CHAPTER 5

IN VITRO BONE REMODELLING PROPERTIES OF VARIOUS PLANT EXTRACTS ON BONE CELLS.

In vivo studies proved osteogenic property of the botanicals. However, little is known about the effect of the botanicals in *in vitro* conditions on bone cells. The aim of this study was to determine the effect of plant extracts on the bone cells for their remodeling property on calvarial co culture system as well as SaOS 2 osteoblastic cells.

INTRODUCTION:

Bone is a dynamic organ constantly remodeled to support calcium homeostasis and structural needs. The osteoclast is the cell responsible for removing both the organic and inorganic components of bone. It is derived from hematopoietic progenitors in the macrophage lineage and differentiates in response to the tumor necrosis factor family cytokine receptor activator of NFκB ligand. αvβ3 integrin mediates cell adhesion necessary for polarization and formation of an isolated, acidified resorptive microenvironment. Defects in osteoclast function, whether genetic or iatrogenic, may increase bone mass but lead to poor bone quality and a high fracture risk. Pathological stimulation of osteoclast formation and resorption occurs in postmenopausal osteoporosis, inflammatory arthritis, and metastasis of tumors to bone. In these diseases, osteoclast activity causes bone loss that leads to pain, deformity, and fracture. Thus, osteoclasts are critical for normal bone function, but their activity must be controlled. Increased bone resorption mediated by osteoclasts is central to pathogenesis of osteoporosis (Qui *et al.,* 2007). In osteoporosis, the formation and function of osteoblasts decreases whereas osteoclast formation and recruitment increases, and this causes a relative increase of bone resorption over bone formation.

The specific bone formation markers are alkaline phosphatase (AlP) , osteocalcin and collagen synthesis; and the bone resorption markers are tartrate-resistant acid phosphatase activity (TRAcP) and multinucleated cell formation (Wu *et al.,* 2009b). Apart from osteoporosis, there is increasing evidence to believe that osteoclasts play a crucial role in rheumatoid arthritis as well. Abundant osteoclasts are found within the synovial tissue at sites adjacent to bone, creating resorption pits and local bone destruction followed by degradation of bone matrix and calcium solublization. Osteoclasts thus play a role in joint inflammation and structural damage. Hence, therapeutic approaches inhibiting osteoclasts and stimulating osteoblasts have been proposed for osteoporosis and rheumatoid arthritis (Penolazzi *et al.,* 2008; Wu *et al.,* 2009b). In the past various scientists reported that plants with osteoprotective effect have been found to have osteoblasts stimulating or osteoclast inhibiting property (Potter *et al.,* 1998; Yamaguchi *et al.,* 2002; Blum et al., 2003; Lai and Yamaguchi, 2006; Yamaguchi *et al.,* 2007; Yan-bin *et al.,* 2009). Lee and co workers (2008) had reported that emodin, a naturally occurring anthroquinone present in the roots and bark

of numerous plants could increase bone formation through activation the mRNA expression of bone morphogenic protein -2 in mouse osteoblastic MC3T3-E1 cell line.

Indian herbal medicine has been widely used for the treatment of many diseases from time immemorial (Khare, 2007; Parikh *et al.,* 2009). *Moringa oleifera* is a plant described in *Ayurveda* (Indian system of medicine) and belonging to the family *Moringaceae* (Khare, 2007; Aney *et al.,* 2009). *M. oleifera* is a highly valued plant, distributed in many countries of the tropics and subtropics. *Moringa* is nature's medicine cabinet (Aney *et al.,* 2009). It is best known as an excellent source of nutrition and is a natural energy booster. Different parts of this plant are being employed for the treatment of various ailments in the indigenous system of medicine, and its fruits are used as food in entire India (Manjari *et al.,* 2007). Moreover, *M. oleifera* is reported to regulate a myriad of cellular activities (Ezeamuzie *et al.,* 1996; Hukkeri *et al.,* 2006; Sukh Dev, 2006; Sutar *et al.,* 2008) and anti inflammatory activity (Hukkeri *et al.,* 2006; Sukh Dev, 2006; Khare, 2007). Further, it has been documented that plants with strong anti inflammatory properties also exhibit osteoclast inhibiting property and can be used for the treatment of osteoporosis and rheumatoid arthritis (Penolazzi *et al.,* 2008). Thus preventing, osteoclasts from activation could be a possible avenue to probe for better management of osteoporosis and rheumatoid arthritis. Recently, this plant has been shown to have osteoprotective effect in Ovariectomy induced osteoporosis (Burali *et al.,* 2010). Thus, the *In vivo* studies will help us in the better understanding its osteoprotective role.

Litsea has been described as promoter of longevity, promoter of semen generation and emollient. Sap of fresh bark or its decoction is prescribed as a remedy for diarrhoea, dysentery, rheumatism, and as an aid to longevity (Devi and Meera, 2010). In our previous in vivo studies it had been proved that *Litsea glutinosa* is having osteoprotective effect by ameliorating ovariectomy induced changes in bone and does not affect the uterus (Parikh *et al.,* 2009; Rangrez *et al.,* 2011). Present studies (chapter 2 and 3) have further proved potent osteoprotective effect of the plant on various biochemical markers and bone histoarchitecture. Thus it might be worth studying the effect of this plant on bone cells and develop a cellular understanding about its osteoprotective role.

Curcuma aromatica, is a common herb used in Indian medicine for treating inflammation and related diseases. It is also used as a flavoring agent, condiment and a source of yellow dye (Ambasta, 2001). The essential oils of *Curcuma* revealed the presence of various mono and sesquiterpenes. Early studies also showed the presence of curcumol in oil. The plant has also widely studied various pharmacological activities like anti angiogenic, cholerctic and cholagougic, anthelmintic, anti microbial, wound healing, antitumour, antioxidant, cytoprotective etc. (Ahmed *et al.,* 2010). *In vivo* studies of Rangrez *et al* (2011) have proved the osteoprotective property of the plant. However, the cellular mechanism behind its role is the objective of the present study.

METHODS AND MATERIALS

Preparation of Extract:

Litsea glutinosa and *Curcuma aromatica* bark powder were purchased from local herbal shop, which was confirmed to be free from any of the fungal or bacterial growth. 20 gm powder was extracted with 500 ml methanol in Soxhlet's apparatus for 48 hours. Methanolic extracts was dried on water bath at 60°C. Percentage yield of the plants were found to be 6.66% for *Litsea glutinosa* and 9.52% for *Curcuma aromatic. M. oleifera* pods were collected from the local cultivators by prior permission near Vadodara city. They were brought to the Laboratory of the Department of .The seed was identified and voucher sample deposited at the herbarium unit of the Department of Botany, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara. Dried fruit powder was prepared by drying complete fruit of *Moringa* in oven at 50°C. 20 gm dried powder was extracted with 500 ml methanol in Soxhlet's apparatus for 48 hrs. Methanolic extract was dried on water bath at 55° C. The plant was found to yield 9.8% extract. The plant extracts were dried and stored at -70° C. Working solution was prepared by dissolving the extracts in Dulbecco's Modified Eagle's Medium (DMEM) obtained from Himedia Chemicals, Mumbai, and filtered using 0.23μ filter (Sartorius, Göttingen, Germany).

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 37° C in Dulbecco's Modified Eagle's Medium (DMEM, High Media,

Bombay) containing 1% anti microbial anti fungal solution (High Media, Bombay), supplemented with 10% FBS (High Media, Bombay). Upon reaching confluence, the cells were detached using trypsin EDTA solution (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. Acridine orange Ethidium bromide staining was carried out to observe any toxic effect of this plant.

