CHAPTER 5

OSTEOCLAST INHIBITING PROPERTY OF VARIOUS COMPONENTS OF *MORINGA OLIFERA IN VITRO*

INTRODUCTION:

Osteoporosis, characterized by loss of bone mass is a major health problem, especially in elderly women. The etiology of human osteoporosis is multi-factorial and is influenced by heredity, hormone excess or deficiency, dietary components and physical activity (Lu-Ping *et al.,* 2003). An increased frequency in osteoporosis correlates with longer life expectancy. Bone loss has been attributed to the imbalance between the bone formation and bone resorption. Many agents such as calcium, estrogen, calcitonin, Vitamin D_3, and ipriflavone have been used clinically in the treatment of this disease (Turner *et al.,* 1994). Osteoporosis is major age related source of morbidity and mortality (Xie *et al.,* 2005). The pathogenesis of postmenopausal osteoporosis is manifested by a drop in ovarian estrogen levels after menopause and increased bone turn over with an increase in bone resorption by osteoclasts, resulting in decreased bone mass which is also associated with an increase in the production of pro-inflammatory cytokines such as IL-1 and IL-6. These pro-inflammatory cytokines contribute to bone resorption by increasing osteoclastogenesis (Xie *et al.,* 2005; Park *et al.,* 2008). Increased bone resorption mediated by osteoclasts is central to pathogenesis of osteoporosis (Sameul *et al.,* 2007).

Osteoclasts constitute the major cell type involved in the degradation of bone not only under physiological conditions but also in disease such as osteoporosis (Pap *et al.,* 2003). The osteoclast is hematopoietic in origin and derived from cells in the monocyte-macrophage lineage which then fuse to form inactive osteoclasts. Osteoclasts are activated to resorb bone and then eventually undergo apoptosis (Fig 1). Both locally produced cytokines and systemic hormones can regulate normal osteoclast formation. Phenotypic characteristics of osteoclasts include that they are large multinucleated cells that contract in response to calcitonin, react strongly with the 23c6 monoclonal antibody that identifies the osteoclast vitronectin receptor, express high levels of tartrate-resistant acid phosphatase, a marker enzyme for osteoclasts, and resorb bone.

In the past, the development of herbal anti-osteoporosis formulas was pursued mainly by scientists in Asian countries, including China, Japan and Korea (Xie *et al.,* 2005; Parikh *et al.,* 2009). Recent evidence suggest that estrogen replacement therapy (ERT) is associated with increased risk of breast, ovarian and endometrial cancer in postmenopausal women, it is now generally recognized that alternative approaches to the prevention and treatment of osteoporosis might be worth exploring (Xie *et al.,* 2005; Parikh *et al.,* 2009;Rangrez *et al.,* 2010). Hence the attention for the phytoestrogens therapy for the treatment of osteoporosis has now become the stronghold.

The modulation of the differentiation and function of osteoclasts has been considered as therapeutic approach in the treatment of osteoporosis (Teitelbaum, 2000; Boyle *et al.,* 2003; Miyomoto and Sudo, 2003; Lorenzo *et al.,* 2008). Indian herbal medicine has been widely used for the treatment of many diseases from time in memorial. There are number of natural agents that have been found to exhibit inhibitory effect on osteoclast differentiation, such as peptides (Kwak *et al.,* 2003), endogenous components (Maran *et al.,* 2006), and natural compounds (Han *et al.,* 2007; Kim *et al.,* 2004). In the course of searching inhibitors of osteoblast differentiation from natural sources, we examined the in vivo activities of different components of MO which showed some promising and positive effects on bone health (Chapter II and III). Further the phytochemical analysis has also proved that the major components are the flavanoids, saponins and alkaloids. (Chapter IV). However, the *in vitro* studies on the same line are lacking and moreover, the cellular changes as well as the means behind this osteoprotective effect were not clear. Hence, it was though pertinent to explore this plant for its effect on bone cells, especially on osteoclastic cells because understanding the mechanism responsible for ostoclast activation in osteoporosis can lead to novel therapeutic approaches for the highly and potentially complication of osteoporosis, as they play a major role in progression of osteoporosis.

MATERIALS AND METHODS

Chemicals: DMEM, 100 X anti-microbial antifungal solutions, 0.1% trypsin EDTA and FBS were purchased for High Media Chemicals. Vitamin D_3, dexamethasone, vitamin A and napthol AS-MX phosphate were purchased from Sigma Chemicals. Methanol, potassium sodium tartrate, triton X-100 were of domestic AR grade products. 0.23µ filter were purchased from Sartorius.

Calcium, Alkaline Phosphatase (ALP) and Tartrate resistant Acid Phosphatase (TRAcP) Kits were purchased from Reckon Diagnostic Kits Pvt. Ltd.

Preparation of Extract: Fruits, leaves and flowers of MO were obtained from wild and was sent to botany department for identification and a herbarium sheet was deposited for further record. Dried powder was prepared by drying MO in oven at 50º C. 100 gm dried powder of each component was extracted with 500 ml methanol in Soxhlet's apparatus for 48 hours . Methanolic extract was dried on water bath at 55º C. The percentage yield of the plant was found to be 9.8%, 6.3% and 7.7% for fruits, leaves and flowers respectively. The plant extract was freeze dried and stored at -70º C. Working solution was prepared by dissolving the extract in DMEM and filtered using 0.23 μ filter.

Osteoclast cell culture

Preparation of bovine cortical bone slices: preparation of Bovines cortical bones were carried out as described previously (Richard *et al.,* 1997). Briefly the bones were collected from local slaughter house in 2X anti-microbial anti-fungal liquid. Bone slices were cut into 8x8x3 mm^3 (lxbxw). The slices were then defatted by sonicating twice for 30 min periods in 5% solution of Triton-X 100 in double distilled water for 30 min and sterilized with 75 % ethanol, dried, and left under ultraviolet light for 15 min before use.

