CHAPTER 6

OSTEOBLAST STIMULATING PROPERTY OF VARIOUS FRACTIONS OF *LITSEA GLUTINOSA*

Phytochemical analysis showed the presence of alkaloids and phytoestrogens (Chapter 4). This study aimed to explore further the subfractions of the plant and to find out their osteoblast stimulating potential on SaOS 2 cells cultivated *in vitro*.

INTRODUCTION

Osteoporosis has now been recognized as a major public health problem associated with substantial morbidity and socio-economic burden. As a result of osteoporosis, one in two women and one in five men over the age of 50, sustain fractures (Dolan and Torgerson, 2000). Postmenopausal women, in particular, are more vulnerable to osteoporosis. After menopause, due to lack of estrogen, the rate of bone turnover increases, resulting in accelerated bone loss. Bone is a tissue maintaining itself through continuous osteogenesis and osteolysis by osteoblasts and osteclasts (Dempster and Lindsay, 1993) respectively. The unbalance between osteoblast and osteoclast activities is caused by the reduction of estrogen in a woman at the menopause, aging, and like. Bone formation is essential for skeletal growth, remodelling and repair; involving differentiation of osteoblasts, synthesis and deposition of mineralizing matrix (Erlebacher *et al.,* 1995). The differentiation and proliferation of osteoblasts can be affected by numerous extracellular factors such as hormones, growth factors and cytokines (Jochum *et al.,* 2009). Osteoblasts are derived from mesenchymal stem cells and are bone forming cells, whose maturation is characterized by expression of membrane associated AlP (Bonnelye *et al.,* 2008). Osteoblasts play a principle role in bone formation. These cells not only regulate bone formation, but also indirectly mediate osteoclastic bone resorption. Osteoblasts are the central player of bone remodelling who maintain equilibrium between bone formation and bone resorption. The maturation sequence of osteoblasts has been divided in to three consecutive phases: proliferation, extracellular matrix maturation, and mineralization (Scherft and Heershe, 1975; Holtrop, 1990). The most widely measure of osteoblast marker is alkaline phosphate, a ubiquitous enzyme which catalyzes the hydrolysis of phosphate esters at an alkaline pH. Osteoblast regulates the mineralization of formed matrix by producing AlP, and hydrolyzes phosphate esters to increase the local phosphate concentration of phosphate and enhance the mineralization (Aubin, 1998). Although osteosarcoma cell lines such as SaOS 2 are transformed, they have a stable osteblast like phenotype in culture, making them a good model for studying the characteristics and functions of osteoblasts (Rodan *et al.,* 1987; Rao and Murray, 2000; Vali *et al.,* 2007).

Clinical trials indicate that phytotherapy is one of the potential treatments for postmenopausal osteoporosis (Xu *et al.,* 2009).The potentially undesirable effects associated with pharmacological treatments have meant that natural alternatives for osteoporosis have

received some attention in recent years (Parikh *et al.,* 2009). Among the hundreds of natural products available, one that may exert a positive benefit on the ability of the bone cells to resist osteoporosis is curcumin. Curcumin is derived from the spice turmeric and purportedly has many biological effects. Curcumin promotes apoptosis and inhibits bone resorption by rabbit osteoclasts (Ozaki *et al.,* 1997, 2000). Our studies have also proved the osteoprotective role of the CA (Chapter 6). It has also been shown in murine cells that curcumin inhibits osteoclastogenesis induced by receptor activation of NFkß ligand (Bharti *et al.,* 2004). Thus curcumin could decrease bone resorption by inhibition of osteoclast proliferation, differentiation and activity as well as promotion of osteoclast apoptosis (Bell, 2003). In addition, curcumin inhibits proliferation and causes the arrest of cell cycle progression in rat calvarial osteoblasts (Notoya *et al.,* 2006). *In vitro* evidence implies that curcumin may have a role in the treatment and/or prevention of osteoporosis (chapter VI).

Moringa oleifera tree has probably been one of the most underutilized tropical crops (Oduro *et al.,* 2008). *Moringa* contains properties which help rheumatism, arthritis and other joint disorders. Biological studies have confirmed that the drum stick leaf has anti-inflammatory,antispasmodic and diuretic activities (Aney *et al.,* 2009). *Moringa oleifera* has therapeutic potential against established arthritis (Mahajan and Mehta, 2007). Ethanolic extract of *Moringa oleifera* prevented OVX induced bone loss in rats. The osteoprotective effect was comparable with estradiol (Burali *et al.,* 2010). The earlier phytochemical analysis has demonstrated the presence of various flavonoides, saponins, sterols, Glycosides, Phytosterols, Phenolic compounds and Proteins. The anti-osteoporotic activity by flavones and steroids has been ascertained in their ability in bone remodeling. *In vitro* experiments in the present studies have also confirmed the osteoprotective role of *Moringa* (Chapter 5).

Phytochemical content of the bark of *Litsea glutinosa* was found to possess various phytochemicals including alkaloids, steroids, triterpenoids, saponins, and tannins (Chapter 4). The most prominent phytochemical constituent of the bark of LG was found to be Alkaloids; presumably responsible for their antibacterial and anti-fungal activity (Chapter 4). *Litsea glutinosa* had been shown to have osteoprotective effect both *in vivo* and *in vitro* (Chapter 1, 2, 3 and 5). Our previous studies had also proved that this plant is having osteoprotective effect in OVX wistar rats by inhibiting the osteoclastic resorption *in vivo* (Parikh *et al.,* 2009). Bioactivity – guided fractionation will throw much detailed information of the plant and will enhance our understanding of the osteoprotective

component. Hence, the present study was undertaken to comprehend the precise component of the plant fraction responsible for its osteoprotective value.

METHODS AND MATERIALS

Preparation of Extract:

Litsea glutinosa bark powder was purchased from local drug market and 20 gm powder was extracted with 500 ml methanol in Soxhlet's apparatus for 48 hours. Methanolic extract was dried on water bath at 60°C. Percentage yield of both the plants were found to be 6.66%. Methanolic extract is on the whole a combination of hydrophobic and hydrophilic constituents. Hence, the Methanolic extract (LG) was further fractionated into water soluble (LG AQ) and water insoluble extracts (LG NAQ). Water insoluble fractions were then further redissolved in methanol and methanol was completely evaporated to yield a paste. These fractions were further re-dissolved in DMSO. Alkaloids (LG ALK) were isolated as described in chapter IV and various alkaloid fractions were dissolved in DMSO and 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 cultures as described in chapter V. Upon reaching confluence, the cells were detached using trypsin EDTA solution (High Media, Bombay) and Loaded in 96 well plates (Merck Scientific, Bombay) for culturing for 96 hours in 4 increasing concentration (10 μg/mL, 20 μg/mL, 100 μg/mL, 200 μg/mL). After 96 hours, MTT and AlP assays were carried out to understand the effect of the plant extract on osteoblastic cells. Those fractions which were found to be active in 96 hour study, were further taken for 14 day culture of SaOS 2 by taking the cells in 24 well plate, as per standard protocol (Vali *et al.,* 2007, Arnet and Henderson, 2006) in 4 increasing concentrations of 10 μg/mL, 100 μg/mL, 200 μg/mL, 400 μg/mL of various fractions. Subfractions of alkaloids were further exposed to SaOS 2 for 96 hour culture for their osteoblast stimulating property.

MTT test

MTT assay was carried out as described in chapter 5.

AlP activity

For estimation of AlP profile, the changing medium was collected and the media was subjected to analysis for AlP activity as described in chapter 5.

