Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 5, Issue 4, 2013

Research Article

PREVENTIVE ROLE OF *EMBLICA OFFCINALIS AND CISSUS QUADRANGULARIS* ON BONE LOSS IN OSTEOPOROSIS

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Received: 27 July 2013, Revised and Accepted: 24 Aug 2013

ABSTRACT

Objective: Osteoporosis, a bone degenerating disorder is one of the most common ailments in old age. Though the treatment with hormone replacement therapy for osteoporosis is effective, but they are often associated with serious side effects. Therefore, in search for more effective natural remedy, we evaluated the efficacy of aqueous extract of *Emblica officinalis (EO)* singly or in combination with the petroleum ether extract of *Cissus quadrangularis (CQ)* for their anti-osteoporotic potential in ovariectomized rat model.

Methods: After the onset of the osteoporosis in bilaterally ovariectomized rats, they were treated with reference doses of Raloxifene, *EO, CQ* singly and/or in combination for 6 weeks.

Results and conclusion: At the end of the experiment, serum ALP, TRAP and hydroxyproline levels were significantly increased in ovariectomized animals, but no significant changes were observed in the calcium levels. Treatment with *EO* and *CQ* significantly increased the serum ALP levels, while the serum TRAP and hydroxyproline levels were significantly restored towards normal level. Loss of bone mass and strength due to osteoporosis was significantly increased with *EO* and *CQ* treatments. Loss of bone mass and strength due to osteoporosis was significantly reduced with *EO* and *CQ* treatments. The results clearly depicts that the individual effects of *EO* and *CQ* are comparable with that of Raloxifene. Further when compared to effects of individual plant extracts, the combined effect of *EO* and *CQ* were equipotent. Therefore, EO and CQ can effectively reduce the bone loss and increase the bone strength, thus they can be used to treat the bone degenerative disorders.

Keywords: *Emblica officinalis, Cissus quadrangularis*, Ovariectomy, Osteoporosis, Treatment.

INTRODUCTION

Osteoporosis is a metabolic bone disease occurs due to the alterations in the bone remodeling where the activity of osteoblasts to form new bone does not match with the bone resorption by the osteoclastic activity. It is a disabling and painful condition, and is characterized by low bone mineral density, structural deterioration of bone tissue, with a subsequent increase in the bone fragility and susceptibility to fractures [1]. Estrogen deficiency in postmenopausal women with ovarian involution is the major contributing factor for bone deterioration with advancing age [2].

Further, there is a growing evidence to link the oxidative stress directly to the osteoporosis [3,4]. Oxidative stress associated with accumulation of ROS will affect the remodeling process by accelerating the osteoclast mediated bone resorption [5]. Experimental animal models of ovariectomy induced osteoporosis have been demonstrated the elevated oxidative stress with subsequent bone loss [4,6]. Majority of the therapeutic agents used to treat osteoporosis include calcitonin, calcium and vitamin D supplementation, bisphosphonate, ipriflavone and sodium fluoride [7]. Amongst, estrogen replacement therapy (ERT) or hormone replacement therapy (HRT) showed the established benefit for the treatment of osteoporosis. But ERT/HRT usage frequently associated with major side effects such as risks of breast cancer, endometrial cancer, heart attacks, strokes, and blood clots [8]. It is necessary to discover potent natural products to replace the current treatment because of high costs and to ovoid the high risk of health hazards associated with long term use of hormone replacement therapy.

One such natural medicine is *CQ,* a vining plant distributed throughout the topical world and it has been widely used medically for centuries. The plant has been used widely used to promote the bone health in the traditional medicine [9,10]. The fracture healing property and anti-osteoporotic activities of *CQ* has been demonstrated in various pharmacological studies [11-20]. The application of *CQ* in fracture healing has been tested in rats, dogs and human subjects [11-14]. Various authors have reported the efficacy of *CQ* in preventing the bone loss in experimentally induced osteoporotic rat models [15-18]. The additional evidence of efficacy of petroleum ether fraction of *CQ* on fetal bone maturation and prevention and reversal of osteoporosis was observed in a series of studies done in our laboratory [17-20].