Isolation, activation of Osteoclasts and MO treatment: Osteoclasts were isolated from young rats as described previously (Richard *et al.,* 1997). For activation of osteoclasts combination of Vitamin $_{D3}$, Vitamin A and dexamethasone was used by slight modification from the method of previous workers (Qin *et al.,* 2003). Briefly, all long bones are removed and freed of adherent muscle; their epiphyses were cut off with a scalpel at the level of the growth plate and discarded. The dissection was performed on a dissecting board using a scalpel and fairly fine forceps to hold the bones. Once clean, the bones are transferred to 35 mm plastic tissue culture plates containing 3.5 ml chilled HBSS without serum. Bones are minced to create a cell suspension, using a scalpel and holding the bones with forceps. The bone debris was then repeatedly sucked in and out of a transfer pipette so as to get maximum number of osteoclasts. The petridish was then tilted at a slight angle so as to allow the bulk of the bone matrix to settle for about 10 seconds into the corner of the dish and the cell suspension was then taken up gently from the top of the liquid into a transfer pipette, trying to avoid pieces of bone. These cells were co cultured with osteoblastic cells isolated from calvarial bones by the method of Liu and coworkers (1995) in DMEM medium containing 10% FBS, 1X anti-microbial antifungal solution, 1 gm/L glucose, L - glutamine, sodium pyruvate, sodium bicarbonate, 25 mM HEPES buffer, Vitamin A and vitamin D_3 (10 nmol/L) and dexamethasone (100 nmol/L) at 37ºC in a humidified atmosphere for 48hours in 24 well culture dishes (1.5 mL per well). After 48hours of activation, culture liquid, dead and non-attached cell were removed. Attached cells were detached from the wells by washing with 0.1% EDTA Trypsin solution to recover cells. The cells were counted to be 1 million cell/mL. 500 µL of these solution was loaded on each bone slices in 24 well plate containing 1 ml culture medium to study the resorption. For cell blank only 500 µl of DMEM was added. The formation of osteoclast-like MNC (multinucleated osteoclasts) was confirmed by the staining of TRAcP and resorptive pit formed on bone slices after 48 hours. Treatment of MO plant extract was given in 6 doses after confirmation of active resorption pits in the culture. 10 µg/ml, 20µg/ml, 50 µg/ml, 100 µg/ml, 200 µg/ml and 400 µg/ml. After 24 and 48 hours of MO treatment, solution was collected for analysis of calcium release and enzyme activity. For histology, the slices were collected at 24 hour and 48 hour and were stained with Toluidine blue stain as described previously (Richard *et al.,* 1997) to detect osteoclastic activity and observed under Leica stereo microscope and photographed using Canon S 7 camera.

Biochemical estimations: Biochemical estimations were carried out in the cell culture media collected at the interval of 24 hours and 48 hours of MO treatment. Cold calcium release assay was carried out as described previously (Meghji *et al.,* 1997). ALP and TRAcP activity were measured using the commercial kits purchased from Reckon Diagnostics Pvt. Ltd. (Vadodara).

Scanning Electron Microscopy: For SEM, the bone slices were fixed in 4% buffered paraformaldehyde and defatted in Triton X 100 and gold coated using JEOL JSM 300 microscope and scanned using JSM 5610 microscope as per our previous study (Rangrez *et al.,* 2009). All the images were analyzed using image J software. Resorption area analysis was carried out using Image J and the data generated was calculated and expressed using Microsoft office excel.

Histomorphometric evaluation: Bone slices were stained with Toluidine blue and observed under Leica stereo microscope and photographed using canon S 7 camera. For image analysis, the images were converted to single color with threshold range of 140 – 170 in image J software. The resultant red cavities were indication of osteoclastic resorption pits formed.

Statistical Analysis: All the statistical analyses were carried out using Graph pad Prism 5, and the test for significance was compared using one-way analysis of variance (ANOVA), followed by Bonferroni post hoc test (Parikh *et al.,* 2009 and Rangrez *et al.,*2011). Images were analyzed using Image J software. Resorption area was calculated using Microsoft office excels.

RESULTS

In the present study the osteoclastic cells were found to be growing adhered to the surface, compared to other bone marrow cells which were free floating. Hence, washing of the wells enabled easy isolation of osteoclasts like cells. After 48 hours, two distinct types of cells were present in cultures, the monolayer of osteoblast-like cells and the free, large cells growing on them. The first type cells were in close to each other with side by side contact. The second type cells were free, sub-round cells characterized by processes and pseudopodia, numerous nucleus and vacuole in cytoplasm. The resorption pits were more like roundish initially but were found to be more irregular with progression of time.

Calcium is one of the chief constituents of our bone and dissolution of hydroxyapetite by osteoclastic resorption leads to release of calcium. Hence, Cold calcium release is one of the important markers of osteoclastic resorption. Of all the three fractions, minimum effect was observed with leaf extract (Figure 1). Treatment of osteoclastic cells did not show any changes in the calcium release from the bone slices. Flower extract did show osteoclasts inhibiting property where it reduced the calcium release (Figure 2). However, this effect of the plant was not found to be statistically significant in initial 24 hours but later it was found to be significant in high dose groups. Compared to leaves and flower, fruit extract of MO was found to be having maximum effect in reducing calcium release from bone slices. It started exerting its effect in low doses and at 24 hour stage (Figure 3). Though not statistically significant in initial 24 hours at low doses, it was found to be highly significant at 48 hour stage. At 48 hour stage with 400 µg/ml treatment, there was 46% reduction in the calcium release from bone slices.

MO plant treatment reduced the calcium release from the solution. However in first 24 hours the data was not statistically significant, but at 48 hours the plant treatment significantly reduced the calcium release in a dose dependent manner indicating the osteoclast inhibiting property of this plant (Table 1).

ALP is one of the most important markers of osteoblastic differentiation. Hence, we tried to explore the changes in ALP activity so as to understand the status of osteoblastic cells in the co culture system. Effect of various components of MO on ALP activity is expressed in Table 2. It was observed that compared to initial stage there is gradual rise in the ALP activity in the co culture system, indicating the normal growth of the culture. However, none of the plant components were found to be having ALP stimulating activity. Flower extract of MO did show increase in ALP activity, but it was found to be non significant (Figure 5). Compared to flower and leaf (Figure 4 and 5), Fruit showed more promising results, although at higher doses it showed decrease in ALP activity (Figure 6).

