.

Results and Discussion

Isolation of fractions by Ion Exchange chromatography

Crude VBE (0.5 g) gave five protein peaks (P1, P2, P3, P4, P5). P4 (tube no. 55) was eluted with 0.2M NaCl and P5 (tube no. 65) was eluted with 0.5M NaCl, possess anti-osteoarthritic activity in male albino rats. Yield of P4 and P5 were found to be 87.93%, 26.10% protein loss was 12.07% and 73.98% protein was recovered by this process (Fig.1a). P4 was named as VB-P4 and P5 was named as VB-P5 for the sake of convenience.

UV Scan, CD- Spectra analysis

UV λ_{max} of VB-P4 was 288nm and VB-P5 was 286nm (Fig.1a). CD-spectra analysis showed 53.44% strong α-helical structure, 5.15% β strand with random coil in VB-P4, whereas VB-P5 showed 1.15% α-helical structure, 10.69% β strand with random coil (Fig.1a).

RP-HPLC profile and SDS-Molecular weight determination

HPLC elution pattern of ion exchange eluted VB-P4 showed one sharp peak within a retention time 5.5 min. VB-P5 showed a sharp peak at the retention time 5.6 min (Fig.1a). On SDS-PAGE, the molecular weight of VB-P4 and VB-P5 was found to be 140kD and 150kD respectively (Fig.1b).

Anti-Osteoarthritic effect of VB-P4& VB-P5-

Effect of VB-P4 & VB-P5 on Physical and urinary parameters

Ankle and knee swellings were significantly decreased after treatment with VB-P4 &

VB-P5 (200 μg.100g^{-1}, 400 μg.100g^{-1}; i.p. x 15 days) as compared with the OA control group.

VB-P4 (200 μg.100g^{-1}, 400 μg.100g^{-1}; i.p. x 15 days) treated groups showed 46.01%, 48.98% decrease in urinary OH-P level and 46.44%, 44.02% decrease in glucosamine level. VB-P5 (200 μg.100g^{-1}, 400 μg.100g^{-1}; i.p. x 15 days) treated groups showed 40.01%, 42.92% decrease in urinary OH-P level and 41.07%, 45.58% decrease in glucosamine level, whereas standard drug (indomethacin) treated group 3 rats showed 42.22% decrease in OH-P and 40.01% decrease in glucosamine level as compared with OA control (Table.1).

VB-P4 (200 μg.100g^{-1}, 400 μg.100g^{-1}; i.p. x 15 days) treated groups showed 40.31%, 44.89% decrease in urinary calcium, 80.56%, 83.19% decreases in urinary phosphorous and 48.76±0.07%, 52.06±0.07% decrease in urinary creatinine level. VB-P5 (200 μg.100g^{-1}, 400 μg.100g^{-1}; i.p. x 15 days) treated groups showed 35.89%, 38.13% decrease in urinary calcium, 72.07%, 74.90% decreases in urinary phosphorous and 32.20%, 38.99% decrease in urinary creatinine level, whereas standard drug showed 46.09%, 70.06%, 35.17% decrease respectively as compared with OA control (Table.1).

VB-P4 (200 μg.100g^{-1}, 400 μg.100g^{-1}; i.p. x 15 days) treated groups showed 50.53% and 56.55% decrease in urinary pyridoline, 69.11% and 75.13% decrease in urinary deoxypyridoline level in OA model. VB-P5 (200 μg.100g^{-1}, 400 μg.100g^{-1}; i.p. x 15 days) treated groups showed 46.40% and 48.32% decrease in urinary pyridoline, 62.49% and 65.42% decrease in urinary deoxypyridoline level in OA model, whereas standard drug (indomethacin) treatment showed 48.49%, 63.48% decrease respectively in urinary pyridoline and deoxypyridoline as compared with the OA control (Fig.2a).

Effect of VB-P4 & VB-P5 on serum parameters

VB-P4 (200 μg.100g^{-1}, 400 μg.100g^{-1}; i.p. x 15 days) treated groups showed significant decrease in serum ACP (45.18%, 49.02%) and serum ALP (36.99%, 47.04%) level. VB-P5 (200 μg.100g^{-1}, 400 μg.100g^{-1}; i.p. x 15 days) treated groups showed significant decrease in serum ACP (36.99%, 47.04%) and serum ALP (36.11%, 40.15%) level, whereas standard drug (indomethacin) treated group3 rats showed 51.01% and 42.17% decrease as compared with OA control group (Table.1).

VB-P4 (200 μg.100g^{-1}, 400 μg.100g^{-1}; i.p. x 15 days) treated groups showed significant decrease in serum calcium (60.78%, 68.17%) and serum creatinine (46.05%, 48.06%) levels respectively. VB-P5 (200 μg.100g^{-1}, 400 μg.100g^{-1}; i.p. x 15 days) treated groups showed significant decrease in serum calcium (48.90%, 64.18%) and serum creatinine (35.98%, 37.05%) levels respectively, whereas standard drug treatment showed 50.67% and 39.19% decreased as compared with OA control (Table.1).

VB-P4 (200 μg.100g^{-1}, 400 μg.100g^{-1}; i.p. x 15 days) treated groups significantly decreased the over production of pro-inflammatory makers TNF-α (50.67% and 52.01%), CINC-1 (40.22% and 45.39%), PGE2 (45.99% and 48.03%), IL-6 (50.73% and 52.12%), IL-12 (43.90% and 47.64%). VB-P5 (200 μg.100g^{-1}, 400 μg.100g^{-1}; i.p. x 15 days) treated groups significantly decreased the over production of pro-inflammatory makers TNF-α (43.21% and 48.09%), CINC-1 (34.22% and 38.23%),

PGE2 (36.05% and 40.45%), IL-6 (50.73% and 52.12%), IL-12 (36.34% and 40.64%), whereas standard drug decreased the levels respectively on TNF-α (48.56%), CINC-1 (39.93±0.08%), PGE2 (37.29%), IL-6 (50.01%), IL-12 (36.92±%) level as compared with OA control (Fig.2b, 2c).

