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TARGETING ANTIOSTEOPOROTIC POTENTIAL OF β-SITOSTEROL IN*AMORPHOPHALLUS PAEONIIFOLIUS*USING OVARIECTOMIZED RAT MODEL

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ABSTRACT

Petroleum ether extract of *Amorphophallus paeoniifolius* (PEAP) and β-Sitosterol were evaluated for their anti-osteoporotic activity in ovariectomized rat model of osteoporosis. Thirty-six female rats (180-220 gm) were randomly divided into six groups (n=6). All groups except sham control were ovariectomized to induce osteoporosis. Treatment with PEAP (100 and 300 mg/kg, p.o) and β-sitosterol (120 mg/kg, p.o) to ovariectomized rats started from 60th day post-ovariectomy for duration of 45 days thereafter. Serum biochemical parameters (Alkaline phosphatase (ALP), calcium, inorganic phosphorous) were estimated on 1st and 45th day of treatment. Biomechanical parameters (Bone mineral density(BMD), bone weight, length and thickness, uterine weight, force at break, bone ash calcium content)and histopathology were performed at end of treatment. Elevated levels of serum ALP, calcium and phosphorous due to ovariectomy were significantly attenuated ($P<0.0001$, $P<0.01$) on treatment with PEAP and β-sitosterol. Post-ovariectomy rise in body weight was prevented in treatment groups. After administration of PEAP and β-sitosterol total femur BMD and biomechanical strength were significantly ($P<0.0001$) improved, confirming re-mineralization of bones. Histological results exhibited increased trabecular thickness and decrease osteoclast formation thus indicating protective activity of PEAP and β -sitosterol through promotion of bone formation and suppression of bone resorption. These results suggest that PEAP has remarkable anti-osteoporotic activity due to rich content of phytoestrogen β-sitosterol. And thus, may prove a promising candidate in treatment of post-menopausal osteoporosis.

KEYWORD: *Amorphophallus paeoniifolius*, β-Sitosterol, Ovariectomy (OVX), Post-menopausal Osteoporosis, and Bone Mineral Density (BMD).

INTRODUCTION

Post-menopausal osteoporosis is the most frequently occurring global health issue associated with chronic worsening of bones afflicting over more than 2000 million people worldwide.[1] As the condition worsens there is an increase in bone fragility that subsequently increases the susceptibility to fractures.[2] Osteoporosis, is a foremost global public health issue which is characterized by disruption of bone microarchitecture and progressive loss of bone mineral density and an increased risk of fractures which causes disability and mortality. Osteoporosis is an area of interest since it affects the age-old people and particularly postmenopausal women because of estrogen deficiency after menopause.[3]

Many synthetic agents have been developed for treating post-menopausal osteoporosis which include vitamin D3, calcium supplement, selective receptor modulator (SERM's) such as Raloxifene, Droloxifene, Tamoxifene and bisphosphonate but their use is limited because of associated side effects such as hypercalcemia, hypercalciuria, endometrial hyperplasia and breast cancer, breast tenderness, thromboembolic events, vaginal bleeding, dyspepsia, hot flashes and gastrointestinal ulcers.etc.[4] To prevail over side effects of synthetic drugs demand of herbal medicine is increasing as they are thought to be safer and healthier.[5] Phyto-estrogen from herbs plays an important role by binding to the estrogen receptor sites and triggering estrogenic activity. There are several phyto-estrogenic classes viz. lignans, isoflavones, and coumestans etc amongst which isoflavone being reported as the most active phytoestrogen.[6] In this study *Amorphophallus paeoniifolius* (AP) being rich with isoflavoneβ-sitosterol is selected to probe anti-osteoporotic effect in ovariectomized rat models.[6,7,8] *Amorphophallus paeoniifolius* commonly known as elephant foot yam is an edible tuber crop more popularly cultivated in tropical and subtropical regions, predominantly in south-east Asia. The corms of elephant foot yam have broad range of therapeutic applications as analgesic, antibacterial, antifungal, cytotoxic, CNS depressant, anti-inflammatory, anti-oxidant, and hepatoprotective.[9,10,11,12] The phyto-constituent reported in AP include carbohydrates, steroid, flavonoid,

terpenoids, alkaloids etc. Quantification studies conducted in our laboratory had revealed higher content of β-sitosterol in petroleum ether extract of AP (PEAP) and hence it was used for *in-vivo* evaluation of anti-osteoporotic activity by using ovariectomized rat model. Excision of ovary in matured female rat during ovariectomy results into estrogen deficiency which simulates post-menopausal condition and hence is an excellent animal model to induce post-menopausal osteoporosis. The aim of the present study is to explore anti osteoporotic activity of PEAP and β-sitosterol present in it.

MATERIAL AND METHODS

Raloxifene hydrochloride tablets (Cipla Pvt. Ltd. Goa, India), β-Sitosterol (Yucca Enterprise), Ketamine hydrochloride injection (Aneket, Neon Laboratories Ltd.), Xylazine injection (Xylo B, Brilliant Biopharma Pvt.Ltd.), Amoxicillin injection (Augmentine injection, Galaxo Smithkline. Ltd, Mumbai), Povidone iodine ointment-USP (Cipladine, Cipla), commercial reagent kits for estimation of Calcium, Alkaline Phosphatase and Phosphorous (Erba Mannheim, Baddi, India), and all other chemicals and reagents were of analytical grade purchased from S D fine-chem Ltd, Mumbai, India.

Plant collection

Fresh corms of *Amorphophallus paeoniifolius* were procured from the local market of Mumbai, India. The corm was identified and authenticated by Prof. Harshad M. Pandit, Formely Head and Associate Professor of Botany, University of Mumbai, India. The corms of *Amorphophallus paeoniifolius* were sun dried, powdered mechanically, sieved(20 #)and used for extraction.

