CHAPTER 6

EFFECT OF DIFFERENT COMPONENTS OF *MORINGA OLIEFERA* ON OSTEOBLASTIC CELLS; SaOS 2

Introduction

Osteoporosis is a multifactorial disease influenced by genetic and various environmental factors. It leads to reduction in a bone mass and microarchitectural deterioration of the tissue, leading to skeletal fragility and possibilities of fracture, worsening the life of the patient. Osteoporosis arises due to loss of bone integrity which mainly depends on complexly coupled activity of osteoblastic bone formation and osteoclastic bone resorption (Rigs and Melton; 1992). Hence, osteoporotic drugs are designed targeting these two basic processes; especially post menopausal osteoporosis. Post menopausal bone loss is the most common cause of osteoporosis in females (Ganate *et al*., 2000). Estrogen Replacement Therapy can relieve the patient from few problems associated with post menopausal syndrome. However, this therapy is not having any compliance as it poses a greater threat of breast and endometrial cancer (Persson *et al.,* 1999). In addition, other alternative therapies developed in last few decades as pharmaceutical agents are not affordable for common man. Thus an alternative approach is required to develop new therapeutic drugs, which are safer, cheaper and easily available on global scale. Of all these alternative therapies, recently herbal medicine is gaining its importance because of its wider availability and cost effectiveness.

Osteoblasts are characterized by their ability to synthesize and secrete collagen like extra-cellular protein molecules and inducing the mineralization of this matrix via secretion of AlP like enzymes (Aubin, 1998; Lian et al., 1999). Hence, AlP activity is considered to be one of the most significant markers of osteoblastic activity. For studying osteoblastic activity *in vitro*, various cell lines have been developed, out of which SaOS 2 cell line has been considered to be the most promising cell line. Though transformed, it expresses all phenotypes of osteoblastic cells making them a good model for studying osteoporosis *in vitro* (Rodan *et al.,* 1987; Rao *et al*., 2000).

After conducting several experiments on MO and understanding its osteoprotective effect, it was learned that this plant is having miraculous effect on bone integrity and it potently prevents osteoporosis. Various workers have indicated that this plant is having various phytochemicals, especially phytoestrogens which can have positive effect on bone. Recent work by Wang and co

workers (2008) has shown that flavinoids can stimulate osteoblastic proliferation and differentiation. In another study, Vali and his collogues (2007) also proved that Epigallocatechin-3-gallate like flavinoids induce bone mineralization and bone nodule formation. As MO flower and fruit are rich in various flavinoids and phytoestrogens, one can speculate about osteoblastogenic potential of this plant. Hence, it was designed to explore the components of MO for their effect on osteoblastic cells.

METHODS AND MATERIALS

Chemicals: Sodium β glyecerophosphate was purchased from Sigma Chemicals. Methanol, Triton X – 100 were of Domestic AR grade products. DMEM, 100 X antimicrobial antifungal solutions, Accutase and FBS were purchased for High Media Chemicals. 0.23µ filter were purchased from Sartorius. Calcium and Alkaline Phosphatase (AlP) Kits were purchased from Reckon Diagnostic Kits Pvt. Ltd.

Preparation of Extract: Fruits, leaves and flowers of MO was obtained and standardized as per our previous studies (Rangrez *et al.,* 2011). 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.

SaOS 2 cell line culture:

SaOS 2 cell line was obtained from NCCS, Pune and cultured as described previously (Thangakumaran *et al.,* 2009). Briefly, the cell line was procured from the national center for cell sciences (NCCS), Pune, India. The cells were cultured in a humidified atmosphere (95% air, 5% CO_2) at 37º C in Dulbecco's Modified Eagle's Medium (DMEM, High Media, Bombay) containing 1% anti microbial anti fungal solution (High Media, Bombay), supplemented with 15% FBS (High Media, Bombay). Upon reaching confluence, the cells were detached using Accutase (High Media, Bombay) and Loaded in 96 well plate (Merck Scientific, Bombay) for culturing for 96 hours in 6 increasing concentration (10 µg/mL, 20 µg/mL, 50 µg/mL, 100 µg/mL, 200 µg/mL, 400 µg/mL) of all three different extracts. After 96 hours, MTT and AlP assays were carried out to understand the effect of the plant extract on osteoblastic cells.

