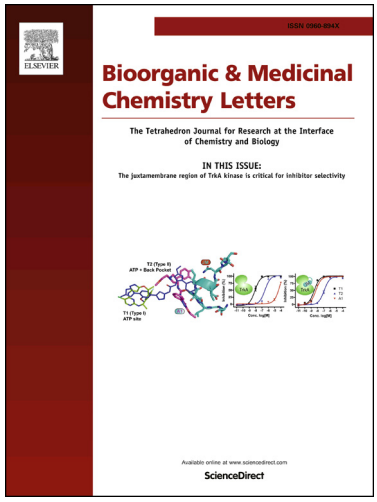
Accepted Manuscript



Design and synthesis of dalbergin analogues and evaluation of anti-osteoporotic activity

Padam Kumar, Priyanka Kushwaha, Naseer Ahmad, Saransh Wales Maurya, Kapil Dev, Vikram Khedgikar, Ibadur Rahman Siddiqui, Ritu Trivedi, Rakesh Maurya

PII: S0960-894X(17)30205-6
DOI: http://dx.doi.org/10.1016/j.bmcl.2017.02.062
Reference: BMCL 24734

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 29 October 2016
Revised Date: 8 February 2017
Accepted Date: 24 February 2017

Please cite this article as: Kumar, P., Kushwaha, P., Ahmad, N., Maurya, S.W., Dev, K., Khedgikar, V., Siddiqui, I.R., Trivedi, R., Maurya, R., Design and synthesis of dalbergin analogues and evaluation of anti-osteoporotic activity, *Bioorganic & Medicinal Chemistry Letters* (2017), doi: http://dx.doi.org/10.1016/j.bmcl.2017.02.062

Bioorganic & Medicinal Chemistry Letters
journal homepage: www.elsevier.com

Design and synthesis of dalbergin analogues and evaluation of anti-osteoporotic activity

Padam Kumar[a,#], Priyanka Kushwaha[b,c,#], Naseer Ahmad[c], Saransh Wales Maurya[a,d], Kapil Dev[a,b], Vikram Khedgikar[c], Ibadur Rahman Siddiqui[d], Ritu Trivedi[c,*] and Rakesh Maurya [a,b,*]

[a]*Medicinal and Process Chemistry Division, CSIR- Central Drug Research Institute, Lucknow 226031, India*
[b]Academy of Scientific and Innovative Research, *CSIR- Central Drug Research Institute, Lucknow 226031, India*
[c]*Endocrinology Division, CSIR-Central Drug Research Institute, Lucknow 226031, India*
[d]*Department of Chemistry, University of Allahabad, Allahabad 211002, India*

ARTICLE INFO

Article history:
Received
Revised
Accepted
Available online

Keywords:
Dalbergin
Aminodalbergin
Ritter Reaction
Post-menopausal osteoporosis
Micro-CT

ABSTRACT

The chemical modifications of the hydroxyl group of dalbergin have been described via the introduction of cyclic amine, ester and amide groups. Among the twenty three prepared novel analogues of dalbergin, compound **4d** (EC_{50} 2.3 µM) showed significantly increased proliferation as assessed by alkaline phosphatase activity and mineralization in calvarial osteoblast cells *in vitro*. Compound **4d**, at a dose of 1.0 mg/kg body weight exhibited the significant osteoprotective effect. It showed a significant increase in osteogenic gene expression RunX2 (~4fold), ALP (~5fold), OCN (~4fold) and COL1 (~4fold) as compared to control group at the same dose *in vivo* assay.

Osteoporosis, a progressive skeletal disease characterized by decreased bone mass with micro-architectural deterioration, which leads to the risk of fractures in both men and women.[1] Clinically, it is called as a silent disease because it progresses without pain and symptoms until the bone fracture occurs. A variety of factors are responsible for osteoporosis e.g. level of sex hormones, calcium supplements, dietary supplements, lifestyle, medication and aging. During bone remodeling, osteoblasts fail to completely refill the resorption pits[2] and hence, cause osteoporosis. Women lose about 50% of their cancellous bone and about 35% of cortical bone over their life span.[3] Estrogen is effective in inhibiting bone resorption and increasing bone mineral density (BMD) by binding to the estrogen receptors on bone and blocking production of specific cytokines that are responsible for increasing the number of osteoclasts and prolong osteoblasts lifespan.[4] Currently available drugs used to treat osteoporosis are very few such as estrogen receptor modulators, calcitonin and bisphosphates, and their effects on increasing or recovering bone mass are relatively small, generally less than 10% over three years. The use of these drugs is associated with several severe side effects such as breast cancer, cardiovascular disease and uterine bleeding.[5,6]

Therefore, there is an urgent need to develop new safe and alternative bone-building agents which can stimulate bone formation and reduce the imbalance between osteoclast and osteoblast cells in osteoporosis.

*Corresponding author.
Dr. Rakesh Maurya: mauryarakesh@rediffmail.com
Dr Ritu Trivedi: ritu_trivedi@cdri.res.in
[#]Authors contributed equally
Tel.: +91-(522)-2772450; Fax: +91-(522)-2771941;
CDRI Communication number

Neoflavonoid is a group of naturally occurring compounds of fifteen carbons and having a 4-phenylchroman type of skeleton. These are structurally and biosynthetically related to flavonoids and isoflavonoids.[7] There are a number of publications available which show the broad spectrum of activities of neoflavonoids such as antimicrobial,[8] antiplasmodial,[9] osteogenic,[10] antifungal,[11] anti-inflammatory,[12] antidiabetic,[13] anticancer[14] etc. The fundamental neoflavonoid dalbergin, has attracted several research groups for chemical modifications due to its enormous biological activities like anti mycobacterium, [15] antidiabetic, [16] and cardiovascular. [17] and cytotoxicity[18]. Due to such interesting biological activities and development of several new synthetic methodologies, make it easy to synthesize derivatives of such precious natural products in good yield. Therefore, herein, we were keenly interested in exploring the chemical modifications of dalbergin and the biological evaluation of synthesized analogues for their osteogenic activity.

Recently, we have reported anti-osteoporotic activity of dalbergin, where it exhibited increased alkaline phosphatase activity and mineralization at a lower concentrations.[10] Thus, these results and also our ongoing research of interest in search of biologically active natural product derived compounds[19] prompted us to isolate dalbergin in large amounts for further chemical modifications for the management of osteoporosis.

Structurally, dalbergin (**1**) has a free hydroxyl group at position 6 which could provide a wide window to explore it for biological importance. Therefore, we designed our synthetic approach for the synthesis of derivatives of dalbergin via the chemical modifications of the hydroxyl group at position 6 (Schemes 1-3).

Initially, we synthesized compound **2** by heating a mixture of dalbergin (1.0 eq.) and 1-bromo-2-chloroethane (1.2

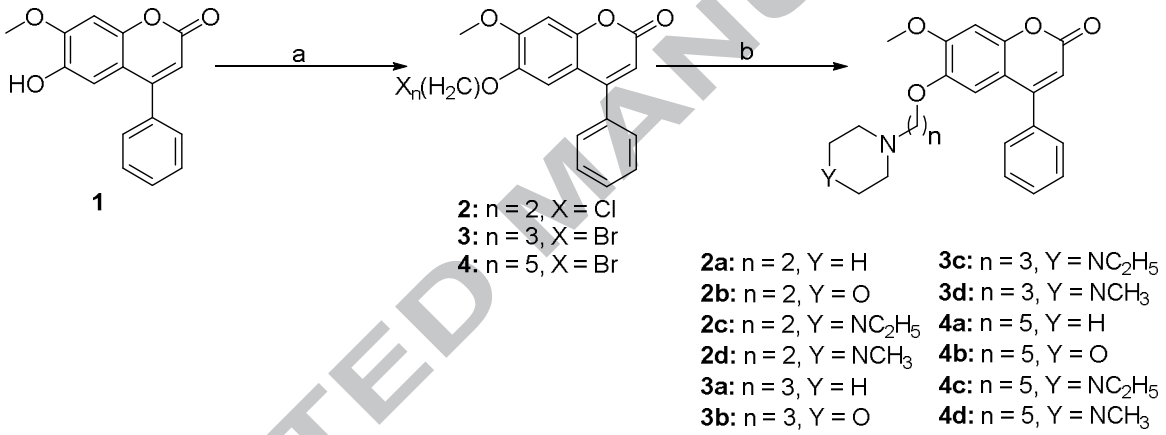
eq.) in presence of base K_2CO_3 (1.5 eq.) in acetone (40 mL). The compound **2** (1.0 eq.) was further refluxed with piperidine (1.1 eq.) in 10 mL ethanol in the presence of triethylamine for 24 h to get the piperidine derivative of dalbergin (**2a**).[20] Similar procedure was utilized to the synthesis of other nitrogenous derivatives (**2b**-**2d**) of dalbergin. Furthermore, to the evaluate the effect of linker chain on the osteogenic activity, we synthesized a series of compounds **3a**-**3d** and **4a**-**4d** containing cyclic amine group with extended aliphatic linker chain (Scheme 1).

As our approach relied on the chemical modification of hydroxyl group of dalbergin at position 6, hence we further synthesized the ester analogues from substituted carboxylic acids. The compound **1** was treated with different carboxylic acid chlorides (1.0 eq.), pyridine (0.4 mL) and DMAP (cat.) in dry CH_2Cl_2, and afforded their respective ester analogues **5a**-**5e** in 74-82% yield (Scheme 2).[21] Taking the consideration of the effect of aliphatic group at position 6, we methylated hydroxyl by the reaction of methyl iodide (2.0 eq.), K_2CO_3 (1.5 eq.) in dry acetone (20 mL), afforded methyl dalbergin **6** in 92% yield.

