CHAPTER 2

Efficacy assessment of *Litsea glutinosa* on the osteoblast Proliferation and Apoptosis using Saos-2 Cell line.

4.0 INTRODUCTION

Osteoporosis is most common disease which is found in post-menopausal women. As per recent reports, it is found that osteoporosis affects around 200 million people globally and costs approximately 14 billion USD annually (Wade et al., 2014; Sozen et al., 2017). Along with the common risk factors of osteoporosis like age, post-manopausal condition, smoking, other identified factors include high alcohol intake, nicotine use, liver disease, underweight, inflammatory joint disorders, diabetes mellitus and hyperthyroidism (Schürer et al., 2015). Main feature of osteoporosis include bone loss, deterioration of microarchitecture of bone tissue. It is very difficult to diagnose the osteoporosis at initial stage. Due to this, it remains undiagnosed and undertreated, which contributes to miss the opportunity to initiate bone modulating therapy prior to any fracture (Bliuc et al., 2013; Lee S.-R et al., 2013).

There are mainly four types of cells being found in bone tissue which includes osteoblasts, osteoclasts, osteocytes and osteoprogenitor cells. In normal condition, there are balance between the activity of osteoblasts and osteoclasts cells. Hampering of activity of either of the cells or changes in the number of cells may lead to detrimental consequences. In addition to that, if osteoclasts increases or the activity of the same increases, there will be increase in bone resorption activity, which will lead to bone loss and will results in increased bone porosity (Yahaya et al., 2018). Osteoclasts and osteoblasts comprise of metabolic cells. These cells are exhibit great influence of sex the hormones like estrogen, progesterone, androgens and other hormone like PTH on them. These hormones are responsible for maintaining balance between the activity and number of osteoclasts and osteoblasts. Deficiency of certain sex hormone leads to negative impact on bone health and its metabolism, post-menopausal condition is an ideal example of this situation where there is a deficiency of estrogen hormone (Thent et al., 2019). Estrogen downregulates the activity and formation of osteoclasts by decreasing the osteoclast proliferation supportive cytokines like IL-6, IL-1, TNF-α. It also regulates the osteoclast

proliferation by responsiveness of osteoclast precursor cell to RANKL (Ayyanan et al., 2011).

Currently there are many treatments and therapies available for osteoporosis. Management of osteoporosis by nonpharmacological approach may include increase in uptake of adequate vitamin D and calcium, weight bearing exercise, termination of smoking, alcohol consumption (NIH, 2000; Tosteson et al., 2008; Das et al., 2013; Cosman et al., 2014; Camacho et al., 2016; Buckley et al., 2017). As per institute of medicine (IOM) calcium intake should be limited to 1000 mg and 1200 mg for men of 50 to 70 years of age and women 51 years and older respectively (Taylor, 2011). Vitamin D is also a key constituent of calcium absorption and bone metabolism. As per IOM 600 IU and 800 IU is recommended per day for 51 to 70 age and older than 70 for both men as well as women respectively (NIH, 2000). However there are some studies which have shown that higher monthly doses of vitamin D is associated with significant risk of falls (Chung et al., 2011; Bischoff et al., 2016). Pharmacological approach can be categorised in mainly two category: 1. Anti-resorptive drugs (inhibiting osteoclasts) and 2. Bone building drugs (stimulating osteoblasts). Bone anti-resorptive drugs include bisphosphonate, Selective Estrogen Receptor Modulators (SERMs). They avidly bind to bone and will be internalized by osteoclasts. Fosamax®, Actonel®, Boniva® and calcitonin are some of the known and marketed BP drugs (Tortora, 2017; Martinis et al., 2019). Bone forming drugs include PTH, estrogen/hormone replacement therapy, Denoumab drug etc. However as per reports, these treatments come with many serious consequences like osteonecrosis of jaws, thromboembolic events, stroke, breast cancer and cholecystitis (Nelson et al., 2002). Because of many benefits like cost effectiveness, safety, availability, herbal medicines are gaining popularity (Sen et al., 2015).