MTT test

Saos-2 were incubated in a DMEM containing 10% FBS under partial pressure of 5% CO_2 at 37 ° 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).

Acridine Orange Ethidium bromide staining of Saos 2:

Acridine orange (AO) is taken up by both viable and nonviable cells and emits green fluorescence if intercalated in to double stranded nucleic acid (DNA). Ethidium bromide (EB) is taken up only by nonviable cells and emits orange red fluorescence. One microlitre of dye mixture (100 μg/mL of AO and 100 μg/mL of EB in miliQ water) was mixed with 9 microlitre of cell suspension on a clean microscope slide. The suspension was immediately examined for fluorescence microscopy at 400X magnification as described previously (Baskic *et al.,* 2006).

Preparation of Bovine Cortical Bone Slices:

Bovine cortical bones slices were prepared as described by Richard and coworkers (1997). The bones were collected fresh from a local slaughterhouse in anti microbial anti fungal liquid, obtained from Himedia Chemicals, Mumbai. Bone slices were cut into 8 x 8 x 3 mm^3 (l x b x w). The slices were then defatted by sonicating for two 30 min periods in 5% solution of Triton X 100 in double distilled water for 30 minutes and rinsed in distilled water, then sterilized with 75 % ethanol, dried and left under ultraviolet light for 15 minutes before use.

Isolation and Activation of Osteoclasts:

Osteoclasts were isolated from young rats as per the standard protocol (Richard *et al.,* 1997). The activation of osteoclasts is achieved by using a combination of vitamin D and dexamethasone (Qin *et al.,* 2003). Briefly, all long bones are removed and freed of adherent muscle and their epiphyses were cut off with a scalpel at the level of the growth plate and discarded. Once clean, the bones are then transferred to 35 mm plastic tissue culture plates containing 3.5 ml chilled Hank's Buffered Salt Solution (HBSS), obtained from Himedia Chemicals, Mumbai, without serum. Bones are then 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 petri dishes were 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 then co cultured with osteoblastic cells isolated from calvarial bones by the method of Liu *et al.,* (1995) in DMEM medium containing 10 % FBS, 1X anti microbial antifungal, 1 gm/l glucose, L glutamine, sodium pyruvate, sodium bi carbonate, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer and 1,25-dihydroxyvitamin D3 (10 nmol/l) and dexamethasone (100 nmol/l) at 37°C in a humidified atmosphere for 48 hrs in 24 well culture dishes (1.5 ml per well). After 24 hrs of activation, culture liquid, dead and non attached cell were removed. Cells were again incubated for 24 hours. After 48 hours attached cells were detached from the wells by washing with 0.1% EDTA Trypsin solution to recover cells. The cells were counted to be 1X 10^9/l. 500 µl of these solution was loaded on each bone slices in 24 well plate containing 1 ml culture liquid 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 TRAP and resorptive pit formed on bone slices. Treatment of *Moringa* plant extract was given in 3 doses *viz.,* 100 µg/ml, 200 µg/ml and 400 µg/ml. After 24 and 48 hrs of culture, solution was collected for analysis of calcium release and enzyme activity. Cold calcium release assay was carried out according to the standard procedure (Sajeda *et al.,* 1997). Alkaline phosphatase (AlP) and tartrate-resistant acid phosphatase (TRAcP) activities were measured spectrophotometrically using the commercial kits purchased from Reckon Diagnostics Pvt. Ltd. Baroda.

Statistical Analysis

All the statistical analyses were carried out using Graphpad 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). Images were analyzed using Image J software.

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 cultured 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 plant extract is represented in Table 5.1, Figure 5.1. The effect of LG was found to be increasing the cell metabolism of SaOS2 cells. With increasing dose of LG in Saos-2 cells, the cell proliferation rate was found to be increased (29% boost at 50 µg/mL compared to control). However; at higher dose it showed reduced cell viability (40% drop at 400 µg/mL compared to control). *Moringa oliefera* showed insignificant increase on the cell proliferation. *Circuma aromatica* inhibited the growth of SaOS2 cells, which was found to be maximum at 50 µg/mL dose (37% drop compared to control).

AlP activity:

As seen in Figure 5.2a and Table – 5.2, AlP activity of the SaOS2 cells cultured with the LG extract there was a significant increase ($p<0.05$) at 10 µg/mL dose and was seen to reach to its peak at the dose of 50 µg/mL of the dose, followed was a concomitant decrease with 200 and 400 µg/mL dose.($P<0.001$)

Figure 5.2b and Table – 5.2, depicts the AlP activity of the SaOS2 cell cultures with CA extract. There was a significant increase ($P<0.01$) in the AlP activity with the increasing

dose at 10, 20 and 50 μg/mL dose. There was a slight decrease at 200 μg/mL dose ($p<0.001$), which was seen to be significantly increased at the dose of 400 μg/mL($p<0.001$).

MO extract when added to SaOS2 cell cultures it exhibited a nonsignificant increase in the AlP activity at all the doses. Except at the 200 μg/mL dose, which expresses a slight decrease (Figure 5.2c and Table 5.2).

Acridine Orange Ethidium Bromide staining:

We examined the viability of cultured SaoS2 cells by staining the cells with the fluorochromes acridine orange and ethidium bromide. Viable cells excluded ethidium bromide but were permeable to acridine orange, which reacts with DNA to yield green nuclear fluorescence. Nonviable cells show red ethidium bromide fluorescence in their nuclei due to ethidium bromide entry. Under control conditions, cultures of SaOS2 cells contained negligible nonviable cells (figure 5.5). MO did not express toxitcity at all the experimental doses (figure 5.6). Treatment with LG at 400 U/mL dose showed as increase the percentage of dead cells (figure 5.7). However CA showed the sight of clear-cut toxicity at 200 and 400 U/mL dose (figure 5.8).

Calvarial bone cell culture/ co-culture:

In the present study we used a homogenous co-population of calvarial osteoblastic cells and bone marrow derived osteoclastic cells to comprehend the osteoclasts inhibiting property of 3 herbals.

Identification of osteoclast

Two distinct types of cells were observed in the cultures, the monolayer of osteoblast-like cells and the free, large osteoclast-like cells growing on them. The osteoblast-like cells were found to lie adjacent to each other in close contact. However, the large osteoclasts-like cells were free, sub-round cells characterized by pseudopodia, numerous nuclei and vacuole in cytoplasm. The osteoclastic resorption pits were more like round initially but was found to be more irregular with progression of time.

Cold calcium release from bone slice:

As illustrated in Figure – 5.3 all the three plants inhibited calcium release from the bone as expected. Nevertheless, LG and CA were far more effectual then MO. LG and CA showed a steep dose dependent decrease in the calcium release from the bone slice. The decline was

observed from 50 µg/mL ($p<0.001$) dose and continued to decrease ($p<0.001$) reaching maximum at 400 µg/mL dose, supporting their osteoclasts inhibitory action. The MO plant extract too showed a steep decrease in the calcium release at a higher dose (100 µg/mL) and reached to the maximum at 400 µg/mL ($P<0.001$) (Table 5.3).

TRAcP activity

All the three plants were found to be effective in reducing TRAcP activity (Table 5.4). CA was found to be the most potent plant and showed a complete dose dependent effect in inhibiting TRAcP levels. LG was also found to be equally effective, but the data was not statistically significant in higher doses of 200 µg/mL and 400 µg/mL. *M. oleifera* treatment significantly reduced the TRAcP levels in the culture medium. However, this plant was not effective in inhibiting TRAcP activity at low doses (Figure 5.4).