Another such potent and widely used medicinal plant in India is *EO*. The fruit extract of *EO* have been demonstrated to possess hepatoprotective, chemopreventive, antiatherogenic, antiproliferative, cardioprotective, hypolipidaemic, anti-inflammatory, antidiabetic, analgesic and antipyretic and adaptogenic activities. It is also used to treat against diseases such as diarrhea, jaundice, inflammation, cerebral insufficiency and mental disorders [21-26]. The aqueous extract of *EO* has been shown to have potent free radical scavenging property and inhibitor of lipid peroxide formation and scavenger of hydroxyl and superoxide radical in vitro [27]. The effects of *EO* on the bone health have not been studied so far. Therefore this study is aimed to examine role of the aqueous extract of fruits of *EO* singly or in combination with the petroleum ether extract of *CQ,* in preventing the osteoporotic changes using ovariectomy induced osteoporotic model.

MATERIALS AND METHODS

Plant material preparation

Fresh stems of CQ plant were collected in Belgaum, Karnataka, India. The fleshy stems were washed and air-dried, and then the dried stems were grinded into powder. The powder (1.3 kg) was then thoroughly extracted with 95% ethanol in a soxhlet apparatus, and the yield was concentrated under reduced pressure. Next, the total yield of ethanol extract (125 g) was suspended in water and partitioned with petroleum ether to obtain petroleum ether extract of CQ (11.4 g; 9.1% w/w). A dry powder of aqueous extract of fruits of *EO* was obtained from the Natural Remedies, Bangalore. The doses of both *CQ* [17] and *EO* [28] were selected based on acute toxicity tests and also based on previous reports. Both extracts were diluted in 0.5% carboxy methyl cellulose (CMC) before administration to animals.

Animals

8 week old healthy female Wistar rats weighing approximately 180 g were housed in the Central animal research facility of Manipal University. All the rats were maintained in well-ventilated, temperature-controlled room on a 12 h light: 12 h dark schedule. The rats were fed with standard balanced rat pellets and drinking water was made available ad libitum. All the experiments were approved and conducted according the guidelines of Institutional Animal Ethical Committee, Kasturba Medical College, Manipal, according to prescribed guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

Experimental design

Female Wistar rats were divided randomly into sham-operated control group (SHAM) (n=8) and ovariectomized group (n=40). The ovariectomized rats were left untreated for 22 days to develop osteoporosis which was confirmed by the histopathological studies. On 22nd post ovariectomy day, the ovariectomized rats were subdivided into five groups: (i) Ovariectomized-control (OVX) group was treated with vehicle normal saline; (ii) Ovariectomized + raloxifene (OVX+RAL) group was given orally with Raloxifene standard drug (Dr. Reddy's Laboratory, Hyderabad, India) at a dose of 5.4 mg/kg/day [17] (iii) Ovariectomized + CQ (OVX+CQ) group was treated with petroleum ether extract of *CQ* at a dose of 500 mg/kg/day [17] (iv) Ovariectomized + EO (OVX+EO) was given orally aqueous extract of *EO* at a dose of 200 mg/kg/day [28] (v) Ovariectomized + CQ + EO (OVX+CQ+EO) were given orally with the equal volumes of both *CQ* petroleum extract (500 mg/kg) and *EO* aqueous extract (200 mg/kg). Sham group rats were treated with saline. All the treatments were continued for 6 weeks. At the end of the experimental period, blood samples from all the groups were withdrawn to assess biochemical parameters. The animals were then sacrificed by cervical dislocation and left femora were collected for histomorphometrical studies and right femora were used for testing the biomechanical properties.

Biochemical analysis of serum calcium, ALP, TRAP, and Hydroxyproline

The commercially available kits were used to estimate the serum calcium, ALP and TRAP levels (Agappe diagnostics). Neuman and Logan method was employed to measure the serum hydroxyproline levels [29].

Measurement of biomechanical properties

Three point bending test of the right femora of all rats was performed to determine the maximum flexure load using Universal testing 3366 machine (Instron Corp, UK). The femur was placed horizontally on two supports in the material testing machine. Then the callus at the middle of the diaphysis was subjected to a load corresponding to the site of fracture with the span length of 10 mm and loading speed of 5 mm/min until the bone was fractured [30].

Histomorphometrical analysis of femur

The lower ends of left femora were fixed with PLP fixative (2% paraformaldehyde containing 0.075 M lysine and 0.01 M sodium periodate solution, pH 7.4, stored at 4°C) for 24 hr at 4°C. After, fixation bones were decalcified by EDTA-G solution [31]. The bone tissues were dehydrated in a graded series of alcohols and then embedded in paraffin wax (58ºC). On rotatory microtome, the lower end of femur were sectioned (5-µm thickness) longitudinally and processed for haematoxyline and eosin staining and then microphotographs were taken at fixed magnification. The cortical bone, trabecular bone thickness and growth plate thickness of lower end of the femur were measured, using image J software.