Herbal medicines are available and can be prepared in various forms like individual phytochemical compound, crude liquid formula, crude solid powder, paste etc. There are various components in the crude like phytohormones, flavonoids, alkaloids which are known to trigger the pathways of proliferation, differentiation of osteocyte, osteoblasts and

apoptosis in osteoclasts. Phytoestrogen (PE) are chemical which occurs naturally in plants. As they have similar structures, they mimic hormones like estrogen and exhibit estrogenic effects and hence anti-osteoporotic properties (Setchell, 2001). PE can be found in fruits, whole grains, vegetables, bark and roots of plants. Studies suggest that specific recommended dose of PE can be useful in reducing the risk of cardiovascular diseases in postmenopausal women (Moreira et al., 2014). There are some PE like DAIDZEIN, EQUOL, GENISTEIN are known for its anti-osteoporotic properties (Kim H.J et al., 2002). These PEs promote differentiation and proliferation of osteoblasts and majority of them work via estrogen receptor pathways (Wilde et al., 2004; Fujioka et al., 2004; GE et al., 2006; Zhang et al., 2016).

Drynaria fortunei (KUNZE) J. SM. is a folk medicine which is used for the treatment of osteoporosis. There are many studies reported to have therapeutic effects of rhizomes of *D. fortune* (Sun J.-S et al., 2002; Chang et al., 2003; Wang X et al., 2011). It has been studied *in-vivo* as well as *in-vitro*. Sun research group has reported in *in-vivo* study that *D. fortune* is improving bone mineral density and bone mineral content of both femur and tibia bones in OVX rats. It also drastically prevented excretion of Ca and P, keeping serum levels of the same unchanged, which indicates balanced mineral homeostasis and lesser bone mineralization. Along with these, it also corrected the increases ALP activity (Sun X. et al., 2020). As per Chang research group data, ethyl acetate-soluble fraction of *D. fortunei* rhyzomes stimulated proliferation of the MCF-7 and ROS 17/2.8 cells in dose dependant manner (Chang et al., 2003). As per *in-vitro* study, bigger polar naringin, compounds from smaller polar fraction of *D. fortunei* promoted proliferation of UMR 106 cells. Flavonoid aglycones in the small polar anti-osteoporotic fraction was observed to promote mineralisation and differentiation of UMR 106 cells *in vitro* via ER signaling pathway (Wang X et al., 2011).

Angelica sinensis is another herbal medicine which is known for its anti-osteoporotic properties. Besides anti-osteoporotic, it has been also known for cleansing blood and enhancing blood circulation. It is also utilized as a cure against menstrual irregularities, anaemia, constipation etc (Song et al., 1999;

Yim et al., 2000; Liu J et al., 2001). It has been established that *A. sinensis* improves ALP activity. It also enhances type-I collagen protein synthesis. It also improves OPC-1 cell proliferation in lower concentration supplement, resulting increase in bone formation process (Yang Q. et al., 2002). Recent *in-vivo* as well as *in-vitro* reports revealed that Ligustilide, a major bioactive component of *Angelica sinensis*, promotes osteoblasts differentiation and also prevents apoptosis of pre-osteoblast. It works through the GPR30/EGFR pathway (Yang F. et al., 2019).

Cissus quadrangularis is the most widely preferred herbal medicine which is prescribed for improving bone conditions. It has been reported that *C. quadrangularis* induces bone nodule formation. It also induces differentiation and matrix mineralisation of Saos-2 cells. It works through RUNX2 pathway by increasing its RNA and protein expression (Muthusami et al., 2011; Tasadduq et al., 2017).

One such herbal, *Litsea glutinosa* (LG) has been studied widely for its various properties like anti-inflammatory, antibacterial, anti-osteoporotic etc. It has already been established that LG improved bone microarchitecture in OVX rat model. It also corrects decreased ALP level and serum Ca level. In exploration process of molecular mechanism of LG (**CHAPTER I)**, it was observed that during MTT assay LG enhances the cell viability of Saos-2 osteoblastic cells. It enhances of gene expression of various transcription factors and kinases. As explained, there are ample articles suggesting many herbal plants increase bone cell proliferation. It was hypothesized that there might be a proliferative effect of LG on bone cells and it is still among unexplored areas of LG.