Table.1 Effect of VB-P4 & VB-P5 on urinary, serum and synovial fluid markers in OA rats

ANIMAL GROUP	Urinary OH-P (µg^{-1}ml^{-1}rat^{-1} day)	Urinary Glucosamine (µg^{-1}ml^{-1}rat^{-1} day)	Urinary Ca^{2+} (mg^{-1}dL^{-1} day^{-1})	Urinary PO_4^{3-} (mg^{-1}dL^{-1} day^{-1})	Urinary CRE (mg^{-1}dL^{-1} day^{-1})	Serum ACP (µmol.PN P.min^{-1})	Serum ALP (µmol.PN P.min^{-1})	Serum Ca^{2+}(mg^{-1}dL)	Serum CRE (mg^{-1}dL)	Synovial fluid CINC-1 (pg.ml^{-1})	Synovial fluid IL-4 (pg.ml^{-1})
Gr.1	3.22±0.12	14.46±0.14	1.89±0.09	0.171±0.05	0.276±0.01	30.24±1.96	091.53±0.95	09.52±0.55	0.52±0.52	60.18±0.93	36.84±0. 69
Gr.2	7.10±0.09*	25.13±0.34*	3.34±0.07*	0.691±0.21*	0.542±0.02*	60.51±0.94*	162.86±0.87*	16.89±0.34*	1.88±0.65*	98.67±0.59*	17.44±0.84*
Gr.3	4.12±0.09*	16.39±0.46*	1.73±0.11*	0.241±0.09*	0.373±0.03*	31.01±1.32*	105.20±1.01*	11.76±0.96*	0.71±0.93*	69.47±0.91*	39.84±0.62*
Gr.4	3.99±0.05*	15.01±0.29*	2.01±0.16*	0.200±0.09*	0.298±0.06*	35.12±0.12*	100.67±0.45*	10.89±0.12*	0.76±0.17*	65.93±0.59*	35.03±0.59*
Gr.5	3.72±0.12*	14.28±0.13*	1.80±0.12*	0.182±0.20*	0.274±0.06*	32.98±0.45*	092.21±0.70*	09.01±0.24*	0.58±0.24*	63.38±1.02*	36.71±0.96*
Gr.6	4.65±0.11*	16.25±0.63*	2.74±0.08*	0.235±0.02*	0.387±0.02*	41.01±1.70*	126.09±0.70*	11.16±0.56*	0.69±0.67*	67.13±0.91*	30.76±1.12*
Gr.7	4.13±0.13*	15.16±0.20*	2.29±0.04*	0.212±0.07*	0.320±0.01*	37.27±0.98*	110.36±0.96*	09.76±0.43*	0.53±0.98*	65.02±0.78*	34.19±0.93*

Data represent the Mean ± SEM (n=6). * $p < 0.05$ when compared to OA control group. Gr.1: Sham control, Gr.2: OA control, Gr.3: Standard drug (indomethacin), Gr.4: VB-P4 (200 µg.100g^{-1}, i.p. x 15 days), Gr.5: VB-P4 (400 µg.100g^{-1}; i.p. x 15 days), Gr.6: VB-P5 (200 µg.100g^{-1}, i.p. x 15 days), Gr.7: VB-P5 (400 µg.100g^{-1}; i.p. x 15 days).

Table.2 Effect of VB-P4 & VB-P5 on hot plate, tail flick, acetic acid pain model, formalin induced writhing reflex, xylene induced ear edema, and carrageenan induced paw edema in albino mice

ANIMAL GROUP	Hot plate (sec)	Tail flick (sec)	Acetic acid writhing (Times)	Formalin induced writhing [Early 5mins] (Times)	Formalin induced writhing [5-15mins] (Times)	Formalin induced writhing [Late phase (15-30 mins)] (Times)	Xylene induced ear edema [Left Ear] (mg)	Xylene induced ear edema [Right Ear](mg)	Carrageenan induced paw edema [Left paw] (mm)	Carrageenan induced paw edema [Right paw] (mm)
Gr.1	15±2.6	4±2.4	-	-	-	-	4.8±0.13	4.6±0.17	2.3±0.09	2.5±0.19
Gr.2	-	-	61±6.6*	-	-	-	-	-	-	-
Gr.3	-	-	-	73±4.7*	7±2.7*	33±4.6*	-	-	-	-
Gr.4	-	-	-	-	-	-	5.5±0.21*	6.2±0.14*	-	-
Gr.5	-	-	-	-	-	-	-	-	2.6±0.11*	4.1±0.13*
Gr.6	97±3.8*	137±6.8*	4±4.1*	12±3.8*	-	-	5.2±0.13*	5.7±0.24*	2.5±0.21*	3.2±0.11*
Gr.7	92±3.6*	124±5.9*	2±1.4*	6±4.4*	-	-	4.6±0.19*	4.8±0.15*	2.6±0.12*	2.9±0.12*
Gr.8	105±7.6*	170±8.2*	1±1.5*	24±3.6*	-	-	4.3±0.24*	4.6±0.18*	2.4±0.18*	2.8±0.16*

Data represent the Mean±SEM (n=6). * $p < 0.05$ when compared to Acetic acid, Formalin, Xylene, Carrageenan control group. Gr.1: Sham control, Gr.2: Acetic acid control, Gr.3: Formalin control, Gr.4: Xylene control, Gr.5: Carrageenan control, Gr.6: Standard drug (Aspirin), Gr.7: VB-P4 (400 µg.100g^{-1}; i.p.), Gr.8: VB-P5 (400 µg.100g^{-1}; i.p.).

Fig.1a DEAE cellulose ion exchange chromatography profile of VBE; Peak P4, P5 (tube no. 55 and 65) was eluted with 0.2 M and 0.5 M NaCl respectively; inset: (a) and (b) HPLC chromatography of VB-P4 and P5. UV Scan of fraction P4 and P5. CD-spectra analysis of VB-P4 (a) & VB-P5 (b). 1b. SDS-PAGE of VB-P4 and VB-P5