Determination of Cell Number and Cell Viability

Determination of cell number was carried out as described in detail by Vali and co workers (2007). Briefly, under sterile conditions, cells were trypsinized and removed from the culture plates. Trypsinized cells were re-suspended in culture media and mixed with equal volume of trypan blue and observed under microscope and counted using Hemocytometer. Cell number was counted manually and converted to cell density per mL.

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).

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. After 6 days of culture, the cells were found to be prominently attached to the floor of the culture plate and found to developing pale yellowish particles inside the cells.

MTT assay:

Methanolic extracts of LG has already proved its osteoblast stimulating action (Chapter 5). In the present studies we have extended the experiment further to have an insight in the osteoblast proliferating potential of various fractions. In the MTT assay using SaOS-2 cells, the cell proliferation rate was found to be increased with LG treatment (Figure 6.1 and Table 6.1). LG showed a significant increase ($P<0.05$) at 100 μg/mL of dose; LG AQ expressed a significant increase, $p<0.01$ at a dose of 100 μg/mL, and $P<0.05$ at a dose of 200 μg/mL. LG NAQ resulted in an insignificant increase till a dose of 100 μg/mL and surprisingly showed a significant decrease in the proliferative rate indicative of its toxic effect. LG ALK was found to be the most potent amongst all fractions and resulted in a marked increase ($P<0.01$) at low dose and reached to the peak ($P<0.001$) at 200 μg/mL.

AlP activity:

AlP is a marker of osteogenesis. The potency in stimulating this osteoblastic marker was different for different fractions (Table 6.2; Figure 6.2. LG showed an increased AlP activity in a dose dependent manner and was maximum at 100 μg/mL ($p<0.001$); but at the dose of 200 μg/mL there was noticeable decrease ($p< 0.01$). On the other hand, LG M AQ did not show a well defined trend; rather it increased first at 10 μg/mL and then decreased at 20 μg/mL dose, and then again increased ($p<0.001$) at 100 μg/mL dose. However, it decreased at a dose of 200 μg/mL. Contrary to this, the LG M NAQ fraction showed significant increase ($p<0.05$and $p<0.001$) at 10 and 20 μg/mL respectively. At the higher dose there was decrease in the AlP activity. LG ALK expressed a dose dependent increase with increasing dose till 100 μg/mL, but it too showed a decrease at the higher dose, in general there was a decrease in the AlP activity at 200 μg/mL dose with all the four extracts when exposed to SaOS2 cells, suggestive of the toxicity expression of the plant extracts at higher dose.

Effect of various alkaloid fractions on Osteoblasts

Table 3 and Figure 3 represent the effect of different fractions of the alkaloids on AlP activity. Of the total eight fractions surprisingly only three fractions showed a positive result; Fractions A, B and C were found to be effective in increasing the AlP activity. Fraction A showed a significant increase ($p<0.001$) at the dose of 20 and continued till 200 μg/mL dose. Fraction B and C showed an increase at a lower dose of 10 μg/mL ($p<0.001$ and $p<0.05$ respectively) and continued till 200 μg/mL.

Microscopic observation and determination of cell Number by Hemocytometer:

Microscopic analysis revealed clear difference between the control and LG fractions exposed cells. The cells which were translucent at the start point of the experiment appeared pale yellow on day 14^{th} with particles appearing in the culture medium (Figure 6.5). Appearance of the particles points to the probable synthesis of the matrix, furthermore authenticating the osteoblastic property of the cell line. LG induced proliferation of osteoblastic cells and the wells were found to be packed with Osteoblastic cells, and were maximum at 100 μg/mL dosage. There was no further increase observed with increase in the dosage (Figure 6.6). LG AQ fraction too induced the growth of osteosarcoma (Figure 6.7) and was more potent compared to NAQ fraction (Figure 6.8). Of all three fractions it was LG AIK which expressed the strongest effectiveness (Figure 6.9). When the cells were counted for density,

it was observed in concordance with the results of MTT. Control wells were found to be having the density of the cells at 7.5 X 10^4 cells/mL. With increasing concentration of the LG MET we observed increase in the cell number ($P<0.05$ at 100 µg/mL and at 200 µg/mL dosage.). Compared to LG extract, LG AQ fraction was more potent in stimulating osteoblastic proliferation compared to LG NAQ fraction (Figure 6.4). However, parallel to the MTT profile, it was observed that LG ALK was the most effective extract and increased the cell number to maximum with 100 µg/mL ($P<0.001$). However, at 200 µg/mL at dose it showed slight reduction, but still showed more number of osteoblastic cells ($P<0.01$). These results supported the findings of MTT and microscopic observation.

DISCUSSION

In this study we have shown that LG increases the proliferation and differentiation of SaOS 2 cells and that the proliferative and differentiating expression is due to the presence of the alkaloids in the herbal. It has helped us in perception of the bioactivity of the plant and has provided the first evidence that the osteoprotective role of the plant. Parikh *et al.,* (2009) in their studies have proved the osteoprotective effect of the plant in OVX wistar rats. Our studies have also proved the osteoprotective potential of the plant *in vitro* conditions (Chapter 5). Osteoblastic differentiation is a complex process of sequential expression of osteoblastic markers such as AlP, osteocalcin, type I collagen etc. The developmental sequence associated with SaOS 2 makes it a useful model for the in vitro study of osteogenic agents. In this study we determined the effect of LG fractions on the osteoblastic cells and checked it for its osteogenic potential. Our results demonstrated that SaOS 2 cells have osteogenic property and their phenotypic expression was dependent upon the duration of culture.

Although SaOS 2 is a transformed cell line, these cells have a stable osteoblast like phenotype. They not only grow rapidly but also form matrix and develop different stages of differentiation with associated increased AlP activity and with advance in the mineralization the AlP activity decreases (Vali *et al.,* 2007). The more differentiated cultures are, they express more and more AlP (Rodan *et al.,* 1997; Madunka *et al.,* 1993; Rao *et al.,* 1994) and thus it can be used as the functional marker of osteoblastic bone formation. Based on different levels of AlP expression, they can qualitatively represent different stages of

osteoblastic phenotype (Rao and Murray 2000; Vali *et al.,* 2007). Our study showed that AlP profile was upregulated in all the plant fraction treated cells, indicating the induction of the proliferation and differentiation of the osteoblastic cells. These results were further supported by MTT assay which revealed that the cell viability was increased to 130% in fraction exposed cells. Further, the increase in the AlP activity is in concurrence with the earlier work (Madunka *et al.,* 1993; Rao *et al.,* 1994; Rodan *et al.,* 1997; Vali *et al.,* 2007). When this data is combined with MTT assay, it suggested that this plant is affecting both proliferation and differentiation of osteoblastic cells. As noticed after the 14 day culture, microscopic results confirmed these results and showed that cells of the plant treated groups were more larger, more dense and more in number. A comparative analysis of the different fractions of the LG exhibited a degree of variation in its action. However, LG AQ was found to possess more stimulatory potency compared to LG NAQ. LG ALK proved to be the most effective fraction as far as the proliferation and differentiation of the cell line in culture medium is concerned.