DISCUSSION

The current study was intended to comprehend the outcome of three diverse herbal extract on the osteoclastic resorption by means of the co-culture and SaOS2 osteoblastic cells. To obtain mammalian osteoclasts, mechanical disaggregation of new born rat long bones has been generally used. However, the number of osteoclast-like cells obtained by this method is very low. Therefore, Akatsu and coworkers (Nicholas *et al.,* 1987; Richard *et al.,* 1997) developed a method to chemically induce the activation and maturation of osteoclast like multinucleated cells *in vitro*. Vitamin D, A and Dexamethasone are effective and used in differentiation process for bone tissue engineering. (Chen *et al.,* 1986; Rickard *et al.,* 1994; Qin *et al.,* 2003; Fromigué *et al.,* 2004; Somjen *et al.,* 2005; Kok *et al.,* 2006) .We also enhanced the efficacy by treating the cells with the combination of vitamin D, Vitamin A and Dexamethasone for 48 hrs. As a result we could harvest more number of osteoclastic cells, which were more active compared to what was observed by earlier workers.

Oteoporosis is usually caused by the unbalance between osteoblast and osteoclasts activities induced by multiple reasons. Thus, medicine, which suppress the osteoclasts activity to delay osteolysis, or facilitate both osteoblast and osteoclasts activities to activate osteogeneiss, are commonly used for the treatment of osteoporosis (Ha *et al.,* 2003). It is accordingly thought that the medicines, which improves the activity of osteoblast to facilitate osteogenesis and inhibits the activity of osteoclasts to delay osteolysis are ideal agents for therapeutic agents

in osteoporosis. In order to analyze the effect of osteoblasts, MTT assay and AlP are used according to activation mechanism of osteoblast (Stein and Lian, 1995). This is the first scientific record of *in vitro* bone remodelling effects of these herbal extracts. The results of the present study discovered without dubiousness that all the three extracts are inhibitors of osteoclastic resorption induced by 25-dihydroxyvitamine D_3, vitamin A and Dexamethasone. However, when osteoblast stimulatory activity is concerned, LG and CA are strong stimulators, whereas, MO had no significant effect on osteoblastic cells. MTT is considered as general mitochondrial function test and indicates the cell viability in the culture medium (Ha *et al.,* 2003). Previous studies reported that MO is having osteoprotective effect in OVX rats (Burali *et al.,* 2010). However, our MTT assay showed that MO had no significant effect on the cell viability or any other osteoblastic function marker in entire range of doses, suggesting that this osteoprotective agent is following some other pathway rather than stimulating osteoblastic cells. CA showed reduction in cell viability and showed reduction in the cell function to 60%. Conversely, there was no dose dependency observed in CA treatment. LG plant showed an initial dose dependent effect like a therapeutic drug; increasing the cell viability by 28%. However, at higher doses of 200 µg/mL and 400 µg/mL it showed toxicity and reduced the cell viability, suggesting that this plant is an osteoblastic stimulator but at higher doses, it is toxic to the osteoblastic cells. AlP is one of the very important markers of osteoblastic function (Sajeda *et al.,* 1997; Parikh *et al.,* 2009). In SaOS 2 culture we observed that both CA and LG are potent stimulators of this marker. CA showed 100% increase in this osteoblastic function marker, suggesting its potent osteoblast stimulating property. This is contradiction to MTT results, suggesting that this plant might be stimulating osteoblast activity rather than stimulating osteoblastic proliferation. Curcumin is a known anti tumor agent and this cell line is a known sarcoma, so inhibitory activity of this plant on MTT might be attributed to curcumin (Sukh Dev, 2006). LG was found to be showing similar profile as that of MTT assay and it showed dose dependent increase in osteoblastic activity till 100 µg/mL but at higher doses it showed no further increase in the AlP activity. Thus, it is possible that at lower doses LG is stimulating osteoblastic proliferation, but at higher doses it induces their differentiation and leading to functionally activated osteoblast which secrete more AlP. It was noticed that LG was more potent compared to CA in stimulating the osteoblastic cells. MO did not show any significant changes in AlP activity.

Acridine orange ethidium bromide staining is a widely accepted protocol for assessing the cell death Baskic *et al.,* 2006). MO did not show any alteration up to 400 µg/mL dose, while CA and LG showed toxicity at 200 µg/mL and 400 µg/mL dosage where some red cells were observed with nuclear condensation. The cell morphology revealed that all the green cells were triangular with green large nucleus in the centre, while red cells were altered in size, showing shrivelled nucleus and irregular shapes. CA was found to have more ethidium bromide stained cells, suggesting that this plant is more toxic to the osteoblastic cells compared to MO and LG. MO plant was found to be least toxic to the SaOS 2 system supporting its wide nutritional usage.

The osteoclast induced bone resorption is reported to be mediated by two different processes - by the formation of new osteoclasts and the resorption activity of osteoclasts (Penolazzi *et al.,* 2006). It is well documented that bone resorbing agents such as 1, 25-dihydroxyvitamine D_3, PTH, and IL-1 markedly stimulate the formation of osteoclasts and enhances the resorption rate (Qin *et al.,* 2003). 1, 25-dihydroxyvitamine D_3 is thought to stimulate osteoclast formation by prostaglandin E2 (PGE2), which is also reported to be a mediator in the progression of osteoporosis (Nicholar *et al.,* 1987). The current experimental model also simulates the development of inflammation like condition seen in the initial stages of rheumatoid arthritis (Akatsu *et al.,* 1992; Qin *et al.,* 2003; Penolazzi *et al.,* 2008). Hence, Vitamin D_3-Dexamethasone activated bone marrow cells are best suited model for studying osteoporosis and rheumatoid arthritis *in vitro* (Akatsu *et al.,* 1992; Qin *et al.,* 2003; Penolazzi *et al.,* 2008).

Osteoclasts are the keys cells that playing the major role in the pathogenesis of osteoporosis (Hidaka *et al.,* 2006). In our study, Compared to MO, LG and CA were highly potent in inhibiting the osteoclastic resorption and favoring the osteoblastic activity. Cold calcium release is the marker of osteoclastic resorption rate and was used to study the resorption rate on the bone slice (Richard *et al.,* 1997). The activity of tartrate-resistant acid phosphatase, one of the marker enzymes of osteoclastic resorption, was studied in order to understand the resorption inhibition property of the botanicals. The activity of tartrate-resistant acid phosphatase is directly related to the activity of bone resorption (Okazaki *et al.,* 1999; Qin *et al.,* 2003; Penolazzi *et al.,* 2008). The analysis of the results revealed that the methanolic

extract of MO retarded the experimentally induced osteoclastic resorption in a dose dependent manner. However, below 100µg/ml, no significant protective effects were observed, suggesting nutraceutical role of this plant. CA and LG showed potent osteoclast inhibiting property even at high doses, suggesting their potent therapeutic role as an osteoprotective agent. All three plants inhibited Cold calcium release and TRAcP; markers of osteoclastic function. However, no significant effects were observed in the AlP marker of the co culture system.

In conclusion, it is confirmed that the plants with potent anti inflammatory property are candidates for the osteoporosis treatment. LG, CA and MO have significant effects on inhibiting activated osteoclast like cells. CA and LG also have osteoblast stimulating property; MO did not have any osteoblastic activity. Our data also proved that CA and LG have therapeutic properties as they evoked response in the cells at lower dosage, while MO has nutraceutical property as it showed activity only at higher doses. Hence, it can be concluded that these botanical extracts could be developed as phytoremedial in preventing osteoporosis and rheumatoid arthritis by inhibiting osteoclastic resorption without affecting osteoblastic cells.