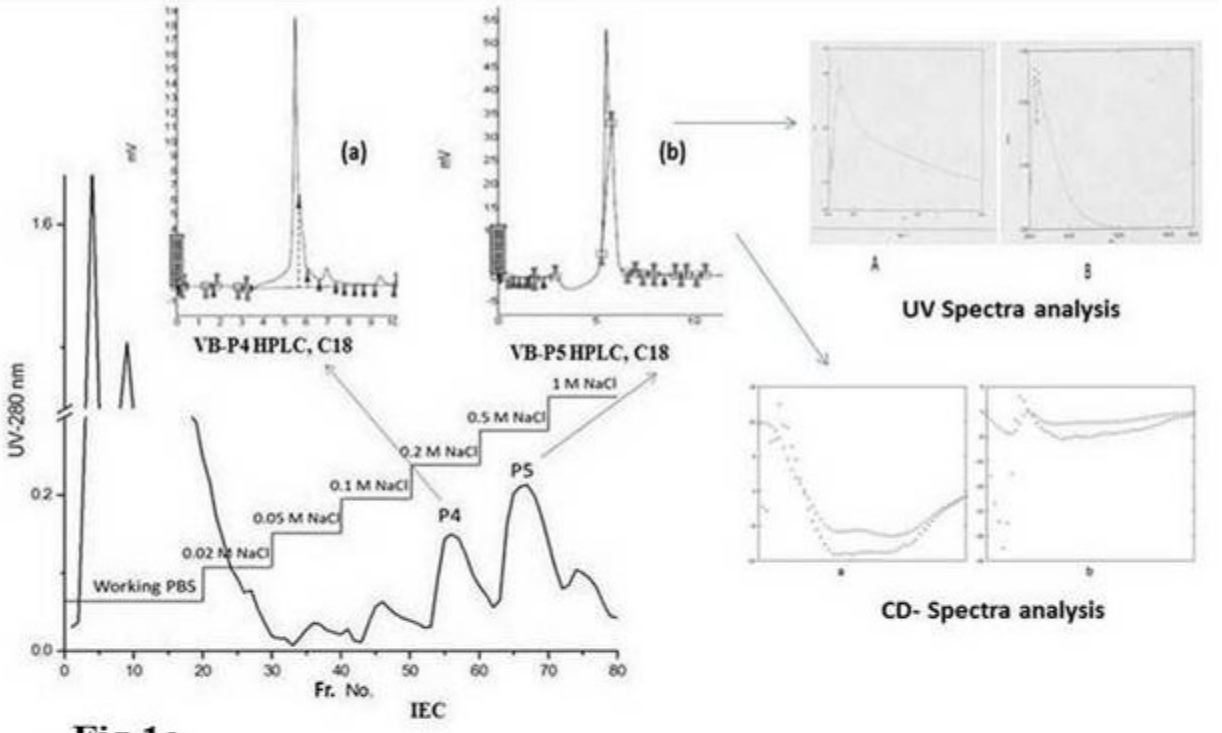
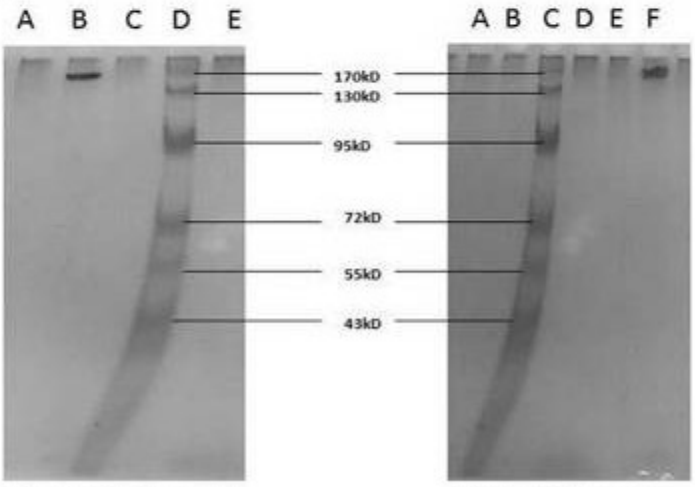


Fig.1a



Lane B: VB-P4; Lane D: MW marker

Lane C: MW marker; Lane D: VB-P4

Fig.1b

Fig.2a Effect of VB-P4 & VB-P5 on urinary pyridoline, deoxypyridoline in Collagenase-induced OA rats. 2b. Effect of VB-P4 & VB-P5 on serum TNF-α, CINC1, PGE2 levels of OA in rats. 2c. Effect of VB-P4 & VB-P5 on serum IL-6, IL-12 levels of OA in rats. 2d. Effect of VB-P4 & VB-P5 on serum IL- 4, IL-10 levels of OA in rats. 2e. Effect of VB-P4 & VB-P5 on serum MMP-1, Cathepsin-K levels of OA in rats. Concentration of TNF-α, CINC1, PGE2, IL-6, IL-12, IL-4, IL-10, MMP-1, Cathepsin-K were expressed in terms of pg/ml. 2f. Effect of VB-P4 & VB-P5 on serum LPO, GSH, SOD, catalase in OA rats. 2g. Effect of VB-P4 & VB-P5 on serum pyridoline, deoxypyridoline, osteocalcin of OA rats. Concentration of pyridoline, deoxypyridoline, osteocalcin were expressed in terms of pg/ml. Data represent the Mean±SEM (n=6). *p < 0.05 when compared to OA control group. Gr.1: Sham control, Gr.2: OA control, Gr.3: Standard drug (indomethacin), Gr.4: VB-P4 (200 µg.100g-1, i.p. x 15 days), Gr.5: VB-P4 (400 µg.100g-1; i.p. x 15 days), Gr.6: VB-P5 (200 µg.100g-1, i.p. x 15 days), Gr.7: VB-P5 (400 µg.100g-1; i.p. x 15 days)

