EVALUATION OF OSTEOPROTECTIVE ACTIVITY OF EMBELIN ISOLATED FROM *EMBELIA RIBES BURM.* IN EXPERIMENTAL RATS

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ABSTRACT:

The systemic degradation of bone mass, strength, and microarchitecture that characterises osteoporosis, which raises the risk of fragility fractures, is an increasing medical and socioeconomic hazard. It has been demonstrated that Embelin (2, 5-dihydroxy-3-undecyl-1,4-benzoquinone) inhibits the NF-kB cell signalling pathway, hence suppressing osteoclast genesis brought on by receptor activator of NF-kB ligand (RANKL) and tumour cells in vitro. Osteoporosis treatment or prevention may involve the use of embelin. The goal of the current study was to evaluate how embelin, a substance derived from Embelia ribes berries, affected rats with osteoporosis brought on by ovariectomy. The results indicate that embelin has anti-osteoporotic properties against ovariectomy-

induced osteoporosis in female rats. A 90-day embelin treatment improved bone biochemical quality by altering trabecular microarchitecture without having a hyperplasic effect on the uterus, and it may be a potential medication for the prevention and treatment of postmenopausal osteoporosis. It also reduced body weight gain and stopped bone loss caused by ovariectomy, and there was a significant reduction in skeletal remodeling, as shown by t-score measurements.

Key words: Ovariectomy, *embelia Ribes*, embelin, osteoporosis and body weight.

INTRODUCTION:

Osteoporosis is an emerging medical and socioeconomic threat characterized by a systemic impairment of bone mass, strength and micro-architecture, which increases the propensity of fragility fractures. Bone quality is defined as the microstructure of bone and by the mineralization and collagen status[1]. About 40% of white postmenopausal women are affected by osteoporosis and with an ageing population.[2] The measurement of bone quality is by DXA (Dual energy absorptiometry)[3] is a valid method to diagnose osteoporosis and to predict the risk of fracture. Other methods for the assessment of the bone are quantitative computed tomography (QCT)[4], radiographic absorptiometry (RA)[5], single X-ray absorptiometry (SXA) and quantitative ultrasound (QUS)[6]. New decision-making methods such as the fracture risk assessment tool (FRAX)[7], predict an individual's 10-year risk of sustaining a hip fracture. The goals of osteoporosis management are to prevent fractures, to decrease the pain when present and to promote bone formation and inhibit bone resorption.[8] The stimulation of new bone formation is considered to be the most effective way of preventing osteoporotic fractures[9]. Embelin (2,5-dihydroxy-3-undecyl-1,4- benzoquinone), a major constituent of *Embelia ribes* Burm. (Family: Myrsinaceae) naturally occurring alkyl substituted hydroxy benzoquinone. The plant is indicated in traditional medicine for the treatment of various diseases[10]. The fruit is bitter in taste, used to

treat fever, inflammatory diseases and a variety of gastrointestinal ailments for thousands of years[11]. Embelin possess anti-inflammatory, analgesic[12], antioxidant[13] and wound healing[14] activities Embelin[15, 16] suppresses osteoclastogenesis induced by receptor activator of NF-kB ligand (RANKL) and tumor cells in vitro through inhibition of the NF-kB cell signaling pathway.[17] The urinary calcium and phosphorus levels were ascertained by using diagnostic assay kits. The bone volume and density also ascertained using Archimedes principles in right femur. The histopathology of left femurs was also identified.

MATERIALS AND METHODS:

Collection of Plant Material and Isolation of Embelin

The berries of Embelia ribes were bought from Abirami Botanicals in Tuticorin, Tamil Nadu, India, and verified by Dr. Siddappa of the botany department of the Sree Siddaganga College of Science in Tumkur, Karnataka, India. The plant's berries were gathered, air dried, and ground into powder. Using the cold extraction method, two kg of coarsely powdered embelia ribes berries were thoroughly extracted with n-hexane. The solvent was decanted and distilled off over a hot water bath after 72 hours. The resulting extract underwent column chromatography over silica gel and was concentrated in vacuo (100 - 200 mesh). As the column was eluted with benzene, an orange-colored powder was produced, which when crystallised with ether produced orange plates of embelin. Both the combined melting point approach and a comparison with an actual sample of embelin on a TLC plate were used to determine the purity of the compound. With Tween 80 (1% v/v), embelin was suspended in distilled water. For oral delivery, two dosages of embelin (10 and 20 mg/kg) were used.