TRAcP is a lysosomal enzyme found in the osteoclastic resorption pit. Levels of TRAcP indicate the resorption rate and hence universally it is accepted as a marker of osteoclastic bone resorption. Both flower and fruit of MO showed convincing results on the activity of this enzyme. However, maximum effects were observed with fruit extracts of MO. Leaves showed no reduction in TRAcP activity, except at the high dose of 400μg/ml, with no statistical significance (Figure 7). Flower extract of MO showed more promising results and both at low and high doses it showed reduction in TRAcP activity (Figure 8). However, significant of the data was not consistent. MO fruit (Figure 9) also exhibited parallel profile with flower, but the statistical significance of the data was consistent, suggesting that flower is having maximum effect in inhibiting TRAcP activity.

From the biochemical profile, it was clear that fruits of MO were having most promising effects as anti-osteoclastic agent. Hence, we explored fruit extract for its effect on histo-morphometric changes induced by osteoclastic resorption. Scanning electron microscopy (SEM) is one of the most promising tools for studying matrix biology. To understand the osteoclastic resorption, we had carried out SEM of de-cellularized bone slices. SEM image of blank slices showed a clear flat surface with no resorption pits (Figure 10). Compared to this, control slides showed excessive erosion of bone minerals (Figure 11). This resorption was indicative of excessive resorption by activated osteoclastic cells. As MO was found to be non-effective in lower doses, SEM was carried out only for higher doses (100 μg/mL, 200 μg/mL, 400 μg/mL). In higher doses of MO, there was significant reduction in the intensity of resorption (Figure 12 and Figure 13). SEM images revealed the dose dependent osteoclast inhibiting property of this plant. At the highest dose of 400 µg/mL, very few resorption pits were observed (Figure 14). To further boost the significance of our results, regression curve analysis was carried out, which compared the resorption area on the bone slices with blank as reference (100%). The regression curve for the decrease in the resorption area was found to be $y = -12.25x + 53.50$; $R^2 = 0.987$. In 400 µg/ml MO treatment the resorption area was reduced to 6.209 % compared to 42.338 % in control (Figure 15). Results of SEM proved that MO fruit had direct effect on the resorption area of osteoclast.

To further support these results, Histological analysis was carried out. Histological features also confirmed the osteoclast inhibiting property of this plant. In first 24 hours though resorbing cells were seen in all the treatment groups, after 48 hours, there was clear inhibition of resorbing cells. In control there was marked increase in the resorption from 24 hours to 48 hours. This excessive resorption was clear when the threshold analysis was carried out using image J software. Dark red and brownish patches were indicative of excessive resorption areas. These areas were markedly reduced with MO treatment. MO extract was found to be potent in reduced these dark pits in a dose dependent and time dependent manner (Figure 16 – Figure 24).

4. Discussion:

In the present study it was proved that the osteoprotective effect of MO is mainly due to its osteoclast inhibiting property. Of all the components of MO, maximum effect was observed with fruit extracts. Osteoclastic bone resorption is thought to be mediated by two different processes: one is the formation of new osteoclasts, and the other is the resorption activity of osteoclasts. It was reported that bone resorbing agents such as $VitD_3$, PTH, and IL-1 markedly stimulated the formation of osteoclasts (Qin *et al.,* 2003). Vit D_3 is thought to stimulate osteoclast formation by a common mode involving prostaglandin E_2 (PG-E_2), which is also found in osteoporosis (Akatsu*et al.,* 1992). Hence, in this co culture system, conditions were mimicked to that of natural onset of osteoporosis and we tried to explore the osteoprotective effect of MO in this system.

Osteoclasts, which are responsible for bone resorption, are rare cells with only 2-3 cells seen per 1 mm3 of bone. However, the loss of function in osteoclasts, problems with their differentiation and decrease in their number leading to bone osteosclerosis/osteopetrosis. On the other hand, an increase in their number or function induces bone osteoporosis, indicating that osteoclasts play a pivotal role in bone homeostasis. It has been demonstrated that bone destruction and hypercalcemia induced by metastatic tumors are carried out by osteoclasts activated by the tumor cells, and the inhibition of osteoclast formation prevents the bone destruction and even bone metastasis.

Abnormal osteoclast function is closely related to various diseases. Furthermore, osteoclasts are indispensable in forming bone marrow to produce blood cells, and the absence of osteoclasts causes osteopetrosis, resulting in extramedullary hematopoiesis. Although the physiological roles of osteoclasts are well described, the mechanisms of their differentiation remain to be elucidated. Recently, RANK (receptor activator of nuclear factor kappaB) and its ligand (RANKL) have been identified and their essential roles in osteoclastogenesis have been demonstrated, which has provided new insights into the osteoclast differentiation pathway. We have established an in vitro osteoclast culture system by isolating osteoclast precursor cells and culturing them in the presence of macrophage colony stimulating factor (M-CSF) and soluble RANKL (Suda *et al.,* 1999; Lorenzo *et al.,* 2008; Del, Fattore *et al.,* 2008 and Young *et al.,* 2011). This system has enabled us to analyze the regulation mechanisms in osteoclast formation.

Tartrate resistant acid Phosphatase is one of the marker enzymes of osteoclastic resorption and is directly related to activity of bone resorption (Qin *et al.,* 2003). Of all the fractions of MO, fruit extract reduced the osteoclastic resorption in a dose dependent manner and it was most significant in high doses after 48 hours of treatment. Parallel with these results were the results of calcium release; one of the important indicators of resorption. Fruit extract also inhibited calcium release, supporting its role as an anti-resorptive agent. Though flowers also showed some effectiveness in lowering these markers of resorption, its potency was quite low compared to fruits, which was evident from the statistical significance of the data. Development of osteoclasts also depends on various paracrine factors secreted from osteoblastic cells and they are also associated with bone formation. Hence, those agents who are exerting their effect on osteoblastic cells may affect the osteoclastic activity. Hence, to explore whether MO components are directly exerting their effect on osteoclastic cells or not, we carried out ALP activity as well, which is an established marker of osteoblast activity. ALP activity, showed no significant variation, with any of the components of MO, suggesting that MO components target osteoclastic cells.