Further analysis of alkaloid fractions revealed that out of 8 fractions, A, B and C were having positive role in stimulating the osteoblastic cells. Fraction A is a mixture of phytoestogens (Androsta 1, 4 diene trione, 3, α acetyl 20 keto, 11- pregnene, Androstane 3 17 diol, dihydroandrostarone) and antioxidants (cinnamolaurine, , flourocinnamic acid, crinamine) as well as few proved phytotherapeutic chemicals (Dichloro, acetyl- phenyl piperazine, tetrahydroisoquinoline), (Chapter 4). Probably of all the subfractions, it is the presence of the phytoestrogens, antioxidants as well as the proven therapeutic components leading to the increased AlP levels. Phytoestrogens are proven osteoprotective agents by numerous workers (Cassidy *et al.,* 1993; Gardner *et al.,* 1998; Fang *et al.,* 2003; Yang *et al.,* 2006); moreover recently discovery of ER-beta in osteoblast cells possibly explain the osteogenic role of Phytoestrogens. Our results are in agreement with the earlier studies (Kuiper *et al.,* 1998; Wober *et al.,* 2002; Guerreiro *et al.,* 2007;). Subfraction B is a mixture of 4, 4, 6 triemethyl Thiocoumarin, coumarin and gestonorone. Coumarin derivative has been shown to exhibit estrogen-like effects, preventing postmenopausal osteoporosis in OVX rats (Li *et al.,* 2002) and also been reported to stimulate cell proliferation of osteoblast-like cells (Meng *et al.,* 2004). Present studies too showed the increased AlP activity; a well known marker for the judgment of an increase in the osteoblasts. Kuo and co workers (2005) have reported increased AlP activity through BMP-2-dependent pathway in osteoblasts. Whether our coumarin sub fraction of the LG also increases the AlP activity through the

similar signaling pathway needs further investigation. Gastronorone, another phytochemical of Fraction B of LG is a derivative of progestogen, has been found to have osteoblast stimulating property.

Apart from these findings recent studies have shown that LCPUFA metabolites, specifically eicosanoids are formed by the activities of cyclooxygenases (COX), lipooxygenases (LOX) and cytochrome P450 like epoxygenases as well as non enzymatic oxidation (Poulsen *et al.,* 2007). Studies by Hamid and co workers (1999) showed that these LCPUFA inhibit COX 2 expressions possibly by modulating the toll like receptor signaling pathway. As the alkaloid fraction was found to be rich in tricosane, oleic acid, tetra decanoic acid like LCPUFA, also justifying the anti inflammatory effect of this plant. And as reported previously by Penolazzi and co workers (2008); plants with anti inflammatory activity are found to have osteoprotective effect.

The results of the LG ALK sub fractions A, B and C clearly accelerated the rate of the osteoblast differentiation. Moreover, sub fractions of LG ALK B and H have shown very attention-grabbing results, where after a initial increase in the AlP activity, a decrease is seen. Mineralization of the bone cells generally involves three important stages: proliferation, development of the extracellular matrix and mineralization. During the process of mineralization, the AlP activity is very good marker for quantifying the state of the mineralization. In our studies, initial increase in the AlP activity continues till the end of development process. Once the mineralization set in there is a decrease in the AlP activity (Aubin, 1998). LG ALK subfractions B and H show the similar trend as far as the AlP activity is concerned. Vali *et al.,* (2007) in their studies have reported the increase in the formation of mineralized bone nodules with epigallocatechin-3-gallate the most abundant catechin in the green tea on human osteoblast-like cells. Thus, opening an avenue for further exploration of the plant extract.

The compounds that are beneficial for preventing osteoporosis have long been claimed either to be the phytoestrogens, antioxidants. LG AQ and LG ALK fractions which showed an affirmative osteogenic property thus can be responsible for the beneficial role of the plant in bone health. Long chain poly unsaturated fatty acids have regulatory role in bone metabolism (Poulson *et al.,* 2007). Sub fractions of LG ALK i.e. sub fraction D, E, F and G which shows the confirmed presence of long chain poly unsaturated fatty acids are thus having the additive role and thus potentiating the osteogenic efficiency of the plant.

Under conditions considered optimal for cell growth, all the LG extracts showed SaOS 2 proliferating potential. All the extract showed positive effect on cell number counting and density in microscopic observation. Previous studies by Poulsen and co workers (2007) have shown that agents with osteoblast proliferating potential are beneficial to bone. As LG extracts are increasing the number of osteoblastic cells, it justifies the ostegenic effect of this plant. Of all the extracts of LG, LG AQ was found to be having more potency compared to LG NAQ fraction, suggesting that phytochemicals responsible behind the osteoprotective effect are hydrophilic in nature. LG NAQ fraction showed statistically non-significant osteoblast stimulating potential which could be due to presence of various phytoestrogens in it. Compared to these fractions, LG ALK fraction increased the osteoblast number almost to 3 folds. Phytoestrogens and LCPUFA (Chapter 4) in alkaloid fractions are the probable bioactive molecules responsible for the enhanced osteogenic activity to the maximum. Phytoestrogens have been proved osteogenic agents by various workers (Ge *et al.,* 2006, Pan *et al.,* 2005a,b; Chang *et al.,* 2003; Heim *et al.,* 2004; Chen *et al.,* 2003a; Federici *et al.,* 2004). Recent studies by Poulsen and co workers (2007) have proved that effect of phytoestrogens on bone cells is enhanced by the presence of LCPUFA, indicating that osteogenic potential of this plant is a due to the combined effect of various phytoestrogens and LCPUFAs. Microscopic observation was also made to further confirm the actual increase in the cell number, which confirmed the quantified increase in the cell number. Presence of phytoestrogens with their intrinsic Estrogenic activity may help in partially compensating the lack of endogenous estrogen synthesis particularly in postmenopausal condition. In addition, the anti-inflammatory activities of the phytoestrogen also help in minimizing the bone loss and thus maintaining good bone health. Moreover, the combined effect of the phytoestrogens and LCPUFAs thus can be a greater therapeutic value in preventing postmenopausal bone loss during osteoporosis. However, the effect of the individual sub-fractions on the extracts on the osteoblasts cell lineage needs to be further investigated which will help us in understanding the metabolic effects of the plant In Toto as far as the osteoprotective role for maintaining better bone health during postmenopausal osteoporosis is concerned.

Table 6.1 MTT assay of various fractions of Litsea

Dose	LG	LG M AQ	LG M NAQ	LG ALKALOIDS
10	91.238 ± 1.283	87.129 ± 1.382	88.492 ± 1.390	84.918 ± 2.230
20	98.238 ± 3.980	97.283 ± 1.879	89.921 ± 4.380	114.367* ± 2.574
100	118.000** ± 1.780	119.230* ± 4.293	90.779 ± 9.380	117.987* ± 8.983
200	98.695 ± 4.980	76.128** ± 8.283	63.331*** ± 8.120	146.104*** ± 7.938

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

Table 6.2 Effect of various alkaloid fractions on AlP activity

Dose	LG	LG M AQ	LG M NAQ	LG ALKALOIDS
0	12.273 ± 1.290	12.374 ± 1.890	11.293 ± 1.390	12.392 ± 2.390
10	22.23 ± 2.980	36.239** ± 1.890	40.129** ± 1.390	21.383 ± 2.390
20	28.690* ± 1.290	25.493 ± 2.980	35.129*** ± 3.760	43.192*** ± 4.390
100	30.568** ± 3.290	39.218*** ± 3.850	9.128 ± 4.382	73.192*** ± 4.290
200	28.192* ± 4.198	12.932 ± 4.560	8.128 ± 1.230	25.238 ± 6.120

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

Table 6.3: Effect of various fractions of alkaloids on AlP activity.