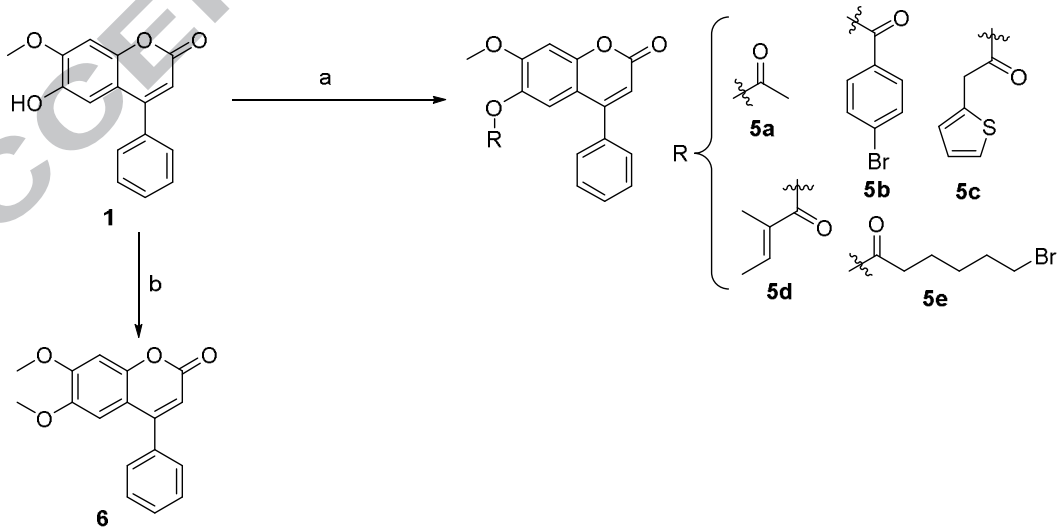
The hydroxyl group of dalbergin provides a wide prospect for the synthesis of the amide derivative of dalbergin via Ritter reaction (Scheme 3). In this consequence, we synthesized compound **7** by stirring the mixture of epichlorohydrin (2.0 eq.), K_2CO_3 (3.0 eq.) in dry acetone for 6h at 50°C.[22] Furthermore, to the synthesis of amide analogue of dalbergin, compound **7** was treated with *p*-methoxy benzonitrile (3.0 eq.) and the dropwise addition of $BF_3.OEt_2$ (0.5 eq.) at 0°C in dry CH_2Cl_2 and the compound **8** afforded as amide after the aqueous work up and chromatographic purification in 54% yield (Scheme 3).[23] All the synthesized compounds were characterized by extensive analysis of 1D and 2D NMR as well as mass spectral analysis and the purity of compounds was determined by HPLC analysis before biological evaluation.

All the synthesized compounds were evaluated for their alkaline phosphatase activity *in vitro*. For the measurement of alkaline phosphatase (ALP) enzyme activity (osteoblast differentiation marker), calvarial osteoblast cells were trypsinized and 2×10^3 cells were plated onto 96 well plates for ALP activity. Treated cells were incubated for 48h in α-MEM supplemented with 10% FBS, 10 mM β-glycerophosphate, 50 µg/mL ascorbic acid, and 1% penicillin/streptomycin (osteoblast differentiation medium). At the termination day, total ALP activity was measured

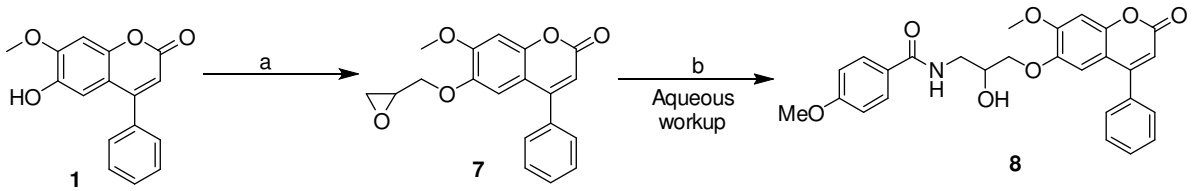
Using *p*-nitrophenyl phosphate (PNPP) as substrate and absorbance was read at 405 nm.[24-26]



Scheme 1: Synthesis of amine analogues of dalbergin at position 6 (**2**, **2a-2d**, **3**, **3a-3d**, **4** and **4a-4d**). Reagents and Conditions: (a) Dihalo alkane (1.2 eq.), K_2CO_3 (1.5 eq.), Acetone (40 mL), 4h. (b) Cyclic amine (1.1 eq.), triethylamine, ethanol (10 mL), reflux, 24 h.



Scheme 2: Synthesis of ester and methoxy derivatives of dalbergin at position 6 (**5a-5e** and **6**). Reagents and Conditions: (a) RCOCl (1.2 eq.), Pyridine (0.4 mL), DMAP (cat.), dry CH_2Cl_2, 0°C-rt, 6–8 h. (b) Methyl iodide (1.2 eq.), K_2CO_3 (1.5 eq.), Acetone (20 mL), 4 h.



Scheme 3: Synthesis of amide analogue (**8**) of dalbergin via Ritter reaction. Reagents and Conditions: (a) Epichlorohydrin (2.0 eq.), K_2CO_3 (3.0 eq.), Acetone (20 mL), 50°C, 6 h. (b) *p*-Methoxybenzonitrile (3.0 eq.), $BF_3.OEt_2$ (0.5 eq.), Dry CH_2Cl_2 (5 mL), 0°C-rt, 2 h.

Out of twenty three compounds, only fifteen compounds showed ALP activity at different concentration [Fig.1]. EC_{50} and E_{max} values were calculated on the basis of ALP activity (Table 1).

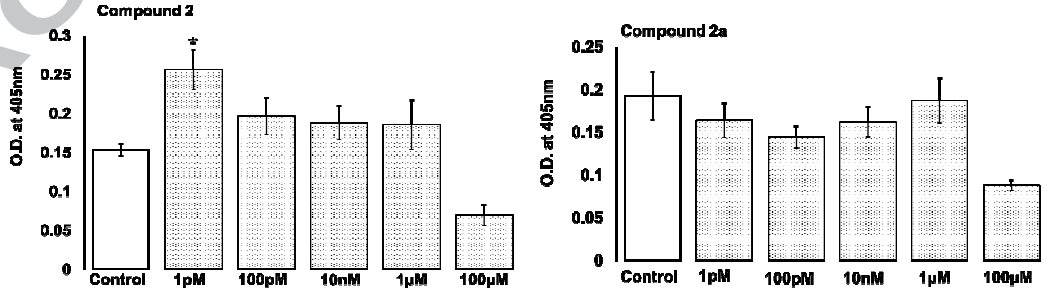
Table 1: E_{max} and EC_{50} of compounds for alkaline phosphatase activity:

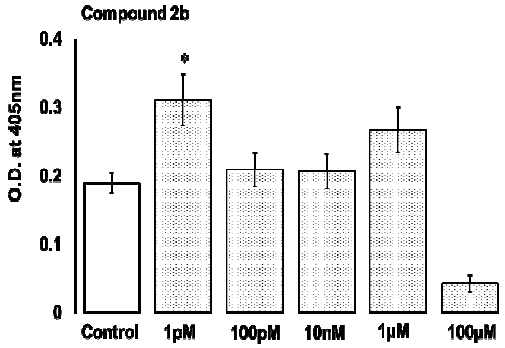
Compound	E_{max}	EC_{50}
2	1 pM	19.4 nM
2a	-	-
2b	1 pM	11.7 nM
2c	1 pM	13.6 nM
2d	1 pM	14.8 nM
3	100 pM	5.9 nM
3a	1 pM	7.6 nM
3b	-	-
3c	10 nM	27.9 nM
3d	100 pM	9.42 nM
4	10 nM	5.7 nM
4a	1 pM	10.8 nM
4b	10nM	0.9 µM
4c	-	12.4 µM
4d	1pM	2.3 µM
5a	-	-
5b	1µM	0.8nM
5c	10nM	7.4nM
5d	1µM	1.1nM
5e	-	-
6	-	-
7	-	-
8	-	-

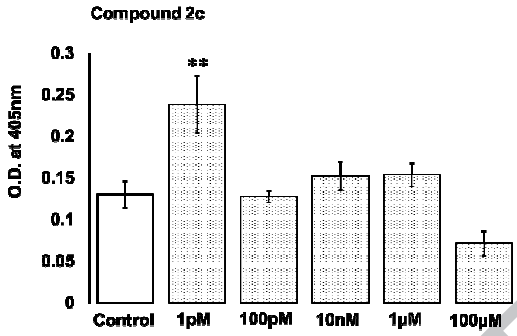
Compounds showing increased alkaline phosphatase were further tested for the ability to form mineralized nodule in mice calvarial osteoblast cells**.** Osteoblast cells were treated with or without active compounds for twelve days and further fixed in 4% formaldehyde. Fixed cells were stained with alizarin red-S stain, extracted and quantified by using acetic acid method.[27,28] Quantification of alizarin extraction showed that the compounds **2b**, **2c, 2d, 3, 3a, 4, 4a, 4b** and **4d** having calcium nodule formation activity [Fig. 2] but compound **2d, 3a, 4a** and **4d** showed maximum calcium deposition at active concentration.