Thus, the study hypothesised that the L. glutinosa may have some components which elevate the proliferation of Osteoblast through its action via different candidate markers like PCNA, Osteocalcin and inhibit the apoptotic markers (Caspase 3, FasL and Cytochrome C) thereby antagonising the effect of Osteoclast cells and maintaining the normal homeostasis. As data is scarce regarding these markers, the present study is inclined towards deciphering the role of the markers upon the action of LG thereby proving its role as a potent anti-osteoporotic agent.

4.1 MATERIAL AND METHODS:

4.1.1 EXPERIMENT REGIME:

For the treatment of LG to Saos-2 cells, methanolic extract of LG was prepared. For the same, bark powder was procured from local market. It was further processed exactly same as described in Material and methods of Chapter 1. After preparing the methanolic extract paste, it was stored at -20 °C. Saos-2 cell line were purchased from National center for cellular science institute (NCCS, Pune). Cells were maintained and propagated same as mentioned in Chapter 1. For the treatment and study, cells between passages P#3 to P#7 were used. LG methanolic extract was dissolved in DMSO to prepare main stock solution and further diluted for respective experiment as mentioned in **CHAPTER I** material and methods.

4.1.2 TRANSCRIPT ANALYSIS:

4.1.2.1 TOTAL RNA ISOLATION & CDNA SYNTHESIS:

Cells from all the flasks were trypsinized. They were resuspended in TRIzol® reagent (15596026, Invitrogen, USA) for total RNA isolation. TRIzol® reagent manual protocol was followed to isolate and purify total RNA. After purification, RNA concentration was estimated by NanoDrop C at 260 nm. First strand of cDNA was synthesized using Thermo cDNA synthesis kit, 5 µg of total RNA was used. cDNA was converted using oligoDT primers using manual protocol (K1612, Thermo; USA). Once cDNA was synthesized, it was then used neat as a template for transcript analysis of desired genes.

4.1.2.2 QUANTITATIVE PCR:

Variable volumes were used as a sample for real time PCR to analyze the expression level of various genes. Different sets of primers (Table C2. 2) were used for transcript analysis using PowerUP Sybr® green master mix (A25742, Thermo, USA). A standard manual PCR conditions (Table C2. 1) were used for amplification. The experiment was performed in triplicates (n=3).

Table C2. 1: Real time PCR conditions

Stage name	Conditions	Cycles/ramp rate
Initial denaturation	95 °C for 5 min	1 cycle
Amplification stage	95 °C for 15 sec	40 cycles
	60 °C for 30 sec	
	72 °C for 45 sec	
Melt curve generation	95 °C for 15 sec	Ramp rate 1.6 °C/sec
	60 °C for 1 min	Ramp rate 1.6 °C/sec
	95 °C for 15 sec	Ramp rate 0.15 °C/sec

*Table C2. 1**Error! Reference source not found.** indicates PCR conditions used for amplify target gene using SyBr green mastermix. Post 40 cycles of amplification, melt curve was run to identify Tm of each gene and checking of non-specific amplification if any.*

Table C2. 2: Primer details

	Gene Name	RefSeq ID	T_m value (°C)	Primer Type	Sequence
1	PCNA	NM_182649.2	65	Forward	5' AAGAGAGTGGAGTGGCTTTTG 3'
			64	Reverse	5' TGTCGATAAAGAGGAGGAAGC 3'
2	CASP3	NM_001354783.2	62	Forward	5' TCGCTTCCATGTATGATCTTTG 3'
			63	Reverse	5' CTGCCTCTTCCCCCATTCT 3'
3	FASLG	NM_000639.3	65	Forward	5' CAGAGGCATGGACCTTGAGT 3'
			65	Reverse	5' GTCTACCAGCCAGATGCACA 3'
4	BGLAP	NM_199173.6	63	Forward	5' GCCTGGGTCTCTTCACTA 3'
			65	Reverse	5' TCACACTCCTCGCCCTATT 3'
5	CYCS	NM_018947.6	51	Forward	5' GGGGTAAACAGTGATACC 3'
			53	Reverse	5' GAATGACTGACAGAATATTTTG 3'
6	ACTB	NM_001101.5	66	Forward	5' GCAACGGAACCGCTCATT 3'
			67	Reverse	5' AGCTGAGAGGGAAATTGTGCG 3'

