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# Esculetin affects the microarchitecture of long bones *in vivo* and the alkaline phosphatase activity and bone nodule formation *in vitro*

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Osteoporosis is a condition of deterioration of bone quality and quantity, making it susceptible to fractures. The current therapy, with its associated risks and also approval for only short period makes the search for better molecules on priority imperative for effective treatment. Esculetin, a phytoconstituent, known to inhibit osteoclast differentiation *in vitro* and increase bone density *in vivo*, could serve to cure osteoporosis. Here, we studied the effect of esculetin on the microarchitecture of long bones in the ovariectomized rat model. The cellular cause for *in vivo* effect was sought by studying its effect on the pre-osteoblastic cell line. Osteopenia was induced by bilateral ovariectomy. Esculetin @1.0 or 10 mg/kg was administered for three months to osteopenic rats. Micro-CT of tibias and femurs was performed. The effect of esculetin on pre-osteoblastic MC3T3-E1 cells was studied. Cell viability in the presence of esculetin was determined by MTT assay. Alkaline phosphatase activity was determined using p-nitrophenol as a substrate. Bone nodules were stained with Alizarin Red S. Esculetin treated groups showed a significant worsening of microarchitecture parameters in the trabecular and cortical regions of the femur and tibia. This deterioration was more evident in the trabecular region than the cortical region. Esculetin augmented bone loss in estrogen-deficient rats without affecting the cell viability of MC3T3-E1 cells up to a concentration of 10 µg/mL. However, it affected the alkaline phosphatase activity and mineralization effect in preosteoblastic cells in addition to its *in vivo* effect in ovariectomized rats.

**Keywords:** Bone imaging, Coumarin, Osteoporosis Ovariectomy, Trabecular

Osteoporosis is a condition of decreased bone mass, which increases the susceptibility to fractures. At present, the drugs are used to decrease bone resorption and/or increase bone formation. As the current therapy is associated with risks and approved for a short period only, the need for newer better molecules for the treatment of osteoporosis is imperative. An ideal anti-osteoporotic agent is not yet discovered, therefore as of now, it is managed by changes in lifestyle along with medicines[1]. Coumarin derivative, Osthole, protects against bone loss in ovariectomized rats[2,3]. Osthole increases osteoblastic activity in human osteoblast-like cell line[4], while imperatorin and bergapten are effective in fetal rat calvariae cells[5]. High glucose levels causes a decrease in osteoclastogenesis and in osteoblastogenesis *in vitro*. This was reversed in the presence of coumarin[6].

Esculetin, a coumarin, is present in plants, such as *Cichorium intybus*, *Fraxini cortex*, *Mori folium*, *Solanum xanthocarpum* Schrad & Wendl, *Artemisiae capillaris herba*[7-10]. On analysing esculetin content of 35 medicinal plants, it was found that *Fraxini cortex* contained the highest concentration of esculetin the mean of which was 3243.8 mg/kg followed by *Mori folium* and *Artemisiae capillaris* containing an average 14.7 and 13 mg/kg, respectively of esculetin[8]. In the uterotrophic assay, the esculetin-treated OVx group showed uterine weight (mg/100 g body wt.) less than the vehicle-treated OVx group[11]. It inhibits RANKL induced osteoclast differentiation. Co-culture of osteoclasts with primary osteoblast preserves the F-actin ring formation with no significant change in resorption pits on the hydroxyapatite plate, though the various transcription factors needed for osteoclast