Statistical analysis

The results were expressed as the mean with the standard error of mean. All data were analyzed using SPSS software version 16. The difference between the groups was set by doing one-way analysis of variance test followed by Bonferroni's test for multiple comparisons and data was represented as mean ± SEM.

RESULTS

Biochemical analysis of serum calcium, ALP, TRAP and hydroxyproline

Biochemical analysis of serum calcium, ALP, TRAP and hydroxyproline revealed the regulatory effects of *CQ* and *EO* on the bone homeostasis in osteoportic conditions. In comparison with SHAM group, no significant changes were observed in the calcium levels in all non-treated and treated groups (Table 1). Serum ALP, the bone marker of osteoblastic activity is significantly increased in OVX group compared to SHAM group ($p<0.001$). This increase may be due to initial compensatory mechanism wherein, existing osteoblast bioactivity increased to compensate the bone loss. Further, treatment with raloxifene, *CQ*, *EO* and *CQ+EO* significantly increased the ALP levels compared to SHAM group ($p<0.001$), indicating the facilitatory effect of these test preparations on the bone formation (Table 1). Serum TRAP, the bone marker of osteoclastic activity significantly increased in the OVX group compared to base level in the SHAM group ($p<0.001$). However, in comparison to the OVX group, TRAP level was decreased in all the treated groups; OVX+RAL, OVX+CQ, OVX+EO and OVX+CQ+EO ($p<0.001$) (Table 1), indicating the inhibitory effect on osteoclast activity in the treated groups. Similarly, serum hydroxyproline, the breakdown product of collagen, was significantly increased in OVX group compared to SHAM group ($p<0.001$) indicating the loss of collagen content in the bone. In comparison with OVX group, such degradation of collagen was significantly decreased in all the treated groups ($p<0.01$) (Table 1).

Table 1: Effect of aqueous extract of *Emblica officinalis* and petroleum ether extract of *Cissus quadrangularis* on serum levels of Calcium, ALP, TRAP and Hydroxyproline in ovariectomized rats

Groups	Calcium (mg/dl)	ALP (U/L)	TRAP (U/L)	Hydroxyproline (µg/ml)
SHAM	9.86±0.05	113.18±3.05	5.49±0.10	0.229±0.008
OVX	10.10±0.1	143.4±2.38***	7.57±0.11***	0.289±0.007***
OVX+RAL	9.87±0.08	174.63±4.09***	4.1±0.14$^{\$\$\$}$	0.230±0.007$^{\$\$\$}$
OVX+CQ	9.80±0.09	157.71±4.46***	4.64±0.13$^{\$\$\$}$	0.238±0.009$^{\$\$}$
OVX+EO	9.82±0.06	148.01±2.05***	4.79±0.19$^{\$\$\$}$	0.241±0.009$^{\$\$}$
OVX+CQ+EO	9.95±0.04	159.81±6.5***	4.32±0.09$^{\$\$\$}$	0.238±0.009$^{\$\$}$

$p < 0.001$ when compared to SHAM group. $^{\$\$\$}p < 0.001$, $^{\$\$}p < 0.01$ when compared to OVX group.

Biomechanical strength

Maximum force required to break the femur was significantly less in the OVX animals compared to SHAM control group ($p<0.001$). However, when OVX animals treated with RAL, *CQ*, *EO*, the force required to break the femur significantly increased indicating the presence of stronger bones in these treated animals ($p<0.001$; Figure 1). Results indicate that all the treatments facilitated the formation of new bone and/or reduced the bone loss in osteoporotic condition.

Thickness of cortical and trabecular bone

Histomorphometrical analysis of lower end of the femur revealed that, the mean thickness of cortical and trabecular bone in the OVX group was significantly less than that of SHAM group ($p<0.001$)

indicating the osteoporotic changes induced by the ovariectomy. However, treatment with Raloxifene, *CQ*, *EO* and *CQ+EO* significantly (p<0.001) improved the cortical bone and trabecular bone thickness in comparison with OVX group (Figure 2 & Figure 3).