Table C2. 2 is indicating details of primers of all the genes along with Tm value and Gene RefSeq ID of each gene from NCBI. β-Actin was taken as a endogenous control & vehicle control was taken as test control in real time PCR to calculate $\Delta\Delta C_T$ and thereby RQ (relative quantitation). $\Delta\Delta C_T$ is calculated from ΔC_T mean (

*Equation **1**). (PCNA: PCNA gene; CAP3: Caspase 3 gene; FASLG: Fas Ligand gene; BGLAP: Osteocalcin gene; CYCS: Cytochrome C gene; ACTB: β-actin)*

4.1.3 FLOW CYTOMETRY CELL VIABILITY ASSAY:

Saos-2 cells were treated with LG Methanolic extract in concentration of 250 µg/mL, 100 µg/mL & 50 µg/mL for 96 hr along with untreated control. After completion of treatment, cells were harvested by trypsinization. These cells were stained with CalceinAM (C3099, Thermo,USA) and Propidium iodide (PI) (P1304MP, Thermo,USA) stains. After staining, cells were washed and analyzed in BD FACSverse™ using green (527/32) and red filters (586/42). A total 10,000 events were run in FACS. All the samples were run in triplicates (n=3). To generate dead cells control, live cells were treated with 0.2% triton-X-100 (Sigma; 93427), incubated for 20 min at 37°C.

4.1.4 STATISTICAL ANALYSIS

All the data were statically analyzed. The statistical analysis was performed using one-way ANOVA using GraphPad Prism 8.3.1 Software followed by Dunnett's multiple comparisons test to test the hypothesis. Significance was analysed for each sample and was compared to control to understand the variance within the experimental groups and was noted at $p<0.05$, $p<0.01$.

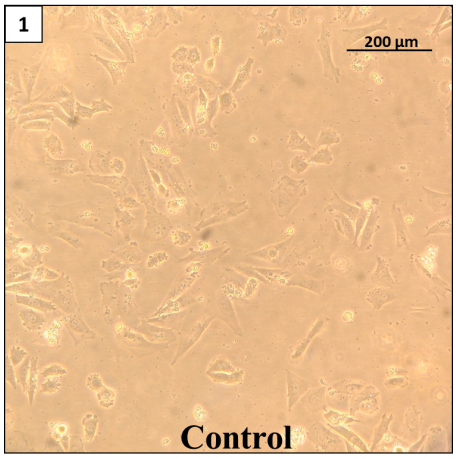
4.2 RESULTS:

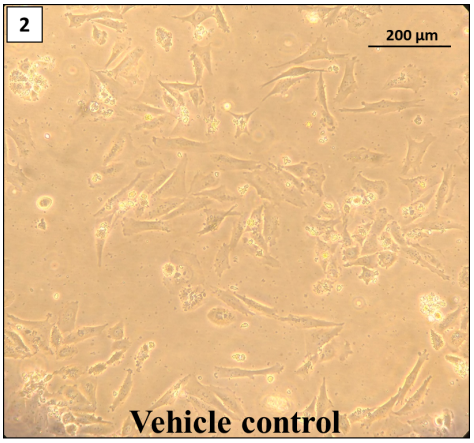
4.2.1 EXTRACTION:

The total yield obtained from LG methanolic extraction was 4.42 gm, which corresponds to ~8.8% total yield.