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*Abbreviations*: B.Ar /T.Ar, Bone area/Total area; BMD, Bone Mineral density; BV/TV, Bone volume/Total volume; Conn.Den, Connective density; Cs.Th, Cross-sectional thickness; DA, Degree of anisotropy; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; MMI, Moment of inertia; OVx, Ovariectomy; RANKL, Receptor activator of nuclear factor kappa-B ligand; TMD, Tissue mineral density; Tb.N, Trabecular number; Tb.Sp, Trabecular separation; and Tb.Th, Trabecular thickness

activity is reduced[12]. It has a bone anabolic effect on zebrafish larvae[13]. Esculetin, when administered at a dose of 384 mg/kg by oral gavage, 6 days per week for three months post-operatively increased the femoral bone mineral density[14]. Esculetin has anti-inflammatory activity[15] and hepatoprotective[16]. It inhibits adipogenesis and increases antioxidant activity[17]. The effect of esculetin on the micro-architecture of bones has not been studied. Micro-CT is the gold standard for *ex vivo* bone analysis[18,19]. As a small amount of esculetin is present in these plants, and when these plants are consumed as part of traditional medicine or as a coffee substitute or part of blended coffee, it reaches the systemic circulation. Therefore, it becomes vital to evaluate the effect of low doses of esculetin on various activities. Concentrations of esculetin 50 and 100 mg/kg cause an increase in uterotropic effect in immature mice[11]. The concentration of esculetin not affecting uterine proliferation would be preferred. In the present study, we evaluated the effect of 1.0 and 10 mg/kg esculetin on the microarchitecture of the bones in the ovariectomized rat model and studied its effect on the preosteoblastic cell line.

## Materials and Methods

Esculetin was purchased from Sigma Aldrich. Commercial oestradiol valerate, the active ingredient of which is 17β-oestradiol (E2) (Progynova®) of Bayer Zydus Pharma Pvt Ltd was purchased, powdered, and given as a suspension intragastrically by oral (*p.o.*) gavage. MC3T3-E1 was cultured in complete media supplemented with penicillin and streptomycin solution. α-MEM and FBS were obtained from Gibco; β-glycerophosphate, ascorbic acid, p-nitrophenylphosphate and 2 amino-2-methyl-1-propranol were obtained from Sigma Aldrich.

### *In vivo* study

The protocol was approved by the Institutional Animal Ethics Committee, Institute of Chemical Technology, Mumbai. The approval number is ICT/IAEC/2013/P16. Twenty female Wistar rats, 3 months old, were acclimatized for one week. After acclimatization, ovariectomy (OVx) was performed for four groups and sham surgery was performed for one group[20]. The rats were allotted randomly equally into Sham+vehicle, OVx+vehicle, OVx+1.0 mg/kg esculetin, OVx+10 mg/kg esculetin, and OVx+E2 groups. After 12 weeks of OVx, two groups of OVx rats were administered either esculetin 1.0 or 10 mg/kg daily orally, E2 group was administered as oestradiol 0.4 mg/kg *p.o.* daily; other groups were administered vehicle (0.2% sodium CMC) *p.o*. On termination of the study, the bones (8 femurs and 8 tibias per group) were collected.

The utility of micro-CT in osteoporotic bone has been established[21]. Micro computed tomography (Micro-CT) of excised bones was carried out using the Sky Scan 1076 CT scanner (Aartselaar, Belgium) at CDRI[22]. Three bones were scanned at a time at a nominal resolution (pixels) of 18 μm and the X-ray source was at 70kV. Reconstruction was done with the help of Sky Scan Nrecon software. One hundred projections were developed over 180°. The trabecular bone's volume of interest was extracted and analyzed by the CT analyzer software. The bone fraction i.e. bone volume/total volume (BV/TV), Trabecular Number (Tb.N), Trabecular Separation (Tb.Sp), Connectivity Density (Conn.Den), Trabecular Thickness (Tb.Th) of the femoral epiphysis and proximal tibial metaphysis was calculated. The cortical bone's volume of interest was extracted and analyzed by the software. For the cortical bones Bone Area/Total Area (B.Ar/T.Ar), Mean Polar Moment of Inertia (MMI), Cross-sectional Thickness (Cs.Th) were measured at the diaphysis of the femur and tibia[22,23].

### *In vitro* study

The cell culture study was carried out at NCCS. Pre-osteoblastic cells MC3T3-E1 were cultured in complete α-MEM media supplemented with penicillin and streptomycin solution. The following parameters were determined using various concentrations of esculetin. The cell viability was determined via MTT assay[24].