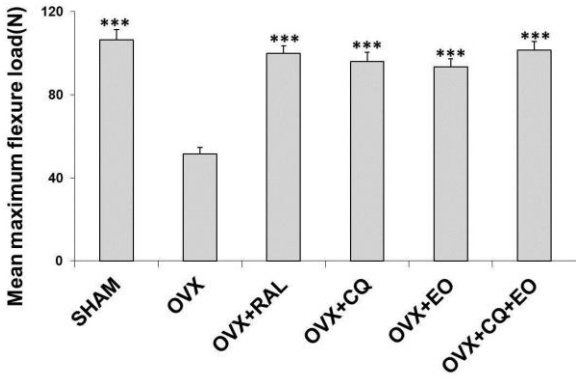

Fig. 1: Effect of aqueous extract of *Emblica officinalis* and petroleum ether extract of *Cissus quadrangularis* on mean maximum flexure load in ovariectomized rats

Mean maximum flexure load in all the experimental groups. The maximum flexor load is significantly decreased in ovariectomized group (OVX) compared to control group (SHAM) (p<0.001). However, treatment with Raloxifene, *CQ*, *EO* and *CQ+EO* significantly (p<0.001) improved the force required to break the femur compared to OVX group. ***p < 0.001 when compared to OVX group.

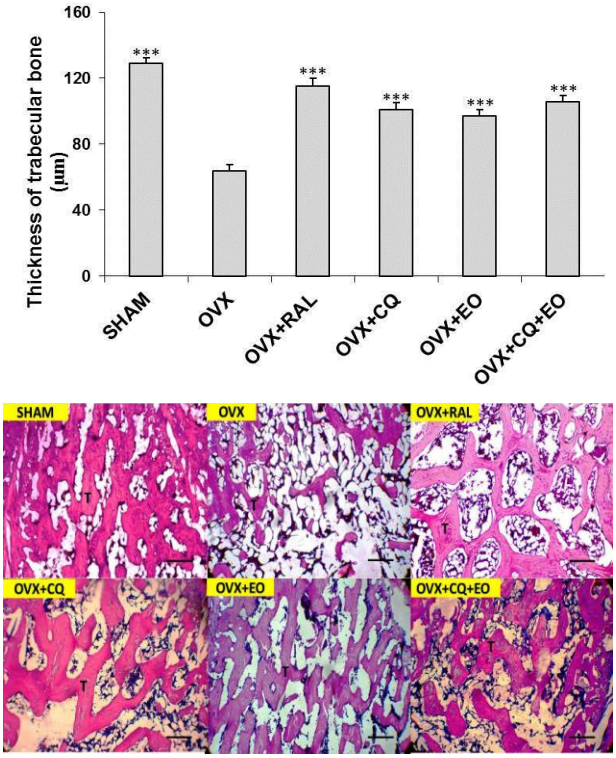

Fig. 2: Effect of aqueous extract of *Emblica officinalis* and petroleum ether extract of *Cissus quadrangularis* on mean thickness of trabecular bone in ovariectomized rats.

Mean thickness of trabecular bone is significantly decreased in Ovariectomized group (OVX) compared to control group (SHAM) (p<0.001). However, treatments with Raloxifene, *CQ*, *EO* and *CQ+EO* significantly (p<0.001) improved the cortical bone and trabecular bone thickness in comparison with OVX group. ***p < 0.001 when compared to OVX group. Scale bar = 150 µm.