Animals

The wistar albino female rats (150-200 g) were used in the present study. The animals were collected from Sree Siddaganga College of Pharmacy Animal House, Tumkur. Animals were maintained at standard laboratory conditions (12 h light and 12 h dark cycle: 25 ± 4 °C: 30-60% humidity) the animals were fed with standard feeding pellets. Prior to oral treatment, albino wistar rats were fasted for 10 and 12 h, respectively. However, water was made available *ad libitum*. All the animals were acclimatized for seven days before the study. The animals were randomized into experimental and control groups and housed individually in sanitized polypropylene cages containing sterile paddy husk as bedding. Animals were habituated to laboratory conditions for 48 h prior to experimental protocol to minimize if any of non-specific stress.

Evaluation of Embelin on ovariectomized induced osteoporosis in rats

40 number of 3 month old female, virgin wistar rats were obtained, Group 1 rats were sham operated, i.e bilaterally operated under ketamine anesthesia (75mg/kg) without causing damage to the blood vessel or ovary in order to maintain the surgical procedure in all the groups. Group 2-5 rats were bilaterally ovariectomized under ketamine anesthesia (75mg/kg) and sterilized conditions. Ovariectomy was made by two dorso-lateral incisions, approximately 1 cm long above the ovaries. With the use of a sharp dissecting scissors, the skin was cut almost together with the dorsal muscles and the peritoneal cavity was thus accessed. The connection between the fallopian tube and the uterine horn was cut and the ovary moved out. The animals were allowed to recover for 15 days and then treated with embelin at different doses for 90 days.

Group 1	Sham operated + 1% Tween 80 v/v was given by oral route.
Group 2	Ovariectomized + 1% Tween 80 v/v was given by oral route.
Group 3	Ovariectomized + treated with Embelin (10 mg/kg) by oral route.

Group 4	Ovariectomized + treated with Embelin (20 mg/kg) by oral route.
Group 5	Ovariectomized + Vitamin D_3 [0.1µg/kg] by oral route.

Body weights of all animals were recorded at the beginning and at monthly intervals throughout the 12 week experimental study. At the end of the experiment total urine excreted over 24-hr period was collected from overnight fasted rats by housing eachrat individually in a metabolic cage. Food was withdrawn during the urine collection (24- hour period). A drop of 6N Hcl was added to each urine sample and stored at freezing temperature. The samples collected were analysed for calcium and phosphorus levels.

The animals were weighed, anesthetized by solvent ether vapours and blood was collected by retro orbital puncture. Serum collected by centrifugation was stored at -20 ^{0}C, till the samples collected were analysed for calcium, phosphorus and alkaline phosphatase levels. Animals were sacrificed by higher dose of pentobarbitone (50 mg/kg). The left and right femur was dissected out. The left femurs were thawed, autoclaved for 15 min at 110 ^{0}C and divested of soft tissue for the measurement of weight, length, volume, density and ash parameters. The right femur was immediately fixed in 10% neutral buffered formalin for histopathological examination.

Serum and Urine analysis

Using a microplate reader and readily accessible reagent kits, calcium, phosphorus, and alkaline phosphatase were biochemically analysed in blood samples as well as calcium and phosphorus in urine samples.

Calcium estimation

Calcium present in samples of serum, urine and bone ash were estimated by using Erba reagent kit [O-Cresolphthalein Complexone (**OCPC**)] method of Moorehead and Briggs.

Phosphorus estimation

Phosphorus present in samples of serum, urine and bone ash were estimated byusing Erba reagent kit (Ammonium Molybdate Method).

Alkaline Phosphatase estimation

Alkaline phosphatase present in serum samples were estimated by using Erbareagent kit (IFCC Method).

Measurement of Femur length and weights

The greater trochanter to medial condyle measurement is referred to as femur length. After being autoclaved, the left femurs were cleansed of the surrounding tissue, dried, and used to measure the length of the femurs with a slide calliper. The femurs were then stored in an oven that had been evacuated and dried for 48 hours at 1100C. Using a digital weighing scale, the dried femurs' weights were calculated.