#### *ALP activity and Bone nodule formation*

Cultured MC3T3-E1 cells reached confluence on the third day. Thereafter, 10 mM β-glycerophosphate and 50 μg/mL of ascorbic acid were added to all wells except for the control without factors. Cells were treated with various concentrations of esculetin every third day from 3rd day onwards. ALP activity was determined on the 7th day and bone nodule formation on the 21st day. ALP activity was determined using p-nitrophenylphosphate[25]. The bone nodules were stained with Alizarin Red S. They were then solubilised in acetic acid followed by neutralization and the optical density was read at 405nm[26,27].

**Statistical analysis**

Statistical analysis was done using one-way ANOVA followed by Tukey's Multiple Comparison Test.

## Results

***In vivo* study**

*Micro-CT of trabecular bone parameters of femur and tibia*

Esculetin treated groups showed a greater deterioration of BV/TV, Tb.N, Tb.Sp, Conn.Den and BMD compared to OVx+vehicle group in a dose-dependent manner. The deterioration was significantly more in the OVx+10 mg/kg group (Tables 1 & 2). A similar effect can be viewed in the 3D model (Fig. 1).

*Micro-CT of cortical bone parameters of femur and tibia*

There was no significant change in B.Ar/T.Ar of the femur and tibia amongst the rats of all the groups. There was a substantial increase in the MMI for OVx+vehicle and OVx+10 mg/kg compared to Sham+vehicle in the case of femur and tibia. There was a significant decrease in Cs.Th in the femur of rats of the OVx+10 mg/kg

Table 1 — Micro-CT bone parameters for femur

| Micro-CT parameters (units) | | Femur | | | | |
|---|---|---|---|---|---|---|
| | | Sham+Vehicle | OVx+Vehicle | OVx+1.0 mg/kg | OVx+10 mg/kg | OVx+E2 |
| Trabecular parameters | BV/TV (%) | 26.68±1.63 | 17.09±1.25* | 12.68±0.91* | 10.83±0.85*$^{\#}$ | 15.58±1.25* |
| | Tb.N (per mm) | 3.02±0.20 | 1.76±0.12* | 1.49±0.09* | 1.16±0.12*$^{\#@}$ | 1.85±0.13* |
| | Tb.Sp(mm) | 0.27±0.03 | 0.52±0.05* | 0.8±0.02*$^{\#@}$ | 0.76±0.05*$^{\#@}$ | 0.56±0.06* |
| | Conn. Den ($mm^{-3}$) | 101.03±11.36 | 46.1±3.35* | 34.67±3.9* | 22.73±3.72* | 47.46±3.93* |
| | Tb.Th (mm) | 0.082±0.000 | 0.088±0.002* | 0.085±0.002* | 0.094±0.004 *$^{\#\$@}$ | 0.084±0.002* |
| | BMD | 0.311±0.026 | 0.218±0.015* | 0.149±0.017* | 0.129±0.013*$^{\#}$ | 0.213±0.028* |
| Cortical parameter | B.Ar/T.Ar | 0.994±0.0007 | 0.9947±0.0003 | 0.994±0.0006 | 0.9946±0.0003 | 0.993±0.0007 |
| | MMI ($mm^4$) | 8.65±0.378 | 12.40±1.28* | 11.02±0.63 | 10.57±0.53 | 11.32±0.4 |
| | Cs.Th(mm) | 0.567±0.014 | 0.554±0.009 | 0.527±0.015 | 0.498±0.007*$^{\#}$ | 0.524±0.014 |
| | TMD | 1.505±0.022 | 1.445±0.028 | 1.440±0.034 | 1.418±0.006 | 1.427±0.018 |