Extraction

The dried powder of AP was extracted using Soxhlet extractor in petroleum ether and methanol. Extracts were 103 re-distilled at 50-60˚C. and were stored in refrigerator at 2-8˚C.[13] The petroleum ether extract was used for *in vivo* studies due to higher β-sitosterol content found during quantification study by HPTLC.

Preliminary Phytochemical Screening

Phytochemical qualitative screening of extract was performed[14,15] for detection of phytoconstituents like alkaloids, sterols, flavonoids, saponins, glycosides, tannins etc.

Identification, Quantification and Validation of β-Sitosterol by HPTLC

Content of β-Sitosterol in extract PEAP was identified and quantified by using HPTLC. 10 mg of standard β-Sitosterol (90%purity) was dissolved in 10 ml of methanol to prepare a stock solution of 1mg/ml. 100 mg of extract was dissolved in 10 ml of methanol to prepare a stock solution of 10 mg/ml. Quantification of β-sitosterol in PEAP was performed using HPTLC method. Standard β-Sitosterol (3µl) and extract solutions (3µl) were applied over pre-coated silica gel 60 F254 plates 130 (stationary phase) using CAMAG HPTLC SYSTEM 100 microlitre Halminton syringe with help of Linomat V applicator. Application distance between two spots was maintained at 8 mm. Plate was run in Mobile phase (Toluene: Ethyl Acetate: Formic acid (50: 15: 5). Plate was placed into CAMAG twin trough chamber for saturation of 20 minutes. After drying and developing plates for 15minutes, plates were scanned using CAMAG TLC Scanner, 1720422 at different wavelengths 254nm and then plate was derivatized by 10% Methanolic Sulphuric acid and observed at 366 nm. Data was analysed using Integrated software vision (CATS). The concentration of β-Sitosterol and Lupeol in the extracts was determined by comparing the areas of chromatograms of extracts with the calibration curve of standard β-Sitosterol. Method of development was validated with PEAP extract using standard β-Sitosterol. Specificity, recovery and reproducibility were considered for the validation of developed method.

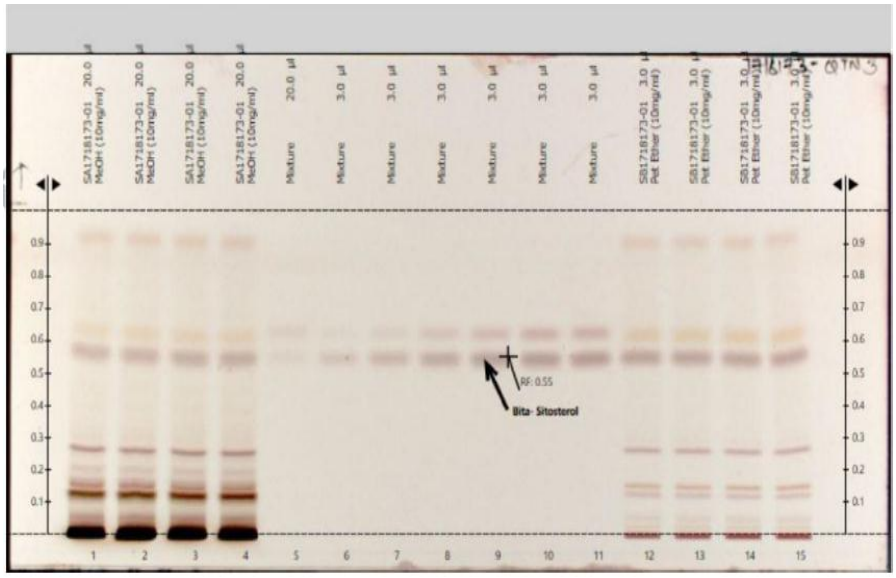


Fig No: Figure illustrates HPTLC fingerprinting of PEAP, MEAP extract and standard B-Sitosterol. (Rf=0.55).

Extract pre-treatment

The weighed quantity of PEAP was suspended in Tween 80 and administered orally to the rats. The suspension of extract was prepared freshly every day.

Experimental animals

Female Albino Wistar rats weighing 180-220g were procured from Shree Dhootapapeshwar Ayurvedic Research Foundation (SDARF), Panvel, a registered breeder, registration no. (136/PO/RcBi/S/99/CPCSEA). The animals were maintained in hygienic conditions in our animal house in clean polypropylene cages containing husk bedding. The animals were fed with standard pellet diet (provided by SDARF, Panvel) and water *ad libitum*. Animals were allowed to acclimatize to our animal house conditions for 8-10 days former to the experiment under standard conditions of temperature (24ºC±2ºC), relative humidity (50%±5%) and light (12 h light/ 12 h dark cycle) in institute's animal house. The institution's animal house is registered with Govt. of India, registration no. (25/PO/ReBi/S/99/CPCSEA Dtd-10/03/1999). Experimental protocol was reviewed and approved by Institutional Animal Ethics Committee (Protocol no- KMKCP/IAEC/06/2017). Guideline of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) India were followed for use and care of experimental animals in research.

Dose selection

Doses selected for PEAP were 100 and 300 mg/kg according to reported study in literature. Toxicity study of PEAP conducted by et al Ghosh A, Das S, 2009 has revealed that the PEAP was safe up to 2000 mg/kg in rats. Dose selected for β-sitosterol is 120 mg/kg as per amount of β-sitosterol (0.040% w/w) quantified in extract PEAP.

