ISSN (Online) 2249-6084 (Print) 2250-1029

International Journal of Pharmaceutical and Phytopharmacological Research (eIJPPR)
[Impact Factor – 0.7826]

Journal Homepage: www.eijppr.com

Research Article

Pharmacological Investigation of Bonton Capsule for Anti-osteoporotic Activity in Ovariectomized Rat

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Article info

Article History:
Received 17 Aug 2013
Accepted 26 Aug 2013

Keywords:
Osteoporosis, Bonton capsule, Ovariectomized rat, Bone strength

Abstract

Osteoporosis is a bone disease characterized by low bone mass, disturbed bone micro-architecture, increased fragility and consequent increase in fracture risk. The objective of present study was to evaluate Bonton capsule for its anti-osteoporotic activity in ovariectomized rat model at two different dose levels i.e. Therapeutic Effective Dose (TED) 180 mg/kg/day and Double to Therapeutic Effective Dose (TEDx2) 360 mg/kg/day. 24 healthy female wistar rats were divided into 4 groups and each group was containing 6 animals. Group 1 was considered as a normal control group fed with 1% Carboxy Methyl Cellulose (CMC) suspension. Group 2 was considered as Disease control group which was ovariectomized (OVX) and was fed with 1% CMC suspension. Group 3 and 4 were orally treated with Bonton capsule at TED (180 mg/kg/day) and TEDx2 (360 mg/kg/day) respectively. The present study was evaluated using biochemical parameters like serum alkaline phosphatase (ALP), serum calcium, femoral bone parameters, bone breaking strength, body weight and histopathological study of the bone. The treatment with Bonton capsule at TED (180 mg/kg/day) showed significant decrease in serum ALP and significant increase in serum calcium level. It also showed significant changes in femoral parameters and histopathological study of bone where TEDx2 (360 mg/kg/day) was observed moderately significant. Hence, it can be inferred that Bonton capsule at experimented therapeutic dose levels provides good anti-osteoporotic activity against ovariectomized rat.

1. INTRODUCTION

Osteoporosis is one of the major health problems associated with aging. It is a disease of bones that leads to an increased risk of fractures. In that, the bone mineral density is reduced and bone micro-architecture deteriorates[1]. It is defined as a skeletal disorder characterized by compromised bone strength predisposing a person to an increased risk of fracture. Here bone strength is influenced by bone density and bone quality[2]. There is a clear correlation between decrease in bone mineral density (BMD) and the risk of fracture. Osteoporosis is defined as BMD T-score as (-) 2.5 or lower[3, 4].

As defined by the World Health Organization (WHO), in 25% of women at age of 65 and in 70% of those above age of 80 suffering from osteoporosis and related problems[5]. Although osteoporosis usually makes its appearance late in life, its roots can be tracked back into adolescence. Particularly during periods of rapid bone growth, dietary calcium levels are of high importance. Other factors that contribute to development of osteoporosis are lifestyle, genetic problems and hormonal attributes. Reduced physical activity increases the rate of bone loss and muscle contraction is the prevailing source of skeletal loading. Regarding hormonal factors, women especially in the decade after menopause, can show a severe reduction of bone mass, which explains the high incidence of osteoporotic fractures in women as compared to men[6].

In conclusion, osteoporosis leads to bone with less tensile strength and significantly more susceptibility to fracture even at less force. Osteoporosis affect on connectivity of trabecular plate and rods[7, 8]. The bone loss affects cortical and trabecular bone more predominant in typical postmenopausal osteoporosis[9]. Bone remodeling rate doubles at menopause and remains elevated in condition of osteoporosis. This change contributes to increases in age-related skeletal fragility in women[10].

Being a natural, Herbal products are widely perceived as safe.[11] Due to their long historical clinical use and reliable therapeutic efficacy, Traditional Indian System of Medicine is getting global attention. Many pharmaceutical companies are using traditional medicine as an excellent pool for discovering novel natural bioactive compounds[12].

Bonton capsule contains extract of *Cissus quadrangularis* (Hadjod) Stem[13-15], *Terminalia arjuna* (Arjun) Bark[16], *Litsea chinensis* (Medasak) Root[17] and powder of Abha Guggulu[18], Lakshadi Guggulu[19], Kukundantwak Bhasma[20], Shukti Bhasma[21] processed with *Curcuma amada* (Aamragandhi Haridra) Rhizome extract[22].

Bonton capsule is manufactured and marketed by Vasu Healthcare Pvt. Ltd., Vadodara. Majority of ingredients of Bonton capsule are well reported in Ayurvedic texts and scientific research publications for anti-osteoporotic, anti-inflammatory and antioxidant activity. However, no such evidence was found which proves the efficacy of such combination.