[Sham operation was performed for one group. For all other groups, ovariectomy was done. Different groups of rats were given vehicle or 1mg/kg or 10mg/kg esculetin *p.o.* for 3 months. Values are Mean±SEM(n=4). SEM=Standard Error of Mean. Statistical analysis was done via ANOVA followed by Tukey's multiple comparison test. Statistical significance * $P$ <0.05 when various groups were compared to Sham+vehicle group; # $P$ <0.05 when various groups were compared to OVx+vehicle group; @ $P$ <0.05 when various groups were compared to E2+vehicle group; $ $P$ <0.05 when various groups were compared to OVx+1.0 mg/kg]

Table 2 — Micro-CT bone parameters for tibia

| Micro-CT parameters (units) | | Tibia | | | | |
|---|---|---|---|---|---|---|
| | | Sham+Vehicle | OVx+Vehicle | OVx+1.0 mg/kg | OVx+10 mg/kg | OVx+E2 |
| Trabecular parameters | BV/TV (%) | 29.27±0.97 | 18.71±1.47* | 14.55±1.22* | 12.33±0.74*$^{\#}$ | 16.71± 0.97* |
| | Tb.N (per mm) | 3.32±0.10 | 1.74±0.10* | 1.57±0.14* | 1.22±0.07*$^{\#@}$ | 1.90±0.07* |
| | Tb.Sp (mm) | 0.22±0.01 | 0.46±0.05* | 0.54±0.05* | 0.63±0.04*$^{\#@}$ | 0.38±0.02* |
| | Conn. Den ($mm^{-3}$) | 87.32±5.57 | 30.92±2.29 | 25.16±3.03* | 16.42±2.11*$^{\#@}$ | 34.30±1.53 |
| | Tb.Th (mm) | 0.088±0.001 | 0.109±0.01* | 0.093±0.003 | 0.101±0.002 | 0.088±0.002$^{\#}$ |
| | BMD | 0.404±0.025 | 0.315±0.035 | 0.217±0.027* | 0.193±0.005*$^{\#}$ | 0.271±0.025* |
| Cortical parameters | B.Ar/T.Ar | 0.968±0.002 | 0.970±0.002 | 0.964±0.003 | 0.967±0.002 | 0.970±0.002 |
| | MMI ($mm^4$) | 3.865±0.12 | 5.290±0.539* | 4.245±0.214 | 5.233±0.324* | 4.889± 0.240* |
| | Cs.Th(mm) | 0.429±0.016 | 0.434± 0.005 | 0.395±0.011 | 0.396± 0.006 | 0.420±0.014 |
| | TMD | 1.387±0.040 | 1.364±0.008 | 1.324± 0.018 | 1.333±0.020 | 1.353±0.023 |

[Sham operation was performed for one group. For all other groups, ovariectomy was done. Different groups of rats were given vehicle or 1.0 or 10 mg/kg esculetin *p.o.* for 3 months. Values are Mean±SEM(n=4). SEM=Standard Error of Mean. Statistical analysis was done via ANOVA followed by Tukey's multiple comparison test. Statistical significance * $P$ < 0.05 when various groups were compared to Sham+vehicle group; # $P$ <0.05 when various groups were compared to OVx+vehicle group; @ $P$ <0.05 when various groups were compared to E2+vehicle group]

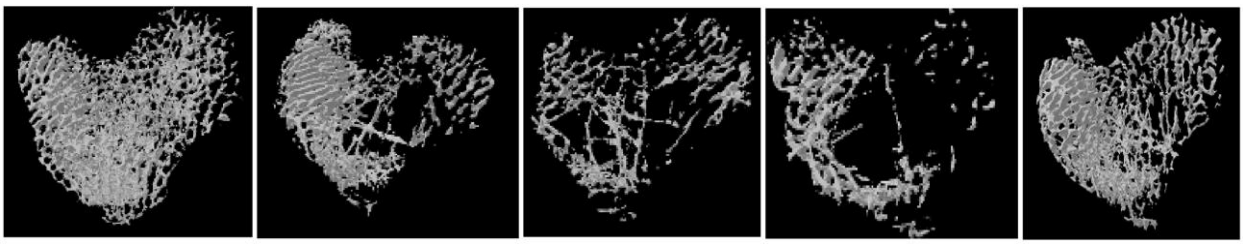

Fig. 1 — 3D model of tibial metaphysis. [Left to Right: Sham+Vehicle, OVx+Vehicle, OVx+1.0 mg/kg, OVx+10 mg/kg and OVx+E2. Model was prepared on Sky Scan 1076 CT scanner]

esculetin group compared to the Sham+vehicle and the OVx+vehicle group.