MTT test

Saos-2 were incubated in a DMEM containing 15% FBS under partial pressure of 5% CO_2 at 37 º C. MTT assay was carried out by placing each cell into each well of 96-well plate; adding 0.05% DMSO containing samples into each well and incubating them for 72 h; adding 0.5 mg/mL MTT into each well and further incubating them for 4 h; dissolving produced formazan crystals in DMSO; and measuring their absorbencies at 550 nm using ELISA reader as described previously (Ha *et al.,* 2003).

Determination of Hydroxyproline and Calcium Content

For estimation of hydroxyproline and Calcium in the culture, After 14 days of culture, the culture was lysed in 6N HCl for 24 hours as described previously (Woessner et al., Roveri *et al.,* 2000). After 24 hours, the solution was filtered, concentrated on sand bath to yield a white crystalline powder. This powder is then dissolved in Tris buffer pH 8.8 for estimation of Calcium and hydroxyproline.

Estimation of Hydroxyproline

Hydroxyproline estimation was carried out by the method of Neuman and Logan. Briefly, The hydrolyzed samples were evaporated to dryness in a boiling water bath to remove acid, and the residue was dissolved in distilled water and made up to a known volume. It was decolorized with activated charcoal and filtered through Whatman filter paper (Tewksbury, MA, USA). For the determination of hydroxyproline, 1 mL of the clear filtrate was mixed with 1 mL of freshly prepared chloramine-T solution and allowed to stand for 20 min. The samples were further mixed with 1 mL of 3.15 mol/L perchloric acid and waited for 5 min. Finally, 1 mL of freshly prepared pdimethylaminobenzaldehyde was added, mixed well, and placed in a water bath at 60 °C for 20 min. The absorbance of the solution was determined by using a spectrophotometer at 560 nm.

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).

Results

Growth of osteosarcoma

SaOS 2 has been an established cell line for the purpose of bone research (Rodan *et al.,* 1987; Richard *et al.,* 1997). During the experimental period, there was no evidence of toxicity to the cells or no signs of bacterial or fungal contamination on the well chamber. The cells were found to be growing well in the culture medium after passaging.

MTT assay: The general principle for the detection of cell growth or cell kills via the MTT cytotoxicity assay is the conversion of the tetrazolium salt (MTT) to the coloured product formazan. The formation of formazan takes place via intact mitochondria. An advantage of using cell lines is to investigate fundamental aspects of drug-metabolism-linked toxicity. For MTT assay the effectiveness of the agent in causing death, or changing metabolism of cells, can be deduced through the production of a dose-response curve. The effect of all the three components is represented in Table 1. Of all the 3 components, MO leaf extract did not show any osteoblastogenic potential. In addition it was found to be toxic at higher doses, where it reduced AlP activity to 62% at 400 µg/mL dosage (Figure 1). Flower extract showed most promising results where it increased the cell viability in a dose dependent manner. However, at very high dose of 200 µg/mL and 400 µg/mL it did not show further increase; suggesting that its therapeutic dose is between 50 µg/mL to 100 µg/mL (29% increase) (figure 2). Fruit extract also showed osteoblast stimulating potential, but its efficacy was lower compared to flower extract, where maximum stimulation was observed with 100 µg/mL dosage (18% increase, p value <0.01) (figure 3). At further higher doses, it lost its osteoblast stimulating potential.

AlP Activity

AlP activity is one of the most established markers of studying osteoblastic activity. Our results indicated that only flower and fruit extract had osteoblast stimulating potential; while Leaf extract was found to be inhibiting osteoblastic activity (Table 2). Though at lower doses leaf extract did show insignificant increase in osteoblast activity, at higher doses it was found to be inhibiting AlP activity (Figure 4). Flower extracts showed more promising results where it increased AlP activity in a dose dependent manner; showing almost 4 fold increase in AlP activity at 400 µg/mL dosage. It started exerting its positive effect on osteoblastic cells at even a very low dose of 10 µg/mL where it showed 2 fold increase in AlP activity(p value<0.05) (Figure 5). MO fruit

showed similar dose dependent effect; where it showed 6 fold increase in AlP activity at 200 µg/mL dosage. However, further dose dependency was not observed with 400 µg/mL dosage (Figure 6). Results of AlP activity showed that both flower and fruit of MO had potent effect on increasing the AlP activity by osteoblastic cells.