Previously established studies have shown that plants with anti inflammatory activity have potent osteoclast inhibitory activity (Vali *et al.,* 2010). MO flower and fruit were established to have anti inflammatory activity by (ref). In extension of those results, we confirmed that MO flower and fruit are having osteoclast inhibiting property. Furthermore, Young and his team in 2011 showed that osteoclast inhibiting property of *weigela subsesillis* species was because of the presence of terpinoids and flavanoids in it. Our previous studies (Chapter IV) have shown that both flower and fruit are rich in terpinoids as well as flavanoids justifying the reason behind osteoclast inhibition. However, one needs to explore these terpinoids further for understsanding the probable mechanistic pathway for the same.

Scanning electron microscopy not only demonstrated the surface appearance of osteoclasts, and their predilection for spreading on various substratum components, but has also been used as an adjunct in resorption assays in which areas of resorption lacunae are measured as indicators of cell activity. Results of SEM analysis further boosted our hypothesis and showed that MO fruit has direct effect on osteoclastic resorption. MO potently inhibited the resorption and significantly reduced the area of resorption to only 6 % in high dose group. Histology and image analysis also confirmed these results, showing that the number and size of resorption pits formed were much less in MO fruit extract treated groups and least was found in 400 µg/ml plant treatment group. MO treatment not only reduced the number of osteoclastic resorption pit, but it also reduced the intensity of resorption. Results of the co-culture studies clearly proved that MO fruit is having significant osteoclast inhibiting activity as it ameliorates the markers of osteoclastic resorption, as well as it reduces the bone resorption in a dose dependent manner.

When the findings of the studies were summarized, it justified the previous reports (Burali *et al.,* 2010) of osteoprotective activity of fruits of MO. Further, it gave first insight into the cellular mechanism behind the osteoprotective activity of this plant and showed that it is mainly attributed to its osteoclast inhibiting property. Though it had no osteoblast stimulating potential, it was safe to osteoblastic cells. This study offers a first understanding about the cellular means behind the osteoprotective effect of this plant component.

Conclusion:

In conclusion, MO fruit significant prevented the osteoclastic resorption, decreased the TRAcP activity, cold calcium release and prevented the bone slices from induced resorption by osteoclast like cells. MO flower extract also exhibited similar profile, but it was not comparable to the efficacy of fruits. Leaves of the MO did not show any pharmacological effect in inhibiting or stimulating osteoclastic cells. These results confirm that MO fruit has potent osteoclast inhibiting property and supports its ethnobotanical usage as an osteoprotective nutritional plant. Contrary to this, MO had non-significant effects on osteoblastic cells. The findings of this study suggest that, *in vivo* osteoprotective effect of MO fruit, is mainly due to its osteoclast inhibiting property. Hence, consumption of this MO fruits can be helpful in ameliorating the osteoporosis as this plant inhibits osteoclastic resorption and lowers the bone degradation.

Table 1 Effect of Different component of MO on cold calcium release (mg/l)

Calcium activity		0 μg/mL	100 μg/mL	200 μg/mL	400 μg/mL
Flower	24	15.400 ± 0.600	14.300 ± 1.230	16.245 ± 0.785	11.234 ± 0.560*
	48	17.800 ± 0.560	15.469 ± 15.469	14.263 ± 0.556	8.562 ± 1.228 **
FRUIT	24	15.40 ± 0.600	11.50 ± 0.878	11.29 ± 0.986	8.99 ± 0.560**
	48	17.80 ± 0.560	13.69 ± 0.480	11.56 ± 0.990*	9.65 ± 0.330**
Leaves	24	15.555 ± 0.606	15.236 ± 2.365	12.456 ± 1.669	15.236 ± 1.656
	48	17.979 ± 0.565	14.695 ± 1.669	17.526 ± 2.001	16.985 ± 1.656

Values were expressed as Mean ± S.E.M. * - p < 0.05; ** - p < 0.01; *** - p < 0.001

Table 2 Effect of Different component of MO on ALP activity (IU/l).

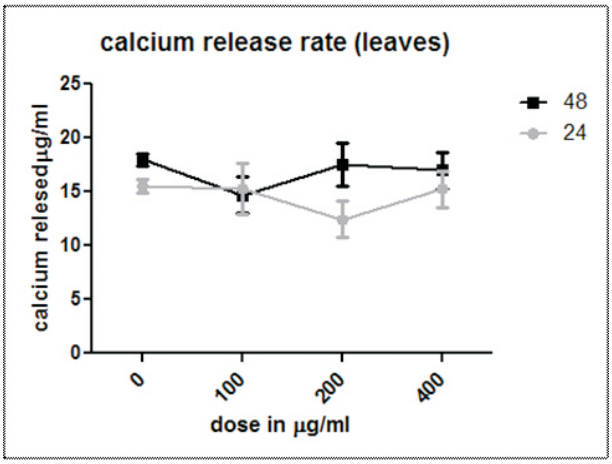
ALP activity		0 μg/mL	100 μg/mL	200 μg/mL	400 μg/mL
Flower	24	36.015 ± 1.260	39.456 ± .3650	42.365 ± 3.5470**	45.369 ± 1.3360**
	48	49.710 ± 1.360	52.652 ± 2.458	59.635 ± 2.336	66.000 ± 1.354**
Fruit	24	36.015 ± 1.260	36.978 ± 2.0	46.319 ± 1.245**	48.971 ± 2.330**
	48	49.710 ± 1.3600	40.939 ± 0.990	52.496 ± 1.3600	39.756 ± 2.120*
Leaves	24	36.015 ± 1.2600	45.632 ± 5.2630	41.236 ± 1.2365	52.360 ± 5.331*
	48	49.710 ± 1.360	42.368 ± 4.2360	54.569 ± 3.888	58.636 ± 3.333*

Values were expressed as Mean ± S.E.M. * - p < 0.05; ** - p < 0.01; *** - p < 0.001

Table 3 Effect of Different component of MO on TRAcP activity (IU/l).