***In vitro* study**

*Cell viability*

The $IC_{50}$ was 65.61 µg/mL. The cell viability was almost similar to control for esculetin up to 10 µg/mL (Fig. 2).

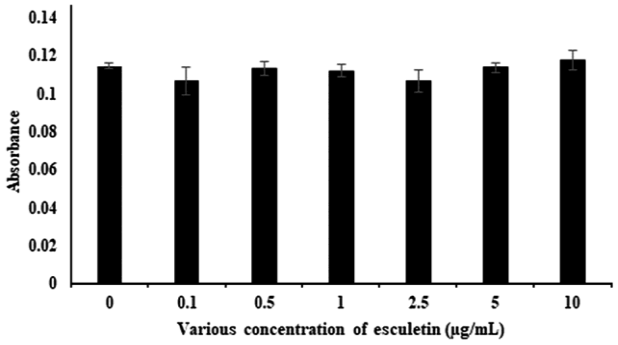


Fig. 2 — Cell viability study. [MC3T3-E1 cells were cultured in complete media supplemented with penicillin and streptomycin solution. [Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for various concentrations of esculetin. . Values are expressed as Mean±SEM(n=4). SEM=Standard Error of Mean. Statistical analysis was done via ANOVA followed by Tukey's multiple comparison test. There was no statistical significance amongst the groups]

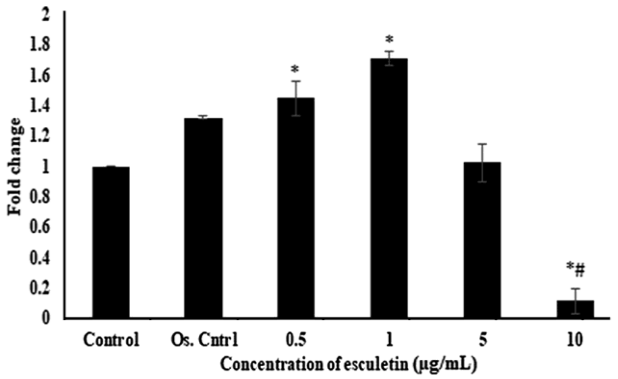


Fig. 3 — ALP activity study. [MC3T3-E1 cells were cultured in complete media supplemented with penicillin and streptomycin solution. [The control group is the group to which neither osteogenic factors nor esculetin was added. Os. Cntrl is osteogenic control to which osteogenic factors (Beta Glycerophosphate and ascorbic acid) were added, however no esculetin was added. All other groups contain osteogenic factors (Beta Glycerophosphate and ascorbic acid) and 0.5 or 1 or 5 or 10 µg/mL of esculetin. ALP activity was determined on the 7$^{th}$ day using p-nitrophenylphosphate as substrate and measured calorimetrically at 405 nm. Statistical analysis was done via ANOVA followed by Tukey's multiple comparison test. Values show a trend seen in three independent experiments. They are expressed as Mean±SEM(n=2). SEM=Standrd Error of Mean. Statistical significance * $P$ <0.05 when various groups were compared to Control group; $^{\#}$ $P$ <0.05 when various groups were compared to Os. Cntrl group]

*ALP activity*

Esculetin @0.5 and 1.0 µg/mL increased the ALP compared to osteogenic control, though not significantly. However, at 5 and 10 µg/mL it decreased the ALP activity in a dose-dependent way (Fig. 3).

