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Original Research Article

Anti-Osteoporosis Activity of Fresh Water Snail (*Viviparous bengalensis*) Flesh Extracted Protein Fraction VB-P4 in Rat Models

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A b s t r a c t	K e y w o r d s
<p>The purpose of the study was to purify and characterize active fraction of fresh water snail flesh extract and to evaluate anti-osteoporosis activity in experimental rat model. <i>Viviparous bengalensis</i> was collected, authenticated and 10% aqueous flesh homogenate (VBE) was prepared in PBS (0.01M, pH 7.2) followed by protein estimation. VBE was purified by IEC followed by RP-HPLC. RP-HPLC purified fraction VB-P4 was analyzed through UV and CD spectra, SDS-PAGE to determine MW through PAS stain, FTIR analysis, EDAX, XRD, PVDF membrane transfer followed by LC-MS/MS for amino acid sequencing. Osteoporosis was developed in female albino rats for biological activity study. Gr.1: Sham control, Gr.2: OSP control, Gr.3: Standard, Gr.4: VB-P4 (200μg.100g⁻¹), Gr.5:VB-P4 (400μg.100g⁻¹). Anti-osteoporosis activity was examined through physical/urinary/serum parameters/bone mineral analysis/bone analysis through SEM. Data represented as mean \pm SEM (n=6), ANOVA was performed, *p<0.05. VB-P4 showed single peak in RP-HPLC and CD-spectra showed α-helical structure followed by β-sheet and random coil having deep purple 128 kDa MW in SDS-PAGE. FTIR analysis confirmed the presence of aliphatic and aromatic compounds. VB-P4 also showed fluorescence activity. EDAX and XRD study confirmed the presence of sodium compound in their structure. PVDF membrane transfer followed by LC-MS/MS study confirmed the presence of 562 amino acids. VB-P4 treatment significantly restored the urinary and serum markers as compared to osteoporosis control rats. Bone ash parameters, scanning electron microscopy of long bone also showed a restoration of structural architecture after treatment with VB-P4 as compared to osteoporosis control groups. It may be concluded that VB-P4 is a high molecular weight glycoprotein, having carbohydrate moiety. VB-P4 possesses anti-osteoporosis activity in rat model. Further studies are warranted.</p>	<p>Bone mineral Fresh water snail Glycoprotein Membrane transfer Osteoporosis</p>

Introduction

Osteoporosis is one of the most common forms of bone related disease to age and sex. It means porous bone. According to International foundation of osteoporosis more than 8.9 millions peoples are affected annually in this disease. In osteoporosis, the bone mineral density (BMD) is reduced, bone microarchitecture deteriorates, and the amount and variety of proteins in bone are altered. Medications like bisphosphonates (alendronate, ibandronate), selective estrogen receptor modulator (SERM), calcitonin, NSAIDs, parathyroid hormone, estrogen replacement therapy are used for treatment of the disease. These medications also increase the chance of stroke, blood clots, breast cancer, gallstones, ovarian cancer, and dementia, skin rash, headaches, nausea, vaginal discharge, fluid retention, weight gain, breast tenderness, spotting or darkening of the skin, particularly on the face.

To avoid the side effects and cost factors alternative treatments are ventured. Ethno-botanical uses against osteoporosis are very common (Hussan et al., 2012; Ko et al., 2012). Animal product therapies and research are ventured all over the world (Gomes et al., 2009; Halder et al., 2009). *Viviparous bengalensis*, a fresh water snail is a common Indian gastropod that is found mainly in the rural area. Local people of low income family use to eat the water snails as a protein source. But no scientific validation was there to establish the effect of fresh water snail against bone disease. Earlier from this laboratory anti-osteoporosis and anti-osteoarthritic activity of crude flesh extract of this fresh water snail has been reported (Sarkar et al., 2013). Anti-osteoarthritic activity of the purified fraction of fresh water snail flesh was also done from this laboratory (Sarkar et al., 2013). The present study was an effort to evaluate the efficacy of fresh water snail flesh extracted purified fractions in experimental osteoporosis rat models.

Materials and methods

Chemicals and reagents

Chemicals, reagents were purchased from Sigma, USA; SRL, India; Merck, India.

Sample preparation

The sample preparation and purification of fraction was done as stated earlier (Sarkar et al., 2013).

Determination of molecular weight

Molecular weights were determined by SDS-PAGE using standard molecular weight marker proteins (Laemmli et al., 1970) followed by PAS staining. Protein bands were visualized and photographed (Sarkar et al., 2013).

UV Scan and CD- Spectra analysis

UV-Scan of VB-P4 at length of 200-400 nm was done. CD-Spectroscopic analysis was done and analyzed through CD spectra analysis software K2D3 online (Sarkar et al., 2013).

Fluorescence absorbance and FTIR analysis

Fluorescence absorbance study was done of VB-P4 through spectrofluorometer.

Desalted sample was lyophilized and with the dry samples FTIR analysis was done of the HPLC purified fraction VB-P4 to identify the organic compound present in the samples.

EDAX, X-RD and LC-MS/MS study

Energy Dispersive X-ray analysis (EDAX) analysis was done of HPLC purified desalted VB-P4 to determine the inorganic metallic and non-metallic compounds present in the samples. X-ray diffraction analysis was done of the HPLC purified desalted VB-P4.

Liquid chromatography–mass spectrometry of HPLC purified VB-P4 was done to confirm the molecular weight of the samples.

Membrane transfer

PVDF membrane transfer of VB-P4 was done for amino acid sequencing through Edman degradation process.

Carbohydrate estimation and Chemical deglycosylation

VB-P4 was further analyzed for carbohydrate content estimation using phenol-sulphuric acid method (Dubois et al., 1956). It was then deglycosylized (Gerken et al., 1992) for paper chromatography.

Paper chromatographic analysis for carbohydrate

Paper chromatography was done with deglycosylated VB-P4 with respect to standard carbohydrate such as glucose, galactose and glucosamine. To confirm the presence of the carbohydrates in VB-P4 Seliwanoff's test, Bial's test, mucic acid test were also done.