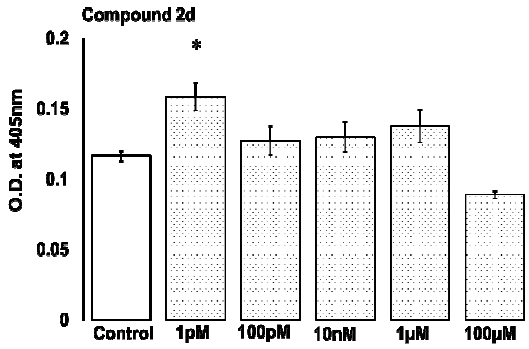
Active compounds **2d**, **3a, 4a** and **4d** were further tested for osteoblast differentiation and mineralization marker genes BMP2, RunX2 and COL1 at the active concentration in calvarial osteoblast cells by Q-PCR. BMP2 (Bone Morphogenetic Protein 2) is the potential inducer of osteoblast differentiation; RunX2 is transcription factor and a key regulator of osteoblast differentiation whereas COL1 is the key component during mineralization. Our results showed that after treatment with active concentration, compound **4d** showed maximum expression of BMP2 (~2fold), RunX2 (~2fold) and COL1 (~6fold) [Fig. 3].[29,30]

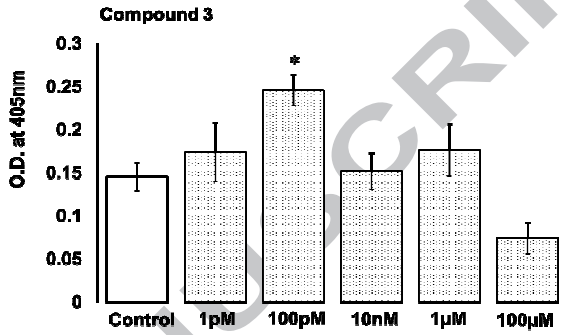
For ALP (alkaline phosphatase) activity, we tested dalbergin and its derivative compounds on calvarial osteoblast cells which are used to investigate the modulatory effects for osteogenic characteristic. Osteoblast cells serve as an important tool to study bone cell proliferation, differentiation and mineralization at the time of bone formation and maturation. The activity of ALP increases at the time of early differentiation and have distinguished characteristic of osteogenic cells in culture that possess the ability to promote bone matrix mineralization. During this phase the osteoblast cells have high levels of the membrane bound enzyme alkaline phosphatase that initiates pre-osteoblast to differentiate into osteoblast lineage. By measuring the ALP activity dalbergin and its derivatives were screened for their ability to induce osteogenesis. In-vitro studies showed that dalbergin and its derivatives such as compound 4d had cell differentiation, proliferation ability in osteoblast cells and compound **4d** showed significant enhancement in ALP activity compared with dalbergin as shown in figure 1.