*Alizarin Red S*

There was a significant decrease in mineral deposited for 5 µg/mL and 10 µg/mL dose when compared to osteogenic control (Fig. 4).

## Discussion

Esculetin inhibits RANKL stimulated osteoclasts differentiation of bone marrow derived macrophages (BMM)[12]. It increases the femoral BMD in ovariectomized rats[14], esculetin has various other effects i.e. anti-inflammatory, anti-adipogenesis, anti-oxidant[15,17]. These effects pose esculetin, a dihydroxy coumarin, as a potential anti-osteoporotic agent. The effect of esculetin on microarchitecture has not been evaluated. Micro-CT is the gold standard for *ex-vivo* analysis of bone morphology[18,19]. We evaluated the effect of esculetin on the microarchitecture in the

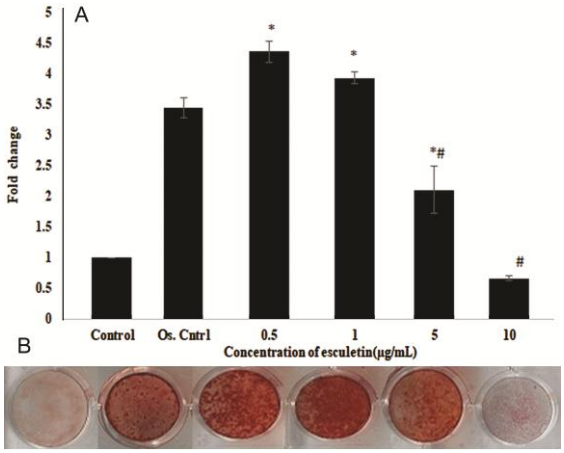


Fig. 4 — (A) Mineralization assay; and (B) Bone nodule formation. [MC3T3-E1 cells were cultured in complete media supplemented with penicillin and streptomycin solution. The Control group is the group to which neither osteogenic factors nor esculetin was added. Os. Cntrl is osteogenic control to which osteogenic factors (Beta Glycerophosphate and ascorbic acid) was added, however no esculetin was added. All other groups contain osteogenic factors (Beta Glycerophosphate and ascorbic acid) and 0.5 or 1 or 5 or 10 µg/mL of esculetin. The mineralized nodules formed were stained on the 21$^{st}$ day with Alizarin Red S. The bone nodules stained with Alizarin Red S were solubilised in acetic acid, then neutralized and the optical density was read at 405 nm. Statistical analysis was done via ANOVA followed by Tukey's multiple comparison test. Values show a trend seen in three independent experiments. They are expressed as Mean±SEM (n=2). SEM, Standrd Error of Mean. Statistical significance * $P$ <0.05 when various groups were compared to Control group; $^{\#}$ $P$ <0.05 when various groups were compared to Os. Cntrl group]