In the present study, an attempt was made to investigate anti-osteoporotic activity of Bonton capsule in ovariectomized rat.

2. MATERIALS AND METHODS

2.1 Preparation of Test Drug

Bonton capsules were emptied to receive Powder which was then triturated with 1 g CMC followed by addition of 100ml distilled water to make suspension.

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2.2 Experimental Animals

Wistar albino female rats of 250-300 g were used and acclimatized to the experimental room having ambient temperature (23±2°C), controlled humidity (55±5%) conditions, and 12 hours light and dark cycle. Animals were caged in polypropylene cages with maximum of three animals per cage. The rats were fed with standard food pellets and water *ad libitum*. Study was conducted after obtaining approval by Institutional Animal Ethical Committee (IAEC) (Babaria Institute of Pharmacy, M.Pharm Sem-IV/12-13/03) as per the guidance of Committee for the Purpose of Control and Supervision of Experiments on Animal (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

2.3 Experimental Design

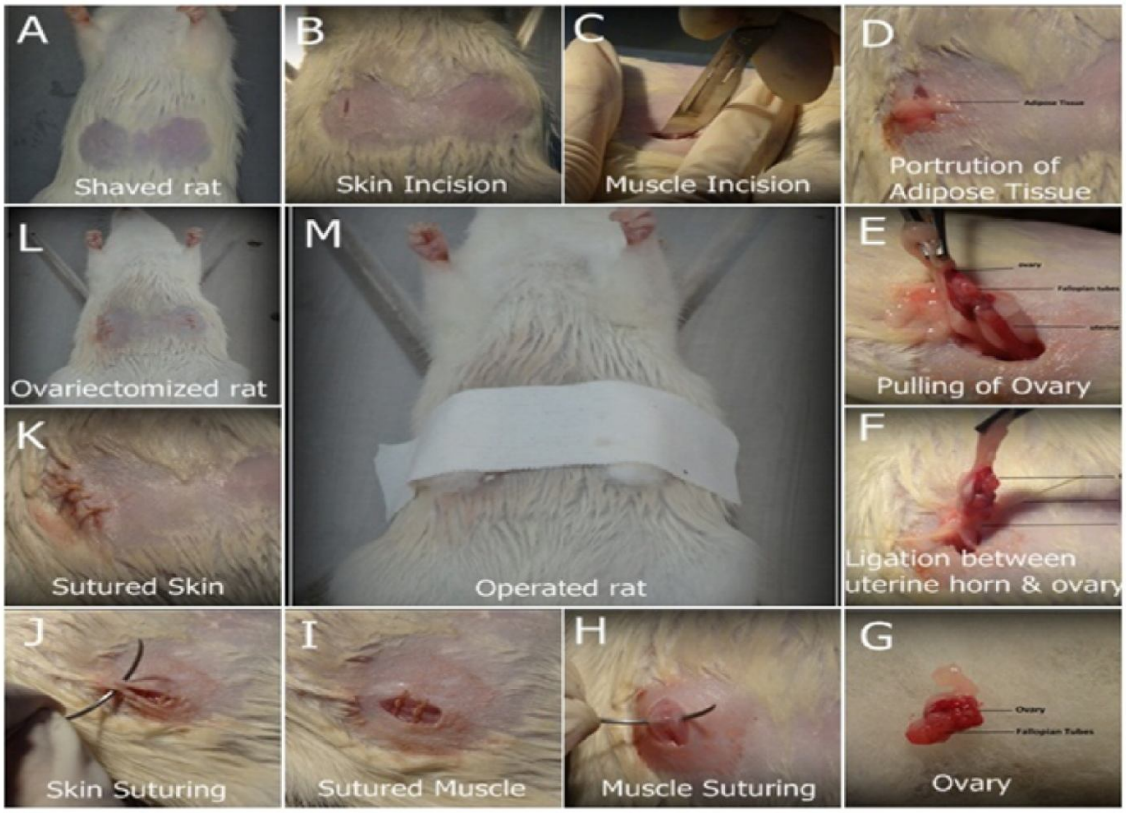
The experimental animals were divided into four groups, containing six animals in each group. Group 1 was considered as Normal control group and was fed 1% Carboxy Methyl Cellulose (CMC) suspension. Group 2 was considered as Disease control group which was ovariectomized (OVX) and was fed with 1% CMC suspension. Group 3 and 4 were ovariectomized and treated with Bonton capsule at TED (180 mg/kg/day) and TEDx2 (360 mg/kg/day) respectively. After the 8th day of ovariectomy, test drug was given to group 3 and 4 till eight weeks.