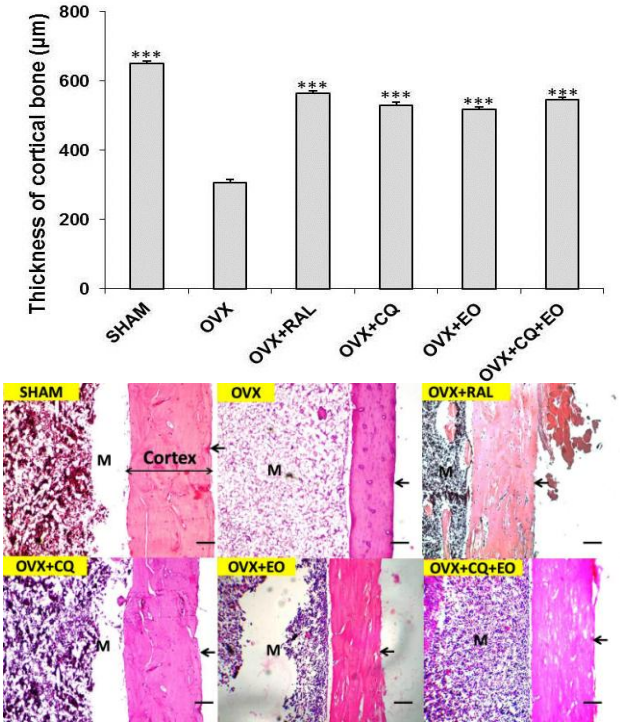

Fig. 3: Effect of aqueous extract of *Emblica officinalis* and petroleum ether extract of *Cissus quadrangularis* on mean thickness of cortical bone in ovariectomized rats.

Mean thickness of cortical bone is significantly decreased in Ovariectomized group (OVX), compared to control group (SHAM) (p<0.001). However, treatments with Raloxifene, *CQ*, *EO* and *CQ+EO* significantly (p<0.001) improved the cortical bone and trabecular bone thickness in comparison with OVX group. ***p < 0.001 when compared to OVX group. M: medullary cavity, arrows point the periosteum, scale bar =100 µm.

Thickness of growth plate

Mean thickness of the growth plate in SHAM, OVX, OVX+RAL, OVX+CQ, OVX+EO and OVX+CQ+EO groups was 111.68±4.8 µm, 115.59±6.5 µm , 118.6±4.9 µm, 114.76±4.7 µm, 113.11±5.35 µm and 120.23±5.3 µm respectively. There was no statistically significant change in the growth plate thickness among the groups (P>0.05) (Figure 4). However, treatment with *CQ*, *EO* singly or in combination, showed darkly stained healthy matrix indicating rich chondroitin sulphate and hyaluronic acid in the matrix.

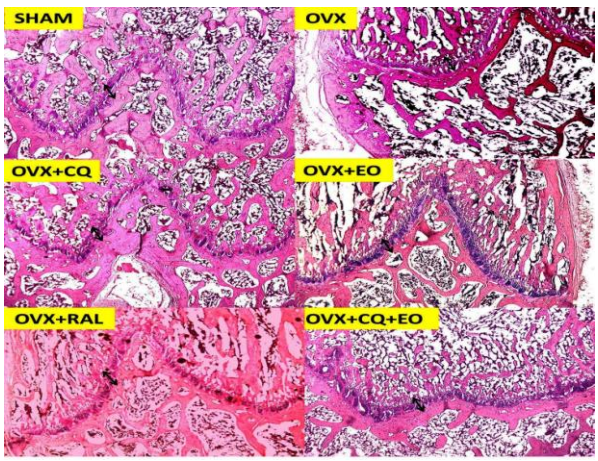

Fig. 4: Effect of aqueous extract of *Emblica officinalis* and petroleum ether extract of *Cissus quadrangularis* on growth plate.

H & E stained photomicrographs of the lower end of femur showing the growth plate in different experimental groups. Note there is no significant change in the thickness of growth plate in ovariectomized rats (OVX), compared to SHAM group. Further, treatments with Raloxifene, CQ and EO did not affect the growth plate thickness.

DISCUSSION

Combination of different plant extracts is often preferred over single extract as there is evidence that a synergism between two or more plant extracts enhances the physiological potential of the bioorganic substances [32]. Studies have shown the beneficial effects of different formulations for the treatment of various disorders. However, very few studies have been attempted the combined effects of two plant extracts on the anti-osteoporotic potential in the ovariectomized rats [33]. Results of the present study show that, the combined effect of plant extracts is not additive but they are equipotent when compared to the individual plant extracts.

Earlier it was shown that the *CQ* is one of the potent drugs which effectively reduce the bone loss and induces the new bone formation [17-20]. Further, this study clearly depicts that in addition to the beneficiary effect of *CQ*, aqueous extract of the *EO* alone can also effectively ameliorate the ovariectomy induced osteoporotic bone damage, and this effect is comparable to that of effect of *CQ*.

In our study, osteopenia in ovariectomized rats was evident with thinner cortical and trabecular bone, decreased biomechanical strength which was significantly lower than the control animals. These findings are consistent with previous studies [34,35]. Treatment with *CQ*, *EO* singly or in combination significantly reversed the bone degeneration and improved the strength of the bones. Further, these results were comparable with that of the standard drug Raloxifene.