TRAcP activity		0 μg/mL	100 μg/mL	200 μg/mL	400 μg/mL
Flower	24	24.230 ± 0.690	23.656 ± 1.236	15.363 ± 3.225	20.369 ± 1.223
	48	33.690 ±0.680	29.556 ± 1.363	22.362 ±1.999**	21.656 ± 2.668*
Fruit	24	24.230 ± 0.690	22.630 ± 0.560	20.630 ± 0.650	15.360 ± 0.660 **
	48	33.690 ± 0.680	26.360 ± 0.950	18.360 ± 0.450**	17.630 ± 0.810**
Leaves	24	24.230 ± 0.690	26.364 ± 2.336	22.456 ± 5.336	24.636 ± 5.662
	48	33.690 ± 0.680	36.525 ± 2.336	38.363 ± 2.565	30.565 ± 4.425

Values were expressed as Mean ± S.E.M. * - p < 0.05; ** - p < 0.01; *** - p < 0.001



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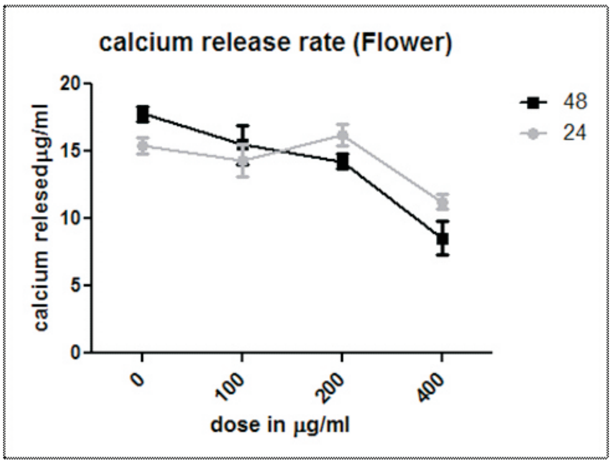

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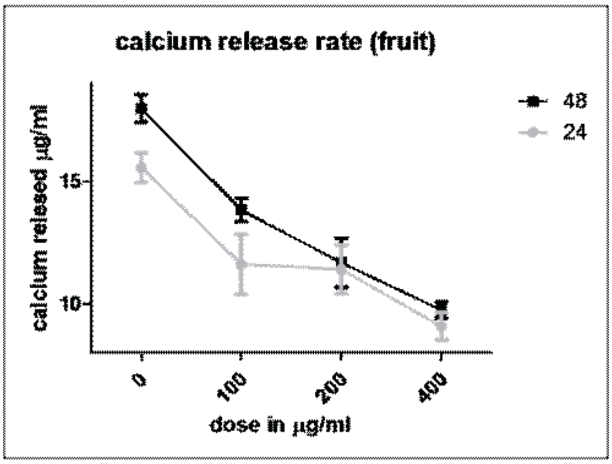
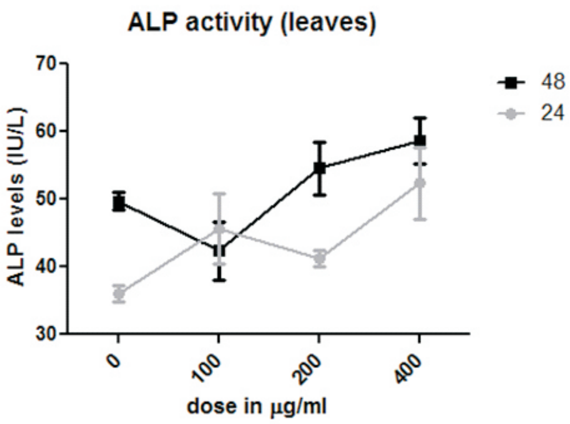


Figure 3

Figure 1: Cold Calcium Release rate (leaves); Figure 2:Cold Calcium Release rate (Flower); Figure 3: Cold Calcium Release rate (Fruit)



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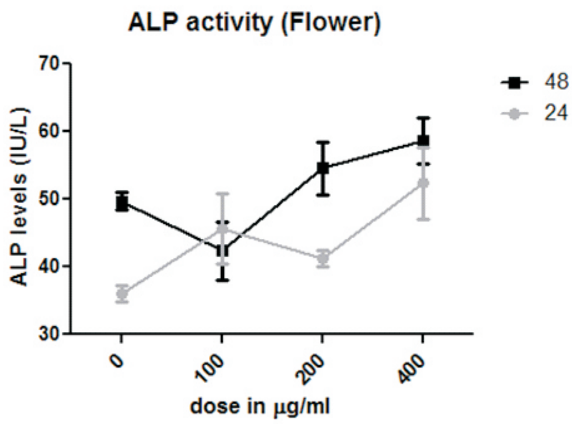

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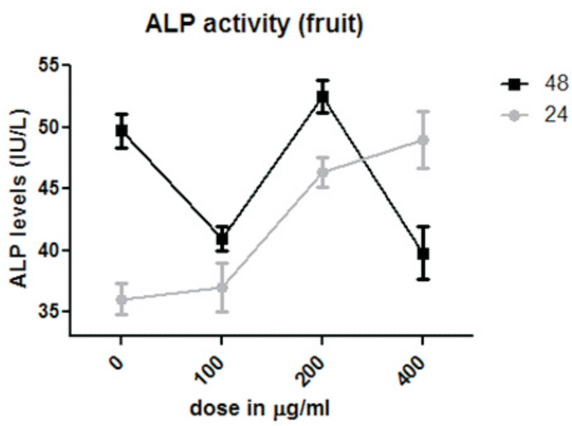
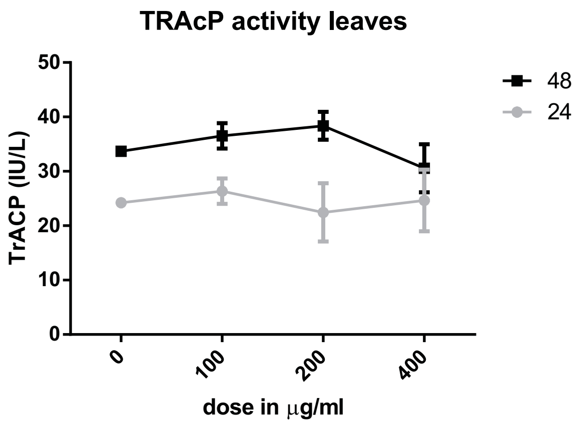