Development of osteoporosis model

Osteoporosis was induced in Wister strain female albino rats (28-30 week old, 120 ± 10 g). Rats were anaesthetized with sodium pentobarbitone (35 mg.kg^{-1} , i.p), and bilateral ovariectomy were done aseptically. Sham operation was done in the same manner but only exposing the ovaries. They were given prophylactic ampicillin (4000 IU.kg^{-1} , i.p). The ovariectomized rats were treated with methyl prednisolone (30 mg.kg^{-1} , i.p) on 12th day. Animals were provided with low calcium diet, green vegetables and tap water *ad libitum*, up to 25 days of ovariectomy (Gomes et al., 2009) (Animal Ethical Committee approval no. of CPCSEA: PHY/CU/IAEC/20/2008).

Treatment schedule

On day 1, animals were divided into Gr.1: Sham control, Gr.2: Osteoporosis control, Gr.3: Standard, Gr.4: VB-P4 ($200 \text{ } \mu\text{g.}100\text{g}^{-1}$; i.p. x 15 days), Gr.5: VB-P4 ($400 \text{ } \mu\text{g.}100\text{g}^{-1}$; i.p. x 15 days). Animals of Gr.3 was treated with standard anti-osteoporosis drug vitamin D₃, arachitol (200 mg.kg^{-1}) and calcium (1500 mg. kg^{-1}) x 15 days, i.p. Animals of all the groups provided with normal diet and water *ad libitum*. On the day 39, urine was collected and urinary parameters were analyzed. On day 41, animals were sacrificed and serum, blood parameters were done. Liver was also cut and liver homogenate was done using homogenizer and liver pro and anti-oxidant markers were analyzed.

Assessment of osteoporosis through physical parameters

Osteoporosis was assessed through physical parameter- body weight. Change of body weight was observed using weighing pan and recorded on day 0, day 2, day 5, day 10, day 25, day 30 and day 40.

Biochemical markers of urine and serum

24 h urine was collected and OH-P (Neuman and Logan, 1950) and glucosamine (Elson and Morgan, 1933) and minerals $\text{Ca}^{2+}/\text{CRE}/\text{PO}_4^{3-}$ were measured. Urinary pyridoline and deoxypyridoline were measured through ELISA kit. Serum enzymes ACP/ALP/TRAP (Mitchell et al., 1970) and minerals $\text{Ca}^{2+}/\text{CRE}$ were measured. Serum TNF α /IL-1 β /CINC-1/PGE2/IL-4/IL-6/IL-10/IL-12/pyridoline/deoxypyridoline/Osteocalcin, hormones like Estrogen/Progesterone/FSH/Calcitonin were measured using ELISA kit. Serum pro and anti-oxidant parameters LPO (Buege and Aust, 1978), GSH (Ellman, 1959), SOD (Beers and Sizer, 1952), Catalase (Altman and Marcussen, 2001) were estimated.

Bone ash mineral estimation and SEM analysis of long bone

Bone ash was prepared and minerals were estimated through ICP-MS. Long bones of osteoporosis induced animals were cut in small pieces, dried without decalcification and scanning electron microscopic study was done (100 X magnification).

Statistical analysis

Data were expressed as mean \pm 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