Calcium content in the lysate

It was observed from the previous studies that MO components exert their effectiveness only at higher doses of 100 µg/mL, 200 µg/mL and 400 µg/mL, Hence, calcium estimation in the given lysate was carried out only for these 3 doses. Table 3 shows variation in calcium content due to MO extracts treatment. As expect, leaf extract did not show any increase in the calcium content. At 400 µg/mL it decreased the calcium content; but the data was non-significant (Figure 7). Flower extract showed increase in the calcium content; with maximum calcium content observed with 400 µg/mL extract treatment (33% increase) (figure 8). Fruit extract was found to be most potent in increasing the calcium content in the lysate. At 200 µg/mL dosage it showed almost 50% increase in the calcium content, suggesting its mineralizing potential (figure 9). However, the data was not found to be dose dependent, as at 400 µg/mL dosage it showed non-significant increase in calcium content.

Hydroxyproline content in the lysate

Hydroxyproline is one the most important amino acids that play a key role in collagen assembly in the bone matrix synthesis. Effect of various components of MO is shown in table 4. Leaf extract had negative effects on hydroxyproline content in the lysate. At 400 µg/mL dosage it showed negative effect on hydroxyproline content; where it reduced hydroxyproline levels to 50% compared to vehicle (figure 10). Flower extract showed non-significant increase in the hydrxyproline levels (figure 11). Fruit extract showed promising results; where it boosted the hydroxyproline levels and maximum effect was observed at 100 µg/mL dosage where it almost doubled the hydroxyproline content in the lysate (Figure 12). At higher doses of 200 µg/mL and 400 µg/mL also it showed increase in the hydroxyproline content.

Discussion

In the present study we have shown that MO, one of the most commonly used food plant in India is having osteoblast stimulating property. Of all the three components of MO, fruit and flower

extract were having significant osteoblast proliferating and stimulating property. Our data provided first evidence about the cellular means of the osteoprotective effect of this plant observed in previous studies. It has already been established that MO is having positive effect on bone and it protects bone from ovariectomy induced bone loss (Burali et al., 2010). In a recent study by vali and coworkers (2007) it was shown that certain flavinoids can have positive effect on bone nodule formation *in vitro*. As MO is rich in certain flavinoids (ref); they might be the one playing a key role in stimulating osteoblastic cells. In the previous studies (Burali et al., 2010) where MO fruit extract was shown to be having positive effect on bone loss; it was also shown to be having positive effect on calcium balance. Apart from this our previous studies in vivo confirmed these results and showed that MO prevents bone loss. However, whether it promotes bone formation or prevents bone loss, was solved by the findings of these study; which showed that MO flower and fruit extract not only increased the AlP activity; it also increased the calcium content in the culture; suggesting that MO increases the osteoblastic activity; leading to more bone formation.

Though various studies have established the role of MO fruit extract on different cell types; very few studies have been conducted till now on flower extract. In this study, we also discovered that MO flower is not only increasing the activity of osteoblastic cells; it also promotes the osteoblastic cell division, making it a more potent osteoprotective agent. At higher doses where MO fruit extract showed lower activity, flower extract was found to be having dose dependent activity on all parameters considered for this study. To our knowledge, this study is the first to explore various components of MO for their osteoprotective effect *in vitro* on osteoblastic cells SaOS 2.

Osteosarcoma cell line SaOS 2 serves as an ideal replacement for primary culture of Human Osteoblastic cells obtained by either human explants technique (Kung et al., 1995) or human bone marrow (Kassem et al., 1991). Primary culture of human osteoblastic cells is a tedious process and they can be used only once after first passaging (Robey et al., 1985). Moreover they grow very slowly and require long times for a confluent growth (Wong et al., 1990). Hence, their maintenance is costly and they change their phenotype after few passaging. Because of their heterogenous nature, response of human osteoblastic cells is also age dependent and hence in last decade, transformed cell lines are gaining importance (Matsuyama et al., 1990). Of all the transformed cell lines, SaOS 2 is considered to be the best; because of their stable phenotype, fast

growth and expression of all osteoblastic features; including bone nodule formation (Manduka et al., 1993).