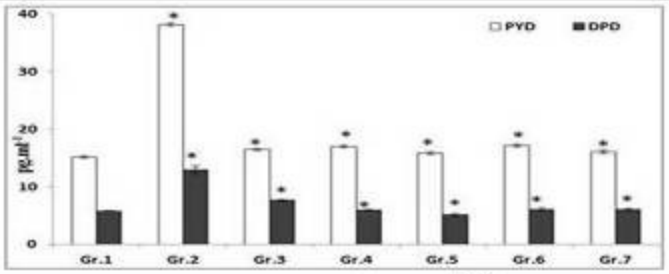


Fig.2a

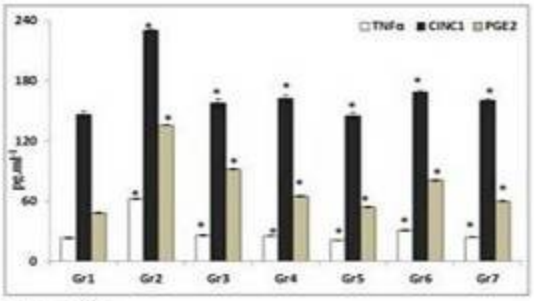


Fig.2b

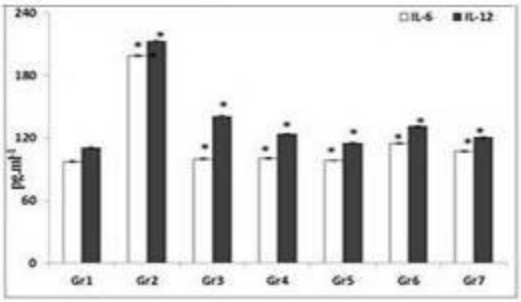


Fig.2c

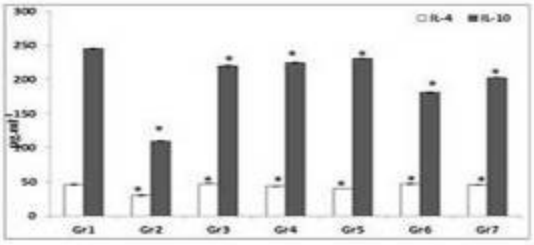


Fig.2d

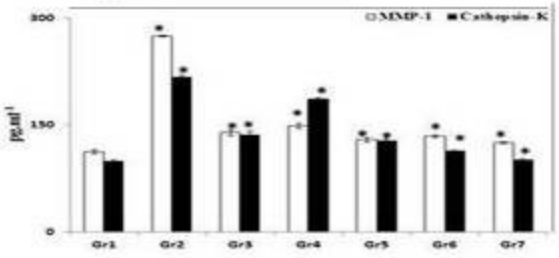


Fig.2e

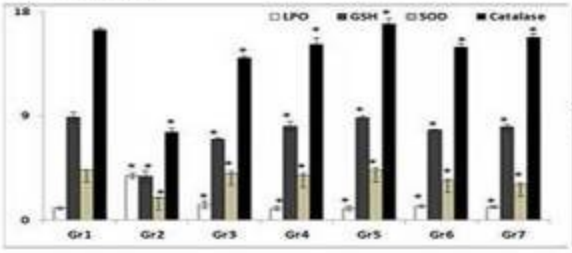


Fig.2f

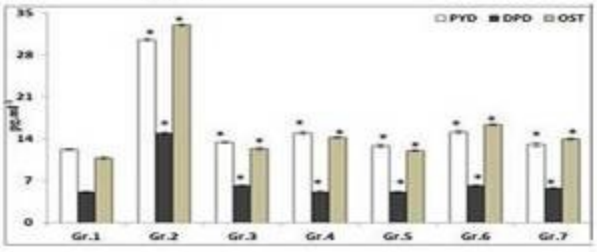


Fig.2g

Fig.3a Flow cytometric analysis of effect of VB-P4 and VB-P5 on blood anti-CD4 activity in OA rats

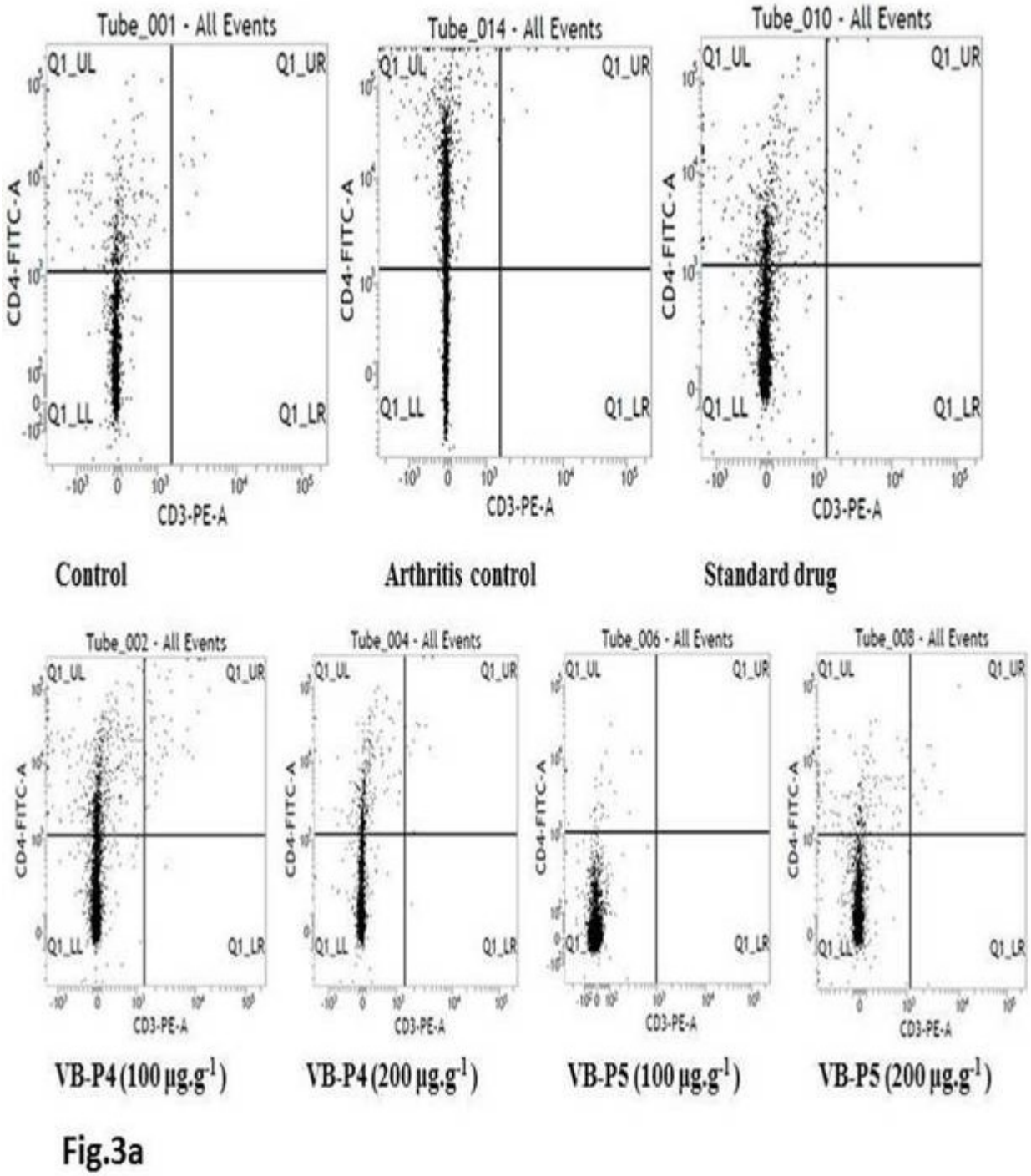


Fig.3a

Fig.3b Flow cytometric analysis of effect of VB-P4 and VB-P5 on blood anti-CD25 activity in OA rats