Measurement of Bone volume and density

Femur bone volume and density were determined by Archimedes principle. Briefly, each bone was placed in an unstoppered vial filled with deionized water, and then 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. Then the bone was removed from the vial, blotted with tissue, weighed.

Ash weights and mineral content of bone

The left femurs after bone volume and density measurements were placed in tared fused silica crucibles weighed and dried to a constant weight at 110^0C and ashed for 24 hr at 650^0C. The ash weights were determined before dissolving in 1ml conc. Hcl. The samples were suitably diluted (20 µl diluted to 20 ml) with distilled water and assayed for calcium, phosphorus content using micro plate reader technique.

Histopathological Examinations

The right femurs underwent decalcification and paraffin method processing. Sections of 5 thickness were cut, stained using the standard H & E procedure, and inspected under a microscope at various magnifications for pathological conditions. For findings, femur sections were cut from the same location in each group.

Statistical data

Data were presented as mean ± S.E.M. Statistical differences between control and treated groups were calculated using one way ANOVA followed by Tukey's test wherever applicable.

RESULTS:

Table 1: Effect of three months treatment of Embelin or VitD$_3$ on biochemical parameters in serum and urine of OVX rats

Treatment groups (mg/kg, p.o.)	S-Calcium (mg/dl)	S-Phosphorus (mg/dl)	U-Calcium (mg/dl)	U-Phosphorus (mg/dl)	ALP (IU/L)
Sham Group	1.649±0.119***	2.546±0.0879***	1.923±0.0574**	173.2±10.73***	1053±67.23**
OVX Control	2.765±0.112	3.336±0.1385	3.455±0.2174	249.5±6.530	1643±147.7
OVX +E-10	2.079±0.1524*	2.841±0.09094*	2.390±0.2715*	178.4±3.955***	1023±29.04**
OVX+E-20	1.795±0.2599**	2.839±0.1097*	2.408±0.1989*	165.7±5.870***	1119±111.8**
OVX+Vit D3	1.828±0.1539**	2.281±0.06005***	1.835±0.2865***	170.4±8.534***	708.1±8.362***

Values are expressed in mean ±SEM (n=6) Data's were analyzed by using ANOVA fallowed by Tukeys multiple comparison test. *P<0.05 when compared with OVX control, **P<0.01 when compared with OVX control*P<0.001 when compared with OVX control. OVX-Overiectomized rats; E-10-Embelin (10 mg/kg), E-20- Embelin (20 mg/kg).**

Table 2: Effect of three months treatment of Embelin or Vit D3 on body weight and uterine index of OVX rat

Treatment groups (mg/kg, p.o.)	First month	Second month	Third month	Uterine index (mg/g BW)
Sham Group	193.3±3.333	207.5±2.500***	215.8±2.007***	1.564±0.1174***
OVX Control	208.3±4.773	238.3±4.773	273.3±4.944	0.2925±0.0207
OVX +E-10	198.3±4.773	208.3±4.773***	220.8±4.167***	0.5769±0.0200**
OVX+E-20	200.0±5.774	210.0±5.774**	212.5±5.737***	0.5753±0.0311**
OVX+Vit D3	196.0±5.099	200.0±3.162***	204.0±1.871***	0.7353±0.0631***

Values are expressed in mean ±SEM (n=6) Data's were analyzed by using ANOVA fallowed by Tukeys multiple comparison test. *P<0.05 when compared with OVX control, **P<0.01 when compared with OVX control*P<0.001 when compared with OVX control. OVX-Overiectomized rats; E-10-Embelin (10mg/kg), E-20- Embelin (20mg/kg).**

Table 3: Effect of three months treatment of Embelin or Vit D_3 on femur physical parameters of OVXrats

Treatment groups (mg/kg, p.o.)	Length (mm)	Weight(g)	Volume(ml)	Density(g/cm$^{3)}$
Sham Group	32.63±0.126**	0.5367±0.01856***	0.7350±0.02363***	0.8383±0.04557***
OVX Control	30.29±0.097	0.2733±0.01801	0.4660±0.01077	0.4467±0.0105
OVX +E-10	33.01±0.574***	0.4442±0.02740***	0.6900±0.03674**	0.7767±0.03533***
OVX+E-20	32.27±0.326*	0.4130±0.01538**	0.6000±0.06124	0.7600±0.05083***
OVX+Vit D3	33.67±0.456***	0.4580±0.03430***	0.7040±0.02040**	0.7392±0.04522***