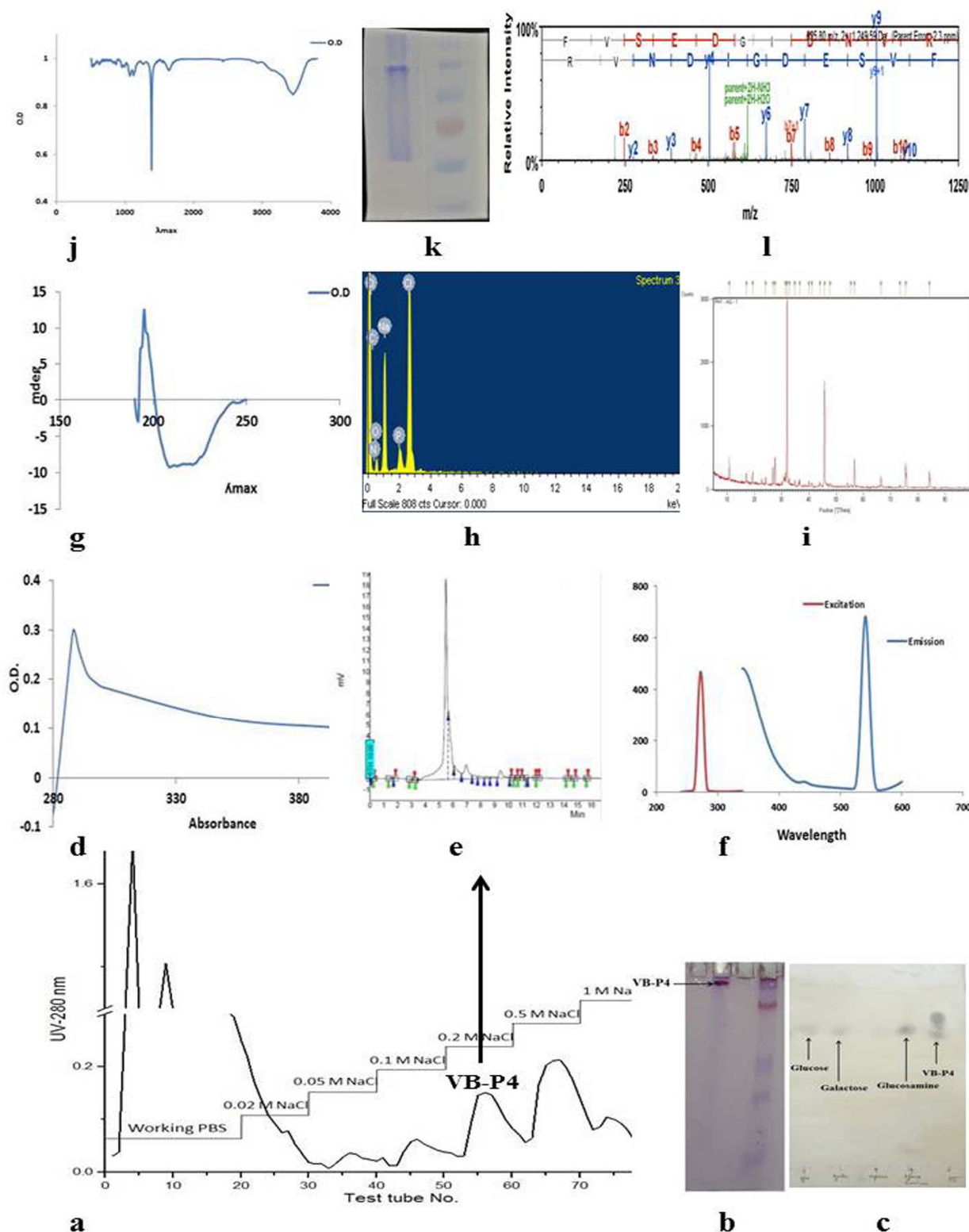
UV Scan, CD- Spectra analysis of VB-P4

UV λ_{max} of isolated and purified fractions VB-P4 were 288nm respectively (Fig.1a). CD-spectra analysis showed 53.44% strong α -helical structure, 5.15% β strand with random coil in VB-P4 (Fig. 1) (Sarkar et al., 2013).

RP-HPLC profile and SDS-Molecular weight determination

VB-P4 showed one sharp peak within a retention time 5.5 min in RP-HPLC (Fig.1a). On SDS-PAGE, MW of VB-P4 was found to be $\sim 128 \text{ kD}$ respectively (Fig. 1) (Sarkar et al., 2013).

Fig. 1: (a) DEAE cellulose ion exchange chromatography profile of VBE; Peak P4 (tube no. 55) was eluted with 0.2 M NaCl respectively. (e) HPLC, (d) UV scan, (g) CD-spectra, (b) SDS-PAGE, (f) Fluorescence absorbance, (h) EDAX, (i) XRD, (j) FTIR, (k, l) LC-MS and Western Blot analysis, (c) paper chromatography of VB-P4.



Fluorescence absorbance and FTIR analysis

Fluorescence spectroscopic analysis of VB-P4 showed an excitation peak at wavelength of 272 nm and emission peak at 540 nm (Fig. 1). FTIR study of VB-P4 showed large number of aliphatic and aromatic compounds in their structures (Fig. 1).

EDAX and X-RD analysis

EDAX analysis of VB-P4 showed presence of sodium, phosphorous, chlorine, nitrogen, oxygen in the sample. According to EDXA graph, VB-P4 having sodium metal (Fig. 1).

Amino acid sequencing of VB-P4:

MEKSTMSAILLLVHLFLVLLQLQYSEVHSLATTSNHDFSYLRFAYDATDLELE	51
GSYDYVIVGGGTSGCPLAATLSEKYKVLVLERGSLPTAYPNVLTADGFVYN	102
LQQEDDGKTPVERFVSEDGIDNVRGRVLGGTSMINAGVYARANTSIYSASG	153
VDWMDLVNKTYEWVEDTIVFKPNYQPWQSVTGTAFLAAGVDPNHGFSL	202
DHEAGTRITGSTFDNKGTRHAADLLNKGNSNNLRVGVHASVEKIIIFSNAPG	254
LTATGVIYRDSNGTPHRAFRVRSKGEVIVSAGTIGTPQLLLSGVGPESYLSSL	307
NIPVVLSHPYVVGQFLHDNPRNFINILPPNPIEPTIVTVLGISNDFYQCSFSSLPFT	363
TPPFSFFPSTSYPLPNSTFAHFASKVAGPLSYGSLTLKSSSNVRVSPNVKFNYY	417
SNPTDLSHCVSGMKKIGELLSTDALKPKYKVEDLPGIEGFNLGIPLPKDQTDDA	470
AFETFCRESVASVWHYHGGCLVGKVLGDGFRVTGIDALRVVDGSTFPYTPA	521
SHPQGFYLMGLGRYVGKILQERSASDLKILDSLKSAASLVL	562

Paper chromatographic analysis of carbohydrate

Phenol-sulphuric acid test showed deep blue color in both VB-P4, which confirmed the presence of carbohydrate in the fractions. VB-P4 has 470 $\mu\text{g.mL}^{-1}$ of carbohydrate content. Paper chromatography of VB-P4 showed presence of glucose, galactose and glucosamine in both the compounds (Fig.1), as silver nitrate stain gave dark brown color formation.

Biochemical confirmation of carbohydrate

Seliwanoff's test of VB-P4 confirmed the presence of aldo (glucose and xylose) group in samples. Bial's test gave a blue-green colored complex in VB-P4, which confirmed the presence of aldopentose, i.e., xylose.

Anti-osteoporosis activity study

Effect of VB-P4 on physical parameter

VB-P4 (200 $\mu\text{g.100g}^{-1}$, 400 $\mu\text{g.100g}^{-1}$; i.p. x 15 days) treated groups showed 8.21% and 14.06% increase in

XRD analysis of VB-P4 showed a sharp peak of sodium i.e. p63/mmc named as Gmelinite-Sodium (Na_2Ca) $\text{Al}_2\text{Si}_4\text{O}_{12}.6 (\text{H}_2\text{O})$ having molecular weight of 552.45 and also a peak of sodium chloride (NaCl) (Fig. 1).

Membrane transfer and LC-MS/MS study of VB-P4

Membrane transfer followed by LC-MS/MS showed that it has 2 subunits having 61 kDa molecular weight each and rest of the compound having carbohydrate moiety. Moreover it resemble (100%) to R-mandelonitrile lyase1 (hydroxynitrile lyase1 or oxynitrilase) (Fig. 1).

body wt. whereas standard drug (arachitol, calcium) treatment showed 5% increase as compared with OSP control group.