Bone formation is a 3 staged process, namely; proliferation of osteoblasts; secretion of extract cellular matrix by osteoblasts and mineralization (Aubin, 1998). AlP activity is an established marker of osteoblast activity. We observed that MO flower and fruit extract treatment increases the AlP activity. This rise in the AlP activity with herbal treatment indicates that MO extract differentiates osteoblastic cells towards the differentiated bone forming phenotype. Of all the three components, leaf extract did not show any rise in AlP activity. Compared to leaf extract, both flower and fruit showed positive effect on this osteoblastic function marker. However, flower was found to be less potent in stimulating AlP activity compared to fruit. But when this data was considered with MTT assay, it showed contrasting results where fruit extract was found to be promoting osteoblast cell division. Hence, by combining this two results, one develops an understanding that MO fruit extract increases the number of osteoblastic cells, favoring their proliferation, while fruit extract not only increases the number but also stimulates them to undergo differentiation into mature bone forming cells. Hence, both the extracts, though favoring bone formation, their mechanism of action was different, as flower helps to increase the osteoblast number, while fruit increases its activity and mineralization as well.

It is an established fact that osteoblast number or AlP alone does not account for the bone formation. Hence, we used both calcium and hydroxyproline estimation to confirm the osteoblastic activity. Calcium is the chief constituent of the bone mineral hydroxyapetite, while hydroxyproline is an important aminoacid required for the formation of collagen; constituent of the bone matrix (ref.). Our results, however, showed positive activity on both the parameters, suggesting that these plant components are having positive effect on bone osteoblastic cell growth, their activity, synthesis of bone matrix and its mineralization.

In summary, our results demonstrated that MO components are having positive effect on osteoblastic cells. Of all the three components; leaf, flower and fruit, leaf extract was not found to be having any effect on osteoblastic cells. Compared to leaf, flower and fruit extracts were found to be having positive effect on bone cells. Flower extract was found to be increasing the number of osteoblastic cells; while the fruit extract was having more elaborative effect as it increased AlP activity, induced bone formation, increased Collagen content and bone mineral formation. This

study explains that MO fruit and flower extract are having positive effect on bone cells and justifies its previous reports as a potent osteoprotective agent.

Table 1 Effect of different components of MO on SaOS 2 cell viability using MTT assay.

Dose	**Vehicle**	**10 µg/mL**	**20µg/mL**	**50µg/mL**	**100 µg/mL**	**200µg/mL**	**400µg/mL**
Leaves	98.236 ± 5.360	102.360 ± 5.310	106.360 ± 9.360	101.362 ± 8.983	112.489 ± 7.938	93.263 ± 7.793	62.369*** ± 3.384
Flower		104.260 ± 3.690	108.560 ± 4.230	112.456 ± 9.360	129.256*** ± 8.451	124.845** ± 8.100	129.450*** ± 4.236
Fruit		96.453 ± 4.123	106.230 ± 9.180	110.120 ± 3.245	118.423** ± 6.360	100.230 ± 5.360	103.810 ± 5.890

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

Table 2 Effect of different components of MO on SaOS 2 AlP activity

Dose	**Vehicle**	**10 µg/mL**	**20µg/mL**	**50µg/mL**	**100 µg/mL**	**200µg/mL**	**400µg/mL**
Leaves	9.711 ± 1.529	6.474 ± 2.411	13.000 ± 0.658	12.568 ± 2.662	6.235 ± 3.957	4.336** ± 2.733	4.356** ± 4.181
Flower		17.536* ± 0.223	14.360 ±3.471	17.356 ± 5.636	29.135*** ± 3.690	35.690*** ± 3.706	26.459*** ± 3.384
Fruit		12.325 ± 3.552	14.230 ± 3.471	25.360** ± 4.953	55.033*** ± 4.022	39.380*** ±7.793	33.373*** ± 3.384

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

Table 3 Effect of different components of MO on Calcium content in the lysate

Dose	**Vehicle**	**100 µg/mL**	**200µg/mL**	**400µg/mL**
Leaves	29.256 ± 2.354	24.256 ± 4.256	26.563 ± 4.528	22.158 ± 3.265
Flower		34.125 ± 1.236	31.256 ±3.669	39.225** ± 2.335
Fruit		39.254* ± 4.112	45.118** ± 4.226	35.263 ± 3.125

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

Table 4 Effect of different components of MO on Hydroxyproline content in the lysate