Dose	0	10	20	100	200
A	13.892 ± 1.390	21.296 ± 1.370	31.293*** ± 2.392	78.300*** ± 3.280	93.302*** ± 3.489
B	11.290 ± 1.890	38.295*** ± 2.980	29.394** ± 3.850	43.296*** ± 4.561	39.295*** ± 4.581
C	11.294 ± 1.390	33.302** ± 3.760	41.295* ± 4.383	62.398*** ± 1.230	71.338** ± 1.230
D	12.392 ± 2.390	21.296 ± 4.391	19.212 ± 4.291	18.295 ± 6.121	17.195 ± 4.971
E	13.295 ± 2.019	14.192 ± 1.293	9.880 ± 2.280	12.394 ± 1.293	12.394 ± 1.283
F	12.392 ± 2.392	14.295 ± 1.293	9.284 ± 2.192	18.295 ± 1.293	17.295 ± 2.398
G	12.394 ± 1.293	12.394 ± 1.290	12.396 ± 1.890	9.830 ± 1.289	19.295 ± 2.390
H	12.902 ± 1.938	13.292 ± 1.290	19.295 ± 1.570	31.294*** ± 2.380	19.292 ± 3.290

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

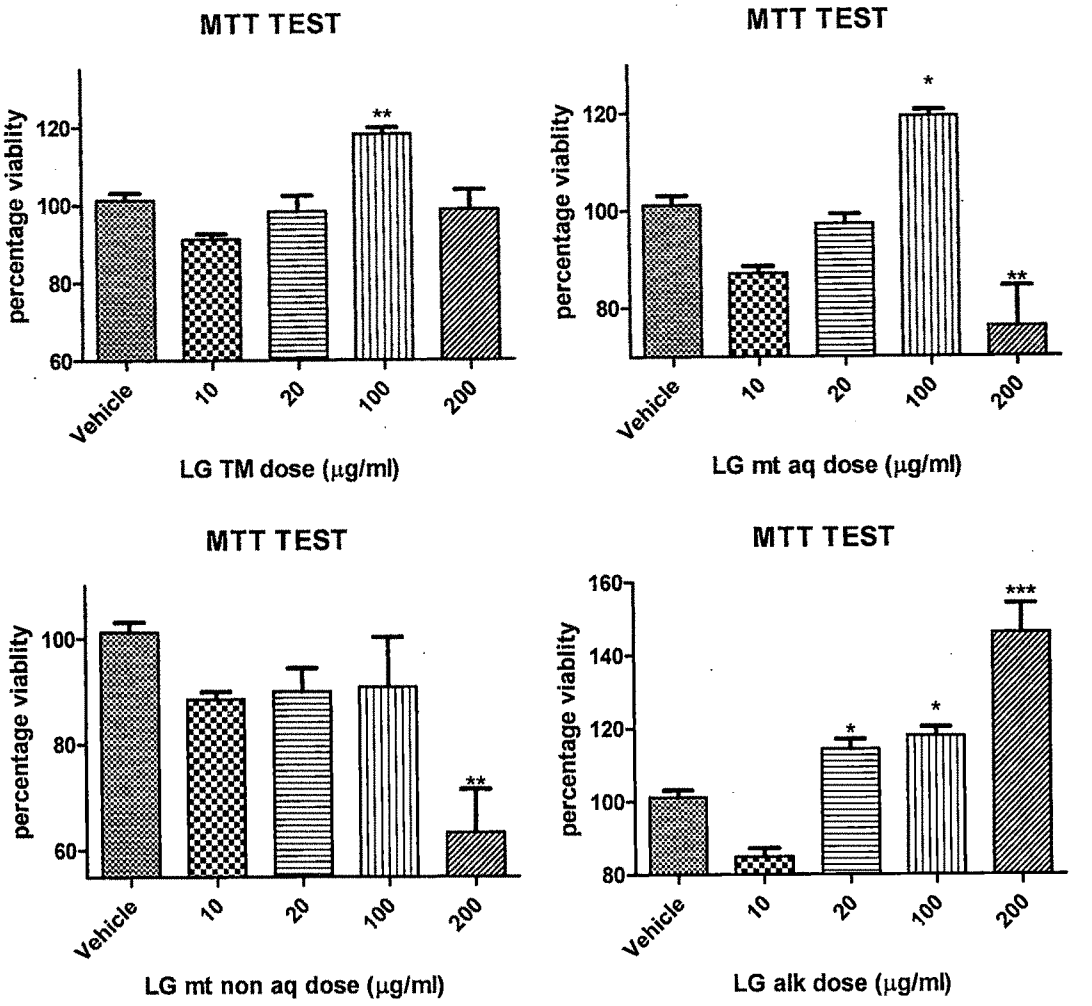


Figure 6.1 graphical representation of effect of various extract of LG on MTT.

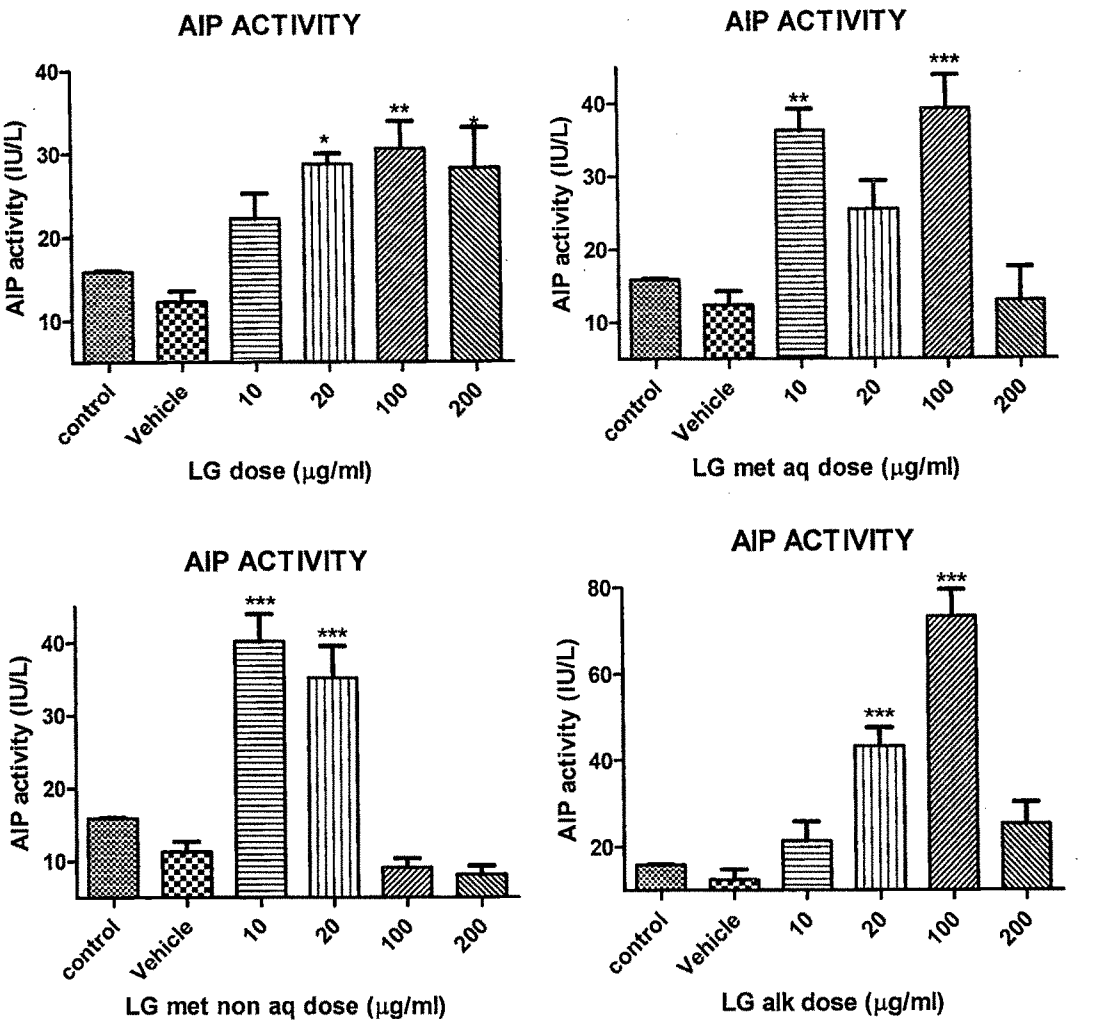


Figure 6.2 graphic representation of effect of various extract of LG on AlP activity.