Effect of VB-P4 on urinary parameters

VB-P4 (200 $\mu\text{g.100g}^{-1}$, 400 $\mu\text{g.100g}^{-1}$; i.p. x 15 days) treated groups showed 61.05% and 58.53% decrease in urinary OH-P and 49.30% and 58.90% decrease in glucosamine level respectively, whereas the standard drug (arachitol, calcium) treatment showed 52.83% decrease in OH-P and 54.32% decrease in glucosamine level as compared with the OSP group (Table.1).

VB-P4 (200 $\mu\text{g.100g}^{-1}$, 400 $\mu\text{g.100g}^{-1}$; i.p. x 15 days) treated groups showed 48.62% and 52.92% decrease in urinary calcium, 69.22% and 81.06% decrease in urinary phosphorous, 42.17% and 47.81% decrease in urinary creatinine level in OSP model; whereas standard drug (arachitol, calcium) treatment showed 48.49%, 63.48% and 13.15% decrease respectively in urinary calcium, phosphate and creatinine as compared with the OSP control (Table 1).

VB-P4 (200 $\mu\text{g}.100\text{g}^{-1}$, 400 $\mu\text{g}.100\text{g}^{-1}$; i.p. x 15 days) treated groups showed 40.87% and 51.52% decrease in urinary pyridoline, 69.09% and 81.36% decrease in urinary deoxypyridoline level in OSP model; whereas standard drug (arachitol, calcium) treatment showed 48.49%, 63.48% decrease respectively in urinary pyridoline and deoxypyridoline as compared with the OSP control (Fig. 2).

Effect of VB-P4 on serum enzymes

Serum ACP, ALP and TRAP level showed respectively 50.76%, 45.80% and 51.91% decrease after treatment with VB-P4 (200 $\mu\text{g}.100\text{g}^{-1}$ i.p. x 15 days), 83.76%, 54.80% and 82.91% decrease after treatment with VB-P4 (400 $\mu\text{g}.100\text{g}^{-1}$ i.p. x 15 days); whereas standard drug (arachitol, calcium) treatment showed 40.05%, 41.11% and 49.82% decrease respectively as compared with OSP control (Fig. 2).

Effect of VB-P4 on serum calcium, creatinine levels

VB-P4 (200 $\mu\text{g}.100\text{g}^{-1}$, 400 $\mu\text{g}.100\text{g}^{-1}$; i.p. x 15 days) treated groups showed 41.87% and 38.52% decrease in serum calcium, 69.09% and 40.36% decrease in serum creatinine level in OSP model; whereas standard drug (arachitol, calcium) treatment showed 35.49%, 43.48% decrease respectively in serum calcium and creatinine as compared with the OSP control (Table 1).

Effect of VB-P4 on serum interleukins

VB-P4 (200 $\mu\text{g}.100\text{g}^{-1}$; i.p. x 15 days) treated groups showed 48.92%, 38.12%, 43.06%, 50.44%, 42.05% decrease in pro-inflammatory makers TNF- α , CINC1, PGE2, IL-6, IL-12 and VB-P4 (400 $\mu\text{g}.100\text{g}^{-1}$; i.p. x 15 days) treated groups showed 58.92%, 49.32%, 53.06%, 52.44%, 58.05% decrease; whereas standard drug showed 56.03%, 44.78%, 44.32%, 48.90%, 49.03% decrease respectively on TNF- α , CINC1, PGE2, IL-6, IL-12 level as compared with OSP control (Fig. 2).

VB-P4 (200 $\mu\text{g}.100\text{g}^{-1}$, 400 $\mu\text{g}.100\text{g}^{-1}$; i.p. x 15 days) treated groups significantly increased the less production of anti-inflammatory markers IL-4, IL-10 (40.61%, 45.71% and 51.09%, 56.89%); whereas

standard drug increased 30.23% and 50.41% respectively on IL-4, IL-10 level as compared with OSP control (Fig. 2).

Effect of VB-P4 on serum hormones

VB-P4 (200 $\mu\text{g}.100\text{g}^{-1}$, 400 $\mu\text{g}.100\text{g}^{-1}$; i.p. x 15 days) treated groups significantly decreased the serum hormone estrogen (VB-P4 200 $\mu\text{g}.100\text{g}^{-1}$ - 82.06%, VB-P4 400 $\mu\text{g}.100\text{g}^{-1}$ - 89.37%), progesterone (VB-P4 200 $\mu\text{g}.100\text{g}^{-1}$ - 46.11%, VB-P4 400 $\mu\text{g}.100\text{g}^{-1}$ - 53.89%), LH (VB-P4 200 $\mu\text{g}.100\text{g}^{-1}$ - 49.28%, VB-P4 400 $\mu\text{g}.100\text{g}^{-1}$ - 51.67%), FSH (VB-P4 200 $\mu\text{g}.100\text{g}^{-1}$ - 49.28%, VB-P4 400 $\mu\text{g}.100\text{g}^{-1}$ - 51.67%) levels where as standard drug decreases the levels respectively on estrogen (68.82%), progesterone (51.62%), LH (47.36%) and FSH(45.53%) levels.

Only calcitonin level is decreased after treatment with VB-P4 (200 $\mu\text{g}.100\text{g}^{-1}$ - 72.19%; 400 $\mu\text{g}.100\text{g}^{-1}$ - 82.64%); whereas standard drug decreases the level 80.93% (Fig. 2).

Effect of VB-P4 on serum pro-oxidant and anti-oxidants

VB-P4 (200 $\mu\text{g}.100\text{g}^{-1}$, 400 $\mu\text{g}.100\text{g}^{-1}$; i.p. x 15 days) treatment showed 48.34% and 51.03% decrease; whereas standard drug treatment showed 49.32% decrease with compared to OSP group.