4.2.2 CELL IMAGES:

During LG treatment, post 96 hrs, cells were examined microscopically to cell architecture and growth. It was observed that cell number (Figure C2. 1) increased visually in a dose dependent manner compared to control and vehicle control.





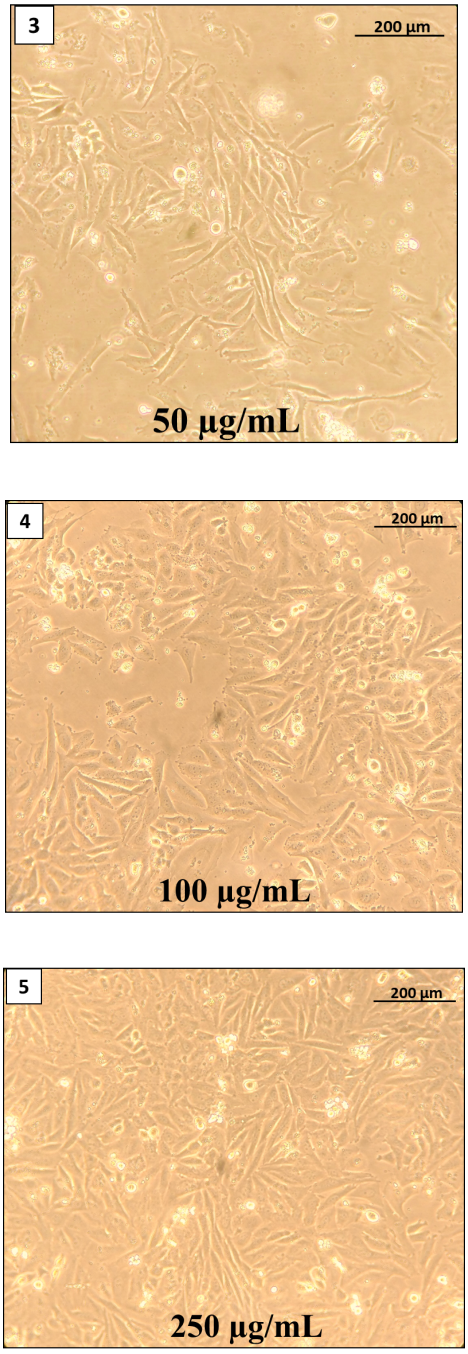


Figure C2. 1: Cell images after 96 hr of treatment with LG methanolic extract.

Figure C2. 1 indicates that there is high cell density in 50 µg/mL compared to control and gradually getting increasing till 250 µg/mL which is in dose dependent manner (n=3). Each image is designated with respective dose concentrations 'Control' indicates untreated cells; 'vehicle control' indicates cells treated with DMSO. (Microscopic resolution 200x)

4.2.3 REAL TIME PCR

The quantitative PCR results showed that expression of the proliferative markers like PCNA and osteocalcin were found to be upregulated significantly ($p<0.01$ for each) in the range of 2.1 – 2.5 with compared to control. This observations were in dose-dependent manner. Whereas apoptosis markers like Caspase 3, cytochrome C ($p<0.01$) were down regulated upon treatment in 0.4 – 0.8 range when treated with 250 µg/mL concentration in dose-dependent manner. However, there was a change in the Expression of FasL in LG treatment, but it was not non-significant Table C2. 3 shows the fold change (relative quantification) of candidate genes compared to untreated control and vehicle control along with Standard error (Table C2. 4). Refer Figure C2. 2 which indicates graphical representation of expression profile.

Table C2. 3: Relative quantitation of genes using Real time PCR

RQ (Relative quantitation)					
	Control	**Vehicle control**	**50 µg/mL**	**100 µg/mL**	**250 µg/mL**
PCNA	1.00	1.00 ± 0.00	1.37 ± 0.01	1.56 ± 0.05	2.56 ± 0.09
Osteocalcin	1.00	1.03 ± 0.06	1.35 ± 0.11	1.65 ± 0.19	2.11 ± 0.11
Caspase 3	1.00	1.02 ± 0.05	0.83 ± 0.06	0.67 ± 0.07	0.41 ± 0.06
CytochromeC	1.00	0.99 ± 0.02	0.90 ± 0.08	0.87 ± 0.04	0.80 ± 0.05
FasL	1.00	1.03 ± 0.02	2.10 ± 0.12	0.87 ± 0.35	1.48 ± 0.24

Table C2. 3 indicates relative quantification of all the genes. Table indicates fold change mean values± SD with compared to expression in control sample (n=3).

