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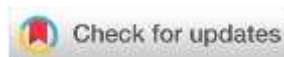
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Research Article

Preventive effect of Total ethanolic extract of *Butea monosperma* on bone loss in osteopenic rats

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Abstract

Aim: The intent of the study was to assess the bone conserving effect of *Butea monosperma* total extract (BTE) prepared from the stem-bark, in ovariectomy (Ovx)-induced bone loss in Sprague Dawley (SD) rats.

Methods: Sprague-Dawley rats were ovariectomized (Ovx) and BTE treatment with three different doses (100mg, 250mg and 500mg/kg body weight) commenced a day after Ovx and continued for 12 weeks. Skeletal parameters including trabecular and cortical bone microarchitecture, bone mineral density, bone mineral content, biomechanical strength, bone turnover markers (serum procollagen 1 N-terminal propeptide/ P1NP and cross-linked C-telopeptide of type I collagen/CTX-1), new bone formation rate (periosteal bone formation rate and mineral apposition rate), and expression of osteogenic genes were assessed. Estrogenicity of BTE was assessed by studying uterus at the end of the treatments.

Results: Ovx rats treated with BTE prevented the alterations in trabecular and cortical microarchitecture, maintained bone mineral density, bone mineral content, and increased bone biomechanical strength in comparison with Ovx rats. Treatment of BTE also enhanced new bone formation rate, increased the serum bone formation marker (P1NP), decreased the serum bone resorption marker (CTX-1), and upregulated the osteogenic genes in bones. BTE was devoid of estrogen-like effect in uterus.

Conclusion: BTE protects against Ovx-induced bone loss by inhibiting bone resorption and increasing new bone formation. The most effective bone conserving dose of BTE was 250mg/kg. This study demonstrates the potential of BTE in preventing/delaying osteoporosis development in postmenopausal women.

Keywords: Butea total extract, ovariectomy, bone formation, bone mineral density.

INTRODUCTION

Bone density is reduced and bones become weaker from the fourth decade of life, and these changes are pronounced in postmenopausal women. Available drugs for treating osteoporosis are classified as antiresorptive and bone anabolic. Bisphosphonates are recommended as the first line treatment for osteoporosis¹. However, besides prevention of bone resorption, bisphosphonates are associated with side effects including osteonecrosis of jaws, atypical femoral fractures, nephrotoxicity and oesophageal cancer^{1,2,3,4}. Hormone replacement therapy (HRT) is effective in preventing osteoporosis in postmenopausal women, however, the side effects include increased risks of breast cancer, cardiac events, and stroke⁵. Selective estrogen receptor modulators (SERMs) including raloxifene and bazedoxifene with bone conserving effect has adverse effects including abnormal vaginal bleeding, swelling of lower extremities and

mood swings⁶. Because of these adverse effects of the clinically used drugs, interest has shifted to alternative therapies from natural sources such as phytoestrogens^{7,8,9}. Phytoestrogens are a class of nonsteroidal plant-derived chemicals that have structural and functional similarities to estradiol¹⁰. Various animal studies have confirmed the bone supporting effects of phytoestrogens¹¹. Among the phytoestrogens, isoflavones are most well-studied.

Butea monosperma (Dhak) is known for its medicinal properties including osteogenesis and fracture healing¹². Ethanolic extract of *B. monosperma* is rich in methoxyisoflavons including cladrin, isoformononetin, formononetin, cajanin and medicarpin. All these compounds promoted bone formation and prevented bone loss in osteopenic animals^{13,14,15}. These phytoestrogens also promoted peak bone mass achievement in growing rats¹⁶. Our

previous study demonstrated that the total ethanolic extract made from the stem-bark of *B. monosperma* (BTE) protected rats from the Ovx-induced osteopenia at 1g/kg dose when given in therapeutic regimen (Ovx rats in which osteopenia was established)¹⁷. As this dose was too high to translate to humans, in the present study, we assessed the possible preventive role of BTE at lower doses administered to Ovx rats in a prophylactic regimen, i.e., dosing commenced right after Ovx. Our study showed the efficacy of BTE in preventing the Ovx-induced decline in bone mass and strength, alterations in the bone turnover markers, and deterioration of bone microarchitecture

MATERIAL AND METHOD

Material

Calcein was procured from Sigma-Aldrich (St. Louis, Missouri, United States). PINP and CTX Elisa kits were purchased from Elabscience (Wuhan, China) and Qayee-Bio (Shanghai, China) respectively. Trizol for RNA isolation was purchased from Takara (Kusatsu, Japan) and cDNA synthesis kit and Syber green were procured from Applied Biosystem (Waltham, Massachusetts, United States).

Methods

Preparation of BTE

Butea monosperma stem bark powder (10.0 kg) was poured in a percolator with ethanol (60 L utilized in four percolations) and allowed to stand for roughly 16 hours at room temperature (overnight). The percolate was taken away. This extraction procedure was carried out four times. The combined extract was filtered and concentrated at 45°C, yielding 900 g (9.0%) BTE¹⁷. Quantification of marker compounds (cajanin, medicarpin, isoformononetin and cladrin) in the standardized extract (BTE) served as the quality control step and showed close correspondence with the previously described extract¹⁷.

Animals Study plan

Animal Ethical approval: Animal experimental study was performed according to current legislation of animal experiments (Institutional Animal Ethical Committee at Central Drug Research Institute) with approval no. IAEC/2021/69/renew-0/dated 29.06.2021.