After 8 weeks, animals from all groups were anesthetized for collection of blood samples for estimation of biochemical parameters. Then, animals of all groups were sacrificed and their femurs were removed for femoral parameters and histopathological study of bone.

2.4 Induction of Osteoporosis by Ovariectomized (OVX) Method

The operating table area was sterilized with alcohol. Sterile surgical instruments were used for operation. For general anesthesia 75 mg/kg ketamine and 10 mg/kg xylazine intra-peritoneally were administered[23]. following anesthesia, animal was placed in lateral position. The left flank of the rats was shaved and furs removed completely (Figure 1A).The shaved area was washed with 70% alcohol. Then rat was transferred to the operation table. 2 cm incision was made on dorso-lateral area from the second either to fifth lumbar vertebrae or middle part of abdomen (Figure 1B). Incision was of minimum length to allow the extrusion of ovaries[24]. Entrance to the peritoneal cavity was made by dissecting the muscle, which revealed the adipose tissue surrounding the ovary (Figure 1C & 1D). The periovarian fat attached with the ovary was gently pulled away (Figure 1E) from the incision site to prevent detachment of a small piece of ovary[25]. After identifying the ovary and uterine horn the ovarian tissue was removed completely in one action (Figure 1F & 1G). The horn was returned to the abdominal cavity and the muscle and skin were sutured (Figure 1H, 1I, 1J) [26]. The procedure was repeated for the right ovary same as the left one. High degree of aseptic procedure was maintained throughout the operation (Figure 1K, 1L). After surgery, the rats were housed individually in polypropylene boxes for a period of one week to allow recovery and then re-grouped in their home cages. There was also some concern regarding site of incision as in case of the ventral approach in rodents, the wound remains almost constant direct contact with the paddy husk bedding, which may result in more frequent wound breakdowns. Hence, it was avoided[27].

Figure 1: Induction of osteoporosis by ovariectomized (OVX) method



2.5 Evaluation Parameters

2.5.1 Body weight

Body weight of every group of rats was recorded weekly and mean was considered to evaluate the effect of treatment[28].

2.5.2 Serum biochemical parameters

The levels of serum calcium[28] and serum alkaline phosphatase (ALP) [28] were determined by colorimetric method and auto analyzer respectively.

2.5.3 Measurement of femoral parameters

a. Femur length: The femur length, defined as the distance between the greater trochanter and the medial condyle. At

the end of the treatment, rats of all groups were sacrificed and the right femur was isolated. The femur length was measured by using vernier caliper[28].

b. Femur weight: The isolated right femurs were kept for drying. After that the femur of all groups were weighed by digital weighing balance.

c. Femur diameter: The external diameter was measured at the femoral mid shaft using vernier caliper[23].

d. Femur volume and density: Bone volume was measured by fluid replacement. Bone volume and density were measured by Archimedes's principle. Each bone was placed in un-stopper vial filled with deionized water, and the vial was put in a desiccator connected to a vacuum for 90 min. The desiccator was agitated periodically to ensure that all trapped air diffused out of the bone, at which time the bone was removed from the vial, blotted with tissue paper, weighed, and returned to the vial containing deionized water. The bone was reweighed in a boat suspended but completely immersed in water previously equilibrated to room temperature, and the density was calculated (grams/volume)[28].

2.5.4 Compression of 5th Lumbar Vertebra (Bone breaking strength)

The fifth lumbar vertebrae (L5) were used to measure the mechanical strength by the compression test. A craniocaudal compression force was applied to the specimen by the hardness tester and the breaking point was considered as a fracture point[29].

2.5.6 Histopathology of femur bone

The left femur was fixed in 10% formalin solution for 48 hours, decalcified in 5% nitric acid for 48hours. After fixation and decalcification, samples were put in paraffin blocks. 5 µm wide sections were taken from paraffin blocks for histopathological examination. After de-paraffinization and rehydration, sections were stained with hematoxylin and eosin stain. After staining the sections were observed under 100X magnification of trinocular microscope. Number of stained osteoblasts and micro-architecture of femur bone were observed[30].

2.6 Statistical Analysis

Data were analyzed by one way ANOVA followed by *Tukey-Kramer* Multiple Comparison Test. All the values were expressed as Mean ± SEM and *P<0.05* was considered as statistically significant.

3. RESULTS

3.1 Effect of Bonton Capsule on Body Weight

The overall body weight analysis revealed that OVX group showed significant decrease (*P<0.001*) in body weight as compared to the normal control group. Significant increase (*P<0.01*) was observed at TED dose level in comparison to OVX group. It was also noticed that body weight was decreased at TEDx2 dose level with respect to TED dose level (Table 1).