Table C2. 4: Standard error and Significance value of Gene fold change

RQ (Relative quantitation)					
	Control	**Vehicle control**	**50 µg/mL**	**100 µg/mL**	**250 µg/mL**

PCNA	1.00	0.00	0.01**	0.03**	0.05*
Osteocalcin	1.00	0.03	0.02**	0.04**	0.07*
Caspase 3	1.00	0.01	0.01 **	0.02 *	0.01 *
Cytochrome C	1.00	0.01	0.01**	0.05*	0.04*
FasL	100	0.01	0.02*	0.02 ^{ns}	0.01*

*Table C2. 4 indicates standard error and significance value of respective gene fold values. *- p< 0.05; **- p<0.01; ns: non-significant. All the data are presented as mean ±S.E. and are representative of three independent experiments (n=3).*

4.2.3.1 GRAPHICAL REPRESENTATION:

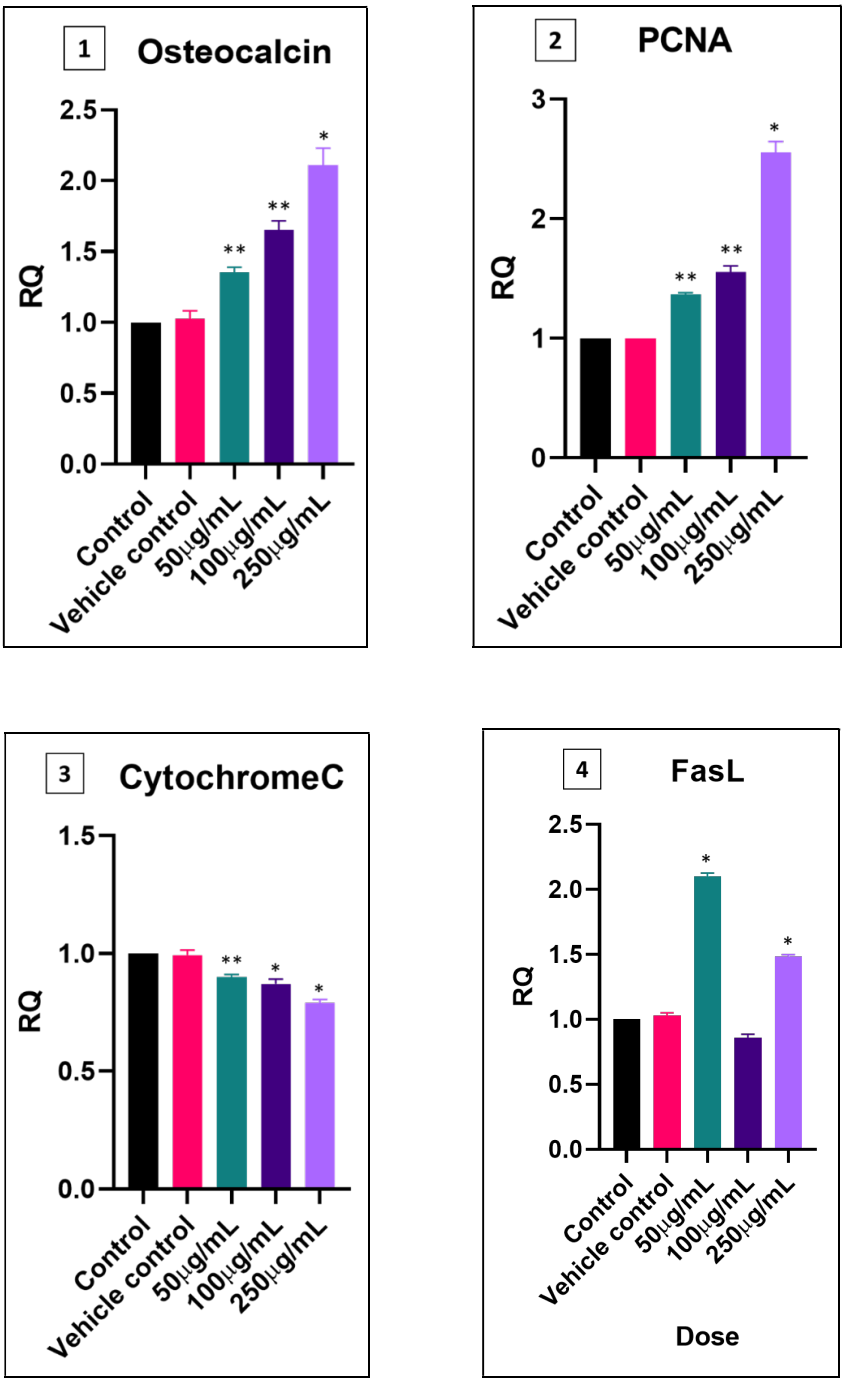


Figure C2. 2: Gene expression profile (transcript analysis) of various genes

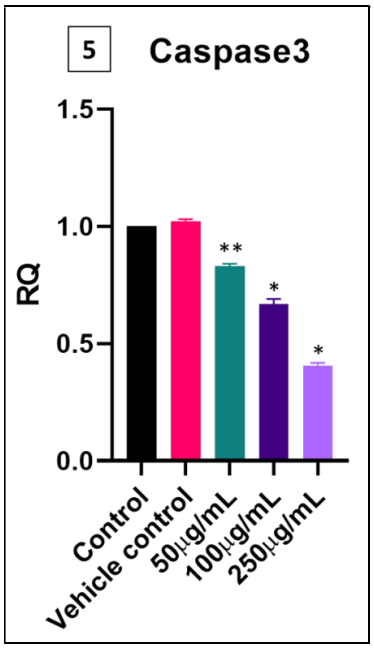


Figure C2. 2: Gene expression profile (transcript analysis) of various genes

*Each graph is showing the expression profile of different genes ((1) Osteocalcin; (2) PCNA; (3) Cytochrome C; (4) FasL; (5) Caspase 3). *- p< 0.05; **- p<0.01; Calculated using one-way ANOVA test followed by Dunnett's multiple comparisons. Data are presented as mean ± S.E. and are representative of three independent experiments (n=3).* Refer Appendix A2.1 figure of Q-PCR amplification.