Values are expressed in mean ±SEM (n=6) Data's were analyzed by using ANOVA fallowed by Tukeys multiple comparison test. *P<0.05 when compared with OVX control, **P<0.01 when compared with OVX control*P<0.001 when compared with OVX control. OVX-Overiectomized rats; E-10-Embelin (10mg/kg), E-20- Embelin (20mg/kg).**

Table 4: Effect of three months treatment of Embelin or Vit D_3 on Ash parameters of OVX rats

Treatment Groups (mg/kg, p.o.)	Ash weight(g)	% Ash	Ash Calcium(mg/dl)	Ash Phosphorous (mg/dl)
Sham Group	0.339±0.020***	59.93±1.92***	4.032±0.75***	1.925±0.03**
OVX Control	0.229±0.006	96.42±4.99	0.825±0.04	0.837±0.05
OVX +E-10	0.289±0.005*	62.90±3.25***	3.086±0.21**	2.165±0.21***
OVX+E-20	0.294±0.016*	71.13±2.73**	2.088±0.17	1.860±0.14**
OVX+Vit D3	0.324±0.018***	67.45±6.66**	2.718±0.11**	2.201±0.24***

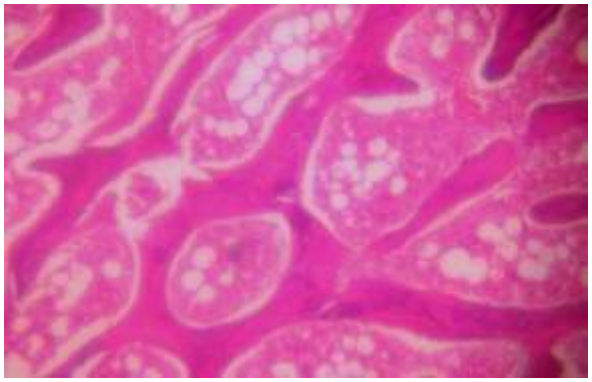
Values are expressed in mean ±SEM (n=6) Data's were analyzed by using ANOVA fallowed by Tukeys multiple comparison test. *P<0.05 when compared with OVX control, **P<0.01 when compared with OVX control*P<0.001 when compared with OVX control. OVX- Overiectomized rats; E-10-Embelin (10mg/kg), E-20- Embelin (20mg/kg).**

Histopathological and other tests

Increased loss of calcium and decrease in phosphorous, in urine are supporting earlier studies on ovariectomized rats. Similarly, higher levels of ALP were observed with respect to decrease in calcium and phosphorous in serum of ovariectomized animals. The decreased bone mineral content was evidenced in reduction of total ash weight, ash percent and ash calcium content, ascertaining its role in the resorption of bone. The increase in bone turnover and enhanced bone fragility, with disruptive changes in the bone architecture was observed in the histopathological study following ovariectomy, is indicative of the development of osteoporosis in rats due to estrogen deficiency and mimics human postmenopausal osteoporosis. The calcium, phosphorus, ALP was evaluated in serum and calcium and phosphorus in urine were analyzed using microplate

reader in ovariectomized rat. The embelin has shown decreased calcium level in urine, increase in serum and increased ash calcium level and has no effect on phosphorus levels and decreased ALP levels in serum and urine respectively. The embelin of 10 mg/kg has shown significant effect similar to standard vitamin D_3 in all parameters.

The calcium levels decreased in urine may be due to increased absorption of calcium by lumen. The ALP levels decreased in serum may be due to osteoblastic activity through new bone formation. Bone density was increased by embelin of 10 mg/kg dose. This may be due to reduction in the bone resorption. In histopathological examination of ovariectomized induced osteoporosis in rats, the embelin of dose 10 mg exhibited when observed for ossification. There was change in structural pattern of profound increase in connectedness, intact bone lamellae and thickness of trabecular bone formation. This has shown significant effect with 10 mg of embelin compared to 20 mg of embelin. This may due to increased calcified cartilaginous deposits. Increase in number of marginal osteoblasts and decrease in the number of osteoclasts. This protective effect by the embelin may be the extent of bone formation and decreased bone resorption.