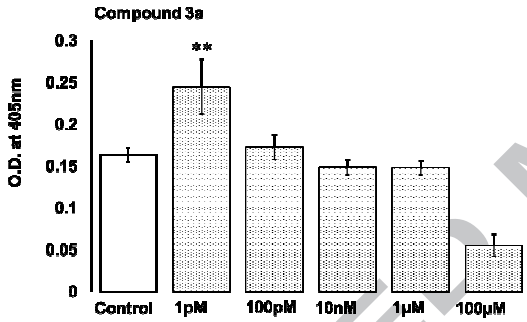


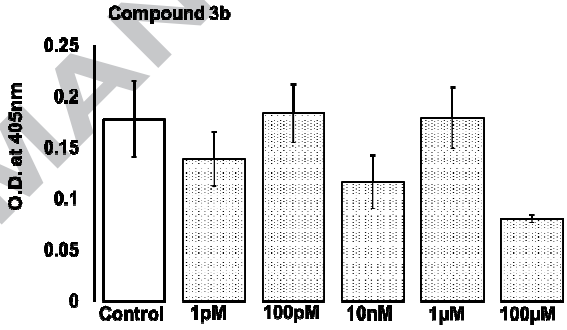


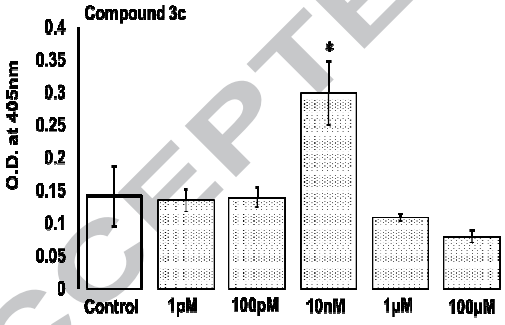


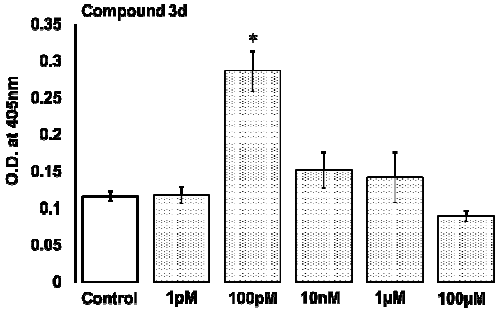


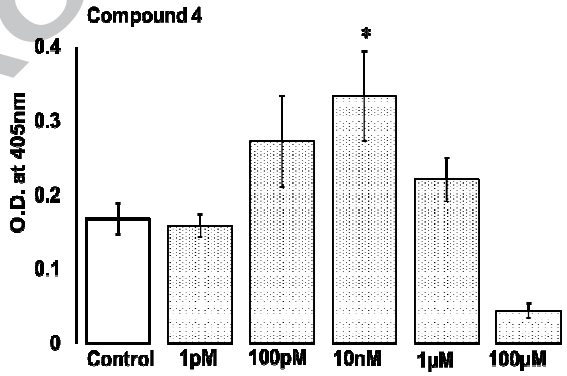


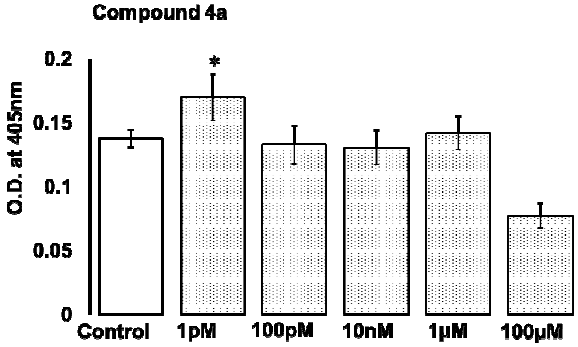


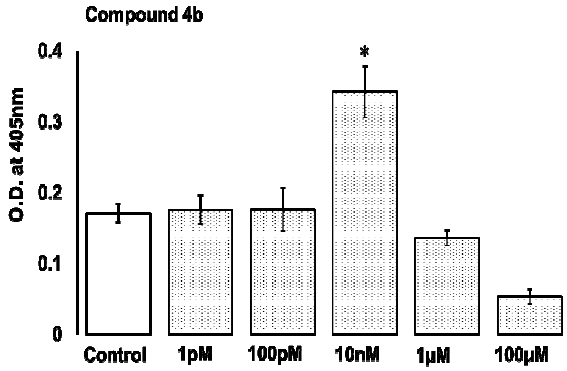


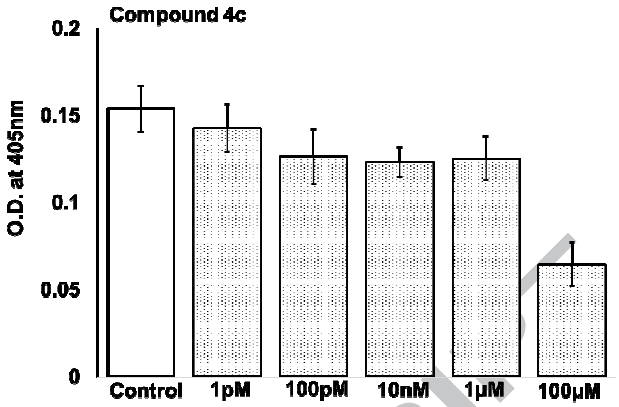


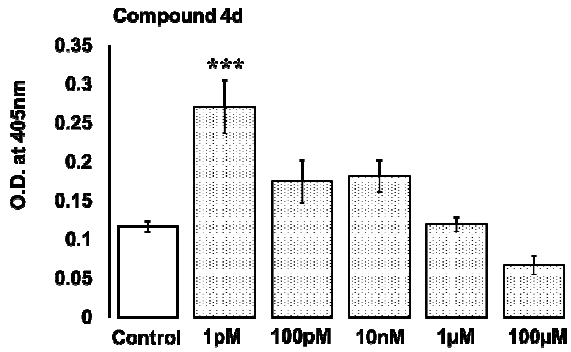


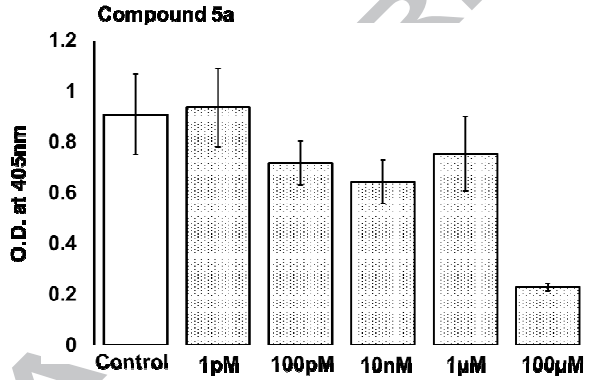


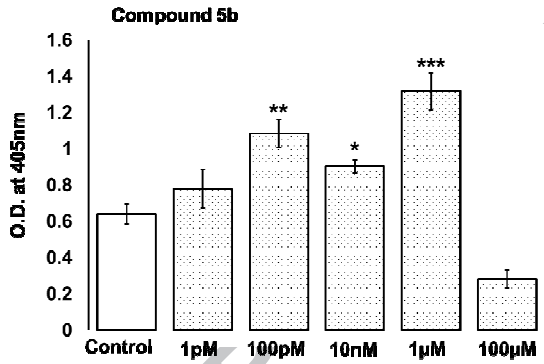


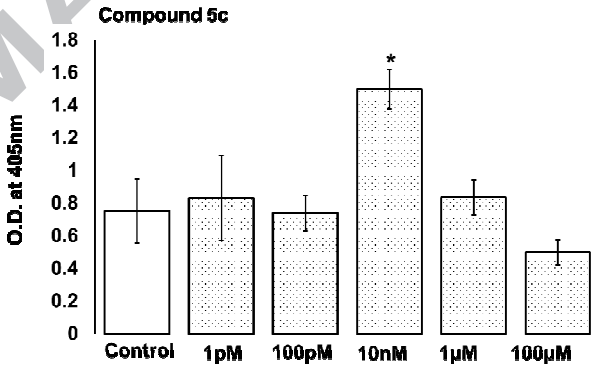


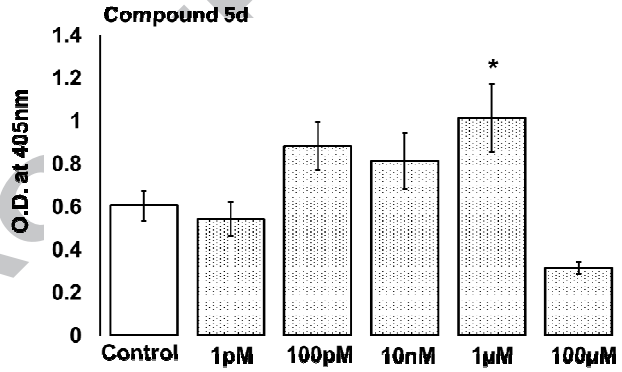


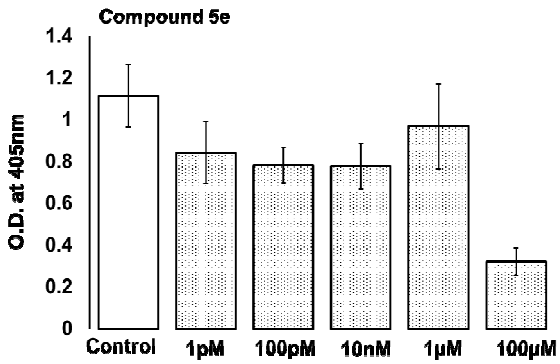












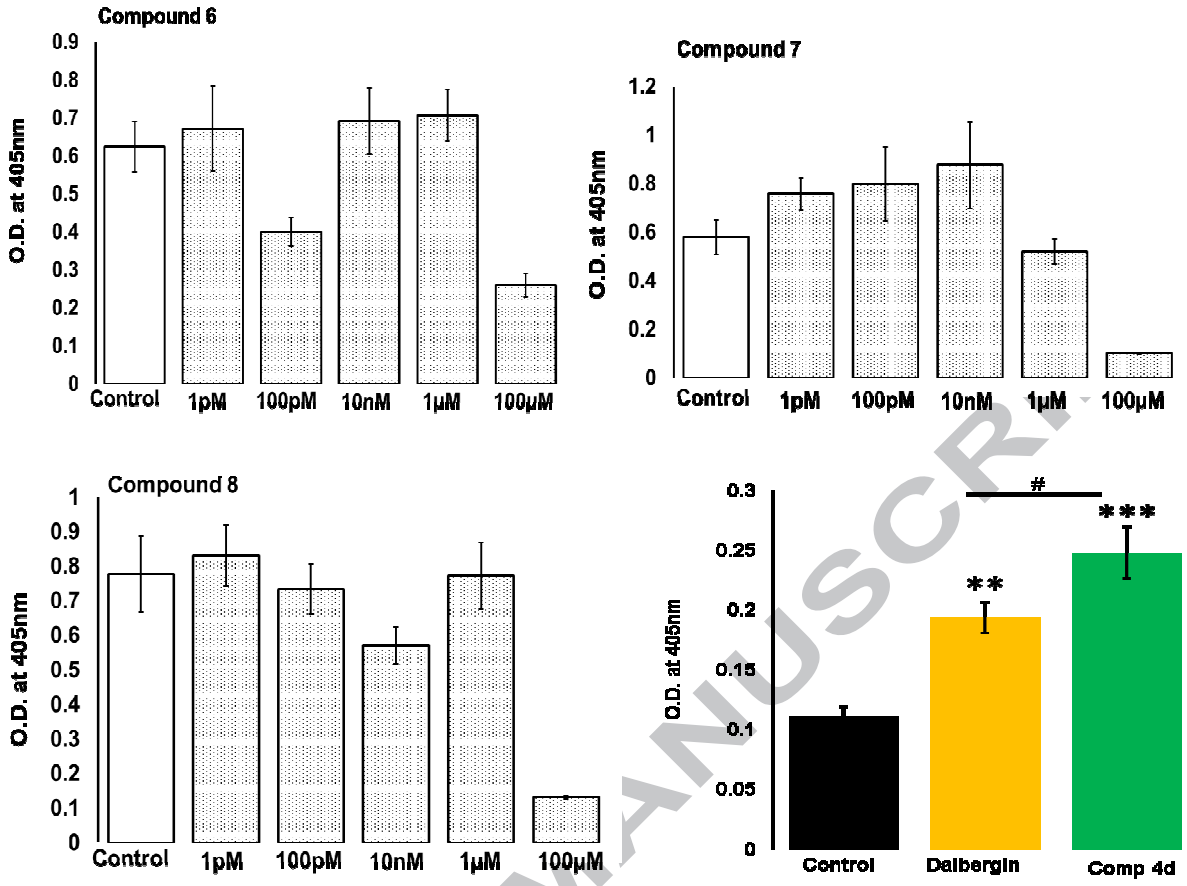


Fig. 1: Dalbergin analogues have osteoblast differentiation property in primary osteoblast cells. After 48h of treatment, ALP activity was measured at 405 O.D. Data was represented as the means ± SE from three independent experiments: ***p<0.001, **p<0.01 and *p<0.05 compared with untreated cells taken as control and #p< 0.05 when compound **4d** was compared with dalbergin.

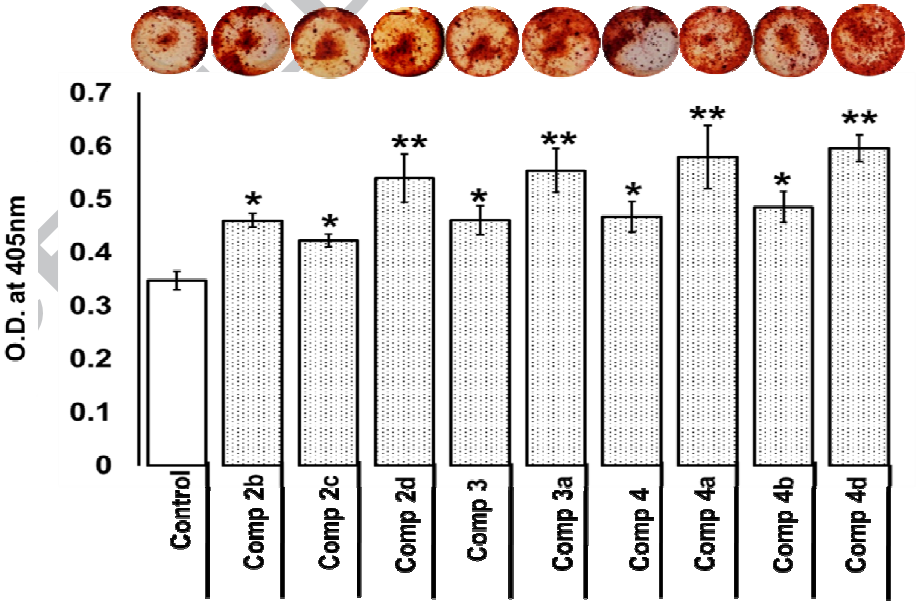


Fig. 2: Measurement of mineralized nodule formation of active compounds by mineralization assay in osteoblast cells. Upper panel shows representative photomicrographs of mineralized nodules formation in active compounds with or without treatment. Bar diagram in the lower panel shows spectrophotometric quantification of mineralized nodule by extraction of Alizarin Red-S dye. O.D. has taken at 405 nm. Data are represented as the means ± SE from three independent experiments: ***p<0.001, **p<0.01 and *p<0.05 compared with untreated cells taken as control.