Fifty 12-weeks old female Sprague-Dawley (SD) rats (230 ±20 gm) were taken for the study. All rats were kept and maintained at controlled temperature (25°C) and humidity (50-70%) in 12h light- dark cycle, and had free access to food and water. Fifty rats were randomly divided into five groups (n=10 rats/group). Animals were anesthetized using ketamine (30mg/kg) and xylazine (10mg/kg). Bilateral ovariectomy(Ovx) was performed to induce osteopenia.

Animal groups were as follows:

1. Sham –Animal with intact ovary, which served as the baseline group
2. Ovx-Ovariectomized animals
3. Ovx +BTE (100 mg /kg/day)- Ovariectomized animals treated with BTE at 100mg/kg dose
4. Ovx + BTE (250 mg/kg/day)- Ovariectomized animals treated with BTE at 250mg/kg dose
5. Ovx+ BTE (500 mg/kg/day)- Ovariectomized animals treated with BTE at 500mg/kg dose

Rats were treated with BTE or vehicle by oral gavage from the second day of surgery and continued for 12 weeks. All rats in

each group were assessed blindly and included in the statistical analysis (n=10). Two doses of intraperitoneal injection of calcein (20mg/kg) were given to all animals, 12 days and 48 hrs before sacrifice. Rats were euthanized, bones and uterus were harvested. Blood was collected through cardiac puncture. During the experimental period, body weight was measured once a week. Isolated bone samples were kept in -20°C for RNA isolation, fixed in 4% formaldehyde and stored in 70% isopropanol and used for microCT and histomorphometry.

Body weight and uterine weight measurement: All animals were weighed every week and body weight was shown at the end-point. Uterine weight was also measured after autopsy.

Micro-CT: Micro-CT scanning of excised bones was performed by Sky Scan 1076 CT scanner (Aartselaar, Belgium) based on previously study¹⁸. Bone samples were scanned in batches of three at a nominal resolution (pixels) of 18µm with 1mm seat filter and 0.8 rotation step. The X-ray source used was adjusted at 70kV and 200Ma. Reconstruction was done by modified Feldkamp algorithm using Skyscan Nrecon software, which enables network distributed reconstruction carried out on four personal computers running simultaneously. For analysing trabecular bone, 100 slices were taken from secondary spongiosa confined 1.5mm away from the distal border of growth plate and ROI was drawn. For Cortical bone analysis, from the region of growth plate, 400slices were discarded to remove trabecular region, and from there 200 slices were taken to draw ROI using Ctan software parameters like fractional bone volume (bone volume per tissue volume (BV/TV), trabecular thickness (Tb. Th), number (Tb. N) and separation (Tb. Sp) were calculated by mean length intercept method.

BMD and BMC Measurements: Micro-CT scans were further used for the determination of cortical and trabecular bone mineral density(BMD), for this VOI was made for cortical and trabecular regions, respectively. BMD was assessed by 4mm hydroxyapatite phantom rods with known BMD (0.25g/cm³ and 0.75g/cm³) as calibrator. Bone mineral content (BMC) was calculated by the formula BMC=BMD×bone volume (BV)¹⁹.

Bone strength analysis: Biomechanical properties of femur mid diaphysis such as force and energy needed to break the bone and stiffness were assessed through three-point bending using bone strength tester (TK-252C; Muromachi Kikai Corporation Limited, Tokyo, Japan) based on previous published protocol²⁰.

Analysis of Serum Bone Biomarkers: Bone turn over markers, Procollagen I N-Terminal Propeptide (PINP) and Cross Linked C-telopeptide of Type I Collagen (CTX) in serum was assessed using enzyme-linked immunosorbent assay (ELISA) kits. Assays were performed according to the manufacturer's recommendations.

Histology of bone sections: Femur bones were fixed in 4% paraformaldehyde decalcified using decalcifying buffer and embedded in paraffin. Paraffin blocks were cut into sections using microtome (Leica microsystem, model number RM2165). Paraffin sections were deparaffinised, processed in different grades of isopropanol and stained with Haematoxylin and Eosin (H&E) for analysis of trabecular bone loss as described earlier²¹. Histological images were taken under fluorescence microscope.

Skeletal dynamic histomorphometry: For assessment of new bone formation *in vivo*, double calcein labelling was performed using previously published protocol¹⁹. Two intraperitoneal injections of calcein (20 mg/kg body weight) were given on 12 days and 48 hrs before autopsy respectively.

During autopsy, femur bone was collected and attached connective tissues were removed. 50µm cross section of undecalcified femur bones embedded in acrylic material were cut using Isomet bone cutter and photographed under confocal microscope (Leica SPSTCS) with the aid of appropriate filters. Image pro software was used to obtain measurements from calcein labelling such as periosteal perimeter, single-labelled surface(sLS), double labelled surface(dLS) and interlabelled width(IrTh). These measurements were used for calculation of mineralizing surface/bone surface (MS/BS), Mineral apposition rate(MAR) and bone formation rate(BFR) by using formula: $MS/BS = (1/2sLS + dLS)/BS$ (%), $MAR = IrTh/12days$ (µm/day), $BFR/BS = MAR \times MS/BS$ (µM³/µm²/day) according to previously published protocol ¹⁹.