Table 5.1 representing the values for MTT assay.

Dose	LG	CA	MO
10	81.23** ± 1.234	81.1935* ± 2.567	92.65 ± 4.98
20	88.1932 ± 2.484	76.43** ± 4.389	89.163 ± 9.18
50	128.7898*** ± 1.876	63.1949*** ± 1.329	109.63 ± 2.84
100	103.3917 ± 3.493	73.393*** ± 2.354	102.43 ± 2.93
200	89.135 ± 3.987	79.124** ± 3.28	89.63 ± 1.89
400	64.9868*** ± 3.29	75.7934** ± 4.23	103.81 ± 5.89

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

Table 5.2 expressing values of AlP activity

Dose	LG	CA	MO
0	15.890 ± 0.120	15.430 ± 0.230	16.010 ± 0.140
10	21.630* ± 0.620	27.190* ± 0.780	18.910 ± 2.110
20	33.910** ± 0.780	28.410 ± 0.180	19.250 ± 1.090
50	34.190* ± 0.591	29.096** ± 0.390	19.370 ± 2.120
100	30.860** ± 0.630	27.536*** ± 0.360	21.780 ± 1.920
200	26.790*** ± 0.370	26.180** ± 0.180	17.260 ± 1.890
400	29.43*** ± 0.170	30.860*** ± 0.980	19.87 ± 1.23

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

Table 5.3 showing values of Cold calcium release

Dose	LG	CA	MO
10	18.120* ± 0.120	18.340 ± 0.230	17.800* ± 0.364
20	16.123** ± 0.620	14.569** ± 0.780	17.120 ± 0.532
50	14.189** ± 0.780	13.196** ± 0.180	16.540 ± 0.129
100	11.640*** ± 0.290	11.289** ± 0.390	17.210*** ± 0.560
200	10.198*** ± 0.630	10.890*** ± 0.360	13.690*** ± 0.480
400	11.293*** ± 0.370	9.892*** ± 0.180	11.560*** ± 0.099

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

Table 5.4 expressing values of TRAcP activity

Dose	LG	CA	MO
10	35.890 ± 1.230	39.180 ± 1.890	33.690* ± 1.210
20	33.890 ± 0.620	32.810 ± 0.780	34.129* ± 0.532
50	29.870** ± 1.230	24.850* ± 1.290	31.190 ± 1.230
100	22.870** ± 1.760	20.870** ± 1.110	27.800** ±
200	26.980* ± 0.630	17.870*** ± 0.360	26.360* ± 0.480
400	19.870*** ± 1.890	16.280*** ± 2.980	18.360** ±