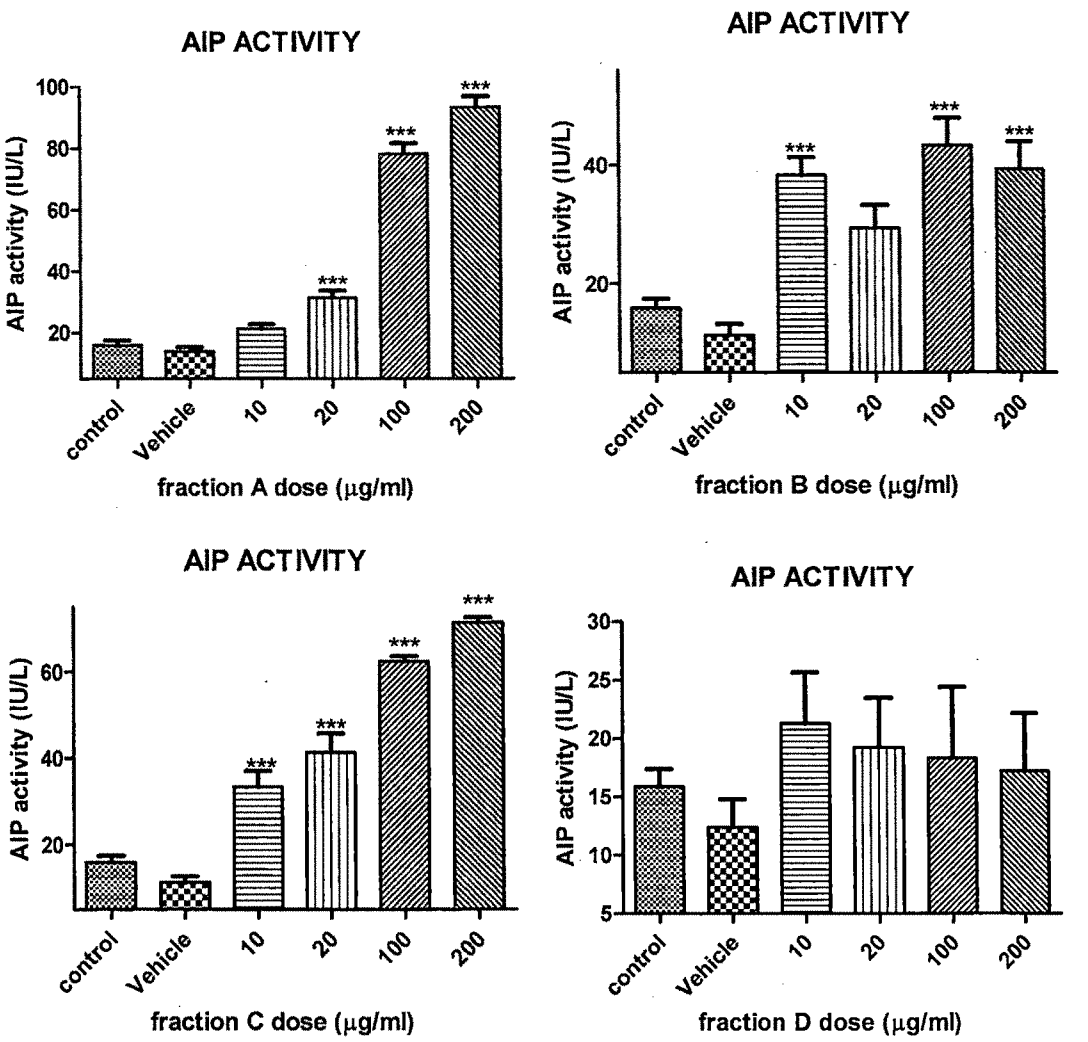


Figure 6.3: effect of various alkaloids fractions on AIP activity.

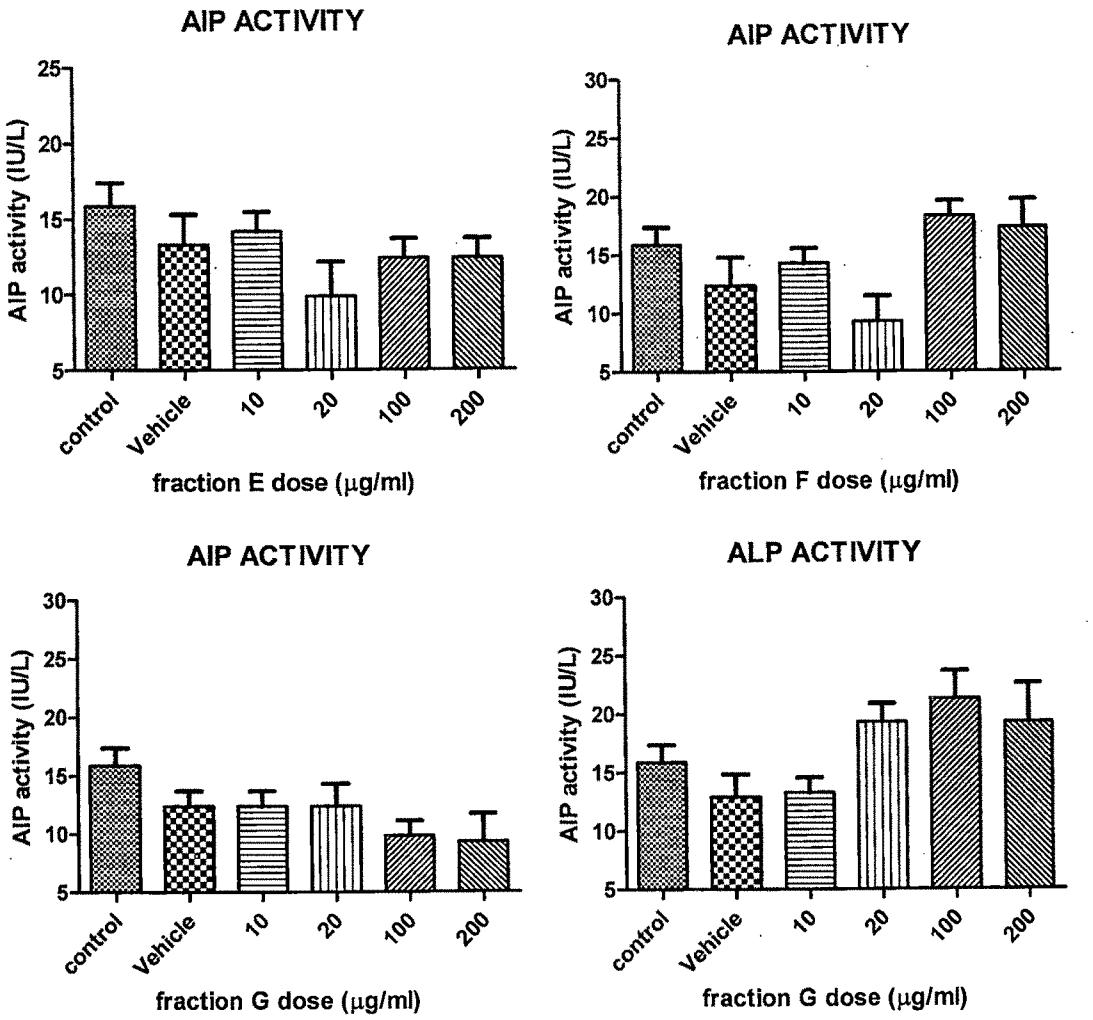


Figure 6.3(cont.): effect of various alkaloids fractions on AlP activity.