Real Time PCR: For gene expression analysis, portion of femoral bone was dissected. Bone tissues were harvested; muscles and attached soft connective tissues were removed. Desired area was frozen in liquid nitrogen and crushed to make powder ²². Total RNA in powder was extracted using Trizol. 1µg of total RNA was used for cDNA synthesis. cDNA was amplified using Light cycler 480(Roche Diagnostics, Indianapolis, USA). Changes in gene expression of osteogenic genes such as Runt related transcription 2(RUNX2) and type 1 collagen (type1col) was quantitatively assessed using Syber green. Primers were designed using Universal probe library (Roche Diagnostic, USA.) GAPDH used as housekeeping normalizing gene. The sequences of primers are given in table 1.

Table1: Primer sequence of genes used for qPCR experiment:

Gene	Primer sequence
Runx2	F- CATGTTTCAGCTTTGTGGACCT R- GCAGCTGACTTCAGGGATGT
Type 1 Col	F-CATGTTTCAGCTTTGTGGACCT R-GCAGCTGACTTCAGGGATGT
GAPDH	F-AGCTTGTTCATCAACGGGAAC R-TTTGATGTTAGTGGGGTCTCG

Uterine histology: Whole uterus was excised and fixed in 4% paraformaldehyde. For histology uterus was processed using xylene and different grades of ethanol according to previously published protocol ¹⁹. Total uterine area, luminal area and luminal epithelial height was measured using image J software.

Statistical Analysis: Data was presented as mean ± SEM and was subjected to one-way ANOVA followed by Newman-Keuls test used to verify differences between different groups using Graph Pad Prism version 5.0 software.

RESULTS

Effect of BTE on body weight and uterine weight: Body weight was significantly higher in Ovx rats compared with sham rats and BTE significantly reduced the Ovx-induced increase in body weight. In addition, uterine weight was significantly reduced in Ovx animals in comparison to the sham group and there was no change in uterine weight in BTE treated groups (Table2).

Table 2: Effect of BTE on body weight and uterine weight in ovariectomized rats

Groups	Initial body weight(gm)	Final body weight(gm)	Uterus weight(mg)
Sham	245.5556±12.125	259.77 ± 9.820613###	823.74±71.96
Ovx	242.2±12.84783	328.4±28.2772	133.42±16.52***
Ovx+BTE(100mg)	238.42±6.078847	290.42±38.86239#	136.57±19.97***
Ovx+BTE(250mg)	244±17.84857	268.87±19.96023###	134.88±15.50***
Ovx+BTE(500mg)	244.9±11.55133	289.6±15.8549##	140.7429±11.09***

Data are expressed as Mean ±SEM. ###p<0.001, ##p<0.01 and #p<0.05 compared with final weight of Ovx. ***p<0.001 in comparison to sham group.

Effect of BTE on femur trabecular bone: Significant improvements in bone quality and reduction in fracture risk is achieved by preserving bone microarchitecture. Effect of BTE on the quality of trabecular bone was assessed by the analysis of trabecular network at femur metaphysis. Erosion of trabecular network was observed in the Ovx group (Fig 1A). However, the trabecular network was maintained in the rats treated with BTE treated groups at all three doses compared with the Ovx group. As shown in Fig 1B-E, bone

volume/trabecular volume (BV/TV), trabecular number (Tb. N) and trabecular thickness (Tb. Th) were reduced and trabecular separation (Tb. Sp) was increased in Ovx group compared with sham. Ovx rats treated with BTE at all doses showed significant reversal in all parameters. However, Tb. Sp. was best preserved at the 250 mg/kg dose of BTE. Femur trabecular BMD and BMC were also reduced in Ovx group compared with sham. However, BTE treatment to Ovx rats at all three doses preserved trabecular BMD and BMC (Fig 1F-G).