Dose	**Vehical**	**100 µg/ml**	**200µg/ml**	**400µg/ml**
Leaves	13.250 ± 1.458	7.236 ± 4.236	11.236 ± 5.360	7.245 ± 4.236
Flower		18.236 ± 1.220	15.362 ± 5.360	11.845 ± 5.636
Fruit		26.152** ± 1.332	22.452* ± 1.336	24.665** ± 6.985

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

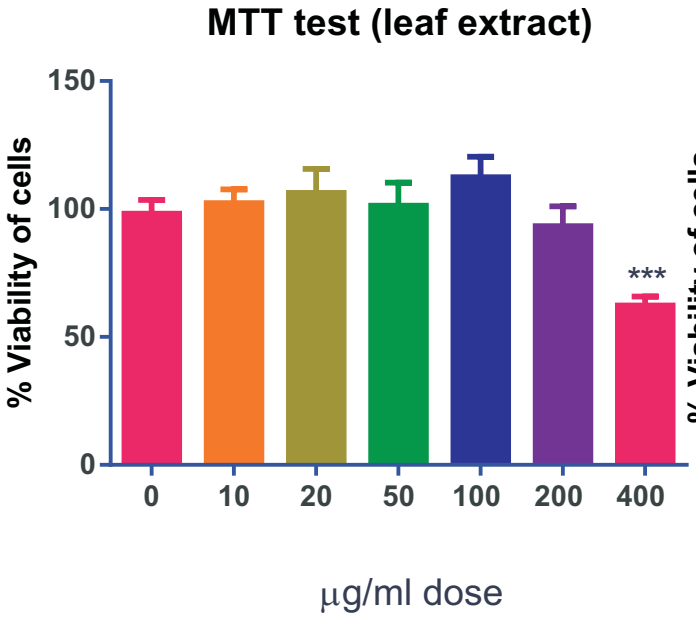


Figure 1

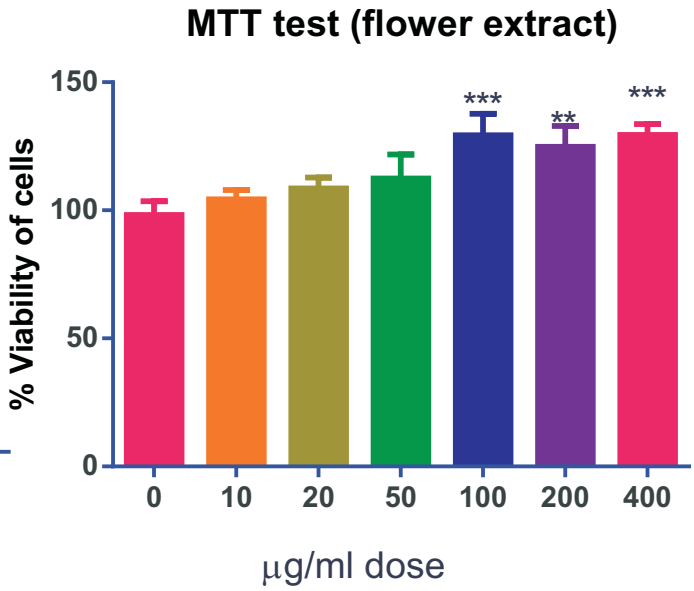


Figure 2

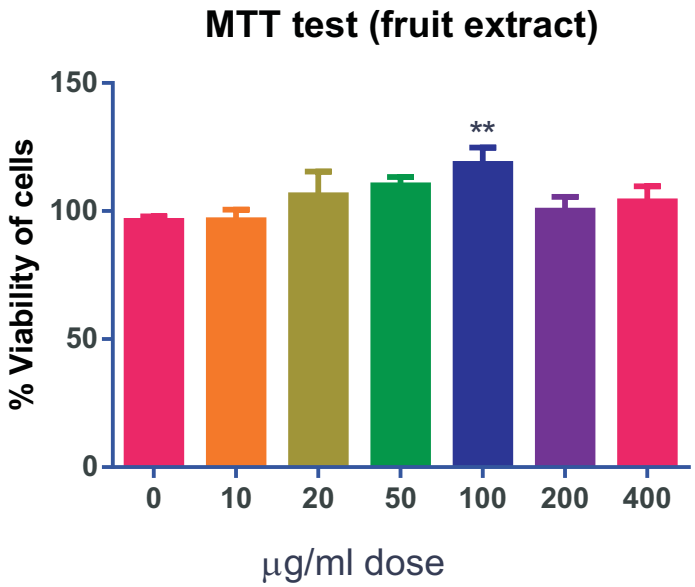


Figure 3

Figure 1: MTT test (leaves); Figure 2:MTT test (Flower);
Figure 3: MTT test (Fruit)