Figure 6.4: effect of various alkaloids fractions on cell number.

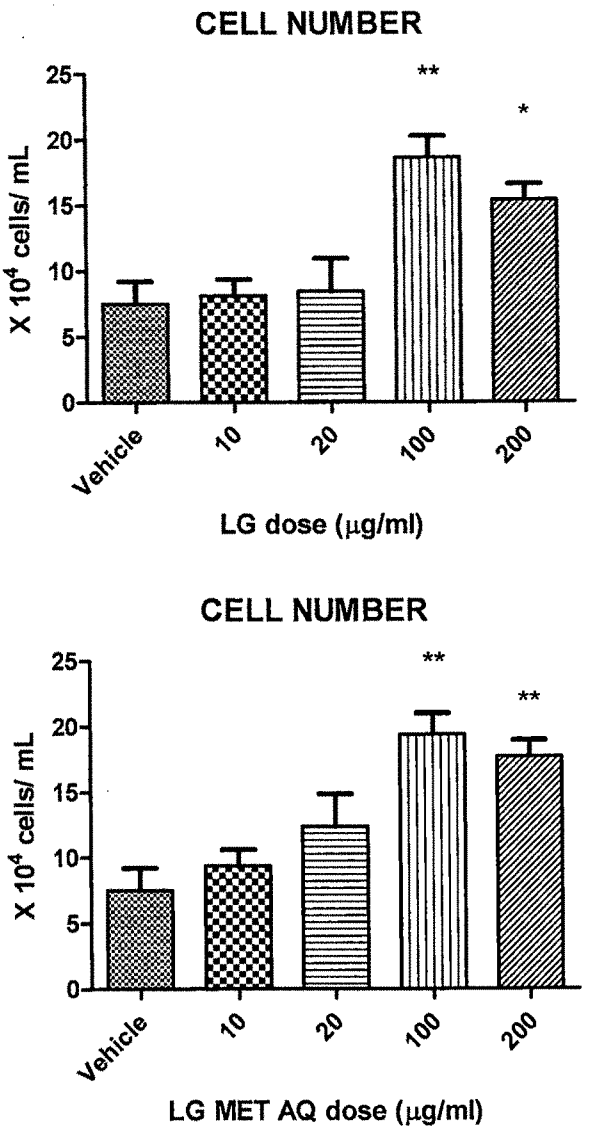
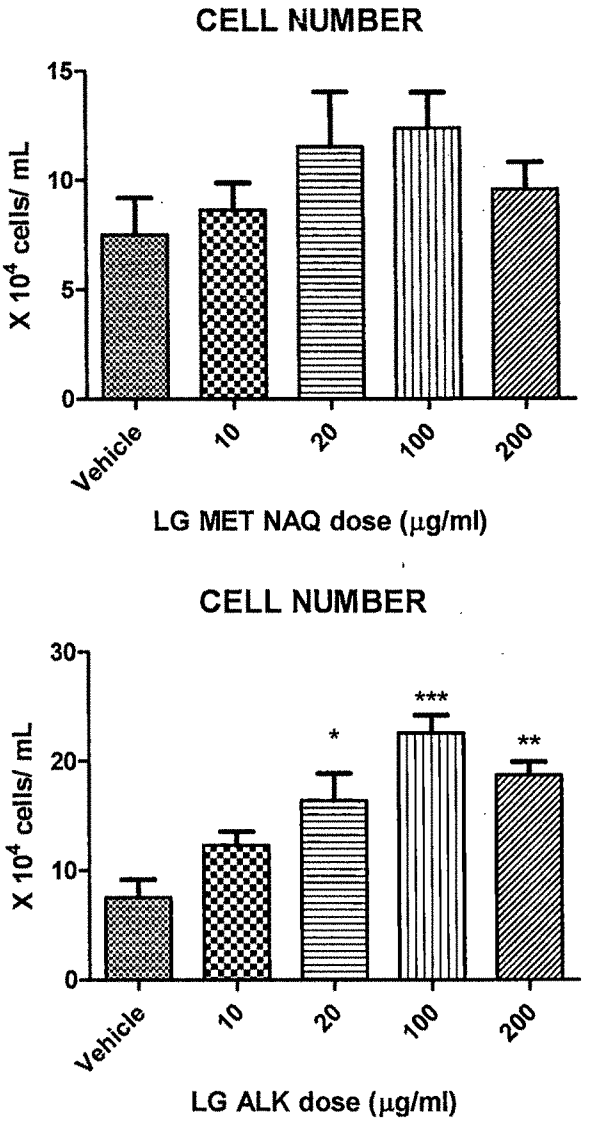
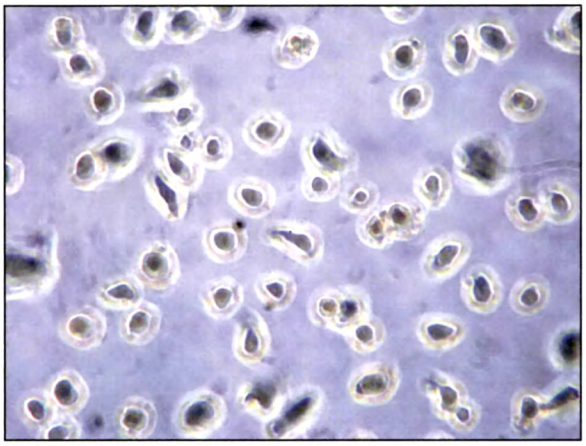
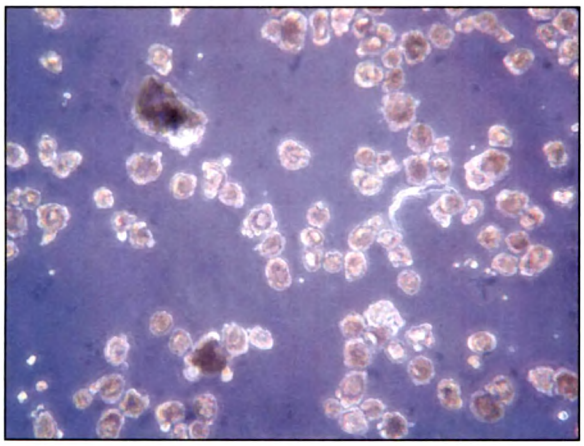


Figure 6.4 (cont): effect of various alkaloids fractions on cell number.