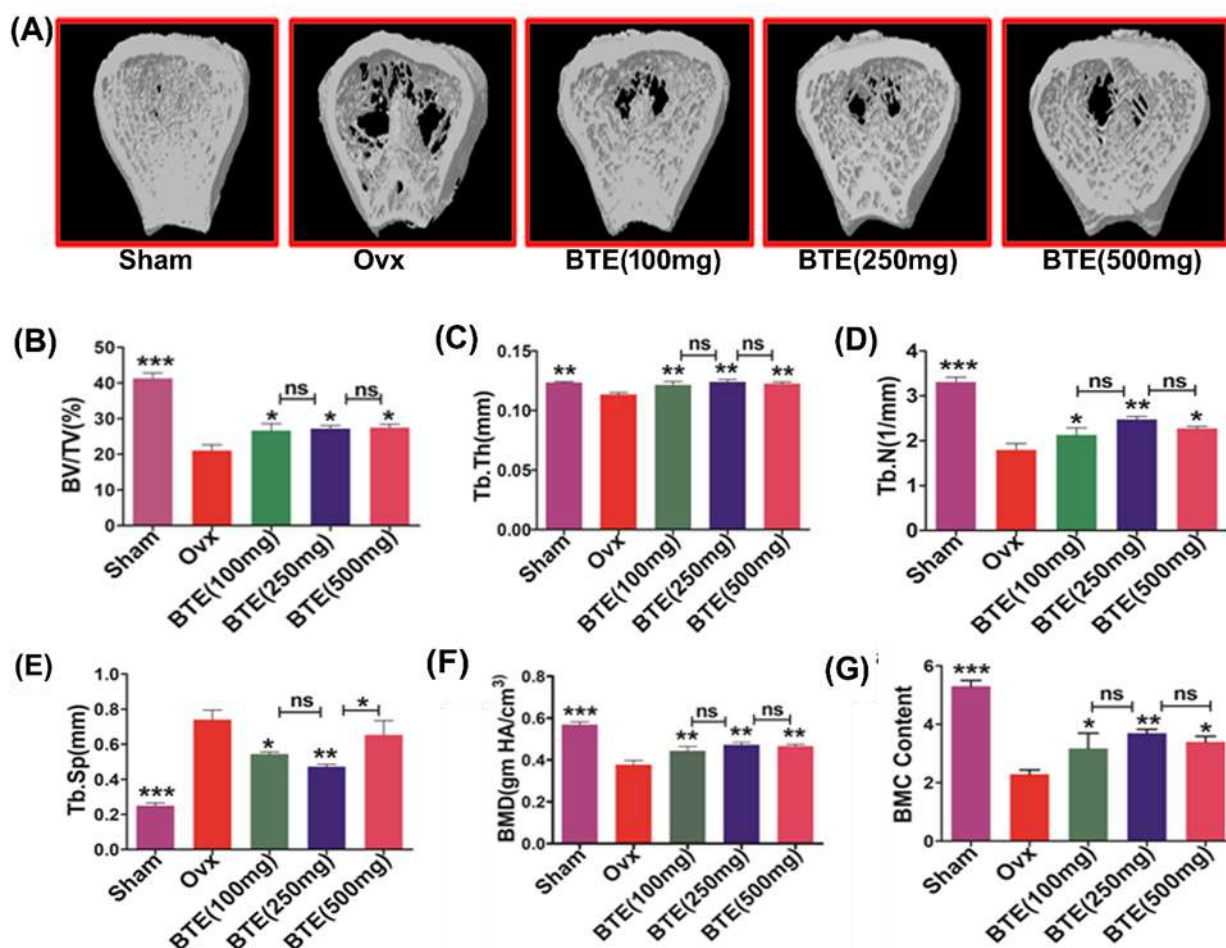


Figure 1: Effect of BTE supplementation on trabecular bone parameters (n=10). **(A)** Representative Micro-CT 3D images of rat femur metaphysis, Quantitative Micro-CT measurements of trabecular parameters including **(B)** BV/TV (%), **(C)** Tb. Th, **(D)** Tb. N, **(E)** Tb. Sp, **(F)** BMD **(H)** BMC. *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ vs. Ovx.

Effect of BTE on femur cortical bone: Effect of BTE on cortical bone at femur mid-diaphysis was determined by 2-D μ CT. In Ovx group, cortical thickness (Cs. Th) was reduced relative to the sham group (Fig 2A). Cs. Th was increased in BTE treated groups at the 100- and 250 mg/kg doses over the

Ovx group (Fig 2B). Bone mineral density (BMD) and bone mineral content (BMC) of the cortical bone was decreased in the Ovx group compared with sham group. Both BMD and BMC were increased by BTE at 250mg/kg dose but not at the other two doses (Fig 2C-D)

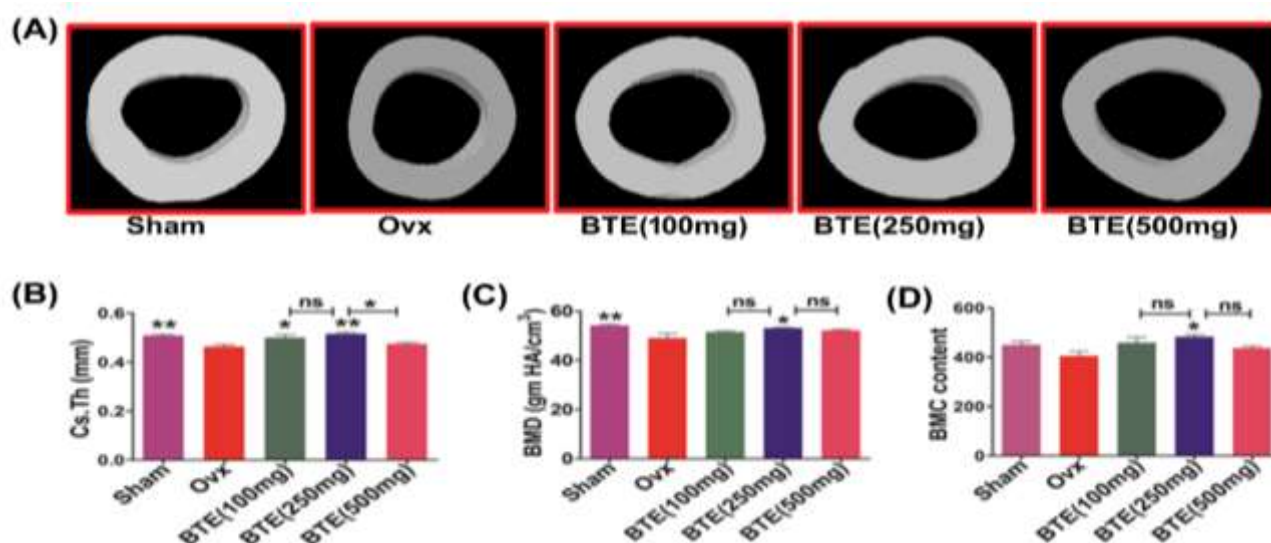


Figure 2: Effect of BTE on cortical bone parameters (n=10). **(A)** Representative images of femur mid-diaphysis of indicated groups. Quantification of cortical bone parameters **(B)** Cs. Th, **(C)** BMD, **(D)** BMC. ** $p < 0.01$ and * $p < 0.05$ vs. Ovx.