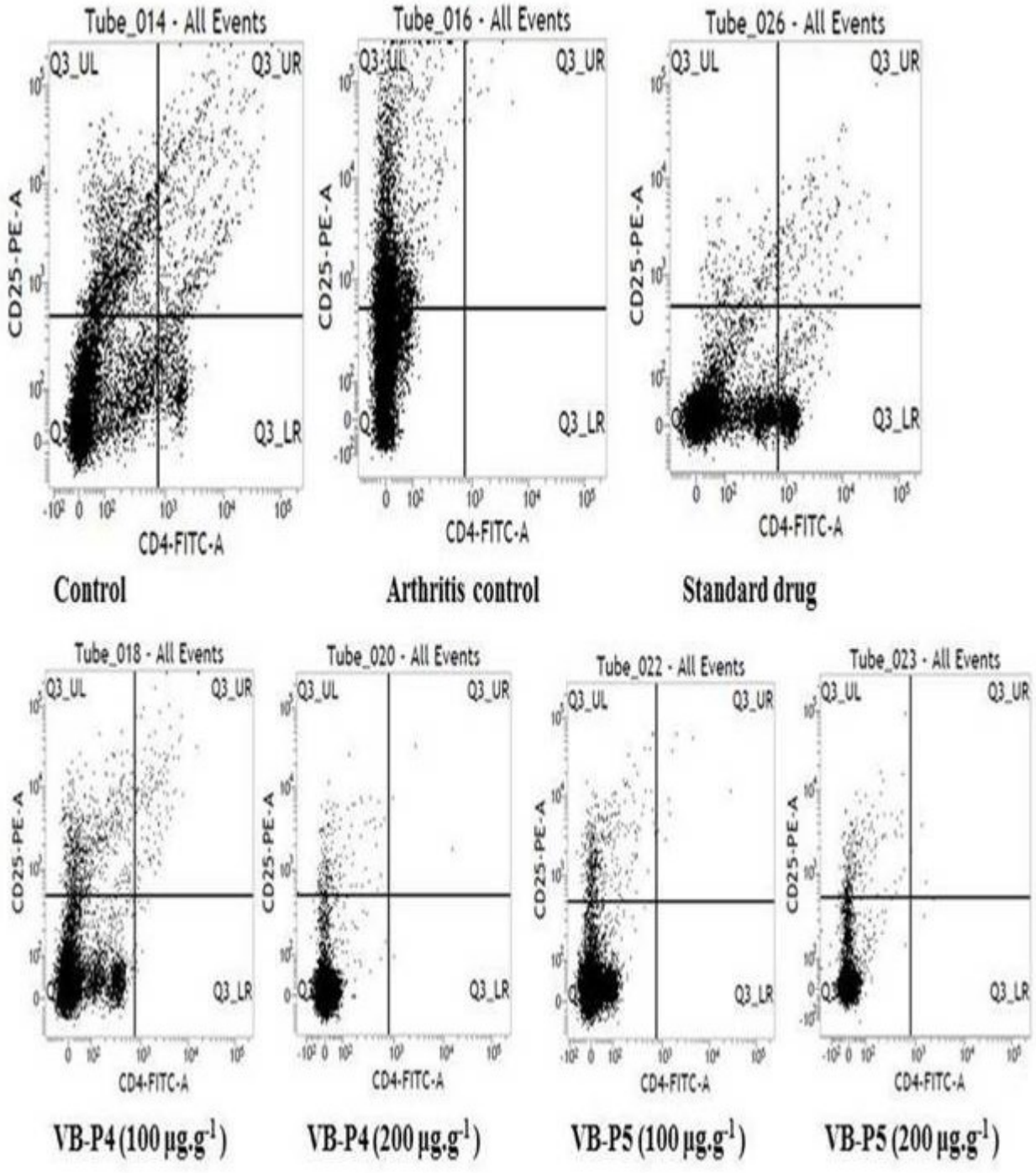


Fig.3b

Fig.3c Flow cytometry analysis of effect of VB-P4 and VB-P5 on blood T cells after BrdU incorporation

Fig.3d Histological analysis of joint after VB-P4 and VB-P5 treatment. A. Normal joint, B. OA

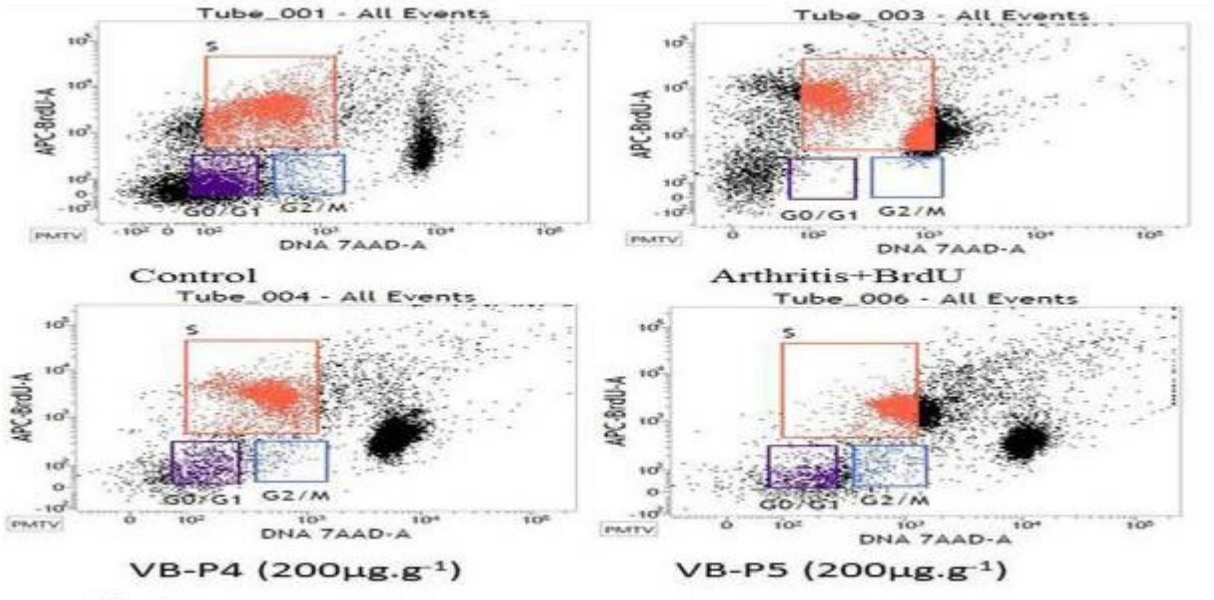


Fig.3c

induced joint, C. Standard drug treated, D. VB-P4 (400 µg.100g-1; i.p. x 15 days), E. VB- P5 (400 µg.100g-1; i.p. x 15 days). 3e. X-ray study of joints after VB-P4 and VB-P5 treatment. a. OA induced joint, b. VB-P4 (400 µg.100g-1; i.p. x 15 days), c. VB-P5 (400 µg.100g-1; i.p. x 15 days)