Induction of osteoporosis by ovariectomy (OVX)

Surgical procedure

After one week of acclimatization, the rats were randomly divided into six groups consisting of six animals each. All groups were anesthetized with the combination of Ketamine (80 mg/kg ip.) and Xylazine (10 mg/kg i.p.). Group I was sham operated and other five groups were ovariectomized. The surgical equipment used were aseptically cleaned with rectified spirit. The operation was carried out by placing each animal on its ventral surface. The fur on the rat abdomen was cleaned with the help of Dettol and rectified spirit and then the fur was completely removed with the help of blade. Ovariectomy was preceded by the midline ventral skin incision (bilateral), 3cm long, approximately half way between the midline of the body and the base of the tail. After peritoneal cavity was accessed, the adipose tissue was pulled away until the uterine tube and the ovary surrounded by the variable amount of fat were identified. A ligation was performed around the area of distal uterine horn with absorbable suture to avoid bleeding before removal of ovary. The connection between uterine horn and fallopian tube was cut and the ovary was excised. Whereas in Sham operated rats' ovary was exposed but not removed. In all ovariectomized groups the uterine horn was returned to the peritoneal cavity after removal of ovaries. The muscle incision was sutured with absorbable suture and skin wounds were closed bilaterally with absorbable catgut.[19,20] Operated animals were given Amoxicillin (Augmentine injection) (25 mg/kg, i.p.) for 5 days. Povidone Iodine solution (Cipladine) was applied topically, and animals were monitored carefully. After two days of surgery, two rats were housed in each polyurethane cage for a period of 1 week to allow recovery and also observed for coprophagy. They were also observed for normal behaviour and then re-grouped in their home cages.[17,18]

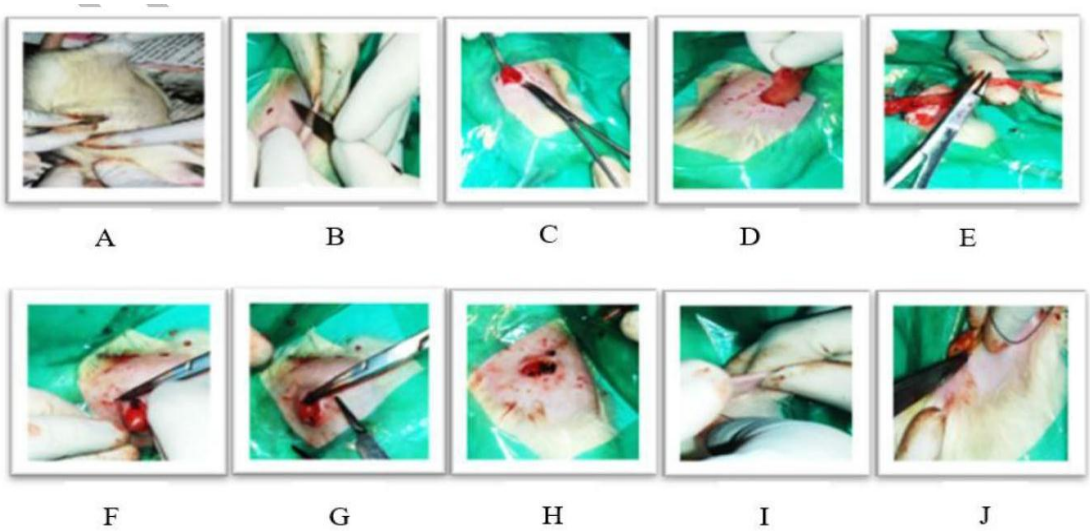


Fig. 1: Steps in ovary removal surgery.

A) Removal of fur by blade (B) Midline ventral incision (Cand D) Assessment of ventral cavity (E) Uterine horns were seen (F) Ovaries were excised (G) Uterine horns were returned to peritoneal cavity (H) Application of iodine solution (I) Suturing with absorbable suture (J) Skin wound were closed bilaterally.

Experimental design

Female Wistar rats of six months old weighing 180-220 300 g were used in the study. All the operated rats were 304divided into 6 groups (n=6) and treated for 45 days.

Group I-Sham operated (distilled water 2ml/kg,p.o)
Group II- OVX control (distilled water 2ml/kg,p.o)
Group III- OVX + Standard Raloxifene (5.4 mg/kg,i.p)
Group IV, V- OVX + PEAP(100 mg/kg and 300 mg/kg, p.o.), respectively.
Group VI- OVX+β-Sitosterol (120 mg/kg, po.)

On 45th day of treatment blood was withdrawn from retro orbital plexus and centrifuged (Eltek refrigerated centrifuge RC 4100 D) at 3000 rpm for 10 minutes at -4˚C to obtain clear serum. Levels of ALP, calcium and inorganic phosphorous in serum were estimated using ERBA diagnostic reagent kits. At the end of the study animals were sacrificed in carbon dioxide chamber and femur bones, each from left and right limbs were removed and were cleaned with the help of papain juice. Uterus was excised to measure uterine weight. Three right femur bones from each group were used for estimation of bone ash calcium levels and remaining three were used for histopathological study. Left femur bone was used to evaluate BMD and force at break for each group.

Evaluation parameters

Measurement of length, thickness and weight of femur bone[19]

The length was measured as the distance between greater trochanter and medial condyle and thickness at the femoral midshaft was also measured using Verniercalliper (Sando). Then the bones were kept in an oven and dried at 100˚C and weights of the dried bones were determined by using a digital weighing balance (Metler).

Measurement of body weight and uterine weight[19]

The body weight of each rat from all the groups were monitored in a weekly basis for 105 days. At the end of the study all the rats were sacrificed by using carbon dioxide chamber and uterus was removed and weighed by using a digital weighing balance(Metler).

Serum biochemical markers[20,24]

The calcium and phosphorous (serum) and alkaline 348 phosphatase (serum), were estimated using Erba diagnostic kits.

Analysis of Bone mineral density (BMD)[24,26]

Femurs were scanned with μ-CT (Tri-Foil imaging) at a resolution of 21μM by applying the following settings:

X-ray voltage 60 kV and electric current 130μA. Bone mineral density (BMD), bone mineral content (BMC), trabecular thickness (Tb.Th), and trabecular space (Tb.Sp) were analyzed using Micro View v. 2.0 362 Software.

Femur bone Biomechanical strength (Force at break)[27]

This experiment was conducted on the right femur of the rats. The fracture force for the given femur sample was measured by using a digital hardness tester. (Praveen enterprise Bangalore.) The right femur was isolated and the surrounding tissues were cleaned. The fresh bone was placed in digital hardness tester, then compressed until bone gets fractured and the reading was recorded in Newton's (kg/cm2).