Figure 6

Figure 4: ALP activity (leaves); Figure 5: ALP activity (Flower);
Figure 6: ALP activity (Fruit)



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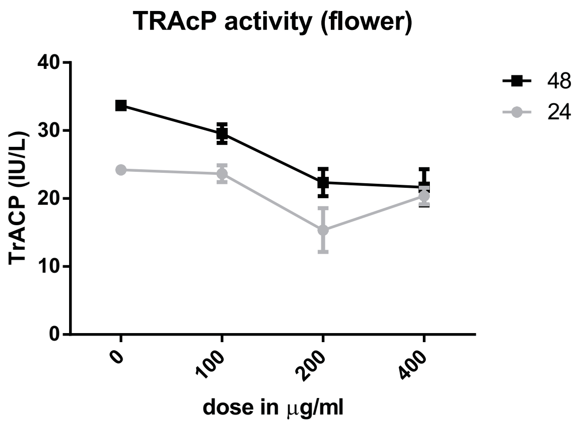

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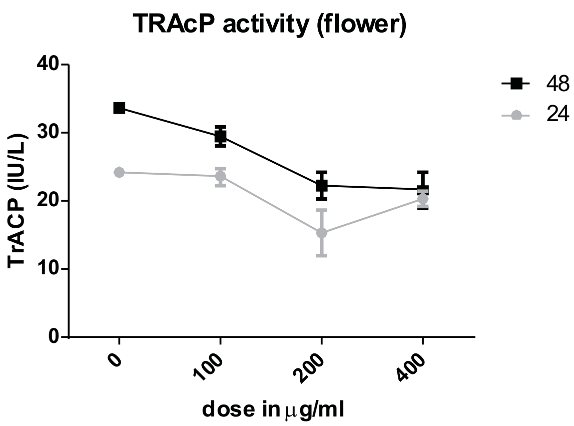
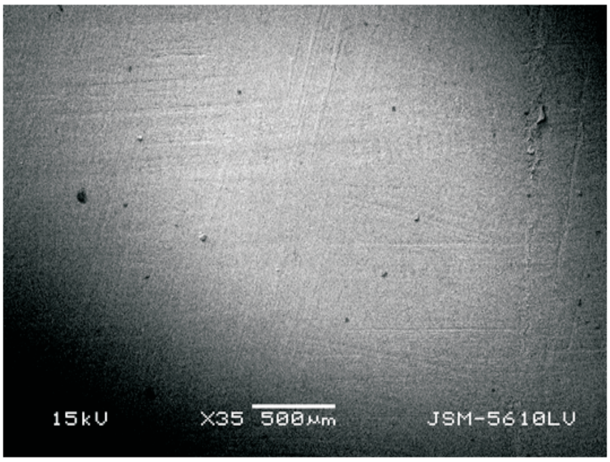


Figure 9

Figure 7: TrACP activity (leaves); Figure 8: TrACP activity (Flower);
Figure 9: TrACP activity (Fruit)



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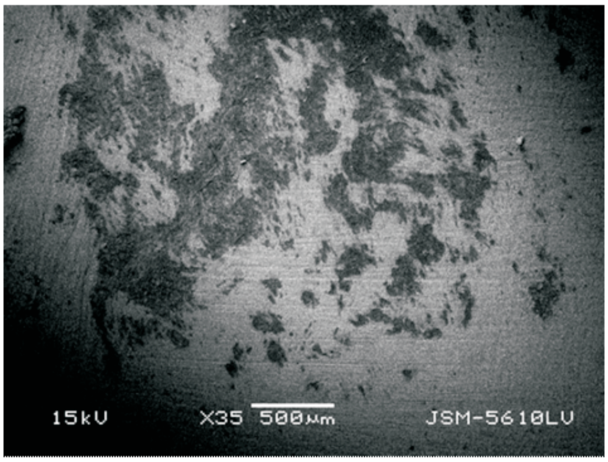

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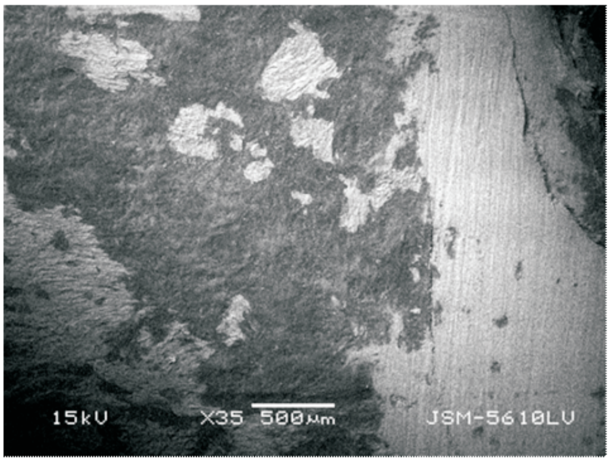