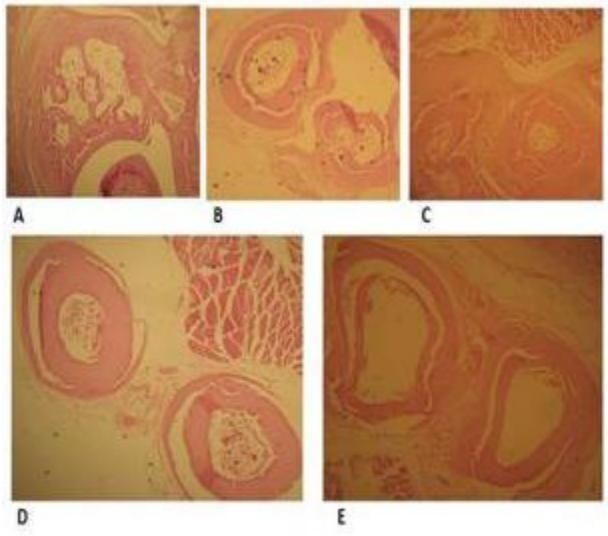


Fig.3d

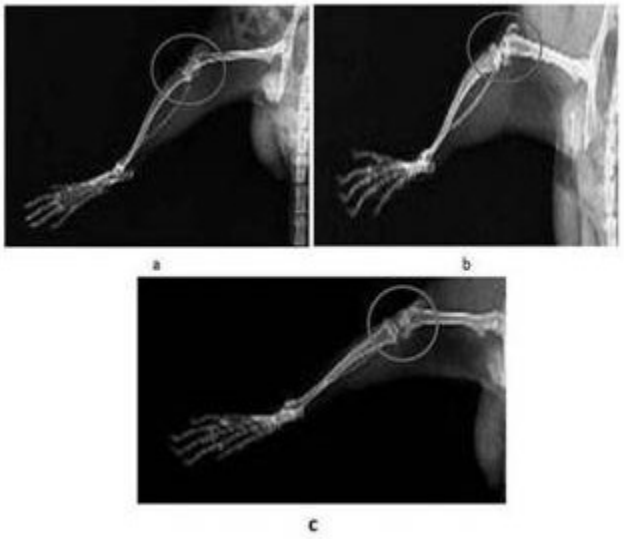


Fig.3e

Fig.4 Probable mechanism of action VB-P4 and VB-P5 in management of arthritis and inflammation

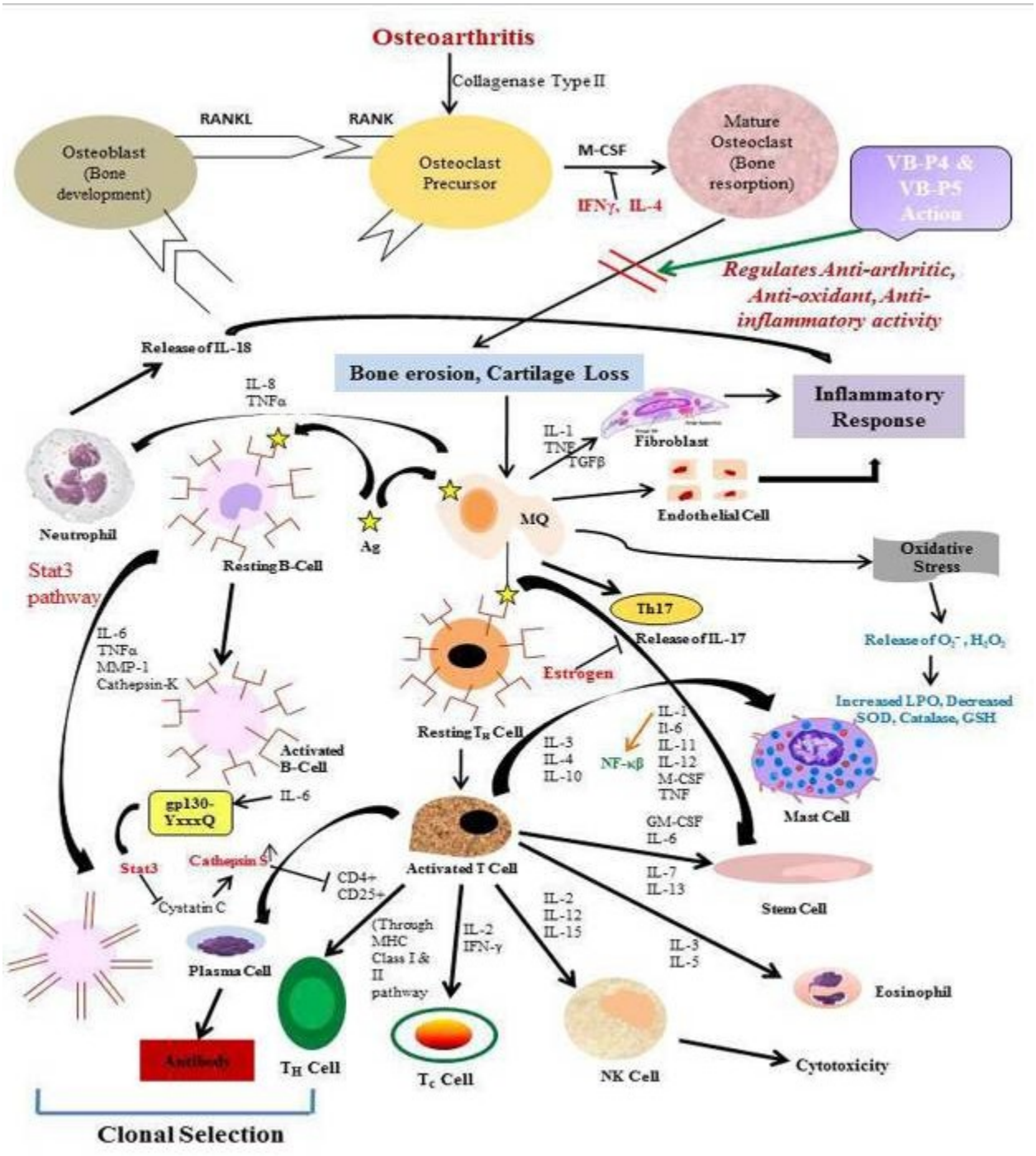


Fig.4

VB-P4 (200 μg.100g^{-1}, 400 μg.100g^{-1}; i.p. x 15 days) treated groups significantly increased the less production of anti-inflammatory makers IL-4 (35.02% and 38.73%), IL-10 (75.92% and 81.64%) and VB-P5 (100 μg.g^{-1}, 200 μg.g^{-1}; i.p. x 15 days) treated groups significantly increased the less production of anti-inflammatory makers IL-4 (30.96% and 32.67%), IL-10 (84.78% and 88.02%) whereas standard drug increased 30.23% and 32.41% respectively on IL-4 (38.99%), IL-10 (73.98%) level as compared with OA control (Fig.2d).