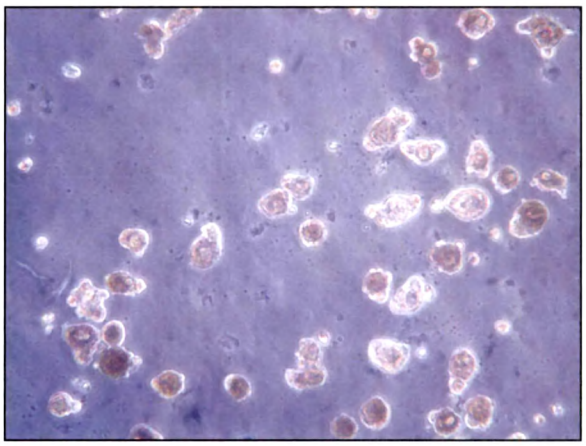




Control SaOS 2 cells (0 hours of culture) (400X)

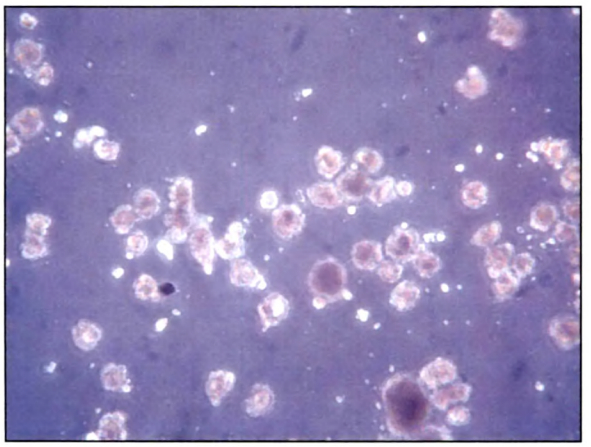


Control SaOS 2 cells (400X)

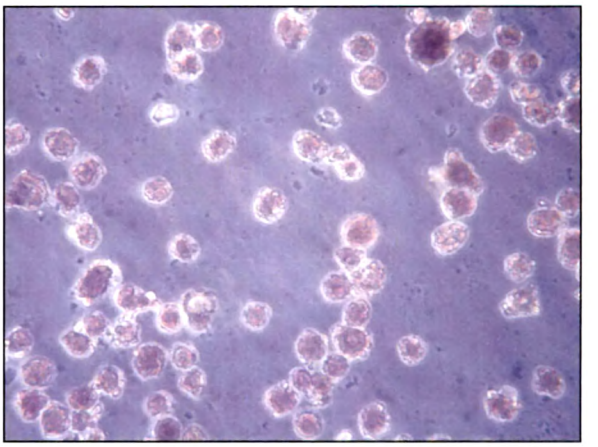


Vehicle treated cells (400X)

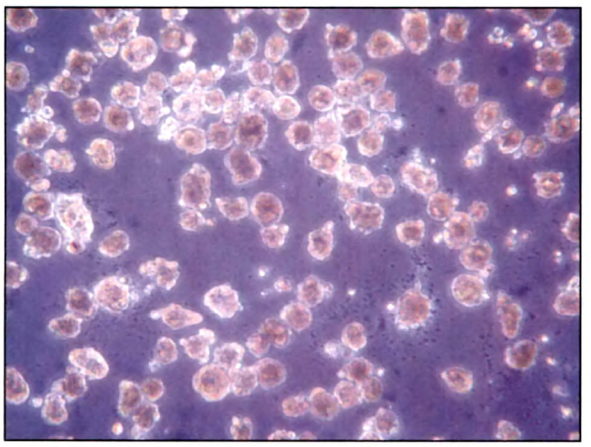
Figure 6.5 Growth of SaOS 2 cells after 14 days of culture.
Cells were found to be synthesizing matrix; No signs of toxicity were seen in Vehicle treatment.



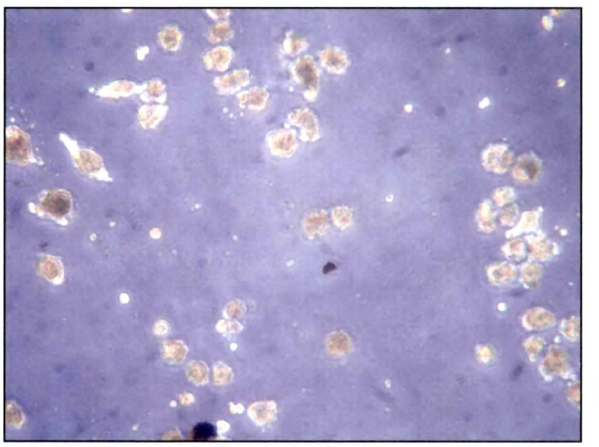
LG TM 10 µg/ml (400X)



LG TM 20 µg/ml (400X)

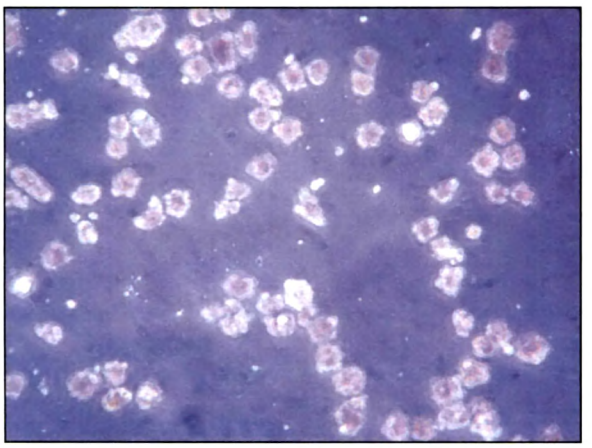


LG TM 100 µg/ml (400X)

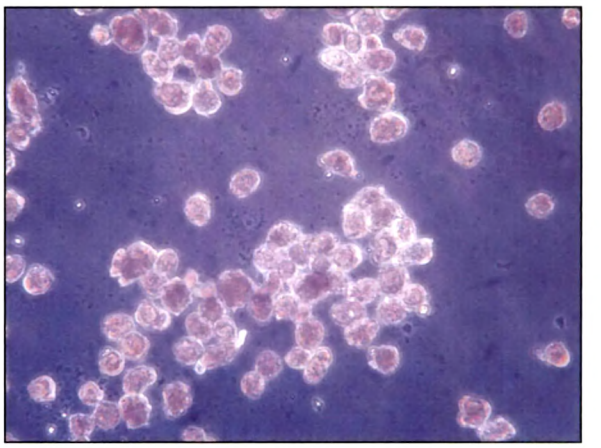


LG TM 200 µg/ml (400X)

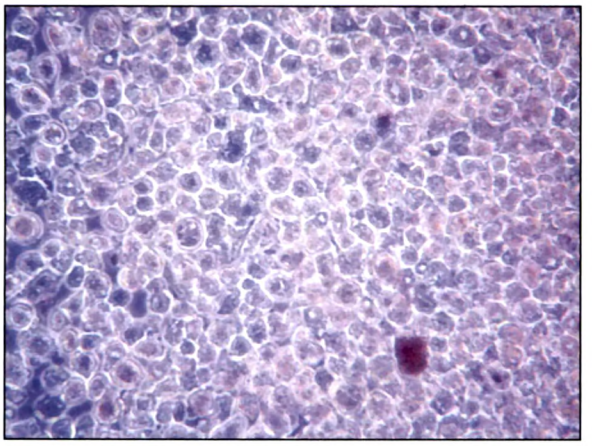
Figure 6.6 showing dose dependent growth of SaOS 2 cells with LG MET treatment



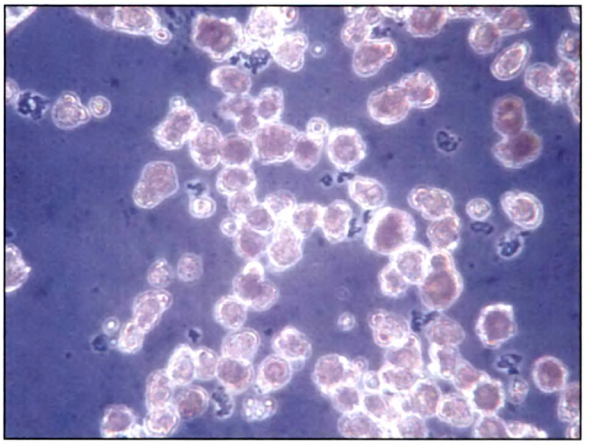
LG MET AQ 10 µg/ml (400X)



LG MET AQ 20 µg/ml (400X)