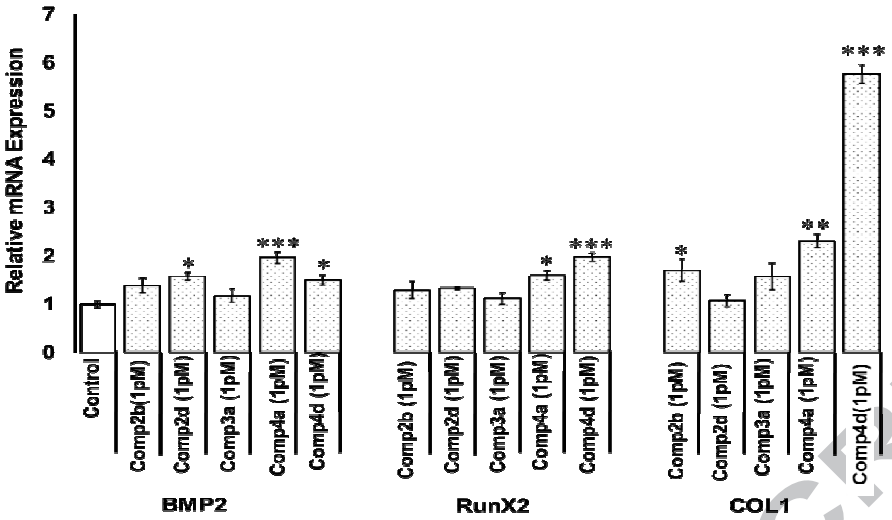


Fig. 3: Relative m-RNA expression of osteogenic genes Bmp2, RunX2, and COL1 of active compounds **2b**, **2d**, **3a**, **4a** and **4d** in mice calvarial osteoblast cells. Calvarial osteoblasts were treated with compound **2b**, **2d**, **3a, 4a** and **4d** at 1 pM for 24h and compare with control. GAPDH is used as internal control. All data represents the means ± SE from three independent experiments: ***p<0.001, **p<0.01 and *p<0.05 compared with untreated cells taken as control**.**

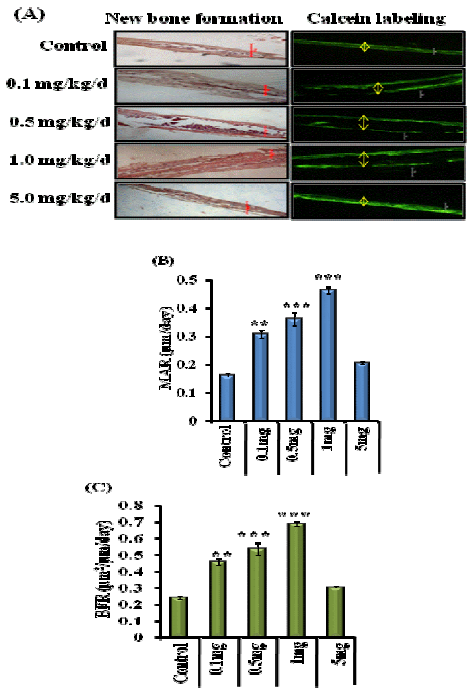
To confirm the gene expression data and bone formation property of compound **4d**, we performed *in vivo* study. In this study, three-week-old Balb/c mice were injected subcutaneously over the calvarial surface with or without the treatment of lead compound **4d** for five consecutive days at the doses of 0.1, 0.5, 1 and 5 mg/kg per day (3 mice per group). All mice were euthanized after treatment and calvaria were dissected, fixed in 4% formaldehyde, and embedded in paraffin. Histologic sections were cut and stained with hematoxylin and eosin (H&E).[31]

For fluorochrome labeling and bone histomorphometry, each mouse received intraperitoneal administration of fluorochromes tetracycline (20 mg/kg) and calcein (20 mg/kg), following a previously published protocol.[25] Histomorphometric analysis of bone formation, such as for the determination of mineral appositional rate (MAR) and bone formation rate (BFR), was performed using Leica-Qwin software (Leica Microsystems Inc., Buffalo Grove, IL, U.S.) as described in our previously published protocol.[32,24]

It was observed that after subcutaneous injection of **4d** at 0.5 mg/kg/d and 1.0 mg/kg/d per day into mouse calvaria significantly stimulated bone formation. Above result was confirmed as shown by histological analysis of calvaria samples harvested after the last injection of tetracycline and stained with H and E. Histomorphometric analysis revealed that compound **4d** showed a significant effect on bone formation [Fig. 4(A)]. This effect, however, was not observed when compound **4d** was used at a higher dose of 5.0 mg/kg per day. For further confirmation of bone formation capability of compound **4d**, we used dynamic histomorphometric analysis followed by calcein double labeling at a higher dose of 5.0 mg/kg per day. Results showed that there was significant difference between control and compound **4d** at 1.0 mg/kg dose which is demonstrated by an increased mineral appositional rate (MAR) at 0.1 mg ($p<0.01$), 0.5 mg and 1.0 mg ($p<0.001$) significantly and the bone-formation rate (BFR) at 0.1 mg ($p<0.01$), 0.5 mg and 1.0 mg ($p<0.001$) increased significantly in calvarial osteoblast *in vivo* [Fig. 4(B) & 4(C)].

In addition, at 1.0 mg/kg dose of compound **4d** showed enhanced osteogenic gene expression RunX2 (~4fold), ALP (~5fold), OCN (~4fold) and COL1 (~4fold) as compared to control group and play role in bone formation [Fig. 4(D)].

Eight-week old adult female Balb/c mice were used for the analysis of trabecular bone formation in ovariectomized post-menopausal model of mice. Animals were housed at 21°C in 12h light/12h dark cycles. Normal chow diet and water were provided ad libitum. Three groups were taken for the further study. The animals were ovariectomized and left for 6 weeks for osteopenia to develop. Thereafter, the animals were divided into groups as follows: sham (ovary intact) + vehicle (gum acacia in distilled water), OVx + vehicle, OVx + 1.0 (mg/kg)/day body weight dose of **4d**. Mice were treated with compound **4d** or vehicle once daily for 6 weeks by oral gavage. After the period of 6 weeks, animals were sacrificed and bones were collected for analysis of trabecular microarchitecture. μCT experiments were carried out using Sky Scan 1076 micro-CT scanner (Aartselaar, Belgium) as previously reported.[32]



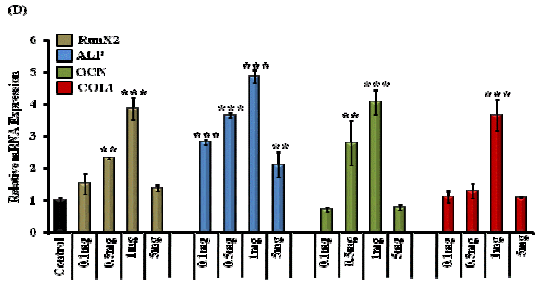


Fig. 4: Compound 4d promotes new bone formation in mice calvaria. (A) Compound **4d** was injected subcutaneously over the surface at 0.1, 0.5, 1.0 and 5.0 mg/kg/d dose of 3 week-old Balb/c mice for 5 consecutive days. The left panel shows longitudinal histomorphometric section treated and control mice calvaria with H and E staining (5µM). The right panel shows fluorochrome labeling with calcein. 1.0mg/kg/d dose showed significantly induced bone formation (Yellow arrow). **(B)** Measurement of mineral apposition rate (MAR) and **(C)** bone formation rate (BFR) in mice calvaria after calcein injection (20 mg/kg). All data represents the Mean ± SE from three independent experiments: ***p<0.001, **p<0.01 and *p<0.05 compared with untreated cells taken as control. **(D)** Relative m-RNA expression of osteogenic genes RunX-2, ALP, OCN and COL-1 in mice calvaria after injecting different dose concentration. Dose 1 mg/kg/d have significantly promoted osteogenic genes expression compare to other doses. All data represents the Mean ± SE from three independent experiments: ***p<0.001, **p<0.01 and *p<0.05 compared with control.

µ-CT analysis of femur bone demonstrated an apparent bone loss in the ovariectomized mice compared with those that underwent sham surgery. Oral dose supplementation of compound **4d** for 6 weeks at 1.0 mg/kg dose significantly reversed the bone loss in OVx mice. Treatment with 1.0 mg dose increased ~1.5 fold bone volume/ tissue volume (BV/TV) compared to OVx group of femoral metaphysis [Fig. 5(B)]. µ-CT analysis of femoral trabecular bone revealed that trabecular number (Tb.No) ($p<0.001$) and trabecular thickness (Tb.Th) ($p<0.05$) also significantly increased as compared to OVx group [Fig. 5C) & 5(D)]. At the same time, trabecular separation (Tb.Sp) ($p<0.001$) and structural model index (SMI) ($p<0.001$) has decreased in treated group [Fig. 5(E) & 4(F)]. Tibia and lumber 5 vertebrae have the same effect (Table 2). Biomechanical strength analysis revealed that femur breaking strength stiffness ($p<0.01$) and energy ($p<0.01$) is significantly increased in 1.0 mg/kg treatment group as compared to OVx. Analysis of vertebrae compressive strength in treated group revealed that stiffness ($p<0.01$) and energy ($p<0.05$) significantly increased as compared to OVx group as shown in Table 2.

Dynamic histomorphometric analysis of femur diaphysis indicated that supplementation of compound **4d at** 1.0 mg/kg dose increased mineral apposition rate (MAR) ($p<0.01$) and bone formation rate (BFR) ($p<0.05$) in the femur bone as compared to OVx. Above data indicate that lead compound **4d** enhanced bone formation at active dose in an osteopenic model as mentioned in Table 2.

Ovariectomy causes the reduction in estrogen, resulted in decreased uterine weight, luminal area and epithelial cell height of uterus shown in Table 2. Administration of compound **4d** at 1.0 mg/kg dose results in no change in uterine parameters. Results revealed that compound **4d** has no estrogenic effect on the uterus.