Effect of BTE on bone biomechanical strength: As BTE treatment in Ovx groups increased the cortical bone mass and thickness, we surmised that it could affect the bone biomechanical property. Data from three-point bending at the femur mid-diaphysis showed that power, energy and stiffness

were reduced in Ovx group compared with the sham (Fig 3A-C). However, power and stiffness required to break the bone were significantly elevated in groups treated with BTE at 250mg/kg dose.

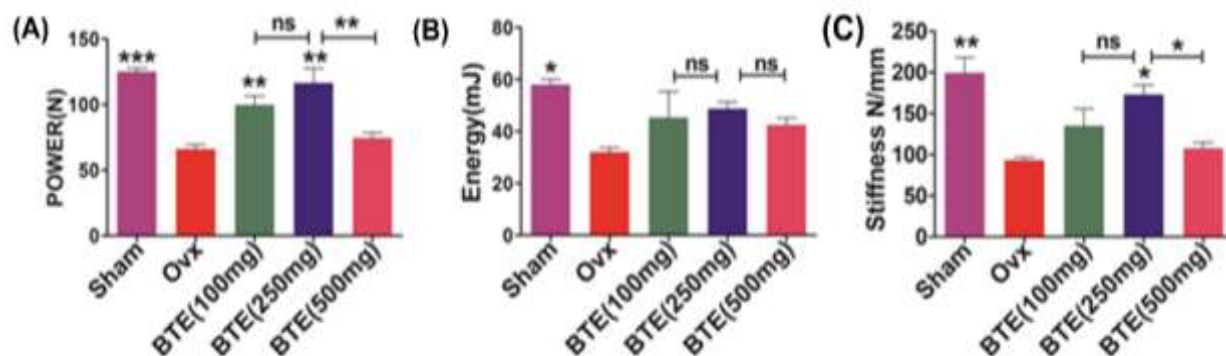


Figure 3: Effect of BTE treatment on biomechanical property of femur bone (n=10). Bone strength parameters (A) Power, (B) Energy, (C) Stiffness. *** $p<0.001$, ** $p<0.01$ and * $p<0.05$ vs. Ovx.

Effect of BTE on bone turnover markers and trabecular structure: Serum P1NP and CTX-1 are two stable and established bone turnover markers. As shown in Fig 4A, serum P1NP (a bone formation marker) was significantly decreased in Ovx group relative to the sham group. Serum P1NP was significantly increased in the BTE group at 250mg/kg dose, though an increasing trend was seen at other two doses. Fig 4B showed that the bone resorption marker (CTX-1) increased

in Ovx group compared to sham. CTX-1 level was significantly reduced in BTE treated group at 100 mg/kg and 250mg/kg doses.

H&E staining of femur metaphysis sections showed that compared to intact trabecular network in the sham group Ovx rats had deteriorated trabecular network. BTE treatment maintained the network integrity. In this regard, BTE at the 250 mg/kg dose was better than the other two doses (Fig 4 C).