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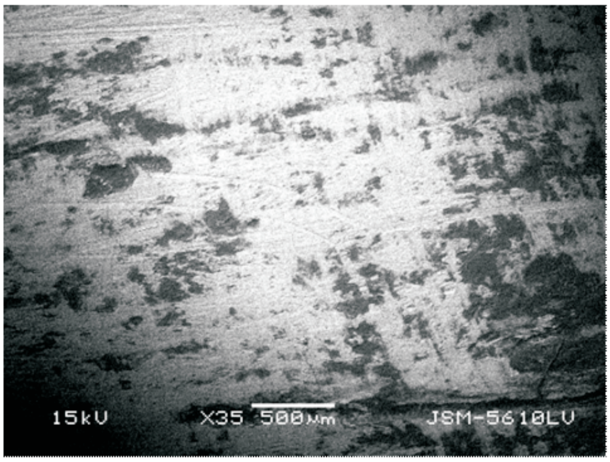

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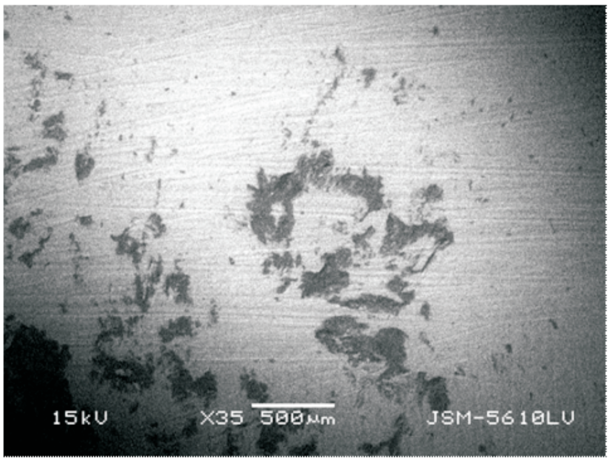

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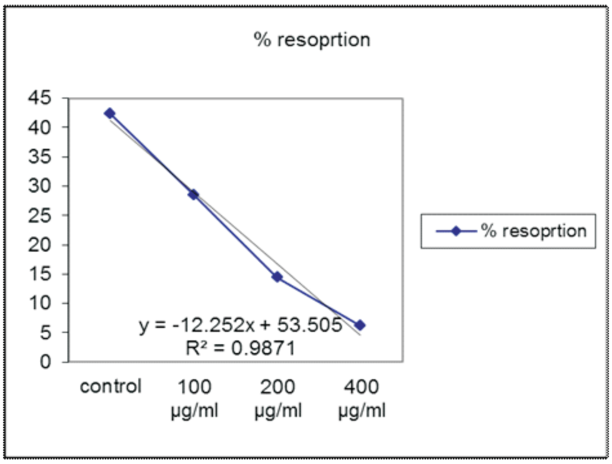
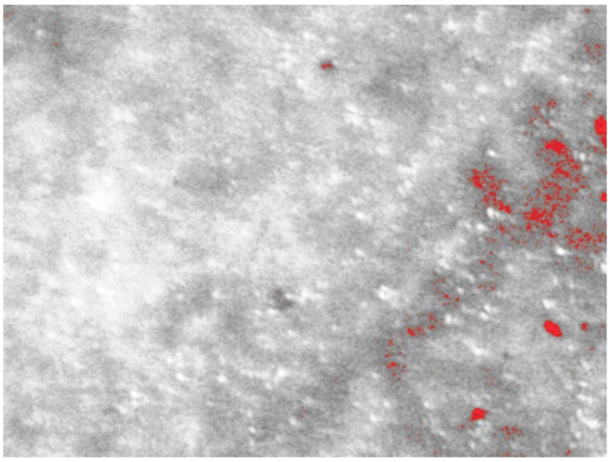


Figure 15

Figure 10 blank bone slice showing no resorption; Figure 11 Control slide showing excessive resorption; Figure 12 100µg/ml MO extract showing reduction in the intensity of resorption; Figure 13 200µg/ml MO extract showing decreased area resorption; Figure 14 400µg/ml MO extract treatment showing marked decrease in area as well as intensity of the resorption; Figure 15: Graphic representation of Decrease in resorption area due to MO treatment



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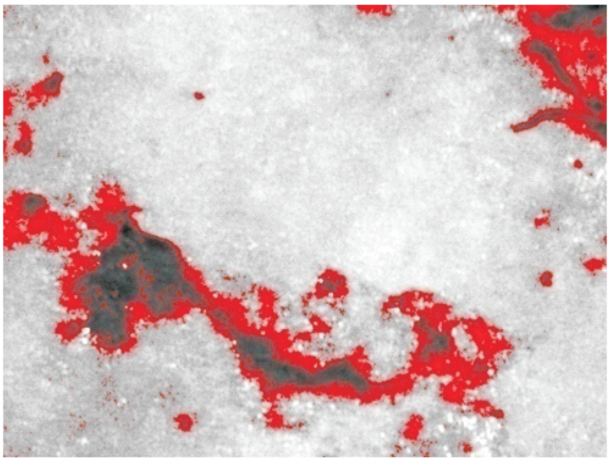

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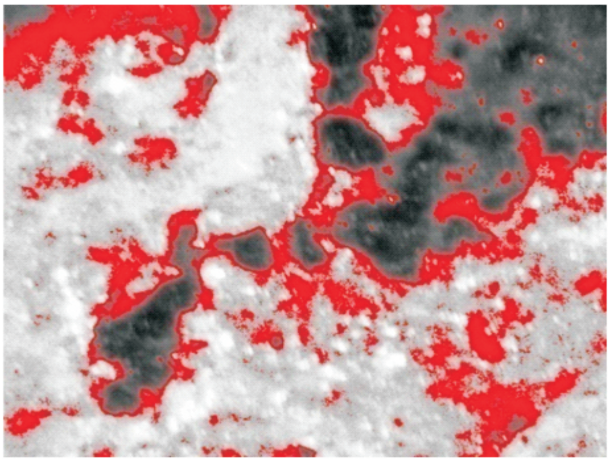