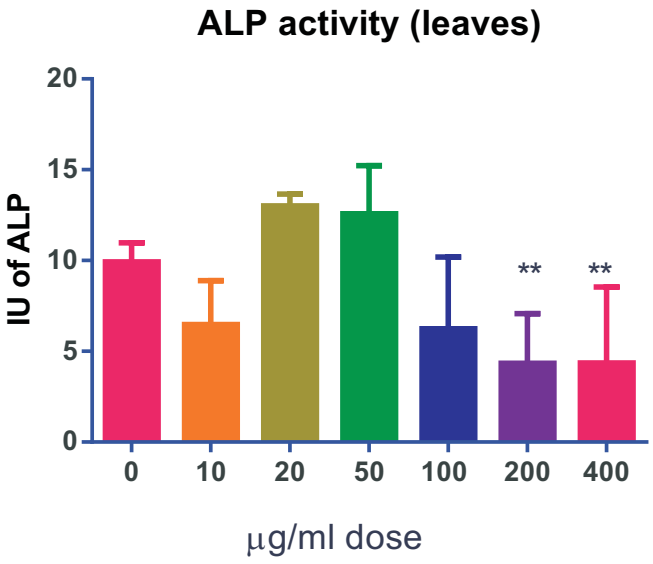


Figure 4

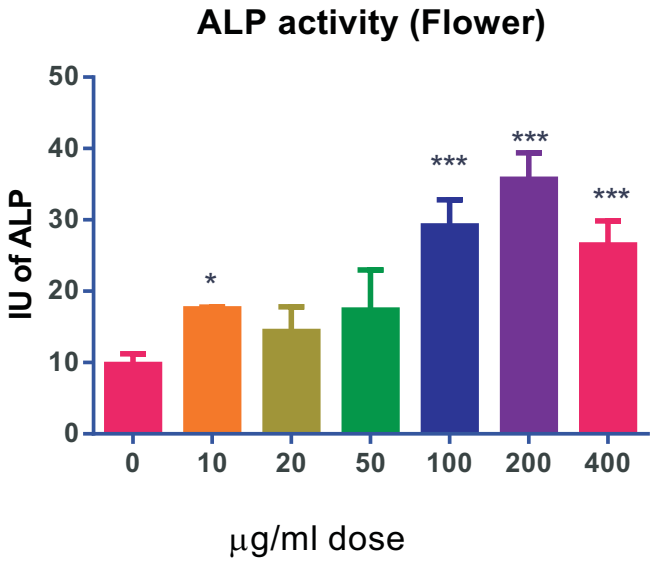


Figure 5

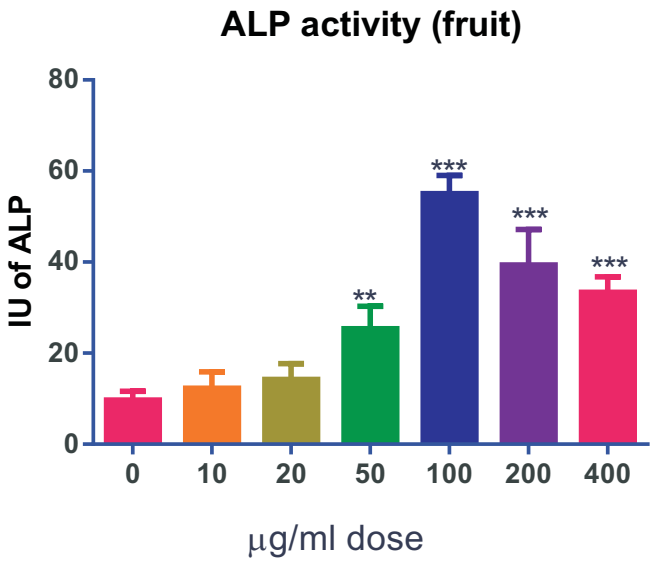
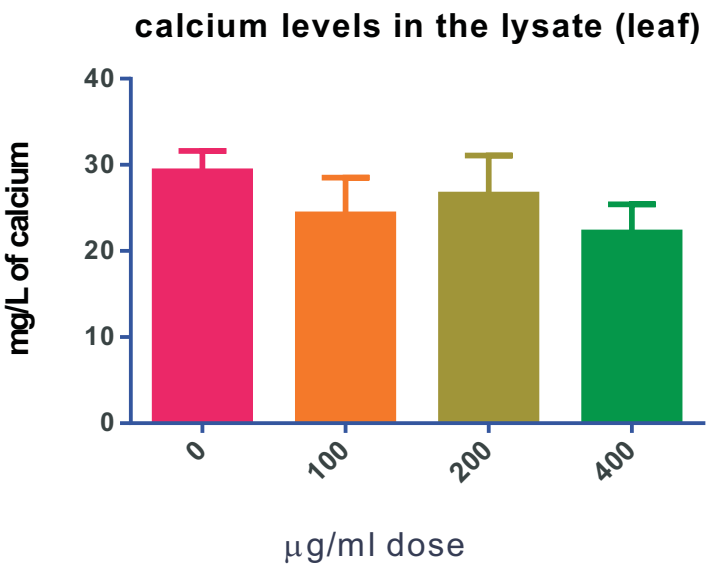


Figure 6

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