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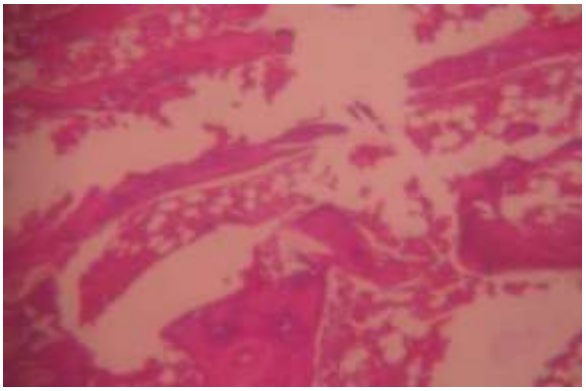
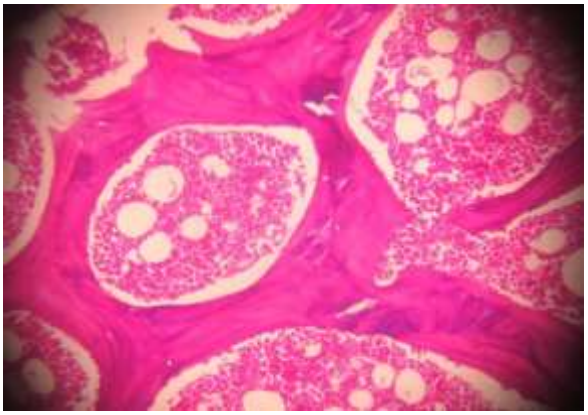
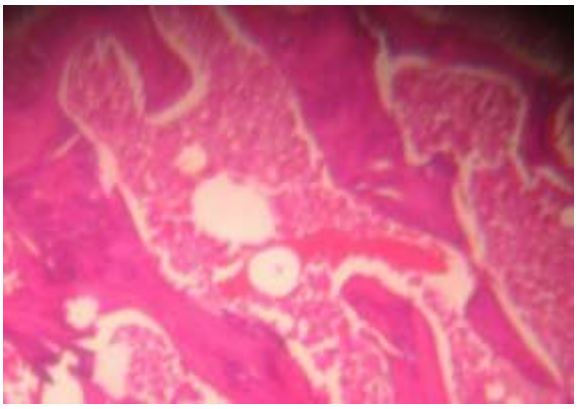


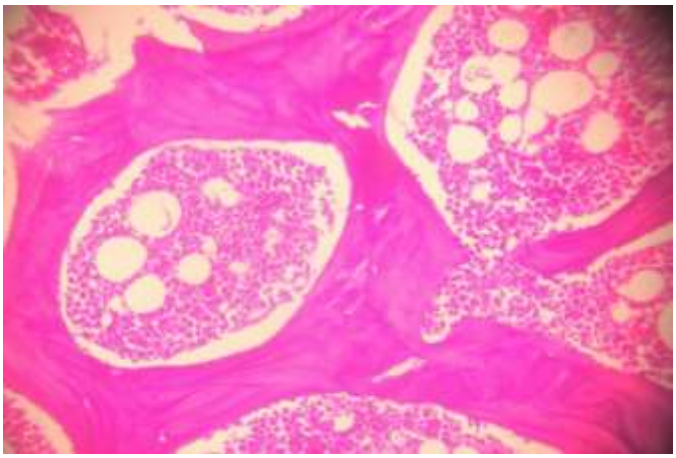
Ovx



Embelin 10



Embelin 20



Vitamin D_3

Fig 1: Histopathological images of OVX induced Osteoporosis in Experimentalrats

CONCLUSION:

The osteogenic characteristic of the embelin isolated from Embelia ribes Burm. berries is accomplished by promoting the formation and mineralization of new bone. Embelin was found to be useful in the research for promoting the formation of new bone. Phytoestrogens, which may work by binding to oestrogen receptors and thereby maintaining the homeostasis of bone minerals, may be the cause of the significant activity of embelin. A novel osteoporosis treatment may be developed with additional research on preclinical trials in animals and clinical trials on human volunteers.

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