VB-P4 (200 $\mu\text{g}.100\text{g}^{-1}$, 400 $\mu\text{g}.100\text{g}^{-1}$; i.p. x 15 days) treatment showed 46.69% and 53.98% increase in GSH level; 48.39% and 53.06% increase in SOD level; 75.48 and 82.04% increase in Catalase level; whereas standard drug increases 42.69% in GSH, 40.76% in SOD and 76.90% in Catalase level (Fig. 3).

Effect of VB-P4 on serum pyridoline, deoxypyridoline and osteocalcin

Serum pyridoline (50.63% and 78.31%), deoxypyridoline (49.34% and 50.89%) & osteocalcin (75.22% and 82.04%) levels in OSP decreased in VB-P4 (200 $\mu\text{g}.100\text{g}^{-1}$, 400 $\mu\text{g}.100\text{g}^{-1}$ i.p. x 15 days) treatment, whereas standard drug (arachitol, calcium) treatment showed 78.56% decrease in pyridoline, 40.76% decrease in deoxypyridoline and 76.45% decrease in osteocalcin level as compared with OSP control (Fig. 2).

Table 1. Effect of urinary and serum parameters after treatment with VB-P4 in OSP rat models.

Animal Group	Urinary OH-P ($\mu\text{g/mL/rat/day}$)	Urinary glucosamine ($\mu\text{g/mL/rat/day}$)	Urinary Ca^{2+} (mg/dL/24h)	Urinary PO_4^{3-} (mg/dL/24h)	Urinary CRE (mg/dL/24h)	Serum Ca^{2+} ($\text{mg}^{-1}\text{dL}^{-1}\text{day}^{-1}$)	Serum CRE ($\text{mg}^{-1}\text{dL}^{-1}\text{day}^{-1}$)
Gr.1	06.10 \pm 0.17	4.58 \pm 0.15	0.122 \pm 0.05	0.088 \pm 0.04	0.390 \pm 0.01	04.90 \pm 0.42	5.20 \pm 0.42
Gr.2	12.59 \pm 0.15*	9.63 \pm 0.05*	0.332 \pm 0.11*	0.664 \pm 0.02*	0.624 \pm 0.02*	12.12 \pm 0.52*	9.88 \pm 0.58*
Gr.3	08.12 \pm 0.13*	5.44 \pm 0.09*	0.171 \pm 0.02*	0.250 \pm 0.02*	0.485 \pm 0.03*	08.89 \pm 0.35*	7.91 \pm 0.95*
Gr.4	07.59 \pm 0.20*	5.01 \pm 0.12*	0.192 \pm 0.05*	0.211 \pm 0.03*	0.450 \pm 0.02*	07.88 \pm 0.51*	7.80 \pm 0.50*
Gr.5	06.98 \pm 0.17*	4.13 \pm 0.09*	0.151 \pm 0.02*	0.112 \pm 0.01*	0.409 \pm 0.01*	05.86 \pm 0.20*	7.43 \pm 1.19*

Data represent the mean \pm SEM (n=6). * $p < 0.05$ when compared to OSP control group. Gr.1: Sham control, Gr.2: OSP control, Gr.3: Standard drug (arachitol, calcium), Gr.4: VB-P4 (200 $\mu\text{g.g}^{-1}$; i.p. x 15 days), Gr.5: VB-P4 (400 $\mu\text{g.g}^{-1}$; i.p. x 15 days).

Fig. 2: Effect of urinary and serum parameters after treatment with VB-P4 in OSP rat model. Concentration of ACP, ALP and TRAP were expressed in terms of $\mu\text{mol PNP.min}^{-1}$. Concentration of $\text{TNF-}\alpha$, CINC1, PGE2, IL-6, IL-12, IL-4, IL-10, pyridoline, Deoxypyridoline, estrogen, progesterone, LH, FSH, calcitonin were expressed in terms of pg.ml^{-1} . Data represent the mean \pm 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 $\mu\text{g.100g}^{-1}$; i.p. x 15 days), Gr.5: VB-P4 (400 $\mu\text{g.100g}^{-1}$; i.p. x 15 days).