Table 2: Different parameters evaluated in this study with compound **4d.**

Parameters	Sham	OVx	OVx+1mg
(A) Tibia micro-CT			
BV/TV (%)	14.70 ± 1.16***	6.43 ± 0.47	13.53 ± 0.63***
Tb.No. (mm^{-1})	3.11 ± 0.18***	1.49 ± 0.25	2.70 ± 0.11***
Tb.Th. (mm)	0.053 ± 0.001***	0.0475 ± 0.0005	0.052 ± .0005***
Tb.Sp. (mm)	0.238 ± 0.006***	0.387± 0.038	0.294 ± 0.015*
SMI	1.84 ± 0.04***	2.32 ± 0.08	1.84 ± 0.03***
(B) Vertebrae micro-CT			
BV/TV (%)	15.91 ± 0.61**	13.92 ± 1.66	23.43 ±1.79***
Tb.No. (mm^{-1})	3.61 ± 0.27**	2.24 ± 0.16	4.63 ± 0.33***
Tb.Th. (mm)	0.053 ± 0.001*	0.047 ± 0.001	0.0522 ± 0.0006*
Tb.Sp. (mm)	0.213 ± 0.016**	0.288 ± 0.021	0.167 ± 0.005***
SMI	1.46 ± 0.07*	1.78 ± 0.08	1.07 ± 0.08***
(C) Bone Strength			
(i) Femur breaking strength			
Energy (mJ)	5.6 ± 0.57***	2.6 ± 0.18	4.55 ± 0.02**
Stiffness (N/mm)	2.37 ± 4.18**	34.3 ± 1.84	53.55 ± 2.04**
(ii) Vertebrae compressive strength			
Energy (mJ)	418.25 ± 6.86***	224.75 ± 1.06	262.55 ± 9.12**
Stiffness (N/mm)	468.75 ± 9.32***	345.7 ± 5.08	381.35 ± 10.76*
(D) Bone Histomorphometric analysis			
Mineral apposition rate (µm/day)	0.283 ± 0.016***	0.099 ± 0.01	0.177 ± 0.01**
Bone formation rate ($\mu m^3\mu m^2$/day)	0.32 ± 0.01***	0.098 ± 0.01	0.163 ± 0.009*
(E) Uterine Histomorphometric analysis			
Uterine weight (mg)	0.145 ± 0.008	0.025 ± 0.002###	0.032 ± 0.007###
Total uterine area (μm^2)	4809.6 ± 610.9	837.2 ± 36.09###	958.6 ± 20.44###
Luminal area (μm^2)	431.76 ± 11.61	57.5 ± 1.19###	75.75 ± 4.06###
Luminal epithelial height (µm)	1.39 ± 0.06	0.55 ± 0.03###	0.66 ± 0.03###

Compound 4d treatment improves micro-architectural parameters in tibia (A) and vertebrae (B) of OVx mice. Bone volume/tissue volume (BV/TV), Trabecular number (Tb.No), Trabecular thickness. (Tb.Th), Trabecular separation (Tb.Sp), Structure model index (SMI). All values are expressed as mean ± SE; *P< 0.05; **P< 0.01 and ***P< 0.001 compared with OVx group. **(C) Compound 4d treatment improves bone biomechanical strength of femur (i) and vertebrae (ii) in OVx mice. Compound 4d treated group shows improved femoral and vertebral strength represented by energy and stiffness, respectively**. All values are expressed as mean ± SE; *P< 0.05; **P< 0.01 and ***P< 0.001 compared with OVx group. **(D) Oral administration of Compound 4d has the ability to form new bone as assessed by MAR and BFR (Mineral apposition rate and Bone formation rate).** All values are expressed as mean ± SE; *P< 0.05; **P< 0.01 and ***P< 0.001 compared with OVx group. **(E) Compound 4d treatment exhibited no uterine estrogenic effect in OVx mice as**

shown by uterine weight and histomorhometric parameters (total uterine area, luminal area and luminal epithelial height), which show that compound 4d treated group had histological features comparable to those of OVx mice. All values are expressed as mean ± SE; ###P< 0.001 compared with Sham group.

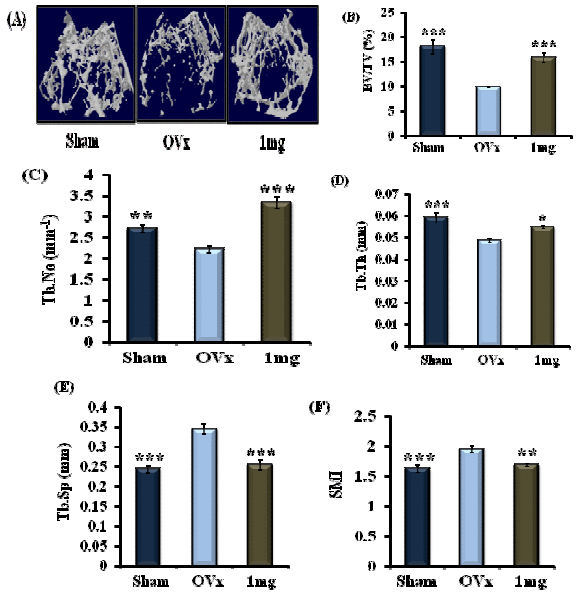


Fig. 5: Compound 4d reversed bone loss induced by ovariectomy in mice without uterine estrogenic effect. **(A)** 3D μ-CT image of trabecular micro-architecture of femoral metaphysis bone **(B)** Assessment of bone microarchitectural parameters including Bone volume/Tissue volume (BV/TV) **(C)** Trabecular number (Tb.no) **(D)** Trabecular thickness (Tb.Th), **(E)** Trabecular separation (Tb.Sp) and **(F)** Structure Model Index (SMI) respectively in various groups. Treatment of **4d** at a dose of 1.0 mg/kg/d to OVx mice improved trabecular micro-architecture, as is clearly visible when compared with OVx group in mice. Data were represented as the mean ± SEM. ***p < 0.001, **p < 0.01, and *p < 0.05 compared with OVx group.

To determine the effect of the compound **4d** on the ability of bone marrow cell differentiation towards osteogenic lineage, ex-vivo culture of BMC's was performed. BMC's from long bone were isolated and cultured for 21 days in osteogenic differentiation media. Fig. 6(A) showed that BMC's harvested after treatment of compound **4d** show significantly enhanced alkaline phosphatase (ALP-Early osteoblast differentiation) activity (about ~2 fold) as compared to OVx group. Further, we analyzed the mineralizing ability of the BMC's that started to form mineralizing nodules at 12th day of culture and maximum calcium nodules were observed at day 21 of culture. At day 21, the number of Alizarin Red-stained calcium nodules [Fig. 6(B)] was increased in compound **4d** at 1.0 mg/kg dose treated group. Extraction and quantification of the dye showed that compound **4d** treated group enhanced mineralization about ~50% (P<0·01) as compared to OVx group as shown in Fig. 6(C). Overall data suggested that compound **4d** has the ability to differentiate BMC's into osteoblast lineage cells.

We further assessed the osteogenic gene expression in long bones. Lead compound **4d** significantly increased BMP2 (p<0.001), RunX2 (p<0.001), Osx (p<0.001), ALP (p<0.01), OPG (p<0.001), OCN (p<0.001) and COL1 (p<0.001) expression as compared to OVx group [Fig. 6(D)].

Ovariectomy resulted in the increase of osteoclast genes expressions (bone resorbing cells) which were reverted back after treatment with compound **4d** at 1.0 mg dose. Expression of RANK (receptor activator of nuclear factor kappa B) (p<0.01), RANKL (receptor activator of nuclear factor kappa B ligand) (p<0.001), TRAP (tartrate-resistant acid phosphatase) (p<0.001) was reduced by **4d** at 1.0 mg dose as compared to OVx group [Fig. 6 (E)].

Serum samples were isolated from blood of control and compound **4d** treated group after autopsy for analysis of bone turnover marker. On the basis of our previously published protocols, serum osteocalcin (mid-portion) was determined by enzyme-linked immunosorbent assay kits by following the manufacturer's protocols (Immunodiagnostic Systems Inc.).[31] During bone remodeling, osteocalcin is produced by osteoblasts and forms matrix component. Osteocalcin levels were increased during rapid bone turnover (OVx model). The OVx-induced bone loss was characterized by high bone turnover rates, as represented by higher levels of serum osteocalcin (OCN) ~2 fold compared with the sham group [Fig. 6(F)]. However, 6 weeks of compound **4d** at 1.0 mg dose treatment to OVx group significantly lowered the level of OCN, indicating that compound **4d** was effective in lowering the serum OCN levels and bone turnover.

Further, on the assessment of compound **4d** in fracture healing model of mice by administrating the 1.0 mg/kg dose for 21 days in mice model, we analyzed the μ-CT parameters at drill hole site of the various group (representative images in fig. 7 A).Quantification of various μ-CT parameters showed that active dose of 1.0 mg/kg improved the bone micro-architecture parameters. Compound **4d** at active dose increased bone volume (BV/TV, p<0.05), trabecular thickness (Tb.Th, p<0.001), trabecular number (Tb.N, p<0.01), and decreased trabecular separation (Tb.Sp, P<0.01) and structure model index (SMI, P<0.001) (Table 3) as compared to control.