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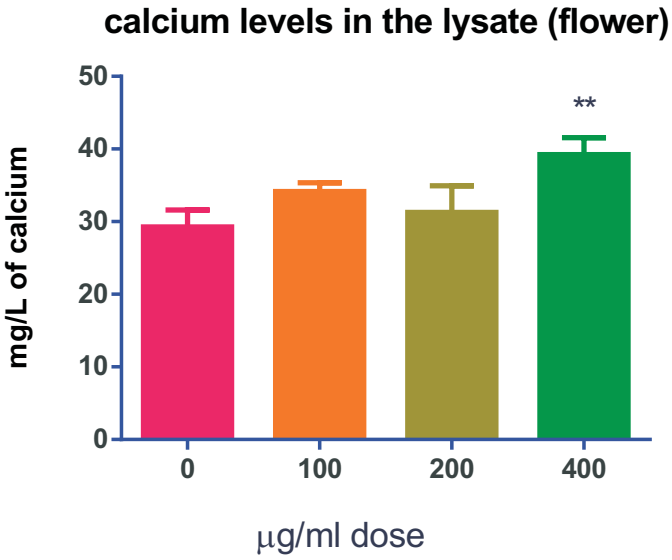


Figure 8

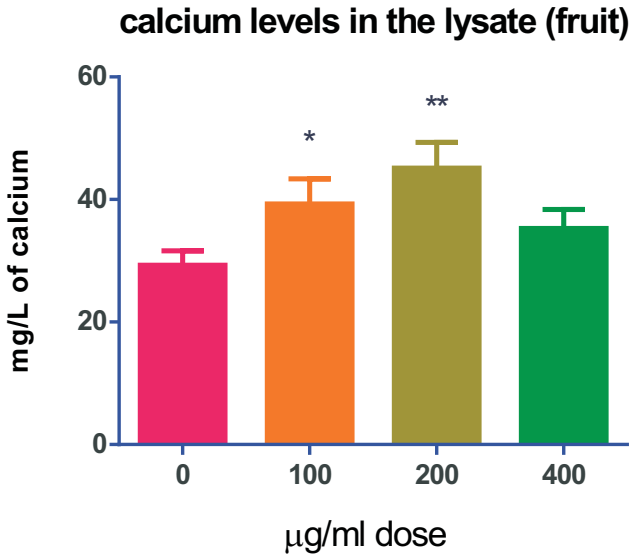


Figure 9

Figure 7: Calcium levels in the lysate (leaves); Figure 8: Calcium levels in the lysate (Flower); Figure 9: Calcium levels in the lysate (Fruit)