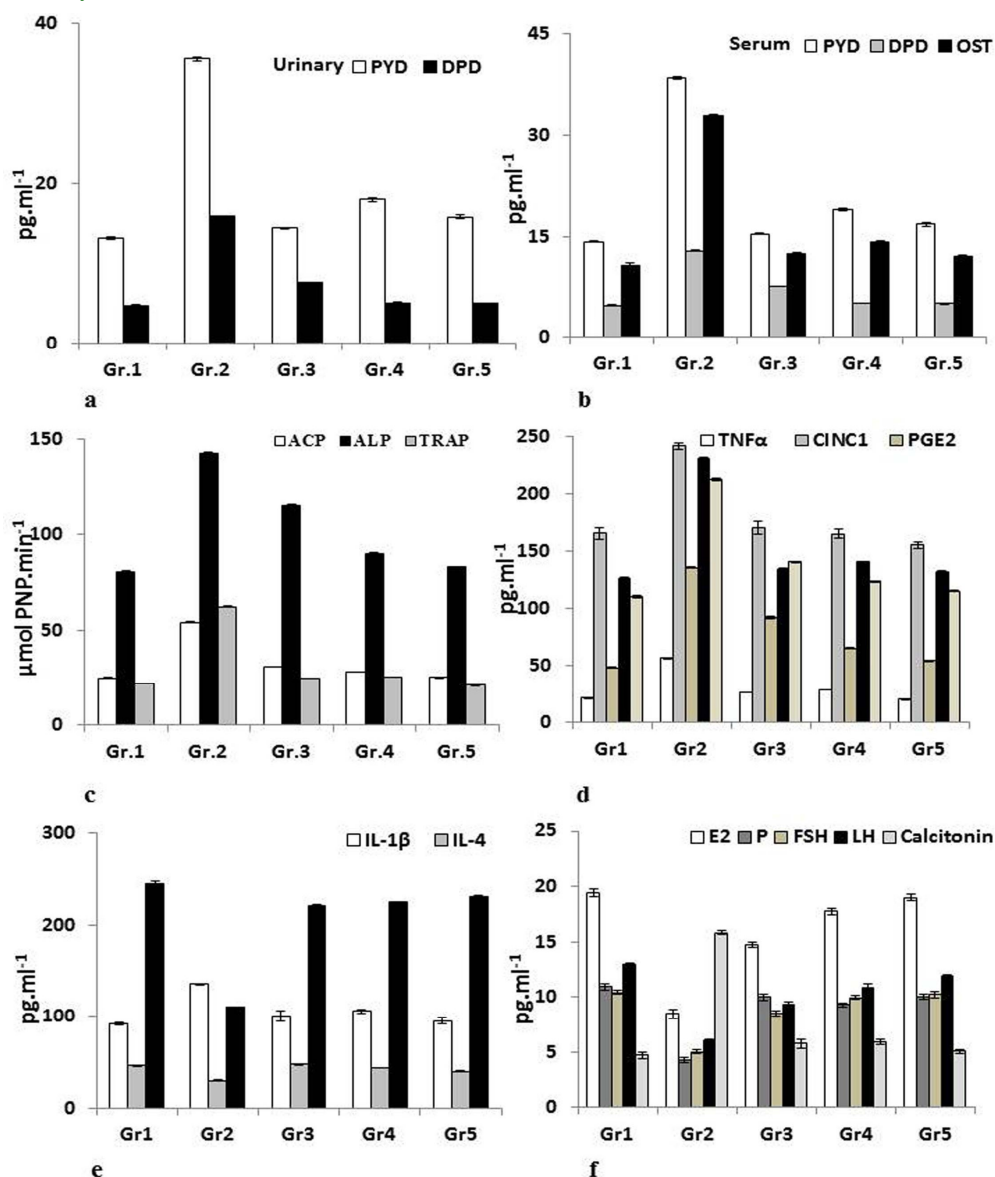
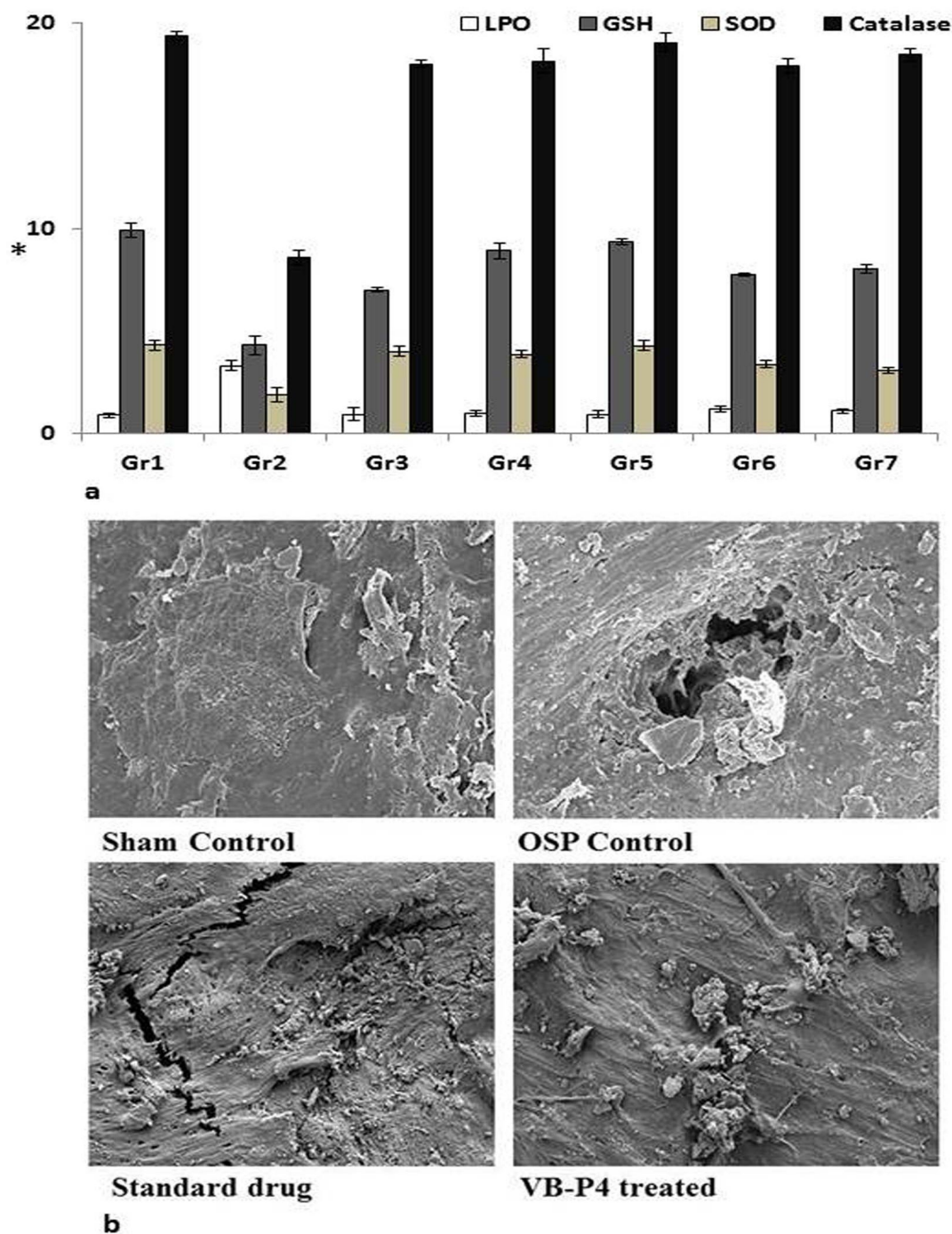


Fig. 3: (a) Effect of serum pro and anti-oxidant parameters after treatment with VB-P4 in OSP rat model. (b) Bone SEM analysis after treatment with VB-P4.