Determination of femoral ash weight, ash percentage and calcium content[28,30]

The soft tissues were cleaned from the femur bones which were then broken into small fragments, placed into a container of ethanol and soaked overnight before being extracted with ethanol in a Soxhlet extractor for 24 h and further extracted with anhydrous ether. After the second extraction, bones were dried at room temperature for 24h. And then placed in tarred fused silica crucibles, kept in muffle furnace, ashed at 600˚C for 24 hrs, then ash weight and percentage ash were determined. Further the ash was used for the calcium assay by titrimetric method using AOAC standard procedures.

Histopathology

Tissues specimens were collected from animals belonging to different treatment groups. After collection the tissues samples were immediately preserved in the 10% neutral buffered formalin for fixation. Tissues were decalcified using 5% Formic acid for 12 days and were subsequently processed by routine method for histological observation. Processed tissue was sectioned (at 5 um) and taken on the clean glass slides and stained by haematoxylin and eosin and observed under microscopes at different magnifications. Skin examined microscopically to check presence of alterations having pathological significance.

Statistical analysis

All the values were expressed as Mean ± SEM. Statistical analysis were carried out by one-way ANOVA followed by Tukey-Kramer post hoc multiple comparison test using Graph Pad Prism version 7.0.

RESULTS

Table No. 1: Effect of PEAP and β-sitosterol on femur bone weight, length and thickness.

Sr. No.	Groups (n=6)	Femur absolute	Femur relative	Length (cm)	Thickness
1	sham control	0.597±0.0327	0.234±0.0015	3.53±0.026	0.450±0.018
2	OVX control	0.418±0.0215###	0.121±0.0017###	3.46±0.018	0.412±0.022
3	OVX+ Raloxifene (5.4 mg/kg, i.p)	0.515±0.0132***	0.173±0.0043***	3.5±0.026	0.425±0.025
4	OVX+PEAP (100 mg/kg, p.o)	0.455±0.0147**	0.156±0.0049**	3.5±0.026	0.425±0.025
5	OVX+PEAP (300 mg/kg, p.o)	0.505±0.0211***	0.171±0.0038***	3.51±0.022	0.437±0.018
6	OVX+β-Sitosterol (120 mg/kg, p.o)	0.543±0.0364***	0.188±0.0029***	3.51±0.022	0.412±0.022

Values are expressed as Mean ± SEM (n=6). Statistical analysis was carried out by One-way ANOVA followed by Tukey – Kramer post hoc multiple comparison test for weight, length and thickness of femur bone. For femur bone:###P<0.0001 vs Sham Control Group; ***P<0.0001, **P<0.01 vs OVX Control Group; For bone length and thickness: data were found non- significant.

The results of absolute and relative weight, length and thickness of the femur bone in animals of different groups are illustrated in table 1. The absolute and relative weight was found to be significantly (P<0.0001) decreased in the OVX control group as compared to sham control. Treatment with PEAP (P<0.01, P<0.0001), β-sitosterol (P<0.0001) and standard Raloxifene (P<0.0001) showed significant increase in femur weight as compared with OVX group. However, there was no significant alteration in bone length and thickness in treated groups.

Table No. 2: Effect of PEAP and B-sitosterol on uterine weight and body weight.

Sr. No	Groups (n=6)	Uterine wet weight (gm) on	Body weight (gm)		% increase in body weight.
		45th day	1st day	45th day	
1	Sham Control	2.358±0.065	208.7±22.26	254.30±23.09	21.84%
2	OVX control	0.835±0.062###	301.2±18.26###	345.30±20.14###	14.61%
3	OVX+Raloxifene (5.4 mg/kg, i.p)	1.613±0.073***	300±18.25$	297.21±18.01***	13.92%
4	OVX+PEAP (100mg/kg,p.o)	1.203±0.091**	303±20.22$	300.34±22.05***	13.02%
5	OVX+PEAP (300 mg/kg,p.o)	1.523±0.054***	300±20.3$	293.71±19.04***	14.94%
6	OVX+ β-Sitosterol (120 mg/kg, p.o)	1.655±0.080***	301.5±18.20$	290.20±23.03***	15.95%

Values are expressed as Mean ± SEM (n=6). Statistical analysis was carried out by One-way ANOVA followed by Tukey-Kramar post-hoc multiple comparison test ###P<0.0001 vs sham control group, $= Non-Significant, **P<0.01, ***P<0.0001, vs. OVX control groups.

In the present study, wet weight of uterus was determined. The uterus weight was significantly (P<0.0001) reduced in OVX control as compared to sham control rats. On treatment with PEAP (P<0.01, P<0.0001), β-sitosterol (P<0.0001) and standard Raloxifene (P<0.0001) significant increase in uterus weight was observed as compared with OVX group. The body weights of the animals were also monitored on weekly basis results of which are shown in table 2. OVX control group showed (P<0.0001) increase in body weight as compared to sham control. On administration of PEAP, β-sitosterol and standard Raloxifene to the ovariectomized animals significant (P<0.0001) increase in body weight was observed as compared to OVX.

Table No.3: Effect of PEAP and β-sitosterol on serum alkaline phosphatase, calcium and phosphorous levels.

Sr. No.	Groups (n=6)	Alkaline Phosphatase (IU/I)	Calcium (mg/dl)	Phosphorous (mg/dl)
1	Sham Control	153.28±14.49	7.603±0.51	6.03±0.68
2	OVX control	258.35±15.53###	9.203±0.53###	7.10±0.87##
3	OVX+Raloxifene (5.4 mg/kg, i.p)	205.06±16.63***	8.107±0.42***	6.18±0.75*
4	OVX+PEAP (100 mg/kg, p.o)	222.35±13.40**	8.58±0.52$	6.94±0.81$
4	OVX+PEAP (300 mg/kg, p.o)	203.63±13.49***	7.90±0.51***	6.13±0.65**
6	OVX+ β-Sitosterol (120 mg/kg, p.o)	199.47±14.56***	8.017±0.48***	6.10±0.71**

Values are expressed as Mean ± SEM (n=6). Statistical analysis was carried out by One-way ANOVA followed by Tukey-Kramar post-hoc multiple comparison test. ###P<0.0001, ##P<0.01 vs sham control group $= Non-Significant, *P<0.05, **P<0.01, ***P<0.0001 vs. OVX control groups.