Serum ALP a biomarker of bone formation, elevated in bone diseases such as osteoporosis due to increased bone turnover [36]. However, further elevation in serum levels of ALP in *CQ*, *EO*, *CQ+EO* and Raloxifene treated group indicates their positive effect on active bone formation. On the other hand, serum TRAP, a marker of bone resorption significantly decreased in treated groups compared OVX group. Thus, these results clearly shows that *CQ* and *EO* extracts singly or in combination effectively prevent the bone resorption and facilitates the new bone formation in the absence of estrogen. There were no significant changes were observed in the calcium levels indicate that serum of calcium levels were maintained by the homeostatic mechanisms despite having alterations in the estrogen levels. Further, decreased levels of hydroxyproline, a marker for collagen degradation in the treated groups compared to OVX animals indicate that *CQ*, *EO* can inhibits the bone resorption activity. Earlier, reports have been demonstrated that ovariectomy induces increase in the thickness of the growth plate [37,38]. In our study, no significant change in the thickness of growth plate was observed in OVX rats. Further, treatments did not affect the growth plate thickness in ovariectomized rats.

The ovariectomy induced bone loss, micro architectural lesion of bone tissue and greater susceptibility to fractures attributed to the effect of deficiency of estrogen on remodeling of bone [39-41]. The deficiency of estrogen also causes the reduction in osteoblast activity which will then automatically stimulate the osteoclast activity due to of the lack of its suppression by osteoblast in a paracrine way. The increased osteoclastic activity may also due to lack of inhibition of stimulating activity of parathormone on osteoclast by the estrogen [39]. Proinflammatory cytokines such as TNF-α, IL-1, IL-6, and IL- 11 are known stimulate the osteoclasts and subsequently increase the bone resorption [42,43-46]. These cytokine levels were found to be significantly decreased in *CQ* treated animals [16]. This could be one of the possible mechanism by which *CQ* ameliorated the bone loss.

Phytochemical analysis of *CQ* revealed the presence of flavonoids, triterpenoids [47-49] stilbene derivatives and resveratrol, piceatannol, pallidol perthenocissin [50-52] and phytosterols [53]. Further, the anabolic steroid isolated from *CQ* found to act on estrogen receptors of bone cells [54]. Further, an in vitro study by Boissy and his co-worker found that, the resveratrol inhibits the osteoclast proliferation and enhances the osteoblast differentiation in multiple myeloma [55]. Studies have also shown that *CQ* promotes the bone mineralization by accumulating mucopolysaccharides at the site of bone formation [56]. In animal studies, *CQ* is also found to increase calcium uptake and mechanical properties [57]. Therefore, the positive effect of the *CQ* observed in the present study could be also due to one or all of the above mechanisms.

It is postulated that elevated free radicals also implicated in the pathogenesis of osteoporosis [3,4]. Growing evidence confirmed the link between reactive oxygen species, estrogen deficiency, and bone loss. Estrogen deficiency increase the ROS levels and activates NF-κB which is said to one of the main signal involved in the osteoclast formation [58]. Further, estrogen deficiency also stimulates osteoblast apoptosis [59]. Lein et al. have demonstrated the reduced antioxidant defense linked with increase osteoclastic resorption, in the absence of estrogen [60]. Earlier, it has been shown that *EO* can induce the programmed cell death of human primary osteoclasts without affecting the process of osteoclastogenesis [61]. *EO* is shown to have antioxidant properties [62] and can modulate the basal oxidative markers and enhances the endogenous antioxidant defense [63]. Therefore, protective effect *EO* against the bone loss as seen in this study may be due its strong antioxidant properties and its direct effect on the inhibition of osteoclastic activity.

CONCLUSION

Though, previous studies have shown the anti-osteoporotic potential of *CQ* in the ovariectomized rat model, the efficacy of the *EO* singly or in combination with *CQ* has not been evaluated. Results of the preset study clearly depict the antiosteoporotic potential of *EO*. Further, the combined effect of *EO* and *CQ,* equivalent to the effects of individual plant extracts, these two may be used to treat the osteoporosis.

ACKNOWLEDGEMENT

We sincerely thank the Central Council for Research in Ayurvedic Sciences (CCRAS), department of AYUSH, Ministry of Health & Family Welfare, Government of India for their financial support for this study. We are thankful to Dr. Satyamoorthy, Director of Manipal Life Sciences Center, Manipal for all the support and facilities provided for the smooth conduction of these experiments.

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