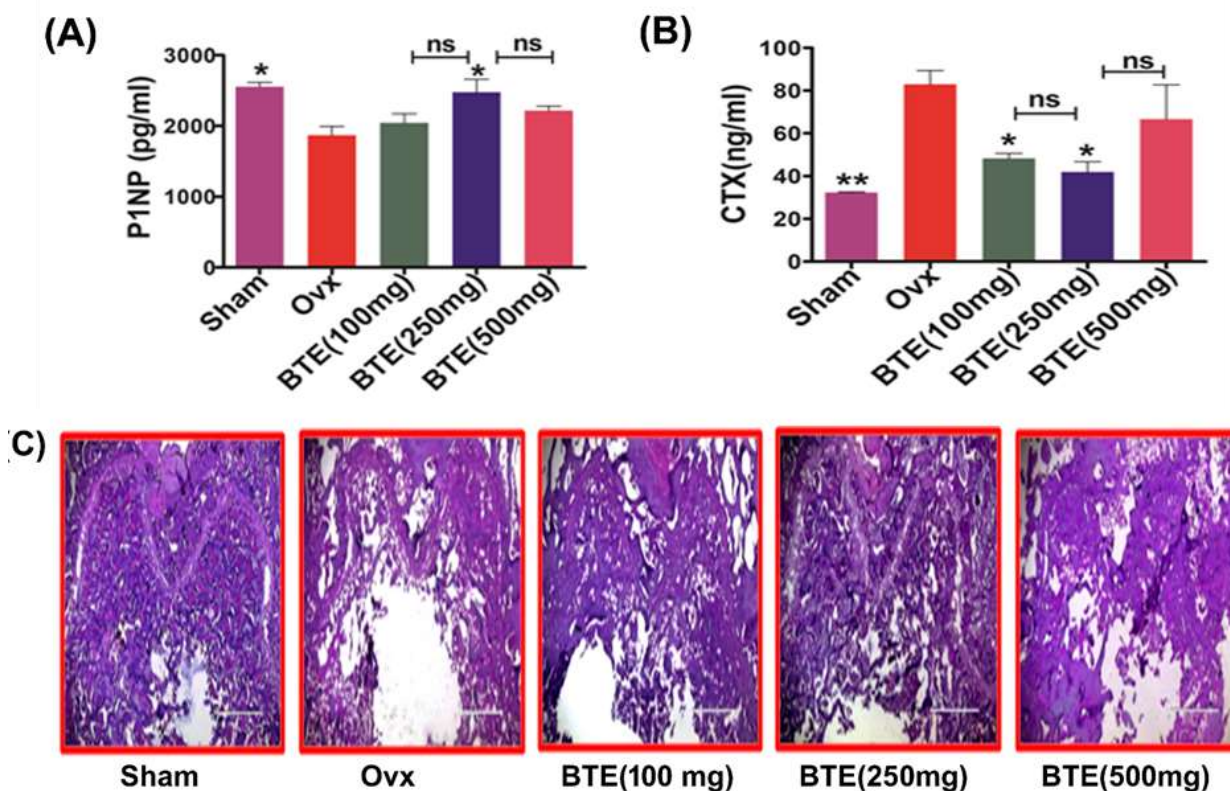


Figure 4: Effect of BTE on serum bone turnover biochemical markers (n=6). (A) serum osteogenic marker (P1NP), (B) serum bone resorption marker (CTX). Effect of BTE on trabecular network integrity, (C) HE staining images of femur trabecular bone (4X). ** $p<0.01$, * $p<0.05$ vs. Ovx.

Effect of BTE on new bone formation: For determination of surface referent bone formation effect of BTE, double calcein labelling study was performed. Fig 5A-D showed that periosteal mineral apposition rate (pMAR), periosteal mineralizing surface per bone surface (pMS/BS) and

periosteal bone formation rate per bone surface (pBFR/BS) were significantly reduced in Ovx group relative to the sham group. These three parameters were increased in BTE treated groups with the best effect observed at 250mg/kg.

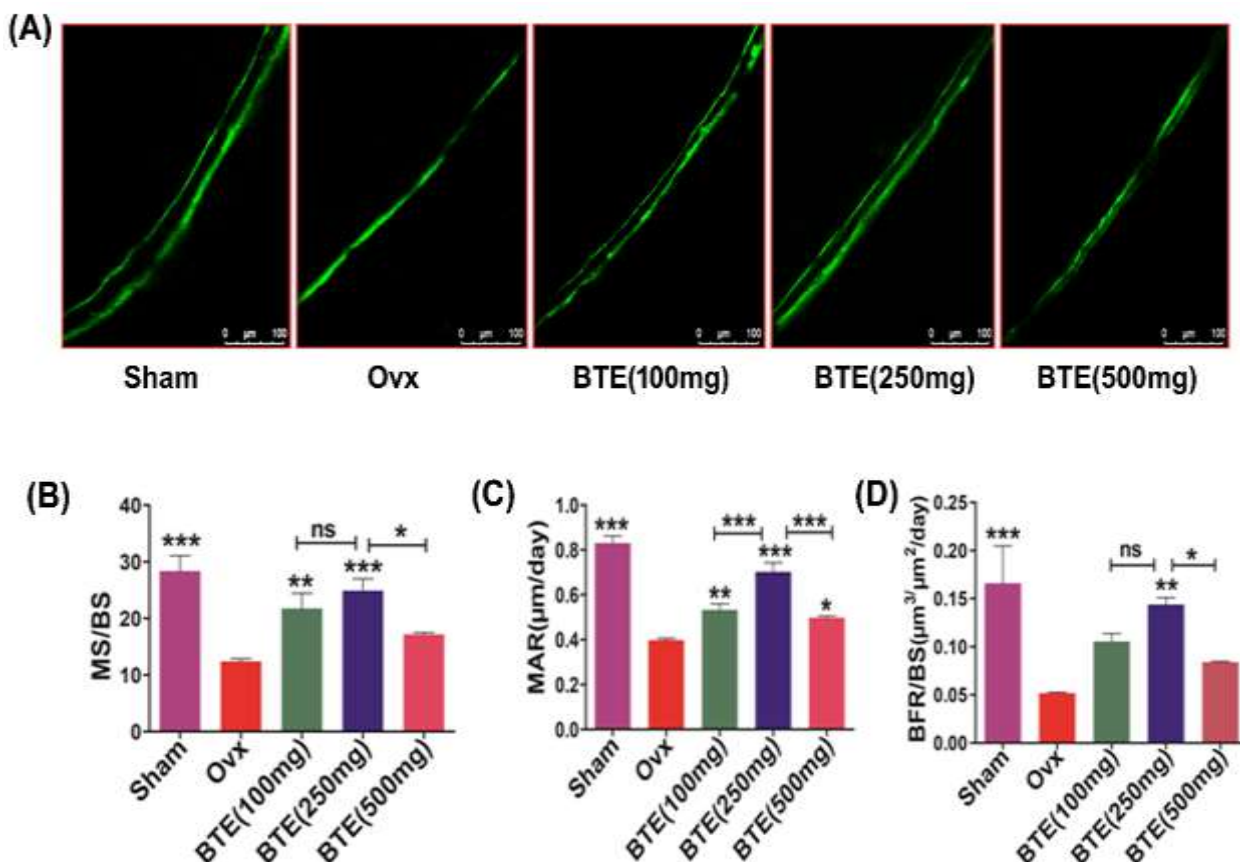


Figure 5: Effect of BTE on bone forming response in Ovx rats (n=6). Assessment of dynamic histomorphometry parameters in femur mid diaphysis (A) Representative calcein double labelling images (20X), (B) pMS/BS (periosteal mineralizing surface per bone surface) (C) pMAR (periosteal mineral apposition rate) (D) pBFR (periosteal bone formation rate). ***p<0.001, **p<0.01 and *p<0.05 vs. Ovx.