The effect of PEAP on serum alkaline phosphatase, calcium and phosphorous, are illustrated in table 3: The activity of serum ALP was significantly (P<0.0001) increased in OVX control when compared with sham control. Whereas, groups treated with PEAP(P<0.01, P<0.0001), β-Sitosterol (P<0.0001) and Raloxifene (P<0.0001) significantly suppressed rise in serum ALP levels.

Serum calcium level was also found to be significantly increased (P<0.0001) in OVX control as compared to sham control. However, PEAP (NS, P<0.0001), β-Sitosterol (P<0.0001) and Raloxifene(P<0.0001) treatment significantly attenuated serum calcium levels as compared to OVX control.

Serum phosphorous levels was also found to be significantly increased (P<0.01) in OVX control as compared to sham control. However, PEAP (NS, P<0.05), β-Sitosterol (P<0.01) and Raloxifene(P<0.01) treatment significantly attenuated serum phosphorous levels when compared with OVX control.

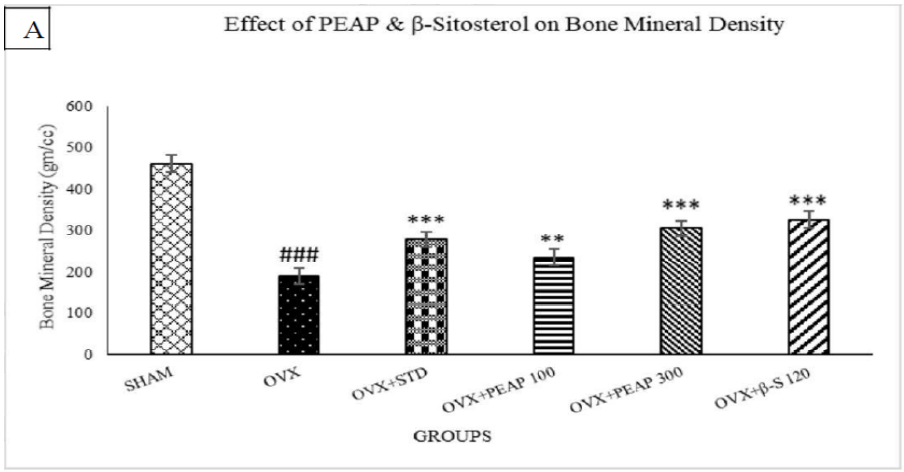


Figure No. 2: Effect of PEAP and β-Sitosterol on Bone Mineral Density and Force at break of femoral bone.

- X axis represent Groups
- Y axis represent Bone mineral density (gm/cc)
- Each column represents mean of 6 readings, n=6
- Vertical errors bars represent Mean ± SEM

Values are expressed as Mean ± SEM (n=6). Statisticallyanalysis was carried out by One-way ANOVA followed by Turkey – Kramer post-hoc multiple comparison test###P<0.0001 vs sham group, ***P<0.0001, **P< 0.01 vs OVX group.

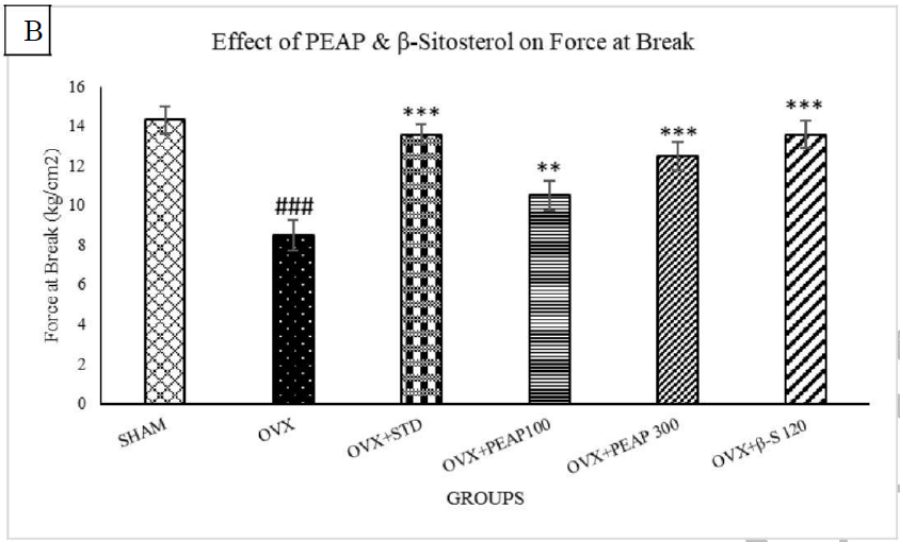


Figure 2: A and B: Illustrates the bone mineral density and force at break on femur.

- X axis represent Groups
- Y axis represent Force at break (kg/cm2)
- Each column represents mean of 6 readings, n=6
- Vertical errors bars represent Mean ± SEM

Values are expressed as Mean ± SEM (n=6). Statistically analysis was carried out by One-way ANOVA followed by Turkey – Kramer post-hoc multiple comparison test ###P<0.0001 vs sham group; ***P<0.0001, **P<0.01 vs OVX.

Bone mineral density in OVX control group was found to be significantly (P<0.0001) lowered when compared to sham control. Whereas, the PEAP (P<0.01, P<0.0001), β-Sitosterol (P<0.0001) and standard Raloxifene (P<0.0001) supplemented groups were seen to significantly recover bone mineral density of the femur bone.