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

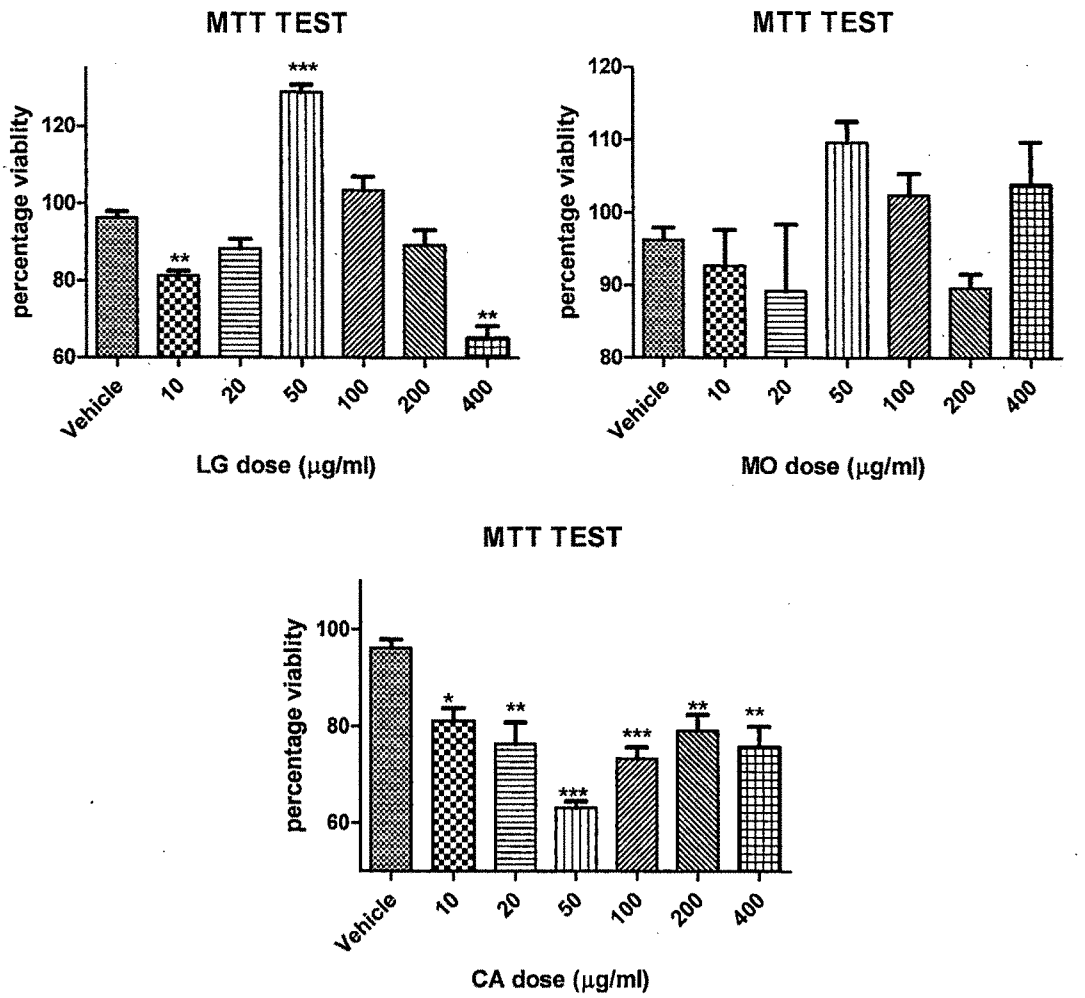


Figure 5.1 represents the MTT test with LG, CA and MO on the SaOS 2 cells

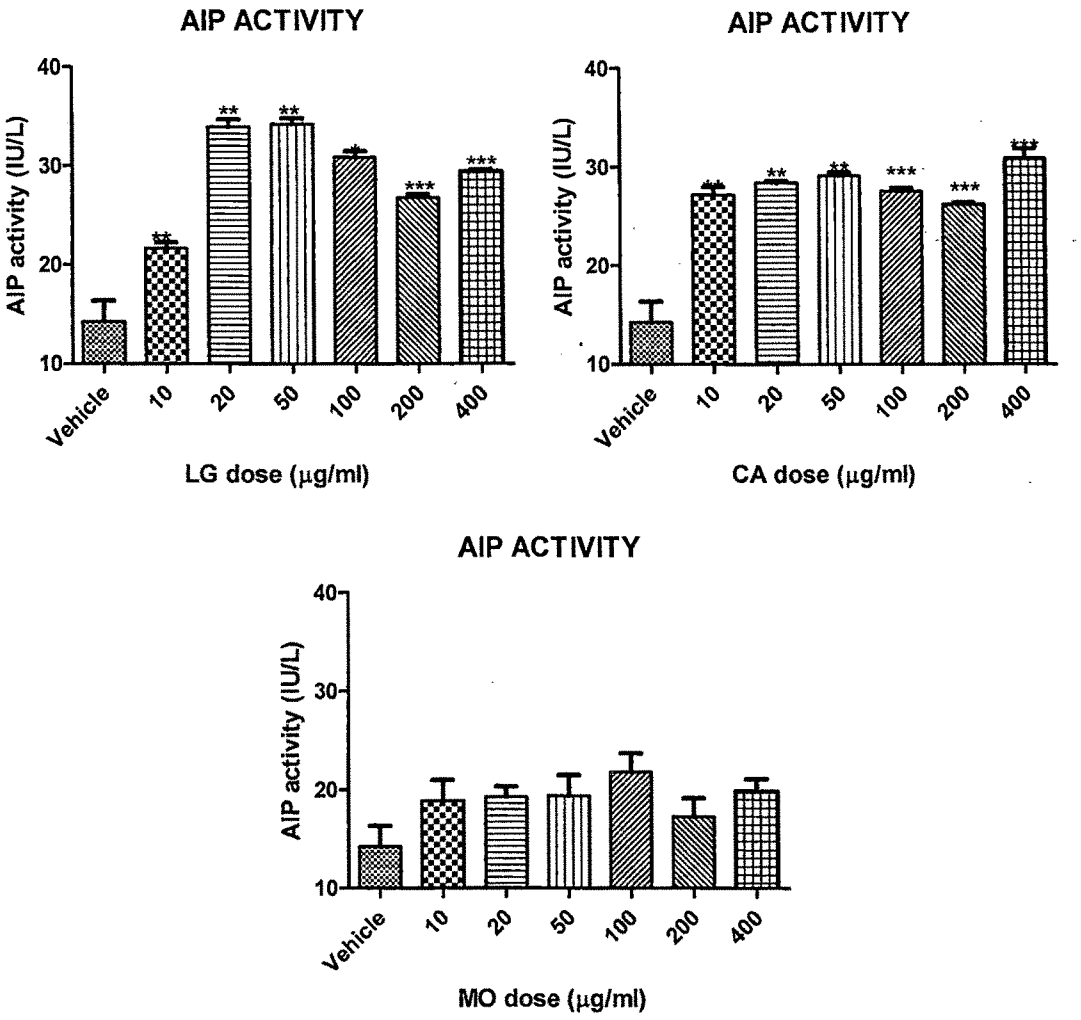


Figure 5.2 effect of different plant extract on AIP activity.

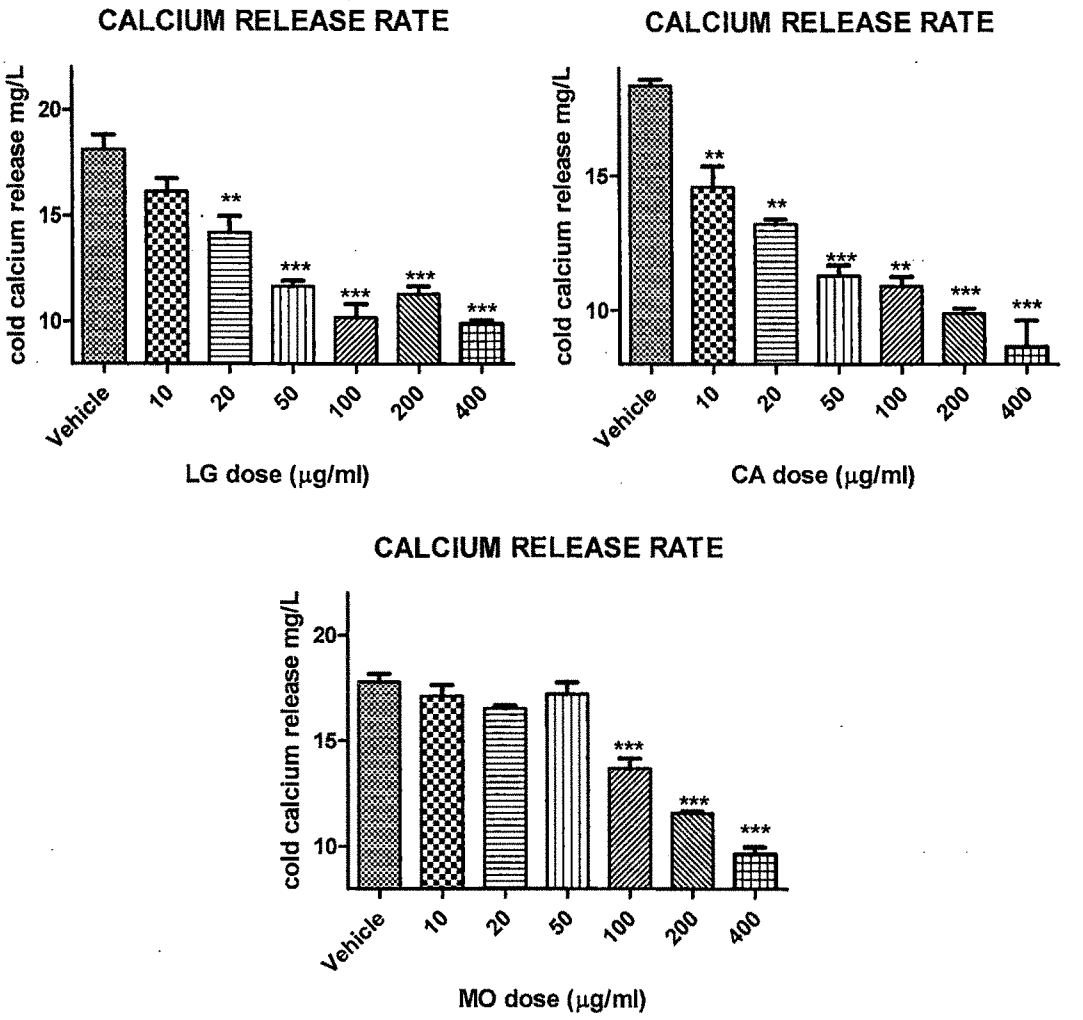


Figure 5.3 showing reduction in cold calcium release with LG, CA and MO treatment.