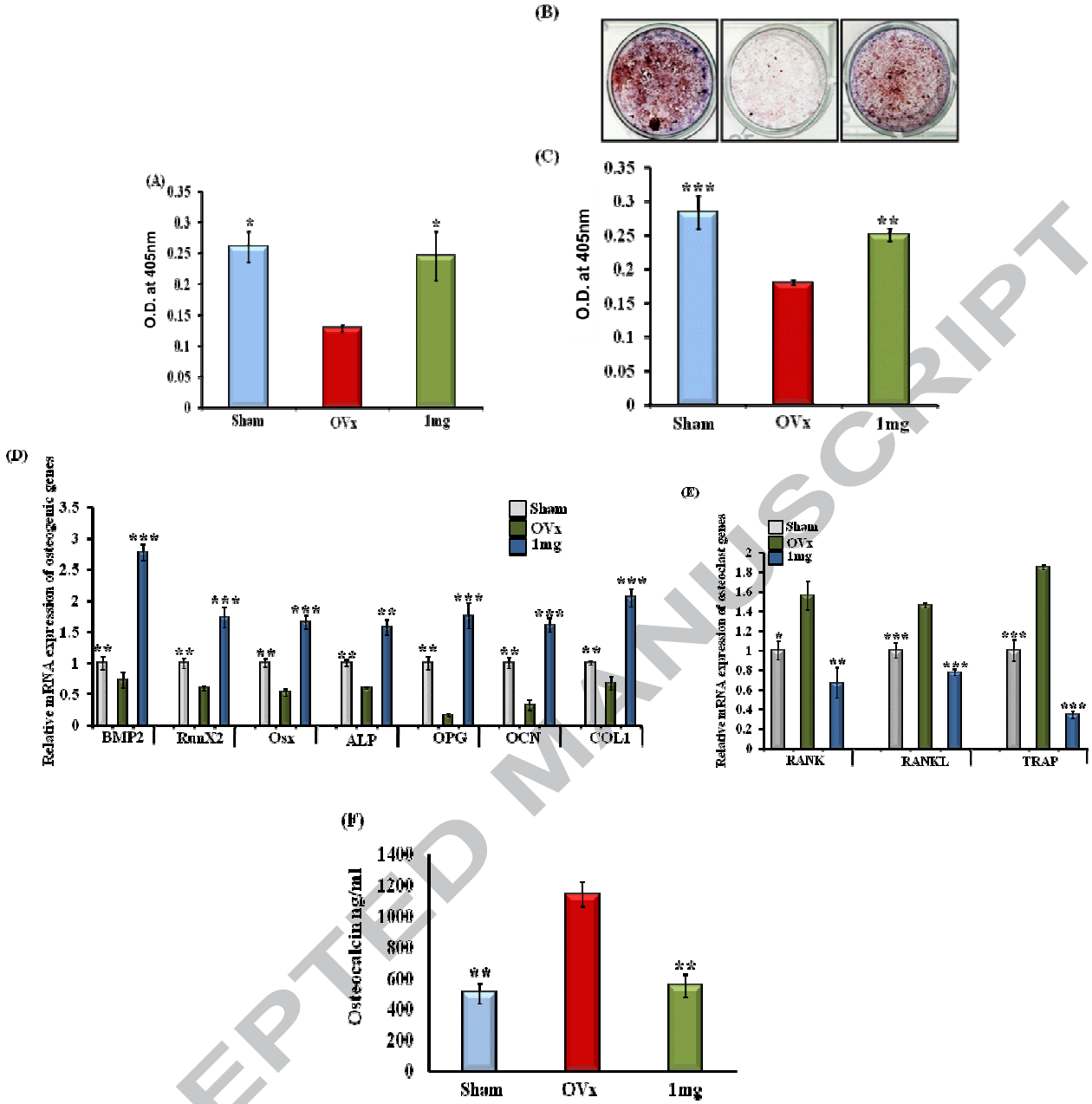


Fig. 6: Compound 4d promotes osteoblast mineralization in BMCs through inhibiting osteoclast gene expression. **(A)** Ex vivo experiments showed that BMCs (Bone marrow cells) were differentiation toward osteoblast lineage measured by ALP activity compared with OVx group **(B)** Oral administration of Compound **4d** has the ability to form mineralized nodule in BMCs compared to OVx group assessed by mineralization assay. The upper panel showed the representative photomicrographs of mineralized nodules formation in various experimental groups **(C)** lower panel showed quantification of alizarin dye. O.D. has taken at 405nM. Both data are represented as the means ± SE. ***p<0.001, **p<0.01 and *p<0.05 compared with OVx group **(D)** Relative m-RNA expression of osteogenic genes in treated and control groups. Compound **4d** induced the m-RNA expression of osteogenic genes Bone morphogenic protein-2 (BMP-2), RunX-2, Osterix (Osx), alkaline phosphatase (ALP), Osteoprotegerin (OPG), Osteocalcin (OCN) and Collagen I (Col-I) in femoral bone. All values were normalized with GAPDH. **(E)** Relative m-RNA expression of osteoclast genes including Receptor Activator of Nuclear Factor k (RANK), Receptor Activator of Nuclear Factor-k legend (RANKL), and Tartrate-Resistant Acid Phosphatase (TRAP) and normalizing with housekeeping gene glyceraldehydes-3-phosphate dehydrogenase (GAPDH). Data was represented as the means ± SEM. ***p<0.001, **p<0.01 and *p<0.05 compared with OVx group. **(F)** Measurement of serum osteocalcin levels (bone turnover marker). Administration of Compound **4d** at 1mg/kg dose modulated the bone turnover in favor of bone formation. Results represent pooled data as means ± SE. **p<0.01, compared with OVx group.

Table 3: Micro-CT analysis of bone at drill-hole site

Parameters	Control	1 mg/kg
BV/TV (%)	32.924 ± 3.806	44.359 ± 1.912*
Tb.Th (mm)	0.111 ± 0.006	0.166 ± 0.010***
Tb.No (mm^{-1})	2.194 ± 0.265	3.442 ± 0.262**
Tb.Sp (mm)	0.219 ± 0.015	0.164 ± 0.007**
SMI	2.585 ± 0.387	0.496 ± 0.081***

Compound 4d (1mg/kg) improves micro-architectural parameters at the drill-hole site. Bone volume/tissue volume (BV/TV), Trabecular thickness. (Tb.Th), Trabecular number (Tb.No), Trabecular separation (Tb.Sp), Structure model index (SMI). All values are expressed as mean ± SE; *P< 0.05; **P< 0.01 and ***P< 0.001.

We further investigated osteogenic and chondrogenic genes expression during fracture healing in bone including Sox9, aggrecan, RunX2, BMP2, BMP4, OCN and COL1. As shown in Fig. 7(B), the results revealed that daily administration of compound **4d** of 1.0 mg dose exhibited increase in the mRNA levels of Sox9 ($p<0.05$), aggrecan ($p<0.05$) and BMP4 ($p<0.05$). RunX2 and BMP2 have increased but not significant. At 1.0 mg/kg dose of **4d** had a robust induction of ~6 folds on the mRNA levels of OCN and mineralizing gene COL1($p<0.05$) over the control.

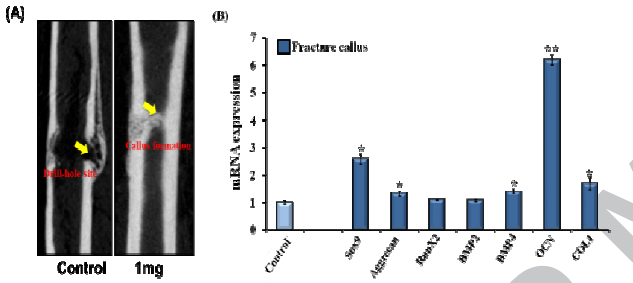


Fig. 7 Compound 4d enhances bone healing at the drill-hole site in the treatment group. (A) Representative µCT 2-D images from different groups showing callus formation at the drill-hole site in femoral bone of mice. **(B)** Relative m-RNA expression of Sox9, Aggrecan, RunX2, BMP2, BMP4, OCN and COL1 in callus at the drill-hole site and compared with control group. All data were represented as the mean ± SE. **p<0.01and *p<0.05 with three independent experiments.

The structure-activity relationship revealed that the compounds having extended aliphatic linker chain the osteogenic activity was significantly increased. The introduction of aliphatic chain from one carbon to five carbon at position 6 of dalbergin enhances the ALP activity. To evaluate the effect of bromo group on ALP mineralization, we replaced it by cyclic amines and a significant increase in ALP activity was observed. Further, the introduction of ester groups instead of the aliphatic chain was found to enable in the enhancement of ALP activity. Similarly, the synthesized amide compound was also found to inactive.

In conclusion, we have synthesized twenty three novel analogues of naturally occurring dalbergin in concise, efficient and economical synthetic routes. Syntheses of these new analogues aim to explore the chemical modification of dalbergin and to investigate their role in the treatment of osteoporosis. Among all twenty three derivatives, four compounds **2d**, **3a**, **4a**, and **4d** showed significant ALP activity and mineralization in calvarial cells. The compound **4d** was found to be most potent and showed a significant osteoprotective effect at 1.0 mg/kg/d in *in vivo* assay.

Acknowledgement

Authors are thankful to CSIR and UGC for providing fellowships. Saransh W. Maurya is thankful to University of Allahabad, Allahabad for providing fellowship and Director CSIR-CDRI for providing opportunity of academic training in CDRI, Lucknow, India. Rakesh Maurya is grateful to CSIR, New Delhi, India, for providing emeritus scientist scheme with reference no. 21(1019)/16/EMR-II. The grant from DBT (Department of Biotechnology GAP0127), CSIR sponsored network project CSC0130, BSC103, AcSIR and CSIR, Government of India are acknowledgment. Authors are also grateful to SAIF division of CDRI for providing spectral data.