Biomechanical strength in OVX control group significantly reduced (P<0.0001) when compared to sham operated. Treatment with PEAP (P<0.01, P<0.0001), β-Sitosterol (P<0.0001) and standard Raloxifene significantly (P<0.0001) improved the mechanical strength of the femur when compared to OVX control.

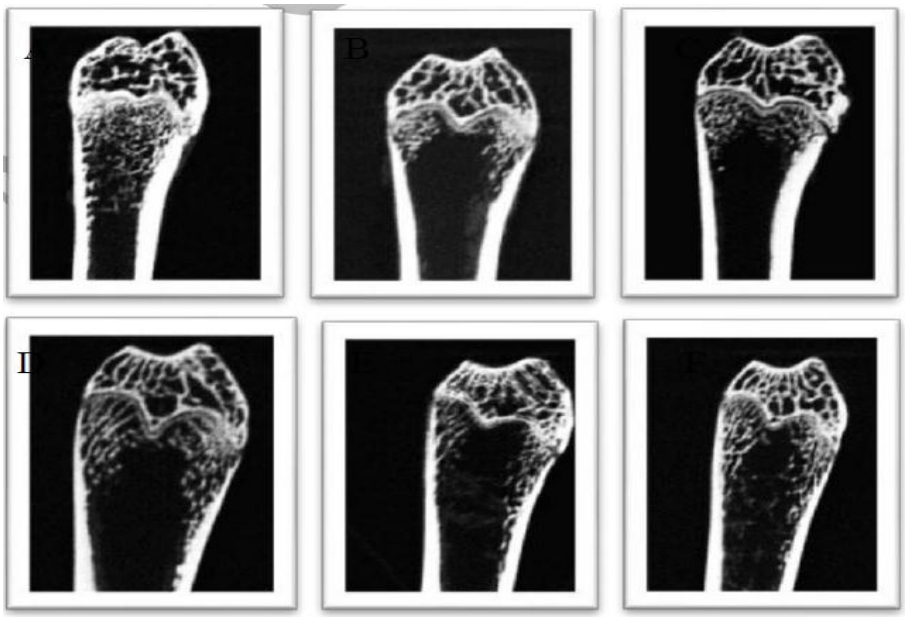


Figure 2.1: Illustrates images of micro-CT analysis of femur bone.

(A): represent sham control, (B): represent OVX control, (C): represent OVX + Raloxifene, (D): represent OVX+B β-Sitosterol, (E): represent OVX+PEAP 100, (F): represent OVX+PEAP 300.

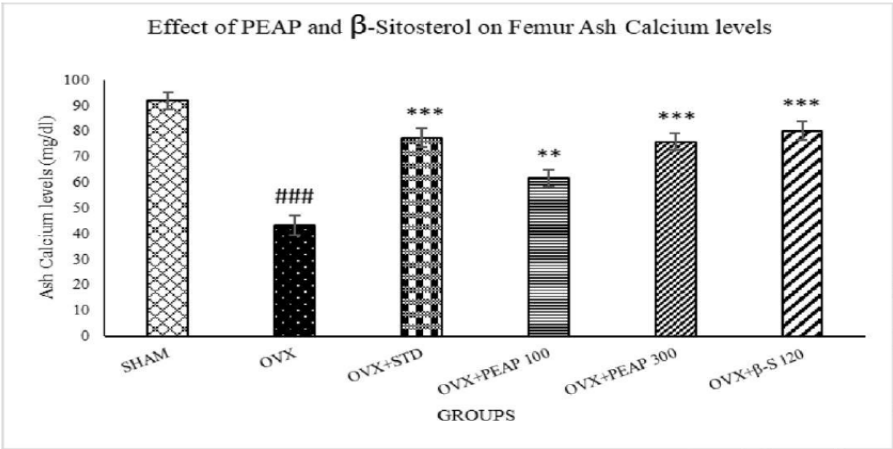
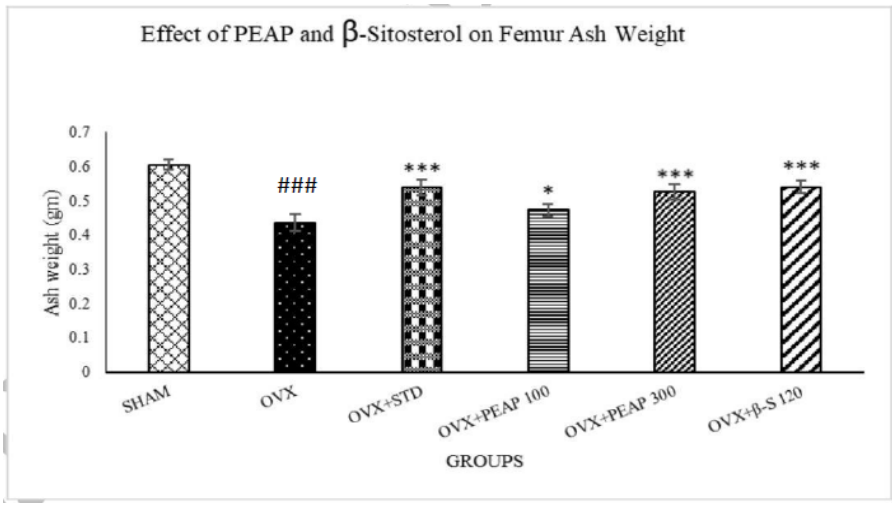


Figure 3: Effect of PEAP and β-sitosterolon Ash Calcium content, Ash Weight, Ash Percent of femoral bone.