Figure 18

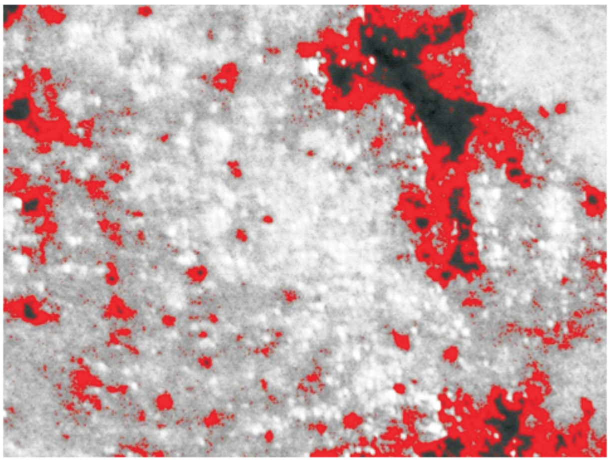

Figure 19

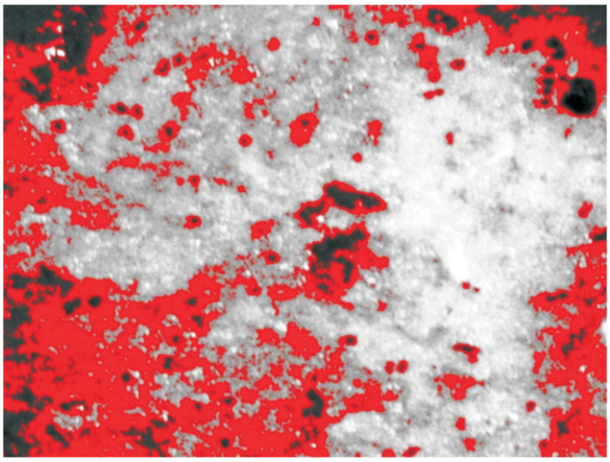

Figure 20

Figure16: control bone slice at 48 hours; Figure 17: 100 ug/ml of MO extract treatment at 24 hours; Figure 18: 100 ug/ml MO extract treatment at 48 hours; Figure 19: 200 ug/ml MO extract treatment at 24 hours; Figure 20: 200 ug/ml MO extract treatment at 48 hours

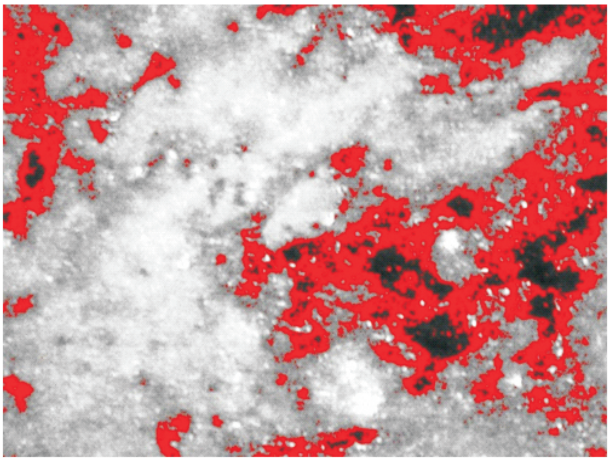

Figure 21

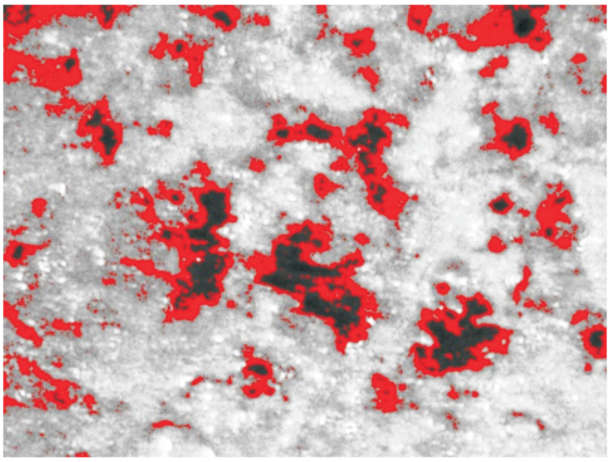

Figure 22

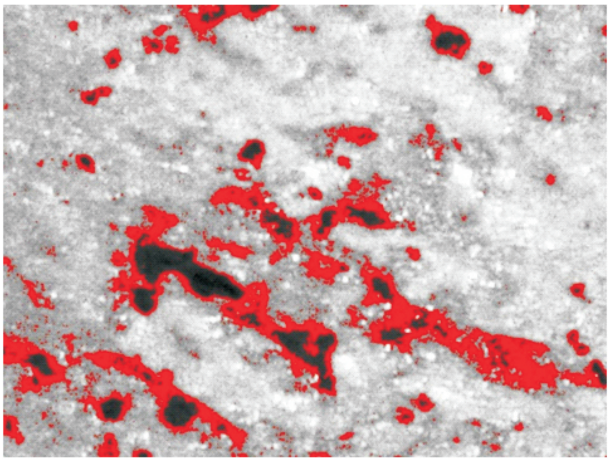

Figure 23

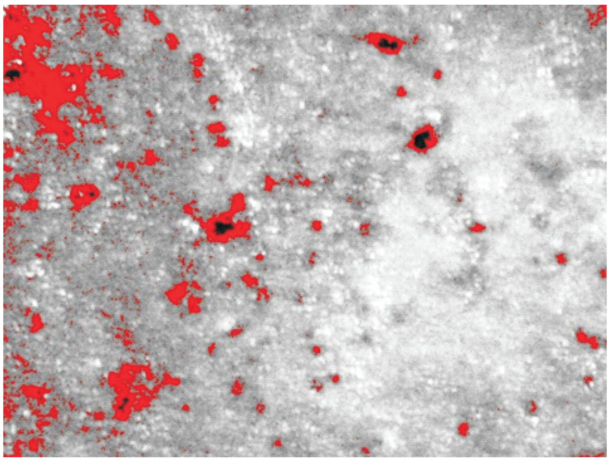

Figure 24

Figure 21: 200 ug/ml MO extract treatment at 24 hours; Figure 22: 200 ug/ml MO extract treatment at 48 hours; Figure 23: 400 ug/ml MO extract treatment at 24 hours; Figure 24: 400 ug/ml MO extract treatment at 48 hours.