References

1. Allen, J. G.; Fotsch, C.; Babij, P. *J. Med. Chem.* **2010**, 53, 4332.
2. (a). Oursler, M. J.; Landers, J. P.; Riggs, B. L.; Spelsberg, T. C. *Ann. Med.* **1993**, *25*, 361. (b). Paul, J. C.; Karen, M. B.; Ben, C. A.; John, H. H.; Carol, A. M.; Le, T. D.; Bradley, P. F.; Carmen, F. M.; Michael, A. G.; George, D. H.; Donald, B. K.; Chih-Tai, L.; Lorraine, L.; Kara, M.; Brenda, P.; Thomayant, P.; Gideon, A.; Rodan, G. A.; Wesolowski, S. B.; Rodan, M. E. D. *J. Med. Chem.* **2004**, *47*, 4829.
3. Riggs, B. L.; Wahner, H. W.; Dunn, W. L.; Mazess, R. B.; Offord, K. P.; Melton, L. J. III. *J. Clin. Invest.* **1981**, *67*, 328.
4. Ettinger, B.; Pressman, A.; Silver, P. *Menopause* **1999**, *6*, 282.
5. Liu, Z. Y.; He, X. B.; Yang, Z. Y.; Shao, H.Y.; Li, X.; Guo. H. F.; Zhang, Y. Q.; Si, S. Y.; Li, Z. R. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 4167.
6. Han, C. Y. E.; Wang, Y.; Yu, L.; Powers, D.; Xiong, X.; Yu, V.; Nguyen, Y.; Jean, D. J. S. Jr.; Babij, P.; *Bioorg. Med. Chem. Lett.* **2009**, *19*, 1442.
7. Donnelly, D. M. X.; Boland, G. M. *Nat. Prod. Rep.* **1995**, *12*, 321.
8. de Lima, O. G., Maia, M. H. D., d'Albuquerque, I. L., de Albuquerque, M. M. F., Borba, M. A. P. *Rev. Inst. Antibiot. Univ. Recife* **1961**, *3*, 61.
9. Shirota, O., Pathak, V., Sekita, S., Satake, M., Nagashima, Y., Hirayama, Y., Hakamata, Y., Hayashi, T. *J. Nat. Prod.* **2003**, *66*, 1128.
10. (a) Kumar, P.; Kushwaha, P.; Khedgikar, V.; Gautam, J.; Choudhary, D.; Singh, D.; Trivedi, R.; Maurya, R. *Bioorg. Med. Chem. Lett.* **2014**, *24*, 2664. (b) Choudhary, D., Kushwaha, P., Gautam, J., Kumar, P., Verma, A., Kumar, A., Maurya, S. W., Siddiqui, I. R., Mishra, P. R., Maurya, R., Trivedi, R. *Biomed. Pharmacother.* **2016**, *83*, 942.
11. Sekine, N., Ashitani, T., Murayama, T., Ogiyama, K.,Takahashi, K. *Mokuzai Gakkaishi* **2009**, *55*, 29.
12. Wu, S. F., Chang, F. R., Wang, S. Y., Hwang, T. L., Lee, C. L., Chen, S. L., Wu, C. C., Wu, Y. C. *J. Nat. Prod.* **2011**, *74*, 989.
13. Zhaoa, C., Liub, Y., Conga, D., Zhangc, H., Yua, J., Jianga, Y., Cuia, X., Sunc, J. *Biomed. Chromatogr.* **2013**, *27*, 1621.
14. Esterbauer, H., Poelsler, G., Fodor, G. *Acta Biochim. Biophys.* **1987**, *22*, 195.
15. Mutai, P.; Heydenreich, M.; Thoithi, G.; Mugumbate, G.; Chibale, K.; Yenesew, A. *Phytochem. Lett.* **2013**, *6*, 671.
16. Chunfang, Z.; Yongqiang L.; Cong, D.; Hui Z.; Yu, Jiang J.; Cui Y.; Sun, X. J. *Biomed. Chromatogr.* **2013**, *27*, 1621.
17. Cheng, Y.; Wang, Y.; Ge, Z.; Li, Y. *Faming Zhuanli Shenqing* **2011**, CN 102133260 A 20110727.
18. Kawase, M.; Sakagami, H.; Motohashi, N.; Hauer, H.; Chatterjee, S. S.; Spengler, G.; Vigyikanne, A. V.; Molnar, A.; Molnar, J. *In Vivo* **2005**, *19*, 705.

19. (a) Kumar, M.; Rawat, P.; Khan, M. F.; Rawat, A. K.; Srivastava, A. K.; Mauya, R. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 2232. (b) Khan, M. F.; Kumar, P.; Pandey, J.; Srivastava, A. K.; Tamrakar, A. K.; Maurya, R. *Bioorg. Med. Chem. Lett.* **2012**, *22*, 4636. (c) Khan, M. F.; Maurya, C. K.; Dev, K.; Arha, D.; Rai, A. K.; Tamrakar, A. K.; Maurya, R. *Bioorg. Med. Chem. Lett.* **2014**, *24*, 2674.
20. Cai, X.; Yang, A.; Zhou, J.; Lu, W.; Hu, C.; Gu, Z.; Huo, J.; Wang, X.; Cao, P. *Bioorg. Med. Chem.* **2013***, 21*, 84.
21. Turan, I. S.; Akkaya, E. U. *Org. Lett.* **2014**, *16*, 1680.
22. Nikalje, M. D.; Sasikumar, M.; Muthukrishnan, M. *Tetrahedron: Asymmetry* **2010**, *21*, 2825.
23. Sarkar, S.; Sonkar, R.; Bhatia, G.; Tadigoppula, N. *Eur. J. Med. Chem.* **2014**, *80*, 135.
24. Kushwaha, P.; Khedgikar, V.; Gautam, J.; Dixit, P.; Chillara, R.; Verma, A.; Thakur, R.; Mishra, D. P.; Singh, D.; Maurya, R.; Chattopadhyay, N.; Mishra, P. R.; Trivedi, R. *Cell Death Differ.* **2014**, *5*, e1422.
25. Sashidhara, K. V.; Modukuri, R. K.; Choudhary, D.; Bhaskara, R. K.; Kumar, M.; Khedgikar, V.; Trivedi, R. *Eur. J. Med. Chem.* **2013,** *70*, 802.
26. Khedgikar, V.; Kushwaha, P.; Gautam, J.; Verma, A.; Changkija, B.; Kumar, A.; Sharma, S.; Nagar, G. K.; Singh, D.; Trivedi, P. K.; Sangwan, N. S.; Mishra, P. R.; Trivedi, R. *Cell Death Dis.* **2013**, *4*, e778.
27. Khedgikar, V.; Gautam, J.; Kushwaha, P.; Kumar, A.; Nagar, G. K.; Dixit, P.; Chillara, R.; Voruganti, S.; Singh, S. P.; Uddin, W.; Jain, G. K.; Singh, D.; Maurya, R.; Chattopadhyay, N.; Trivedi, R. *Menopause* **2012**, *19*, 1336.
28. Dixit, P.; Chillara, R.; Khedgikar, V.; Gautam, J.; Kushwaha, P.; Kumar, A.; Singh, D.; Trivedi, R.; Maurya, R. *Bioorg. Med. Chem. Lett.* **2012**, *22*, 890.
29. Sashidhara, K. V.; Kumar, M.; Khedgikar, V.; Kushwaha, P.; Modukuri, R. K.; Kumar, A.; Gautam, J.; Singh, D.; Sridhar, B.; Trivedi, R. *J. Med. Chem*. **2013**, *56*, 109.
30. (a) Gautam, J.; Choudhary, D.; Khedgikar, V.; Kushwaha, P.; Singh, R. S.; Singh, D.; Tiwari, S.; Trivedi, R. *Br. J. Nutr.* **2014**, *111*, 1811. (b) Kushwaha, P.; Khedgikar, V.; Sharma, D.; Yuen, T.; Gautam, J.; Ahmad, N.; Karvande, A.; Mishra, P. R.; Trivedi, P. K.; Sun, L.; Bhadada, S. K.; Zaidi, M.; Trivedi, R. *J. Biol. Chem.* **2016**, *291*, 3959.
31. Tang, D. Z.; Hou, W.; Zhou, Q.; Zhang, M.; Holz, J.; Sheu, T. J.; Li, T. F.; Cheng, S. D.; Shi, Q.; Harris, S. E.; Chen, D.; Wang, Y. J. *J. Bone Miner. Res*. **2010**, *25*, 1234.
32. (a) Gautam, J.; Kumar, P.; Kushwaha, P.; Khedgikar, V.; Choudhary, D.; Singh, D.; Maurya, R.; Trivedi, R. *Menopause* **2015**, Apr 6. (b) Kushwaha, P.; Khedgikar, V.; Haldar, S.; Gautam, J.; Mulani, F. A.; Thulasiram, H. V.; Trivedi, R. *Bioorg. Med. Chem. Lett.* **2016**, *26*, 3719.

Graphical Abstract

Design and synthesis of dalbergin analogues and evaluation of anti-osteoporotic activity

Leave this area blank for abstract info.

Padam Kumar, Priyanka Kushwaha, Naseer Ahmad, Saransh Wales Maurya, Kapil Dev, Vikram Khedgikar, Ibadur Rahman Siddiqui, Ritu Trivedi and Rakesh Maurya

A series of dalbergin analogues have been synthesized. Among them, compound **4d** showed singnificant increased proliferation as assessed by alkaline phosphatase activity and mineralization in calvarial osteoblast cells in *in vitro* as well as *in vivo*.