- X axis represent Groups
- Y axis represent Ash calcium levels (mg/dl)
- Vertical errors bars represent Mean ± SEM

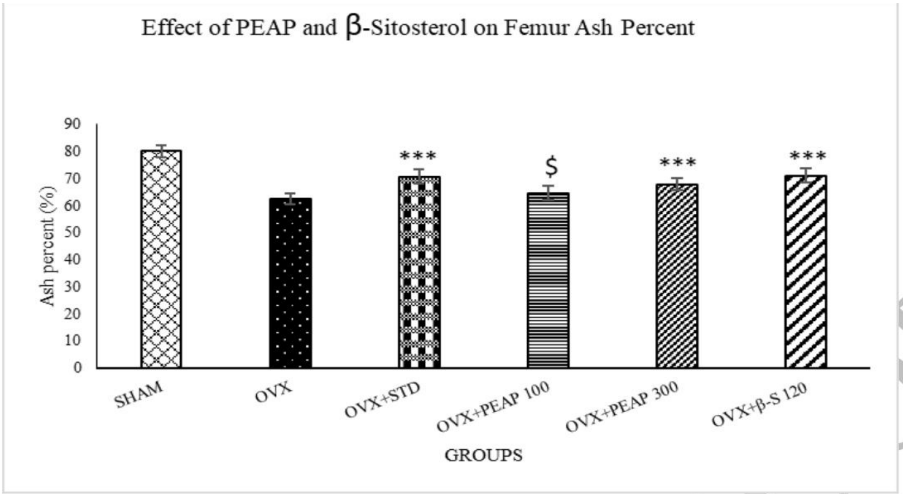
Values are expressed as Mean ± SEM (n=6). Statistically analysis was carried out by One-way ANOVA followed by Turkey – Kramer post-hoc multiple comparison test.

###P<0.0001 vs sham group; ***P<0.0001, **P<0.01 vs OVX control group.



- X axis represent Groups
- Y axis represent Ash weight (gm)
- Each column represents mean of 6 readings, n=6
- Vertical errors bars represent Mean ± SEM

Values are expressed as Mean ± SEM (n=6). Statistically analysis was carried out by One-way ANOVA followedby Turkey – Kramer post-hoc multiple comparison test.
###P<0.0001 vs sham group; ***P<0.0001, *P<0.05 vs OVX control group.



- X axis represent Groups
- Y axis represent Ash percent (%)
- Each column represents mean of 6 readings, n=6
- Vertical errors bars represent Mean ± SEM

Values are expressed as Mean ± SEM (n=6). Statistically analysis was carried out by One-way ANOVA followed by Turkey – Kramer post-hoc multiple comparison test.
$^{###}$P<0.0001 vs sham group; ***P<0.0001, **P<0.01 vs OVX control group.

In figure 3: the bone ash calcium content of the femur was significantly reduced (P<0.0001) in OVX control rats as compared to sham operated. Administration of PEAP (P<0.01, p<0.0001), β-Sitosterol(P<0.0001) and Raloxifene(P<0.0001) significantly increased the calcium content as comparedto OVXcontrol.
respectively.

The total ash percent and ash weight significantly (P<0.0001) reduced in OVX control as compared to sham operated group. There was a significant (P<0.05), (P<0.0001) increase in total ash weight in PEAP (100 and 300 mg/kg), β -Sitosterol and Raloxifene (P<0.0001) treated animals also showed increase in ash weight and ash percent when compared to OVX control.

Histopathology

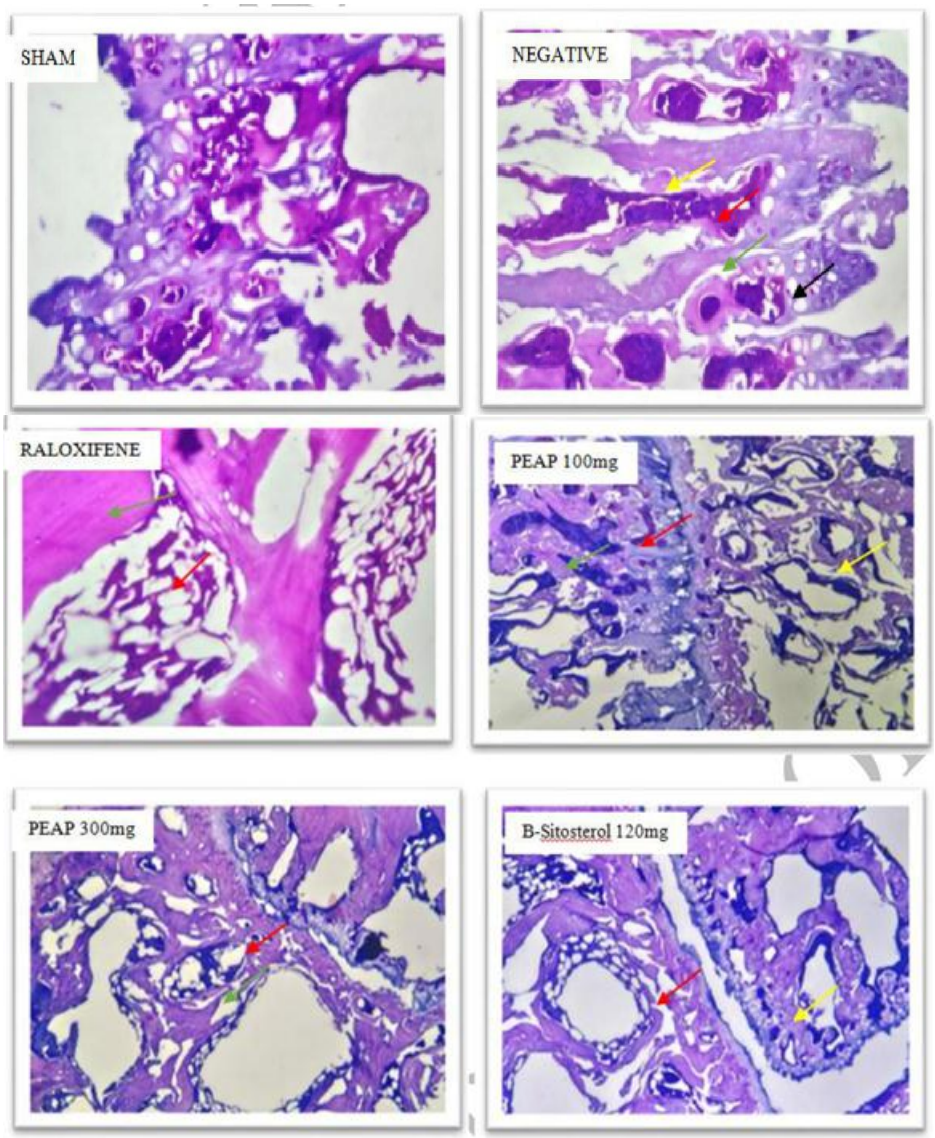


Fig. 4: Histopathology of rat femur bone (H and E).