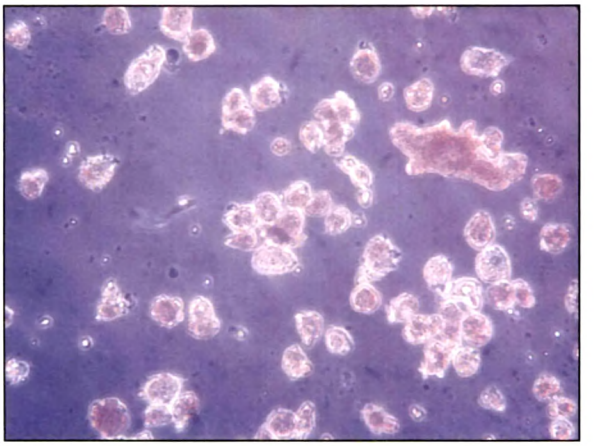


LG MET AQ 100 µg/ml (400X)

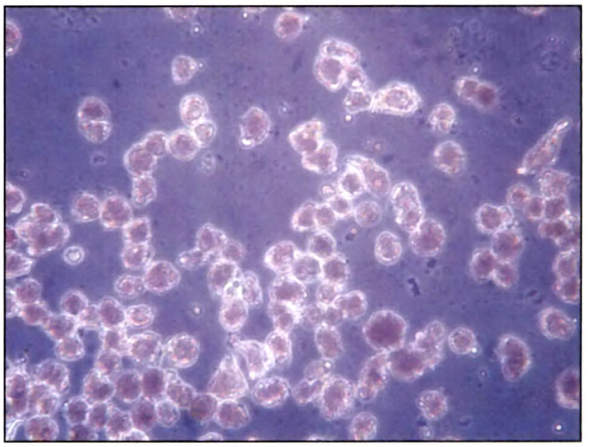


LG MET AQ 200 µg/ml (400X)

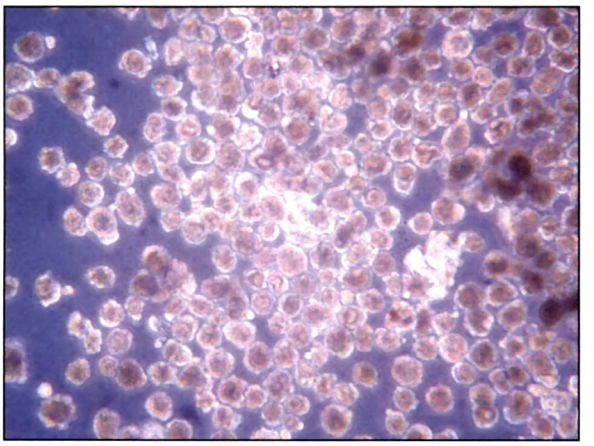
Figure 6.7 showing dose dependent growth of SaOS 2 cells with LG AQ treatment



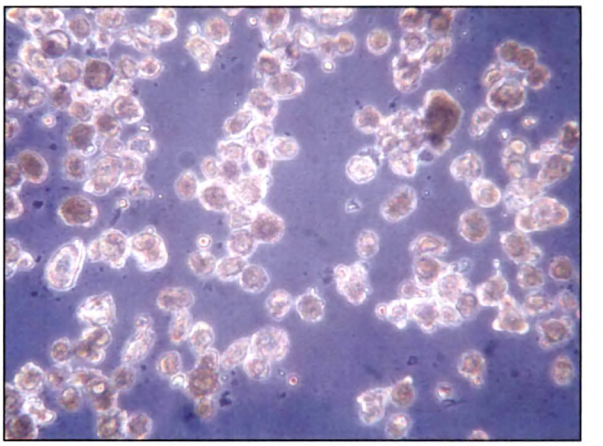
LG MET NAQ 10 µg/ml (400X)



LG MET NAQ 20 µg/ml (400X)

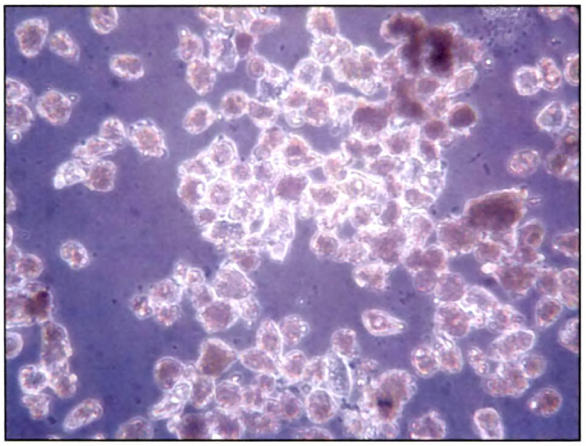


LG MET NAQ 100 µg/ml (400X)

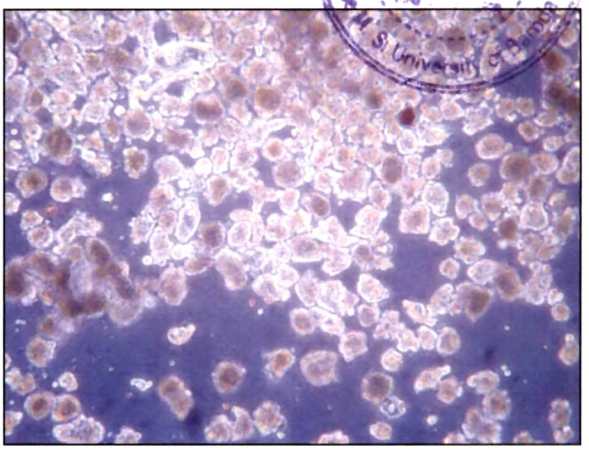


LG MET NAQ 200 µg/ml (400X)

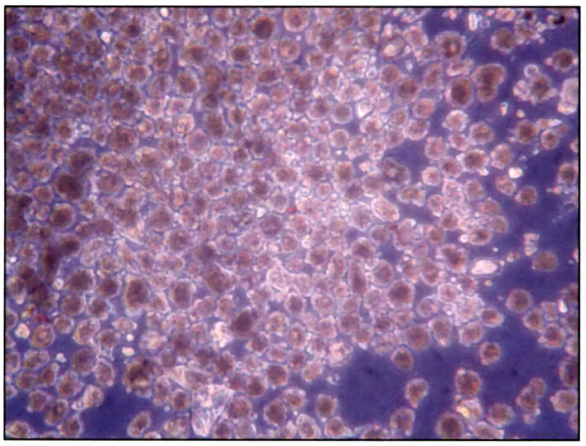
Figure 6.8 showing growth of SaOS 2 cells with LG NAQ treatment



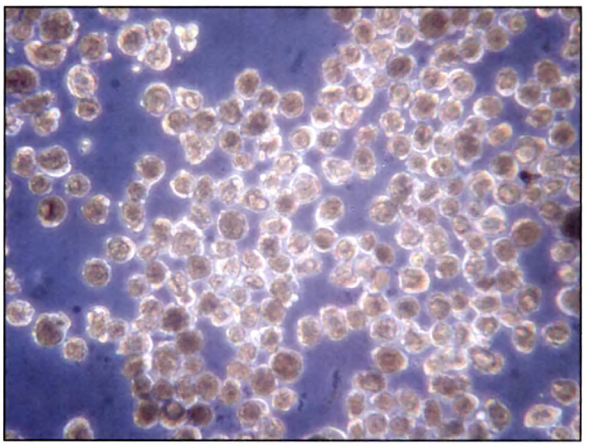
LG ALK 10 µg/ml (400X)



LG ALK 20 µg/ml (400X)



LG ALK 100 µg/ml (400X)



LG ALK 200 µg/ml (400X)

Figure 6.9 showing dose dependent growth of SaOS 2 cells with ALK treatment