3.2 Effect of Bonton Capsule on Serum Biochemical Parameters

A significant decrease (*P<0.001*) was observed in serum calcium level in the OVX group as compared to the normal control group. Serum calcium level was significantly increased (*P<0.05*) in TED group compared to the OVX group. But, there was no any significant change found in the serum calcium level in TEDx2 group (Table 1).

Significant increase (*P<0.001*) was found in serum alkaline phosphatase (ALP) level in OVX group as compared to the normal control group. Significant effect was observed at TED dose level of Bonton capsule (*P<0.001*) but, there was no any significant change was found in TEDx2 group as compared to the OVX group (Table 1).

Table 1: Effect of Bonton capsule on body weight, serum biochemical parameters and bone breaking strength

Groups	Body weight (g)	Serum Calcium (mg/dL)	Serum ALP (IU)	Bone breaking strength (gm/cc^2)
Normal control	239.27 ± 3.75	11.38 ± 0.20	98.50 ± 2.73	7.66 ± 0.33
OVX group	206.10 ± 6.32$^{###}$	10.13 ± 0.13$^{###}$	197.96 ± 7.95$^{###}$	4.00 ± 0.25$^{###}$
OVX + Bonton capsule (TED)	233.43 ± 3.84**	10.90 ± 0.20*	109.87 ± 5.67***	6.66 ± 0.33***
OVX + Bonton capsule (TEDx2)	206.56±6.47	10.33 ± 0.15	212.99 ± 5.34	5.16±0.40*

All the values are expressed as mean ± SEM (n=6) in each group. Where, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$when compared to OVX group. While, $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ when compared to normal control group.

3.3 Effect of Bonton Capsule on Femoral Parameters

Femur length and weight of OVX group were significantly decreased (*P<0.001*) as compared to the normal control group. The TED group showed significant increase (*P<0.001*) in femur length and weight as compared to OVX group. TEDx2 group showed significant increase (*P<0.05*) only in femur length as compared to OVX group (Table 2).

Femur diameter, volume and density of OVX group were significantly decreased (*P<0.001*) as compared to the normal control group. The TED group showed significant increase (*P<0.001*) in femur diameter, volume and density as compared to OVX group. TEDx2 group showed significant increase (*P<0.05*) only in femur volume as compared to OVX group (Table 2).

Table 2: Effect of Bonton capsule on femoral parameters

Groups	Femur length (cm)	Femur weight (g)	Femur diameter (mm)	Femur volume (mL)	Femur Density (gm/mL)
Normal control	3.37 ± 0.01	0.66 ± 0.01	4.38 ± 0.02	0.65 ± 0.01	1.01 ± 0.01
OVX group	2.85 ± 0.01$^{###}$	0.47 ± 0.01$^{###}$	4.17 ± 0.01$^{###}$	0.57 ± 0.01$^{###}$	0.82 ± 0.01$^{###}$
OVX + Bonton capsule (TED)	3.27 ± 0.01***	0.65 ± 0.01***	4.40 ± 0.01***	0.64 ± 0.01***	1.02 ± 0.01***
OVX + Bonton capsule (TEDx2)	2.94 ± 0.03*	0.48 ± 0.01	4.21 ± 0.02	0.61 ± 0.01*	0.79 ± 0.02

All the values are expressed as mean ± SEM (n=6) in each group. Where, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$when compared to OVX group. While, $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ when compared to normal control group.

3.4 Effect of Bonton Capsule on Bone Breaking Strength

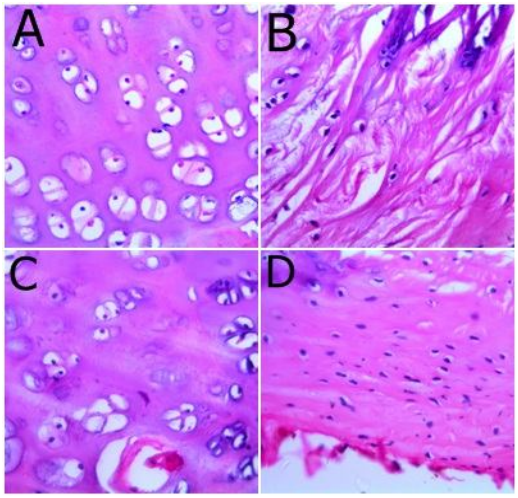
There was significant decrease (*P<0.001*) in breaking strength of 5th lumbar vertebra of OVX group as compared to normal control. The TED group showed significant increase (*P<0.001*) in bone breaking strength as compared to OVX group. TEDx2 group was observed less significant (*P<0.05*) as compared to TED group (Table 1).