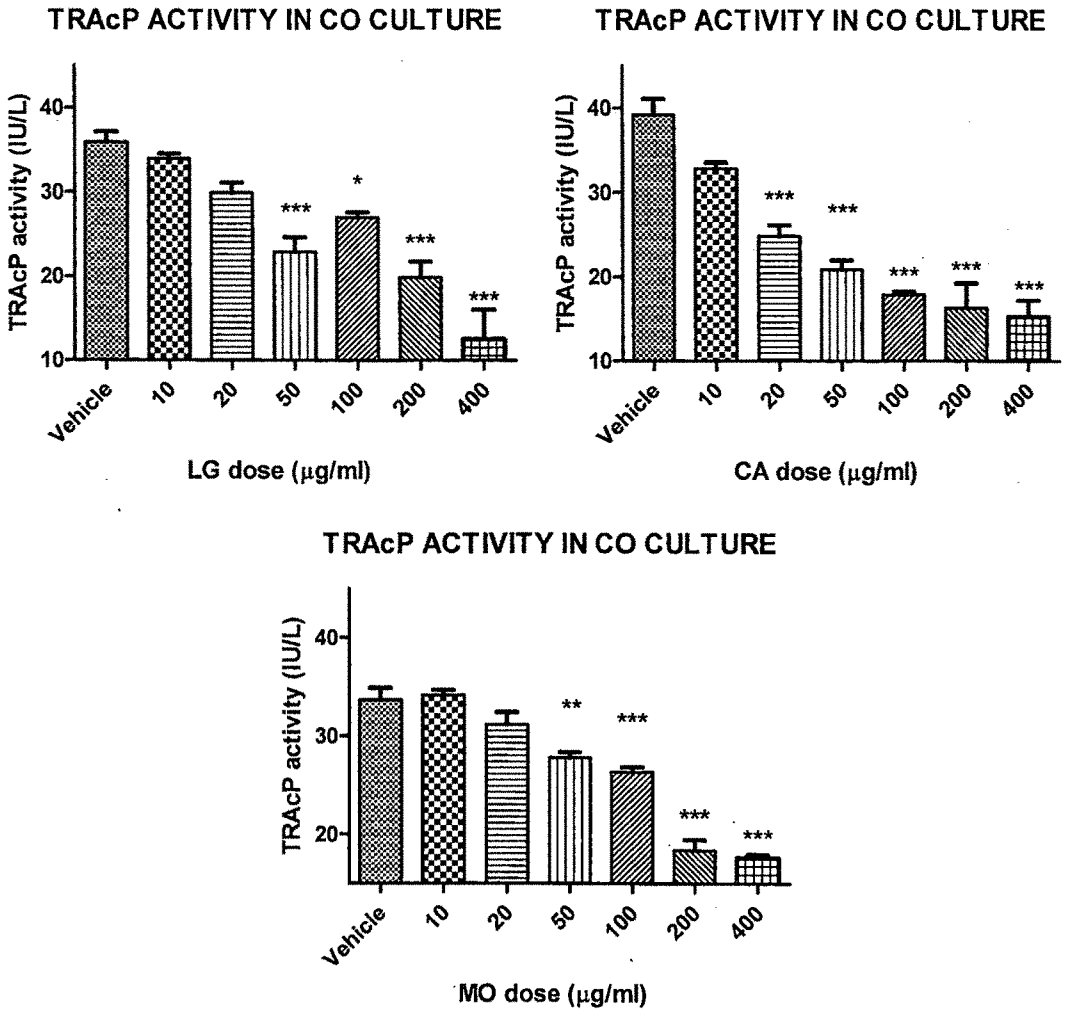
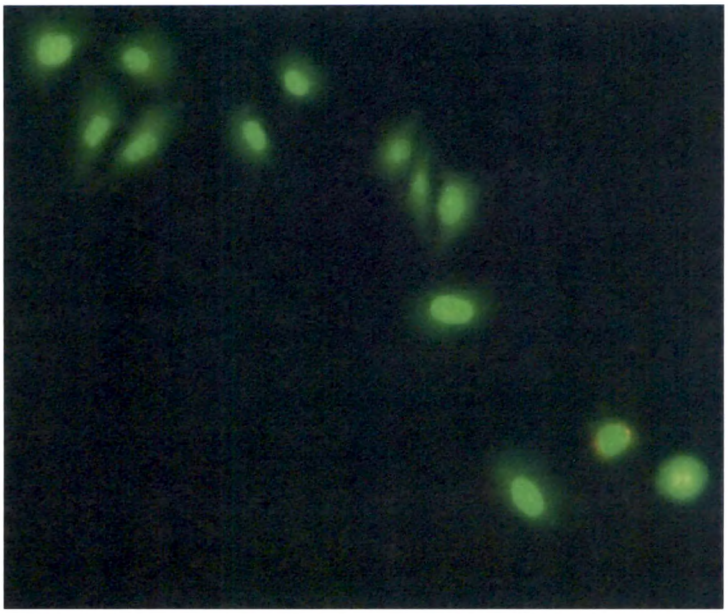
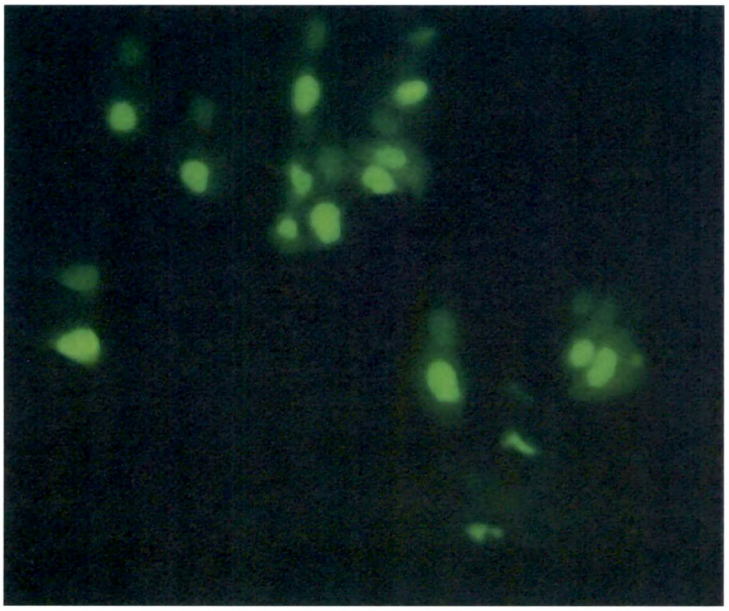


Figure 5.4. Reduction in TRAcP activity with LG, CA and MO treatment

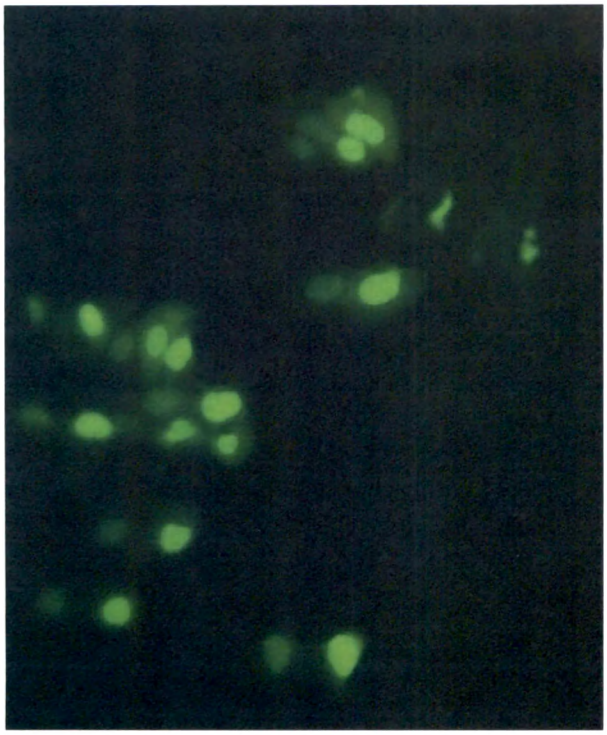


Control SaOS 2 cells (400X)