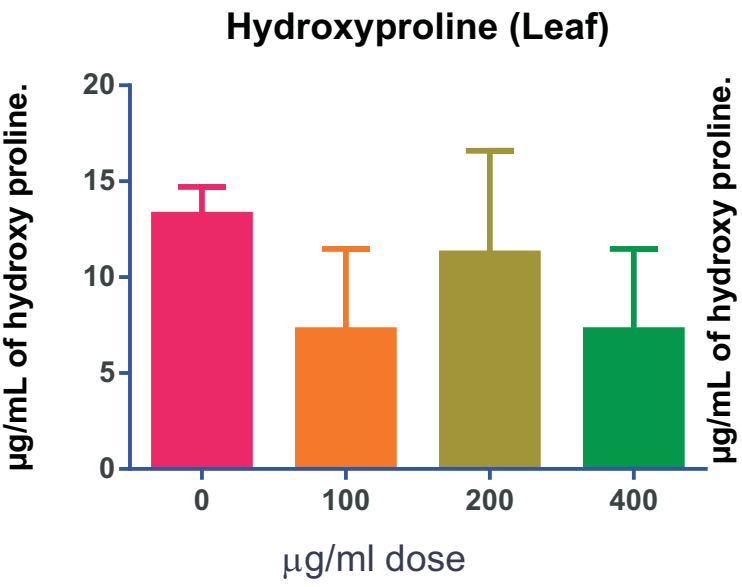
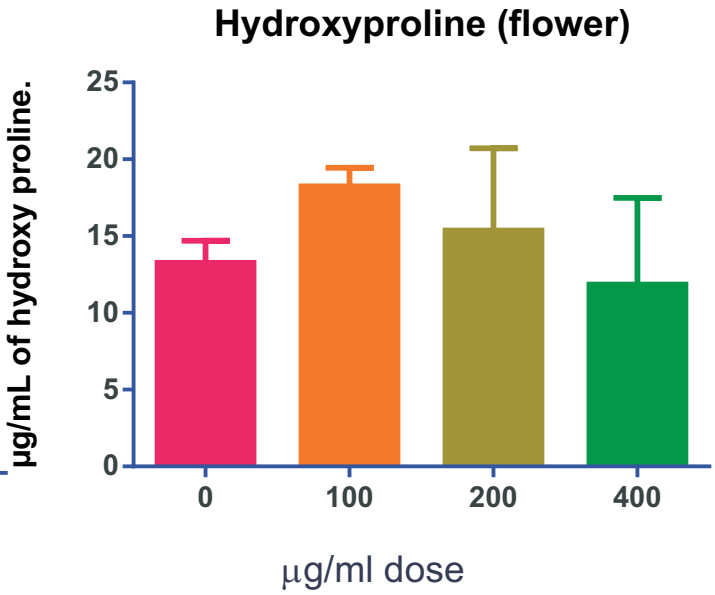


Figure 10



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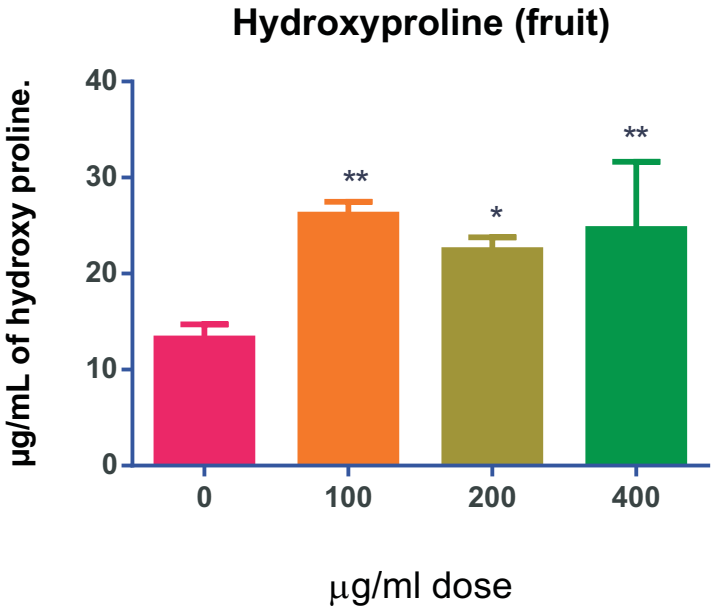

Figure 12

Figure 10: Hydroxyproline levels in the lysate (leaves); Figure 11: Hydroxyproline levels in the lysate (Flower); Figure 12: Hydroxyproline levels in the lysate (Fruit)