3.5 Effect of Bonton Capsule on Histopathology of Femur Bone

Under the microscope, histology of the femur of normal control rat revealed normal size, shape and number of osteoblasts. It also appeared having normal micro-architecture of the bone. OVX group section exhibited sparse, disrupt, spacing enlarged, less number of small size of damaged osteoblasts. The micro-architecture of OVX

femur became disturbed. The Bonton capsule at TED (180 mg/kg/day) treated OVX rats showed significant restorative changes with normal size and shape of osteoblasts. But, the Bonton capsule at $TEDx_2$ (360mg/kg/day) treated OVX rats showed damaged osteoblasts and having disturbed micro-architecture.

Figure 2: Histopathology of femur bone



(A) Normal control group, (B) OVX group, (C) OVX + Bonton capsule (TED), (D) OVX + Bonton capsule (TEDx2)

4. DISCUSSION

Human bone is composed of a mineralized organic matrix and bone cells. Osteoblasts are bone cells that synthesize the organic matrix and regulate the mineralization process whereas Osteoclasts causes bone resorption.[29] Decrease in number of osteoblasts causes decrease in bone mineralization and formation process thus causes osteoporosis. The most common type of osteoporosis is the bone loss associated with ovarian hormone deficiency during and after menopause.[30] The approach of the study was to evaluate the osteoprotective activity of Bonton capsule. In the present study, evaluation was done with parameters such as loss of mechanical strength of bone, reduced serum calcium and increased serum ALP associated with estrogen deficiency in OVX animals. The ovariectomized rat exhibits most of the characteristics of human postmenopausal osteoporosis by developing the deficiency of estrogen.[22] Estrogen deficiency is a well-known causative factor in the pathogenesis of osteoporosis.[31]

The body weight of the ovariectomized rats was comparatively decreased in comparison with the normal control animals. The OVX rats treated with the Bonton capsule restored the body weight which may be due to ameliorating effect on bones.

Regarding bone metabolic marker like serum ALP which is associated with bone formation increases in osteoporosis and other bone metabolic disorders. Similar changes were observed in the present study. ALP is an early indicator of bone formation because it is a byproduct of osteoblasts. Serum ALP levels may double in the post-menopause term, depending on the increase in bone formation cycle.[32] The decrease in calcium level in the ovariectomized rats was created similar condition like postmenopausal women.[33] The treatment of Bonton capsule at TED (180 mg/kg/day) showed significant decrease in serum ALP and significant increase serum calcium level which may be due to enhancement of osteoblastic activity and reduction of osteoclastic activity. Whereas, Bonton capsule at TEDx2 (360 mg/kg/day) did not show much significant changes in serum ALP and serum calcium level as compared to the OVX group.

The biomechanical parameters including, compression test of 5^{th} lumbar vertebra is one of the direct measures of bone strength.[17] In this study the 5^{th} lumbar vertebra breaking strength was decreased in ovariectomized rats and it was restored by treatment of both the dose level of the Bonton capsule however TED (180 mg/kg/day) was found more significant.

The femur diameter, femur volume, femur weight, femur length and femur density were reduced in OVX group which may be due to increase in fragility followed by loss of minerals. This may be due to small stimulatory effect of growth hormone on longitudinal growth.[34] The treatment of Bonton capsule at both dose levels showed significant increase in femur length and femur volume as compared to OVX group. Whereas, Bonton capsule at TED (180 mg/kg/day) only showed significant increase in femur weight, femur diameter and femur density as compared to the OVX group.

Histopathology revealed that normal control possesses more number of osteoblasts as compared to the OVX group. OVX group showed disturbed micro-architecture of the bone as compared to the normal control. The histology of TED (180 mg/kg/day) group showed almost similarity in osteoblasts as compared to the normal control. This may indicate significant effect of test drug on osteoporotic bone.TEDx2 dose level did not showed good significant recovery as TED group.

5. CONCLUSION

On basis of available data, it can be concluded that Bonton capsule at TED (180 mg/kg/day) level was found effective at highly significant level where TEDx2 (360 mg/kg/day) was observed moderately significant. Hence, it can be inferred that Bonton capsule at experimented therapeutic dose levels provides good anti-osteoporotic activity against ovariectomized rat.

6. ACKNOWLEDGEMENT

Authors are sincerely thankful of the management of Vasu Healthcare for providing product sample and also thankful of Babaria Institute of Pharmacy, Vadodara for providing the necessary facilities and technical support for conducting the study.

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