VB-P4 (200 μg.100g^{-1}, 400 μg.100g^{-1}; i.p. x 15 days) treated groups showed significant decrease in serum MMP-1 (64.05% and 78.19%) and serum Cathepsin-K (59.98% and 74.97%) levels respectively. VB-P5 (200 μg.100g^{-1}, 400 μg.100g^{-1}; i.p. x 15 days) treated groups showed significant decrease in serum MMP-1 (72.29% and 84.78%) and serum Cathepsin-K (81.32% and 89.06%) levels respectively, whereas standard drug treatment showed 69.92%, and 70.05% decrease respectively as compared with OA control (Fig.2e).

VB-P4 (200 μg.100g^{-1}, 400 μg.100g^{-1}; i.p. x 15 days) treatment showed 56.01±0.08% and 60.71±0.12% decrease in LPO level; VB-P5 (200 μg.100g^{-1}, 400 μg.100g^{-1}; i.p. x 15 days) treatment showed 51.56% and 53.21% decrease in LPO level, whereas standard drug treatment showed 40.43% decrease as compared with OA group.

VB-P4 (200 μg.100g^{-1}, 400 μg.100g^{-1}; i.p. x 15 days) treatment showed 80.16% and 83.92% increase in GSH level; 47.30% and 52.78% increase in SOD level; 62.42% and 67.03% increase in Catalase level. VB-P5 (200 μg.100g^{-1}, 400 μg.100g^{-1}; i.p. x 15 days) treatment showed 71.53% and 74.34% increase in GSH level; 41.53% and 45.34% increase in SOD level; 57.55% and 60.85% increase in Catalase level, whereas standard drug increased 70.99% in GSH, 40.36% in SOD and 54.92% in Catalase level (Fig.2f).

VB-P4 (200 μg.100g^{-1}, 400 μg.100g^{-1}; i.p. x 15 days) treated groups showed significant decrease in serum pyridoline (60.78% and 64.41%) and serum deoxypyridoline (62.12% and 67.08%) and osteocalcin (76.16% and 80.06%) levels respectively. VB-P5 (200 μg.100g^{-1}, 400 μg.100g^{-1}; i.p. x 15 days) treated groups showed significant decrease in serum pyridoline (54.56% and 58.89%) and serum deoxypyridoline (58.23% and 60.05%) and osteocalcin (67.35% and 72.11%) levels respectively, whereas standard drug treatment showed 61.45%, 58.94% and 77.89% decrease respectively as compared with OA control (Fig.2g).

Effect of VB-P4 & VB-P5 on synovial fluid markers (CINC-1, IL-4)

VB-P4 (200 μg.100g^{-1}, 400 μg.100g^{-1}; i.p. x 15 days) treated groups showed significant decrease in synovial fluid marker CINC-1 (47.15% and 49.19%) and increase in IL-4 (52.32% and 56.41%) levels respectively and VB-P5 (200 μg.100g^{-1}, 400 μg.100g^{-1}; i.p. x 15 days) treated groups showed significant decrease in synovial fluid marker CINC-1 (44.08% and 50.79%) and increase in IL-4 (48.59% and 52.45%) levels respectively, whereas standard drug (indomethacin) treated group3 rats showed 45.01% and 60.21% decrease respectively as compared with OA control (Table.1).

Detection of CD4 and CD25 activity by flow cytometric analysis

In case of CD4 activity study through flow cytometry, lower left (LL) quadrant denotes lower expression of CD4+ activity (lower T

cell expression), upper left (UL) quadrant denotes higher expression of CD4+ activity (higher T cell expression). Flow cytometric data analysis revealed that in arthritis control 70.60% in LL quadrant, 29.30% in UL quadrant. But data showed that after treatment with VB-P4 (100 $\mu g.g^{-1}$; i.p) 92.25 % in LL quadrant, 7.29% in UL quadrant; in VB-P4 (200 $\mu g.g^{-1}$; i.p) 97.60% in LL quadrant, 2.25% in UL quadrant. Result showed after treatment with VB-P5 (100 $\mu g.g^{-1}$; i.p) 99.37% in LL quadrant, 0.60% in UL quadrant; in VB-P5 (200 $\mu g.g^{-1}$; i.p) 97.60% in LL quadrant, 1.51% in UL quadrant; whereas standard drug (indomethacin) showed 94.60% in LL quadrant, 5.18% in UL quadrant (Fig.3a).

In case of CD25 activity study through flow cytometry, lower left (LL) quadrant denotes lower expression of CD25+ activity (lower T cell expression), upper left (UL) quadrant denotes higher expression of CD25+ activity (higher T cell expression). Flow cytometric data analysis revealed that in arthritis control 64.25% in LL quadrant, 35.62% in UL quadrant. But data showed that after treatment with VB-P4 (200 $\mu g.100g^{-1}$; i.p) 91.13% in LL quadrant, 7.43% in UL quadrant; in VB-P4 (400 $\mu g.100g^{-1}$; i.p) 98.18% in LL quadrant, 1.78% in UL quadrant. Flow cytometric data also showed that after treatment with VB-P5 (200 $\mu g.100g^{-1}$; i.p) 94.86% in LL quadrant, 3.07% in UL quadrant; in VB-P5 (400 $\mu g.100g^{-1}$; i.p) 97.75% in LL quadrant, 2.20% in UL quadrant; whereas standard drug (indomethacin) showed 85.21% in LL quadrant, 2.20% in UL quadrant (Fig.3b).

Characterization of T- cell expression through BrdU

The flow cytometer detected BrdU incorporation via T_{reg} cell expression was lower in VB-P4 and VB-P5 treated group with respect to arthritic control group. Where arthritic control group showed 40.67% increase than normal control group in S phase. VB-P4 treated group showed 50.08% and VB-P5 treated group showed 48.90% recovery after treatment with compared to arthritis control group (Fig.3c).