Effect of VB-P4 on bone histology and bone architecture through scanning electron microscopic study

VB-P4 treatment in overiectomized rat showed partial restoration of bone architecture through histological study and scanning electron

microscopic analysis. In OSP control group, deep hollow was observed followed by change in bone normal architecture. The bone surface was disrupted and smoothness was absent there. But VB-P4 treated group showed partial restoration of normal bone architecture followed by restoration of bone surface disruption (Fig. 3).

Effect of VB-P4 on bone ash minerals assessment through ICP-MS

Bone ash minerals were assessed after treatment with VB-P4 in ovariectomized rats. It was shown that bone minerals such as calcium, magnesium, sodium, zinc,

potassium, phosphorus, manganese, iron levels were decreased in OSP control group due to development osteoporosis. These mineral levels showed significant increased level after treatment with VB-P4 near about to the control group as compared to OSP control group (Table 2).

Table 2. Effect of bone minerals after treatment with VB-P4 in OSP rat models.

Animal Group	Calcium (ppm)	Magnesium (ppm)	Sodium (ppm)	Zinc (ppm)	Potassium (ppm)	Phosphorous (ppm)	Manganese (ppm)	Iron (ppm)
Gr.1	200.07±0.12	8.20±0.22	25.44±0.24	122.79±0.14	3.51±0.12	242.11±0.19	0.8±0.02	1.26±0.11
Gr.2	135.07±0.17*	5.98±0.11*	14.78±0.09*	83.91±0.16*	1.24±0.08*	167.80±0.17*	0.3±0.05*	0.60±0.18*
Gr.3	185.80±0.23*	7.32±0.18*	19.33±0.19*	118.41±0.25*	1.51±0.15*	215.11±0.16*	0.5±0.03*	1.11±0.09*
Gr.4	192.28±0.19*	7.87±0.14*	21.06±0.17*	111.98±0.11*	2.16±0.09*	222.48±0.14*	0.5±0.01*	1.10±0.15*
Gr.5	199.14±0.27*	7.99±0.15*	23.95±0.24*	118.91±0.27*	3.49±0.07*	229.47±0.09*	0.6±0.02*	1.17±0.13*
Data represent the mean ± SEM (n=6). * p<0.05 when compared to OSP control group. Gr.1: Sham control, Gr.2: OSP control, Gr.3: Standard drug (arachitol, calcium), Gr.4: VB-P4 (400 µg.g ⁻¹ ; i.p. x 15 days), Gr.5: VB-P4 (400 µg.g ⁻¹ ; i.p. x 15 days).								

Discussion

Osteoporosis mostly happened in elderly population where bone become porous due to loss of calcium inside bone after menopause when the hormonal levels are altered and it leads to pain and inflammation. To avoid these adverse effects and huge cost factors of allopathic drugs, researchers and scientists ventured natural therapies as they are having no such side effects and low cost factor. As natural therapy, plants like ginger (Bliddal et al., 2000; Lee et al., 2003), curcumin (Belcaro et al., 2010), *Andrographis paniculata* (Burgos et al., 2009), pine bark extracts (Cisar et al., 2008), rosehip (Christensen et al., 2008), tea (Ferraz et al., 1991), stinging nettle (Randall et al., 2000), willow bark (Schmid et al., 2001) are common in bone and joint disease. According to folk tradition, rural Indians use several types of animal parts for the treatment and screened for diseases. Fish liver oil (Stammers et al., 1992), green-lipped mussel (Coulson et al., 2012) are also used for treatment of bone and joint diseases. Use of molluscs as zoo therapy is a worldwide phenomenon, starting far back in prehistory. Very few scientific evidences are there to prove the anti-arthritic, anti-osteoporosis, anti-cancer, antioxidant, anti-inflammatory, hepatoprotective activity and so many other health related problems.

In present study, IEC and HPLC purified fraction VB-P4 showed activity against osteoporosis. SDS-PAGE followed by PAS stain confirmed the MW and glycoprotein nature of VB-P4. In UV scan, proteins in solution absorb ultraviolet light with absorbance maxima at 280 and 200 nm. Amino acids with aromatic rings are the primary reason for absorbance

peak at 280 nm. Peptide bonds are primarily responsible for the peak at 200 nm. Fluorescence property also confirmed the presence of tyrosine, phenylalanine and tryptophan in VB-P4, as they are essential to give the fluorescence peak. VB-P4 showed sharp excitation peak at 270-280 nm and emission peak at 530-540 nm that confirmed the protein nature. It also has more α -helical structure. FTIR analysis showed presence a number of aliphatic and aromatic compounds. EDAX analysis showed the presence of sodium metal which was again confirmed by XRD analysis. LC-MS/MS study confirmed 2 subunits in VB-P4, each having 562 amino acids. Presence of carbohydrate was also confirmed through phenol sulphuric acid method and paper chromatography.

In this study, VB-P4 treatment showed restoration of body weights, which may indicate the hormonal and calcium level restoration in animals. Osteoporosis leads to decreased body weight due to hormonal imbalance and loss of calcium from body through urine. VB-P4 treatment increased the body weight which implies the restoration of osteoporotic condition in animal model. VB-P4 significantly restored urinary hydroxyproline (Dull and Henneman, 1963) and glucosamine (Forchhammer et al., 2003) compared with osteoporosis control group. Hydroxyproline is a major component of the protein collagen (Paul, 2011). Hydroxyproline and proline play key roles for collagen stability (Nelson and Cox, 2005). 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 in humans as well as in rats (Felson, 2006; Forchhammer et al., 2003).

Glucosamine ($C_6H_{13}NO_5$) is an amino sugar and a prominent precursor in biochemical synthesis of glycosylated proteins and lipids. Glucosamine makes up the building blocks in body. It exhibit preventive actions on osteoporosis (Nakamura et al., 2007). OSP leads to bone resorption thereby release of calcium, phosphorous into extracellular fluid, then organic matrix resorbed and are excreted through urine. As a result, serum and urinary level of calcium, phosphate and creatinine levels were altered. Pyridoline and deoxypyridoline were found to be released into the blood during bone degradation and rapidly excreted in the urine. For bone remodeling pyridinium based cross-links are very important for extracellular collagen fibrils. Deoxypyridoline or deoxypyridinoline, also called D-Pyridyls, Pyridyls-D, or deoxyPYD, is one of two pyridinium cross-links that provide structural stiffness to type I collagen found in bones (Rubinacci et al., 1999).