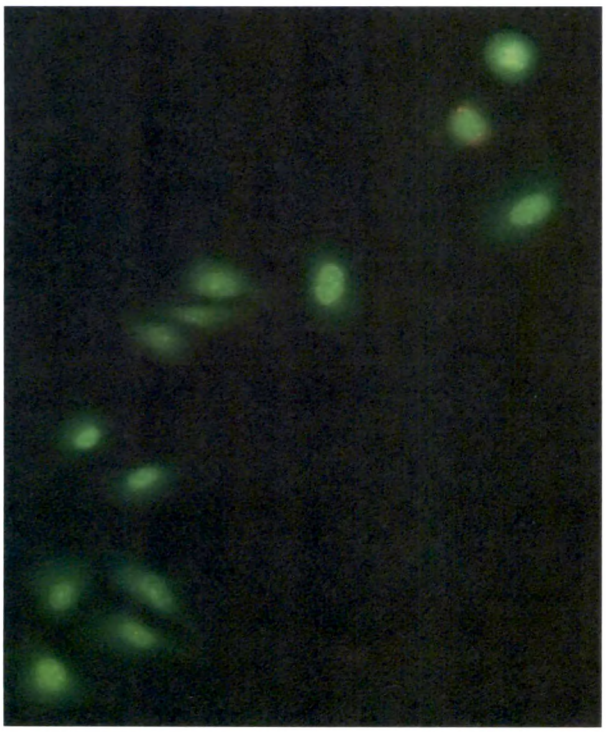
Vehicle treated cells (400X)

Figure 5.5 showing green fluorescence of live cells

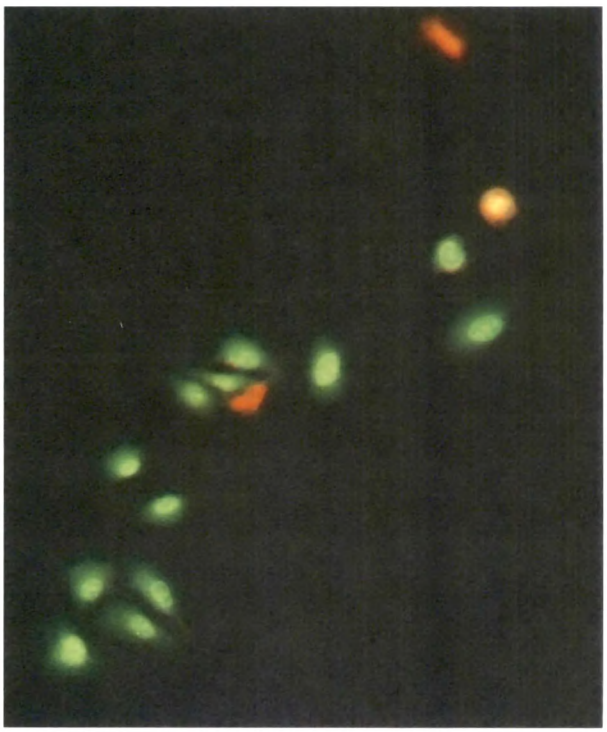


Vehicle treated cells (400X)

MO 100 µg/ml (400X)

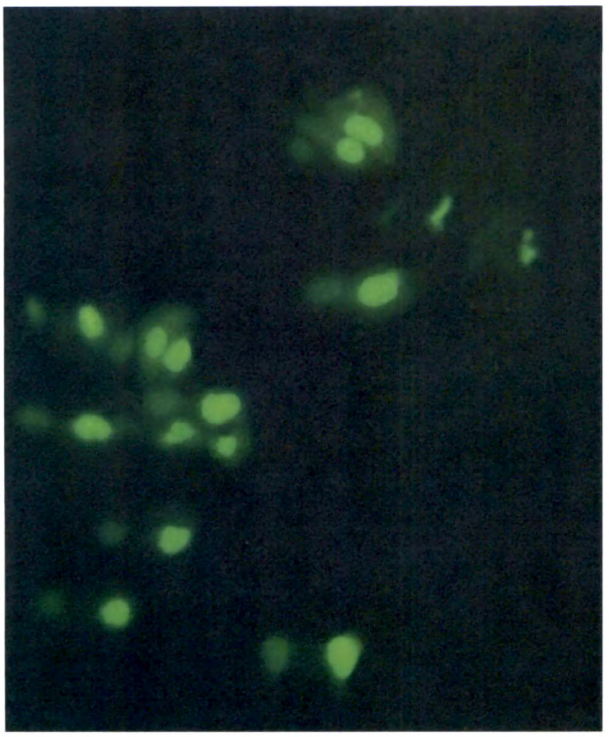


MO 200 µg/ml (400X)

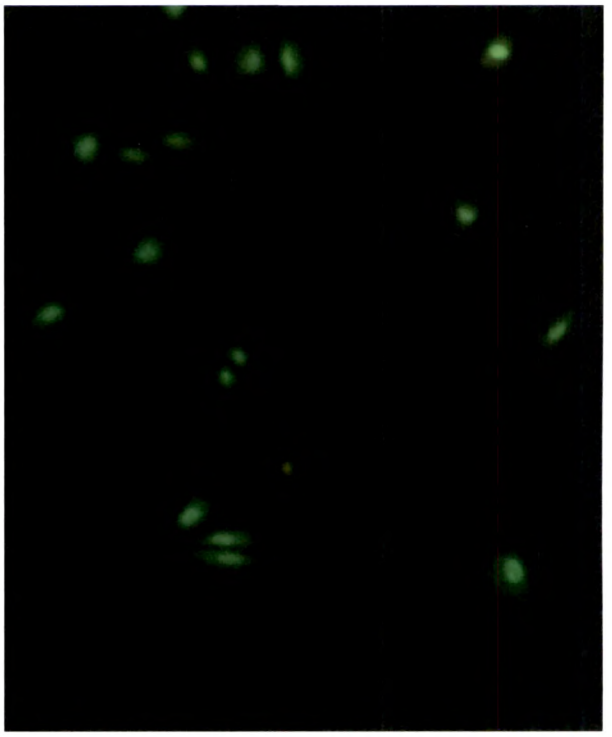


MO 400 µg/ml (400X)

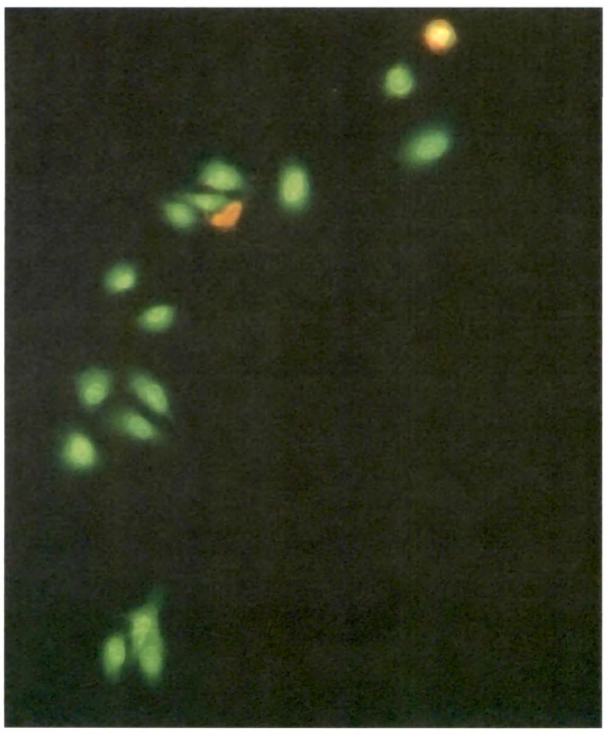
Figure 5.6 Viablity staining of SaOS 2 cells treated with MO; showing no signs of toxicity even at higher doses