The histopathological section studied from the femur bone revealed (a) Sham operated showed depleted bone marrow, (b) OVX control rats revealed increased osteoclast formation with increase bone resorption. Decreased decreased trabeculae thickness, and depleted bone marrow. (c) Standard RLX group exhibited bone resorption with increased trabecular thickness (d) PEAP (100mg/kg) revealed increased bone trabecular thickness, presence of bone marrow and mild bone resorption. (e) PEAP (300mg/kg) and β-Sitosterol (120mg/kg) revealed restoration of trabeculae thickness with improved osteoblastic activity.

DISCUSSION

Osteoporosis is considered a major public health problem which is characterized by decrease bone density, resulting in skeletal fractures.[31] Women are three times more prone to osteoporosis than men, reason being the estrogen deficiency caused by ovariectomy or occurrence of menopause which results into to promotion of bone resorption and rapid bone loss.[19,31] This disease is also influenced by dietary program, adequate nutrition; especially calcium intake plays a major role in averting bone loss and osteoporotic fractures in later life. This research interests towards agents that inhibit bone resorption.[32,33] Estrogen which is important risk factor as its deficiency leads to pathogenesis of osteoporosis. Estrogen replacement therapy, is most effective method to reduce the rate of postmenopausal bone loss but accompanied by side effects such as breast tenderness, bloating or swelling.[34,35]

Phytoestrogen properties of food in human diet has been proved scientifically in the prevention and treatment of osteoporosis.[36,38] by exerting estrogenic actions on bone cells and thus suppressing osteoclastic bone resorption and promoting bone formation. *Amorphophallus paeoniifolius* is found to contain β-Sitosterol and lupeol during HPTLC study which are the isoflavones and terpenoid respectively. β-Sitosterol has structure

similarity to estrogen and has received attention as an alternative to hormones replacement therapy and other allopathic drugs. The present study evaluated the petroleum ether extract of *Amorphophallus paeoniifolius* for its Antiosteoprotic activity in female ovariectomized rats. The ovariectomized rats exhibit most of the characteristics of human postmenopausal osteoporosis.[38] Estimation of serum levels of ALP, Calcium and inorganic phosphorous provides valuable tool for evaluating alterations in bone metabolism and accelerated risk of bone loss in post-menopausal subject.[39,40] Serum alkaline phosphatase (ALP) is one of the most widely used markers for osteoporosis. Human bone is composed of a mineralized organic matrix and bone cells. Osteoblast are active mature bone cells that synthesize the organic matrix and regulate mineralization process. Once osteoblasts are active, they begin to produce large amounts of alkaline phosphatase, a phosphate-splitting enzyme that is released into the osteoid to initiate deposition of minerals. In this study ovariectomy exhibited rise in level of serum. ALP indicating activation of osteoblasts due to ovariectomy induced osteoporosis.[41] However significant decrease in the serum ALP observed in the PEAP and β -Sitosterol treated rats, indicated decrease bone turnover. Bone resorption due to ovariectomy was also indicated by increase serum calcium levels in OVX groups whereas declined serum calcium levels on treatment with PEAP and β -Sitosterol supported decreased resorption of bone.[42] Phosphorus is another major mineral constituting bone mass. Phosphorus in the body is present in the form of phosphates. Both calcium and phosphate are deposited in the bone and are also resorbed together. The maintenance in serum levels of phosphorous is also closely related to that of calcium. On treatment with PEAP and β -Sitosterol maintenance in levels of both calcium and phosphorous in serum thus strongly suggests prevention of bone resorption.[42,43]

Prevention of bone loss and restructuring of osteoporotic bone with respect to bone mineral content was evident by increase in total ash calcium level, ash weight, and ash percent on treatment with PEAP and β -Sitosterol. Bone Mineral Density (BMD) has been described as a substitute measure of bone strength and a primary contributor to bone quality which was observed to be markedly decreased in ovariectomized group (OVX) due to an increase in bone turnover induced by estrogen deficiency.[39,43,44] The phytoestrogens are class of compounds having similar structure to oestradiol and are able to bind the estrogen receptor. β-sitosterol is an estrogen agonist for estrogen receptor (ER) α and β, and preferably binds to ER β and hence been reported to exhibit phytoestrogenic effect.[44] β -sitosterol present in PEAP might have attributed to significant increase in BMD in PEAP and β -Sitosterol treated rats thereby ensuring remodeling of bones and prevention of osteoporosis.

To confirm the micro-architectural changes across bones histopathology was performed. In bones of OVX rats reduced trabecular thickness, and increased osteoclast formation indicated increased bone resorption.[45] On treatment with PEAP and β -Sitosterol restructuring of bone was evident due to increased trabecular thickness and decrease formation of osteoclast. Thus, confirming prevention of osteoporosis due to remineralisation of bone on treatment with PEAP and β -Sitosterol. The anti-osteoporotic effect exhibited by PEAP might be contributed to phytoestrogenic activity of β-Sitosterol present in it.

CONCLUSION

Following treatment with PEAP and B-Sitosterol, exhibited significant re-mineralization of bone in ovariectomised rats thus preventing rise in serum ALP, Calcium and Phosphorous levels. Increase in BMD and calcium content in bone ash supported Anti-osteoporotic effect exhibited by PEAP and β-sitosterol which is equivalent to standard Raloxifene. Thus, PEAP carries vital anti-osteoporotic potential due to presence of phytoestrogen β -Sitosterol.

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