Effect of BTE on osteogenic differentiation: Decline in estrogen level impairs osteoblastogenesis. Osteoblast marker genes including RUNX2 and type 1 col in long bones in all groups were assessed. It was observed that the expression of

these genes was significantly decreased in Ovx rats relative to the sham rats (Fig 6A-B). BTE treatment to Ovx rats exhibited increased expression of RUNX-2 and type 1 Col genes, especially at 250mg/kg dose (Fig6A-B).

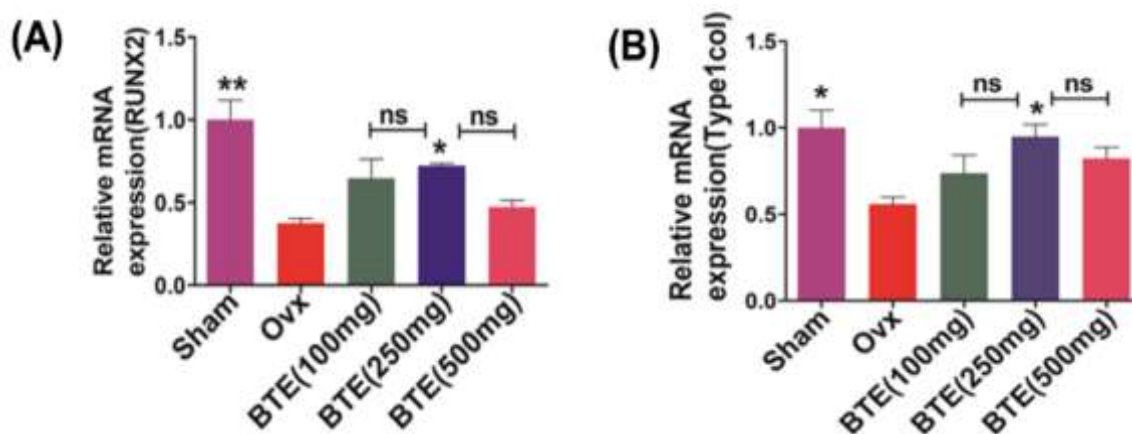


Figure 6: Effect of BTE on osteogenic gene expression in bone (n=3). (A) Relative mRNA expression of RUNX2, (B) Relative mRNA expression of Type1col. *p<0.05 vs. Ovx.

Effect of BTE on uterine parameters: Uterine parameters such as total uterine area, luminal epithelial height and total luminal area were calculated in H&E stained uterine sections (Fig (7)) using Image J software. It was observed that all three parameters were decreased in Ovx group compared to the

sham group. There were no significant changes in uterine parameters in BTE treated groups in comparison to the Ovx group which indicates that the BTE was not associated with uterine estrogenicity (Table 3).

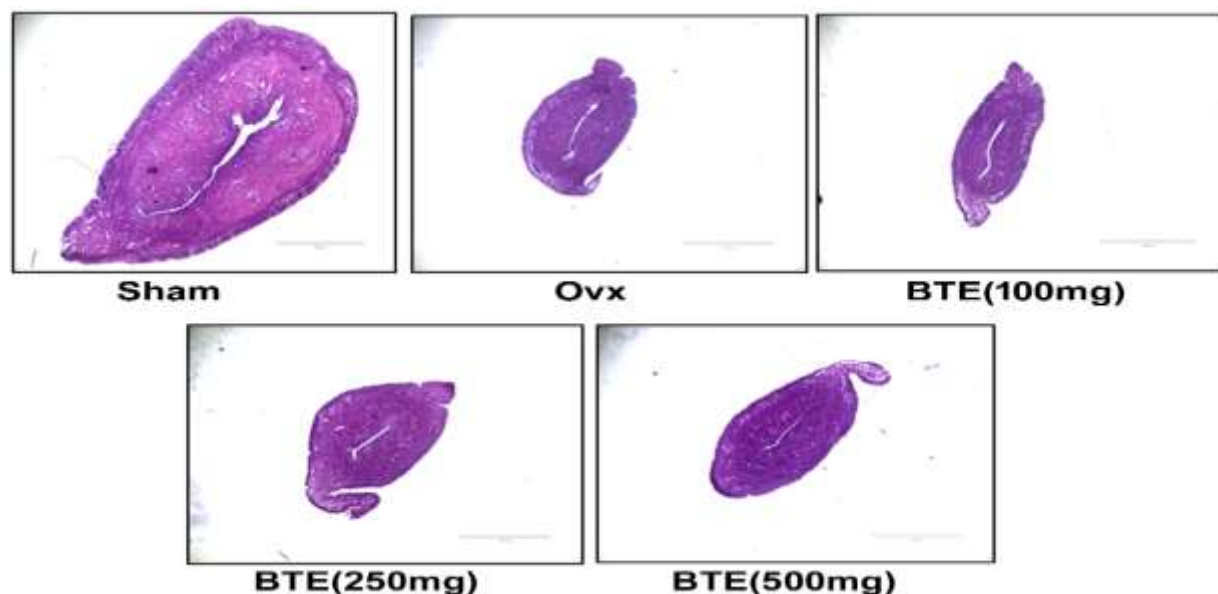


Figure 7: Effect of BTE on uterine parameters: Hematoxylin and eosin stained sections of uterus. Sham Sham-operated control group, Ovx Ovariectomized group, Ovx+BTE(100mg) Ovx+Butea total extract 100mg dose, Ovx+BTE(250mg) Ovx+ Butea total extract 250mg dose, Ovx+BTE(500mg) Ovx+ Butea total extract 500 mg dose.