It is excreted unmetabolized in urine and is a specific marker of bone resorption and osteoclastic activity. It is measured in urine tests and is used along with other bone markers such as alkaline phosphatase, osteocalcin, and N-terminal telopeptide to diagnose bone diseases such as postmenopausal osteoporosis, bone metastasis, and Paget's disease, furthermore, it has been useful in monitoring treatments that contain bone-active agents such as estrogens and bisphosphonates. In the study, after treatment with VB-P4 urinary and serum levels were restored. This may be because of the partial restoration of these cross linked product in bone.

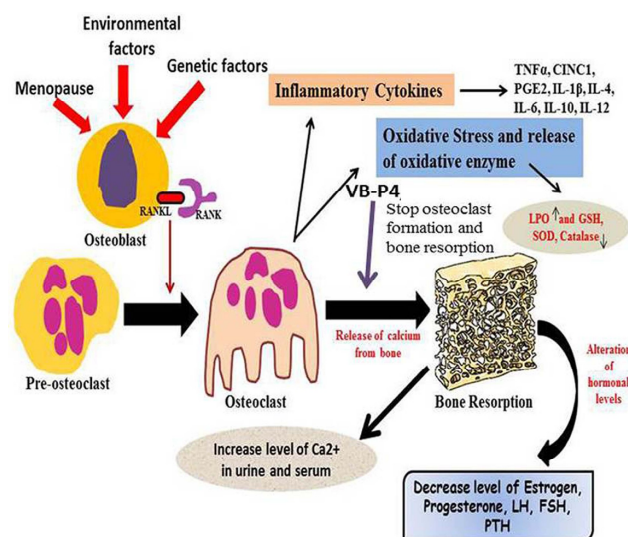
In OSP, ACP, ALP, TRAP levels increased in the serum (Gomes et al., 2009). VB-P4 treatment significantly decreased the enzymes level which indicated restoration of damages of lysosomal membrane integrity (Ljusberg et al., 1999; Ljusberg et al., 2005). Due to T cell activation pro and anti-inflammatory cytokines levels were altered which was restored by VB-P4 treatment. Due to T cell activation TNF- α (Saklatvala, 1986; Probert et al., 1995), IL-1 β , CINC-1 (Takano and Nakagawa, 2001), PGE2, IL-6 (Wong et al., 2006), IL-4, IL-12, osteocalcin levels were significantly increased and IL-4, IL-10 levels were significantly decreased in the osteoporosis and osteoarthritic group (Cannetti et al., 2003) which was restored by VB-P4 treatment. Estrogen, progesterone, LH, FSH, calcitonin are the female sex hormones that altered

during osteoporosis. When excess amount of Ca^{2+} is released from the body, the hormonal levels are decreased, but only calcitonin level alters proportionally to Ca^{2+} release. These hormones are decreased during menopause, so that osteoporosis development occurred. In this study, VB-P4 treatment restored the female hormones levels, which may revealed the protection against osteoporosis and bone loss. During osteoporosis oxidative stress level is increased as ROS generation is increased inside the body. The lipid peroxidase product, i.e. thiobarbituric acid (TBARs) level increased in rats osteoporosis (Jaswal et al., 2003). Glutathione, superoxide dismutase, catalase levels are decreased in osteoporosis (Hassan et al., 2001) due to increased turnover of substrate reactant to prevent oxidative damages. Here also observed a significant restoration of the parameters to prevent oxidative damages after treatment with VB-P4 when compared with OSP control groups. Bone ash analysis in case of osteoporosis revealed a significant restoration of bone minerals after treatment with VB-P4 that also implies a positive effect in case of osteoporosis developed animals. SEM or scanning electron microscopic analysis showed the partial restoration of long bone after treatment with VB-P4 as compared to arthritic control. In case of osteoporosis control group, a clear bone damage followed by hollow formation. Whereas in treated group, the bone damage was significantly lower than osteoporosis control group.

In this study anti-osteoporosis activity of *V.bengalensis* flesh extract purified fractions were observed. In osteoporosis bone mineral density is decreased. Calcium is released from bone due to osteoclast formation. The osteoclast leads to release of huge amount of calcium from bone that leads to disruption of normal bone architecture. Bone becomes brittle at that time. All the released calcium comes in the circulation and excreted through urine. During this time, hormonal levels are also altered. LH, FSH, estrogen, progesterone levels are decreased, because these hormones play a vital role in bone formation and maintenance of bone architecture. Calcitonin hormone level increased significantly because of release of calcium. All the Inflammatory cytokines such as TNF α , IL-1 β , CINC1, PGE2, IL-4, IL-6 etc. levels are altered which is an indication of correlation of OSP and inflammation. Due to oxidative damage also pro and

anti-oxidant enzymes levels are altered in osteoporosis condition. Bioactive components of *V.bengalensis* flesh extract VB-P4 may be stop or minimize osteoclast formation from pre-osteoclast (Fig. 4).

Fig. 4: Probable mechanism of action VB-P4 in management of osteoporosis.



Conclusion

With the advancement of science, lots of medications are there. But most of them are having side effects. To avoid the adverse effect natural therapies from plant and zoo products are ventured. Of them, fresh water snail *V. bengalensis* flesh extract can be used as an alternative therapy. The present study was done to observe the anti-osteoporosis activity of the purified fractions of the flesh extract.

Isolated protein fractions of fresh water snail *V. bengalensis* flesh extract VB-P4 significantly suppressed the development and progression of OSP in experimental animals, which confirmed through physical and urinary parameters. Serum markers also maintained 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. 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.

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