ovariectomized rat model. The OVx rat model, similar to humans, exhibits more cancellous bone loss than cortical bone loss[28]. OVx causes loss of estrogen's protective effect; leading to increase turnover, increase resorption and increase susceptibility to fractures. This was observed via micro-CT. E2 could not restore the bone loss that had already taken place. This is in agreement with previous findings[29,30]. Nevertheless, E2 did not cause any further significant worsening in bone architecture. OVx rats treated with vehicle/E2/esculetin, significantly decreased bone fraction (BV/TV), Tb.N, Conn.Den and BMD compared to Sham+vehicle with a further significant difference in BV/TV, Tb.N, BMD for OVx+10 mg/kg esculetin in the long bones - femur and tibia. This means in the OVx+10 mg/kg esculetin group, the long bones were less able to bear the load and transmit the stress compared to the OVx+vehicle group. There was also a significant increase in Tb.Sp for OVx rats treated with vehicle/E2/esculetin compared to Sham+vehicle. The Tb.Sp increased significantly in the femur of OVx+1.0 mg/kg esculetin rats compared to OVx+vehicle and OVx+E2. This was significantly more for OVx+10 mg/kg esculetin compared to OVx+vehicle and OVx+E2 in femur and tibia. As the Tb.Sp increased this implies the bone's porosity had increased. The more porous the bones, the greater would be its fragility. The Tb.Th increased significantly in the OVx+10 mg/kg esculetin group compared to all the other groups for the femur. The OVx+E2 group had a decrease in Tb.Th compared to OVx+vehicle for the tibia. Also, the tibial Tb.Th of OVx+vehicle was significantly more compared to Sham+vehicle. The thicker the trabeculae, the more would be its load-bearing ability. An increase in Tb.Th could be a homeostatic mechanism by the body to retain bone functionality. As the femurs of rats belonging to the OVx+10 mg/kg esculetin group showed a significant worsening in the micro-architecture, it could be the body's attempt to conserve bone strength by increased trabecular thickness. With the tibia of the OVx+E2 group, perhaps, the deterioration was not sufficient to trigger the homeostatic mechanism, therefore the tibial Tb.Th was similar to Sham+vehicle and less compared to OVx+vehicle. The effects of esculetin on cortical bones were not much, though an increase in the resistance to change was seen by an increase in MMI in the tibia of the OVx+vehicle and OVx+10 mg/kg. There was a significant decrease in femoral Cs.Th. Thus, the cortical bone would also be more prone to fractures. Esculetin @ 1.0 and 10 mg/kg showed dose-dependent pro-osteoporotic effect in the long bones of OVx rats, and also augmented the effects of bone loss in bones due to estrogen deficiency.

Meijie *et al*.[14] observed an increase in femoral bone density, when 384 mg/kg esculetin was administered per day 6 days a week for 3 months post-induction of osteopenia. Whereas, we observed a deterioration of the micro-CT parameters with 1.0 and 10 mg/kg esculetin administered 7 days a week for 3 months. It could be at a higher dose the effect is mainly anti-osteoclastic whereas at a lower dose the effect is anti-osteoblastic. There could be a particular threshold, higher than it esculetin exerts an anti-osteoclastic effect and there could be a ceiling effect for the anti-osteoblastic effect. The measurement method was also different. We used micro-CT which is the standard for measuring bone microarchitecture. The vehicle used in our study was a suspension of esculetin in 0.2% sodium carboxymethyl cellulose. The authors did not specified the vehicle used[14]. This could account for the difference in the absorption and thus the response. Also, esculetin was not administered every day of the week[14]. In our study, we administered esculetin daily for three months without a drug-free period. This may affect the sensitivity of esculetin at the receptor level.

As the effect of esculetin on the osteoblastic cell line, MC3T3-E1 was not investigated, we studied its effect on ALP and mineralization of MC3T3-E1 cell line. At 5 and 10 μg/mL, esculetin reduced ALP activity and mineralization significantly (Figs 3 and 4). Concentrations higher than 10 μg/mL decreased the viability of MC3-T3 cells, whereas low concentrations of 5 and 10 μg /mL reduced the functions of osteoblastic cells i.e ALP activity and bone nodule formation. Therefore, the benefit of decreased resorption would be lost and would result in bone loss due to decreased bone formation. This was the effect we observed *in vivo*.

## Conclusion

We observed esculetin showed a pro-osteoporotic effect in the bilateral ovariectomized rat model at doses of 1.0 and 10 mg/kg body wt. There was a worsening of the microarchitecture of the trabecular bone of the femur and tibia. The deterioration in bone quality and quantity, as seen by the deterioration of the micro-CT parameters, would lead to an increase in

the susceptibility to cause fractures. This effect could be attributed to the osteoblast formation and function. *Cichorium intybus* contains esculetin, thus drinking blended coffee or coffee substitute could be one of the causes of osteoporosis. Also, caution needs to be exercised while consuming plants or food/beverages/herbal medicines containing esculetin.

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## Conflict of interest

Authors declare no competing interests.

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