Table 3: Effect of BTE on uterine parameters in ovariectomized rats

Groups	Luminal area, (mm ²)	Luminal epithelial height, (mm)	Total uterine area, (mm ²)
Sham	0.147±0.31	0.083±0.004	7.29±0.55
Ovx	0.040±0.016**	0.028±0.002***	1.83±0.032***
Ovx+BTE(100mg)	0.046±0.012**	0.024±0.010***	1.84±0.085***
Ovx+BTE(250mg)	0.044±0.009**	0.024±0.004***	1.83±0.051***
Ovx+BTE(500mg)	0.045±0.003**	0.031±0.006***	1.93±0.04***

Data are expressed as Mean ±SEM. ***p<0.001, **p<0.01 compared with sham group

DISCUSSION

Butea monosperma is known for its fracture healing property in Indian traditional medicine. Previous study from our lab showed that total crude extract of *B. monosperma* has *in vitro* osteoblast mineralization activity and this crude extract is rich in different methoxyisoflavones such as cladrin, medicarpin, cajanin and isoformononetin. These phytoestrogens are also known for their bone forming and fracture-healing potential [13, 14, 15, 16, 23, 24]. Our previous study demonstrated bone conserving effect of BTE at 1.0g/kg dose in osteopenic Ovx rats (therapeutic regimen). This dose was too high when translated from rat to human. The goal of the present study was therefore to determine whether a lower dose of BTE has bone sparing effect when administered in prophylactic regimen to Ovx rats. Fall in estrogen levels as occurs after menopause leads to gain in body weight due to fat accumulation [25, 26, 27]. We observed that BTE prevented the Ovx-induced gain in body weight, however, it was devoid of uterine estrogenicity.

Onset of menopause is characterized by rapid loss of trabecular bones due to greater population of osteoclasts at these sites than at the cortical sites. However, if left untreated, cortical bone loss occurs later. Increased osteoclast formation and function coupled with decreased osteoblast formation and function contribute to postmenopausal osteoporosis [28]. The average decline in BMD during the menopausal transition is roughly 10%, although, in a considerable number of women it could be as high as 20%. BTE at all three doses (100-, 250- and 500mg/kg) significantly prevented the trabecular bone loss after Ovx, indicating skeletal protection during the initial rapid phase of bone loss. Increased cortical bone mass and thickness by BTE over the Ovx group further suggested the bone conserving effect of this extract at the cortical site as well.

Bone mass represents the amount of hydroxyl apatite present in the organic matrix which affords mechanical strength or load bearing ability to bone. Besides, bone microarchitecture and biomaterial composition also importantly contribute to bone mechanical strength [29]. We observed that BTE treatment enhanced bone strength parameters over the Ovx group, thus

suggesting that increased bone mass and improved microarchitecture by this extract translated to functional outcome and likely to resist fracture incidence in postmenopausal women. CTX-1 is a clinically used circulating marker of bone resorption^{30,31} and BTE suppressed the Ovx-induced rise in CTX-1 levels maximally at 250mg/kg dose thus suggesting its anti-resorptive effect.

Impairment in bone formation besides increase in resorption in Ovx rats was manifested by significant decline in the surface referent bone formation parameters (MS/BS, MAR and BFR) at the periosteal site of femur diaphysis compared with sham group. BTE treatment resulted in significantly higher bone formation parameters over the Ovx group which suggested enhanced periosteal apposition by this extract. Consistent with its increased surface referent bone formation, BTE also increased serum P1NP (an established and sensitive osteogenic marker) compared with Ovx rats that displayed reduction in P1NP levels compared with sham group. Increased mRNA levels of RUNX2 and type 1 collagen in the BTE treated rats compared to Ovx rats further supported the osteogenic effect of BTE.

CONCLUSION

Our study thus demonstrates that BTE prevents the development of trabecular and cortical osteopenia, and loss of bone strength in a preclinical model of estrogen deficiency-induced model of bone loss. A major implication of this finding is that BTE could delay the onset of osteoporosis if administered in prophylactic mode to postmenopausal women.

Potential Clinical value: Human dose of 250 mg/kg BTE in rats translates to ~40.5 mg/kg based on body surface area-dependent allometric scaling³². For a 60 kg person, the daily dose requirement of BTE comes to ~2.4 g, which could be achieved by dividing the dose equally two times a day (BID). Overall, BTE holds significant promise in conserving bone mass and reducing fracture risk in postmenopausal women when administered prophylactically. Early assessment of response to BTE could conveniently be made by serum bone turnover markers (CTX-1 and P1NP). Although, uterine safety of BTE has been demonstrated, however, necessary regulatory safety studies and bioavailability of marker compounds are contemplated prior to undertaking human studies.

Author contribution: DS & SK designed the biological experiments. SK performed major experiments and wrote the manuscript. AL, RG & KS helped in *in vivo* experiments and in date analysis. RM was involved in extract preparation. DS & NC reviewed and edited the manuscript.

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Conflict of Interest: Author declares no conflict of interest

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