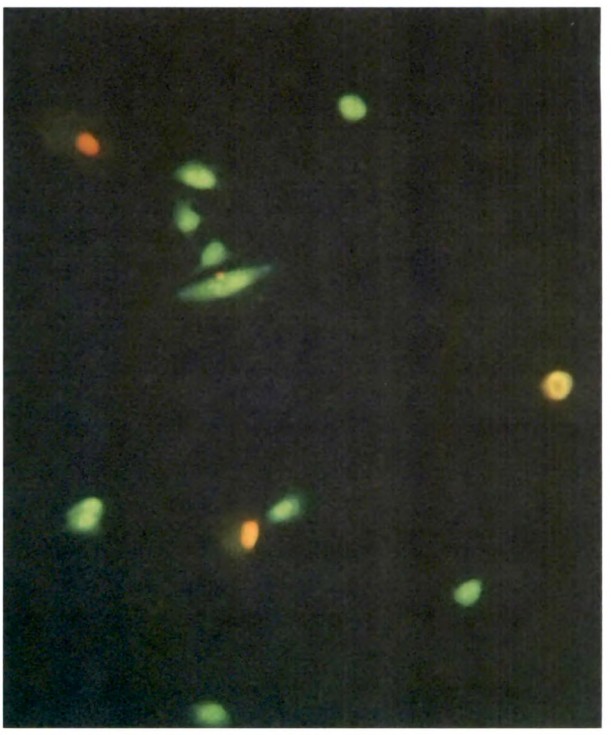
Vehicle treated cells (400X)



LG 100 µg/ml (400X)

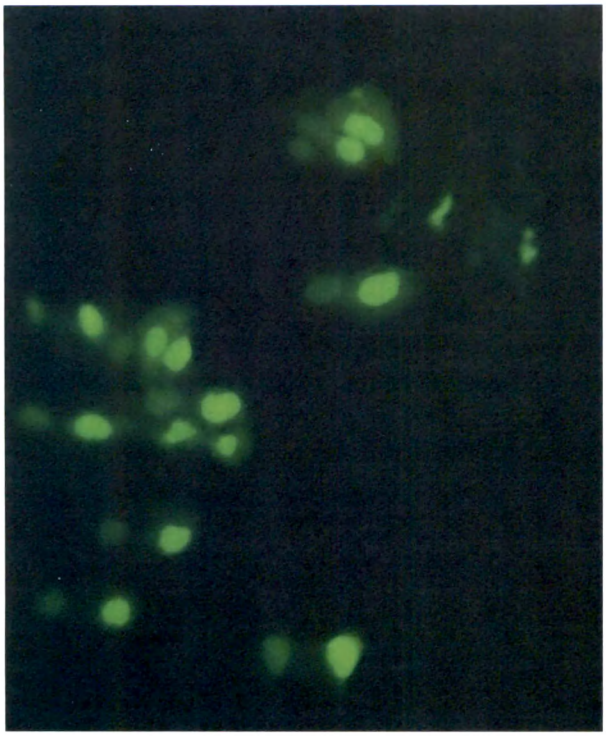


LG 200 µg/ml (400X)

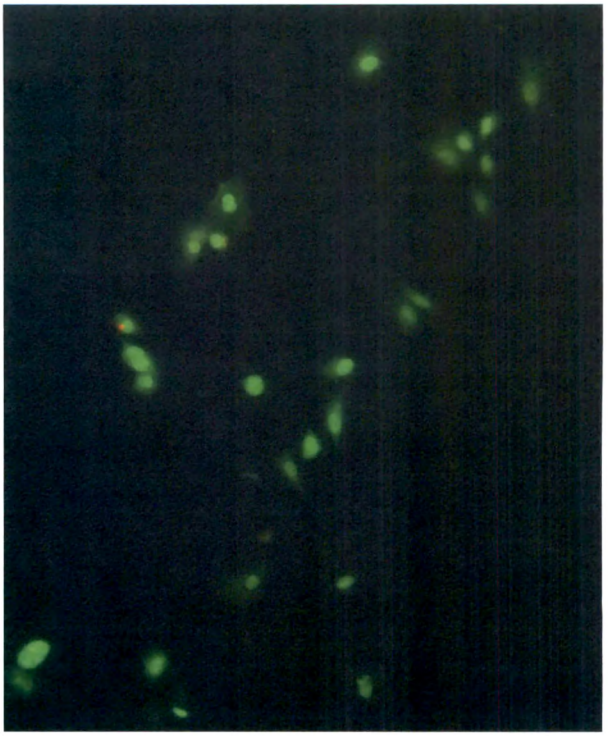


LG 400 µg/ml (400X)

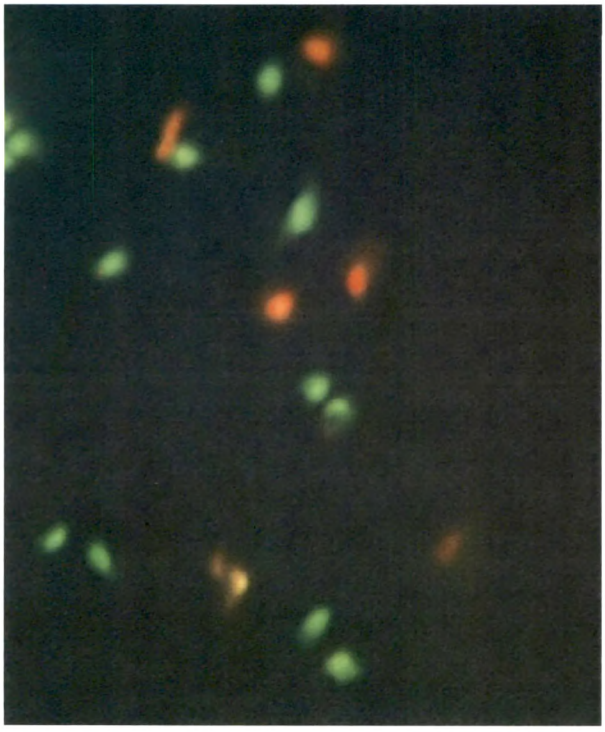
Figure 5.7 Viablity staining of SaOS 2 cells treated with LG showing slight toxicity at higher doses



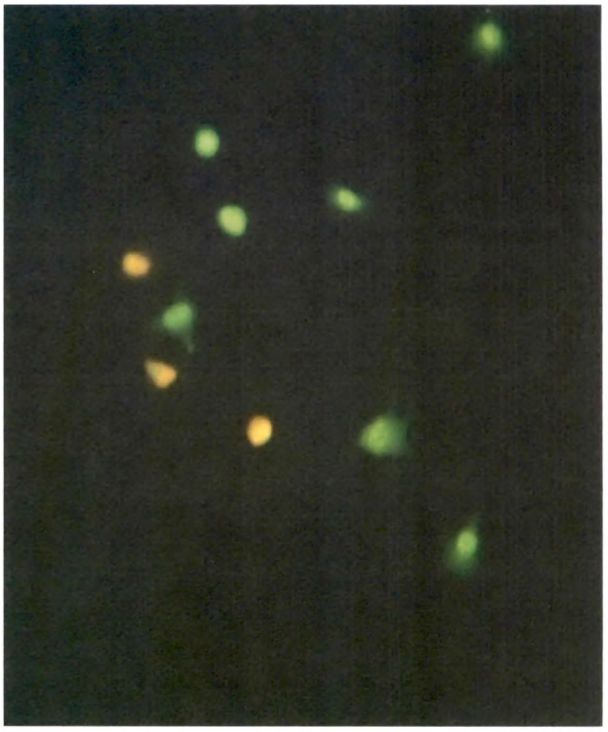
Vehicle treated cells (400X)



CA 100 μg/ml (400X)



CA 200 μg/ml (400X)



CA 400 μg/ml (400X)

Figure 5.8 Viablity staining of SaOS 2 cells treated with CA showing signs of significant toxicity in higher doses