Effect of VB-P4 & VB-P5 on Joint histology and X-ray

Osteoarthritic control Gr.2 rats showed cellular infiltration, increased destruction of synovial membrane, decreased synovial space in ankle joint in case of histological structure and knee joints in X-ray study. Whereas VB-P4 (400 $\mu g.100g^{-1}$; i.p. x 15 days) and VB-P5 (400 $\mu g.100g^{-1}$; i.p. x 15 days) treated groups showed partial restoration of normal architecture of ankle joint in histological slides and knee joints in X-ray study as compared with OA control (Fig.3d, 3e).

Anti-nociception and anti-inflammatory effect of VB-P4 and VB-P5

Effect of VB-P4 and VB-P5 in hot plate, tail flick, acetic acid pain model and formalin induced licking response

VB-P4 (400 $\mu g.100g^{-1}$; i.p) and VB-P5 (400 $\mu g.100g^{-1}$; i.p.) treated groups showed significant increased reaction time of hot plate (74.02%, 82.99%), tail flick model (82.13%, 91.01%). Standard drug treatment (78.29% in hot plate, 88.05% in tail flick) also showed significant changes as compared with control (Table.2).

VB-P4 (400 $\mu g.100g^{-1}$; i.p) and VB-P5 (400 $\mu g.100g^{-1}$; i.p.) treated groups showed significant decrease of writhing response in acetic acid pain model (81.11%, 88.15%) and formalin induced licking response (85.06%, 71.05%) than standard drug

treatment (76.99% in acetic acid pain model, 76.97% in formalin induced licking response) as compared with control (Table.2).

Effect of VB-P4 and VB-P5 in xylene induced ear edema, carrageenan induced paw edema

VB-P4 (400 μg.100g^{-1}; i.p) and VB-P5 (400 μg.100g^{-1}; i.p.) treated groups showed significant restoration of xylene induced ear edema (62.13%, 67.06%) than standard drug treatment (58.02%) as compared with control (Table.2).

VB-P4 (400 μg.100g^{-1}; i.p) and VB-P5 (400 μg.100g^{-1}; i.p.) treated groups showed significant restoration of carrageenan induced paw edema (72.08%, 74.12%) than standard drug treatment (67.92%) as compared with control (Table.2).

RP-HPLC, UV scan and SDS-PAGE cleared that VB-P4 and VB-P5 were protein in nature having higher molecular weight. VB-P4 fraction having more α-helicallical structure and VB-P5 fraction have more β-sheet. Both fractions significantly restored the ankle, knee swellings, urinary markers hydroxyproline (Neuman and Logan, 1960) and glucosamine (Elson and Morgan, 1933; Mitchell *et al.,* 1970) compared with osteoarthritis control group. During osteoarthritis development, a marked increase of urinary hydroxy proline and glucosamine was observed. Significant restoration of urinary hydroxyproline indicated the inhibition of collagen break down and thereby preventing the cartilage damage caused by collagenase induction. Glucosamine has been shown to exhibit preventive actions on osteoporosis and osteoarthritis in humans as well as in rats (Woolfe and McDonald, 1944). OA leads to bone resorption thereby release of calcium, phosphorous into extracellular fluid, then organic matrix resorbed and are excreted through urine. As a result, the serum and urinary level of calcium, phosphate and creatinine levels were altered. In OA, the ACP, ALP level increased in the serum (Aydin *et al.,* 1999). VB-P4 and VB-P5 treatment significantly decreased the enzymes level which indicated restoration of damages of lysosomal membrane integrity. Due to T cell activation pro and anti-inflammatory cytokines levels were altered which was restored by VB-P4 & VB-P5 treatment.

From the increased levels of mAb CD4 and CD25, a connection of activation of T-helper2(T_{H2}) cells and T-helper1(T_{H1}) cells during osteoarthritis may be supposed. The activation of these cells reflects a coordinated action of MHC-class-II and class-I-mediated stimulation of T-cell receptors (Koster *et al.,* 1959; Hunskaar and Hole, 1987).

These results indicated that VB-P4 and VB-P5 treatment shifted the balance of the cytokine milieu in the joints away from the pro-inflammatory cytokines and towards the production of anti-inflammatory cytokines. VB-P4 and VB-P5 also restored the CD4 and CD25 levels in blood (Kou *et al.,* 2005; Winter *et al.,* 1962; Dull and Henneman, 1963; Felson, 2006; Forchhammer *et al.,* 2003; Nakamura *et al.,* 2007).

BrdU incorporation confirmed the increment of T cell expression during arthritic condition (Gomes *et al.,* 2010); whereas after treatment with VB-P4 and VB-P5 T-cell expression is decreased. Joint histology also confirmed the partial restoration of joint architecture, also restored the decreased synovial space during osteoarthritis. VB-P4 and VB-P5 also restored the levels of pain and inflammation in the mice model which

implies that these two protein peaks are also effective in nociceptic and inflammatory animal models.

Above discussion established that VB-P4 and VB-P5 is effective in experimental osteoarthritis and anti-nociception, anti-inflammatory models and also validated the traditional-folk use of snail flesh for the alleviation of bone and joint diseases, especially OA (Fig. 4).

Conclusion

Isolated protein fractions of fresh water snail *Viviparous bengalensis* flesh extract VB-P4 and VB-P5 showed significantly suppressed the development and progression of OA in experimental animals, which was evidenced from its effect on preventing ankle and knee swellings, decreasing the urinary markers, maintaining the lysosomal integrity by suppressing the serum enzymes, maintaining stress molecules during inflammation by restoring the serum pro and anti-oxidant parameters and serum cytokines, also through restoration of T cell expression in blood.

Therefore, the findings suggested that isolated fractions VB-P4 and VB-P5 could be beneficial in the management of osteoarthritis. This study also prove the probable mechanism of action that in osteoarthritis T_H cell activation occurs followed by MHC-class I and II pathways. Pain and inflammation correlates with this arthritis pathway.

From this study it may be concluded that snail flesh proteins are beneficial in bone and joint related disorders as well as it is also useful in nociception and inflammatory actions, a folk-traditional concept is now in reality.

Key Messages

- RP-HLPC purified protein fractions of fresh water snail flesh extract VB-P4 and VB-P5 showed significant restored of development and progression of Osteoarthritis in animal models.
- Both the fractions prevented ankle, knee swellings, decreased the urinary parameters and serum enzymes, serum interleukins, oxidative enzymes.
- The fractions prevented nociception and inflammation in animal model.
- Both fractions were glycoprotein in nature having higher molecular weight.

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