4.2.4 FLOW CYTOMETRY CELL VIABILITY ASSAY:

To study the proportion of live vs. dead cells, flow cytometry cell viability assay was conducted. The viable cells were in the range of 94.6 % to 98.1 % while the apopotic cell were in the range of 5.3 % to 1.9 % in different groups. It was observed that there is a gradual decrease in the proportion of dead cells in dose dependent manner (Table C2. 5; Figure C2. 3).

Table C2. 5: Results of cell viability assay using FACS

Control						
					Total cells	915
Name	All Events	Live	Nonlabled	Dead	% live	93.66
Events	10,000	857	12	58	% dead	6.34
250 µg/mL						
					Total labelled cells	632
Name	All Events	Live	Nonlabled	Dead	% live	98.10

Events	10,000	620	1	12	% dead	1.90
100 µg/mL						
					Total labelled cells	750
Name	All Events	Live	Nonlabled	Dead	% live	96.40
Events	10,000	723	11	27	% dead	3.60
50 µg/mL						
					Total labelled cells	732
Name	All Events	Live	Nonlabled	Dead	% live	94.67
Events	10,000	693	5	39	% dead	5.33

Table C2. 5 indicates proportion of live and dead cells post treatment FACS analysis cell viability assay. It can be observed that proportion of dead cells are getting decreased in dose dependent manner compared to control.

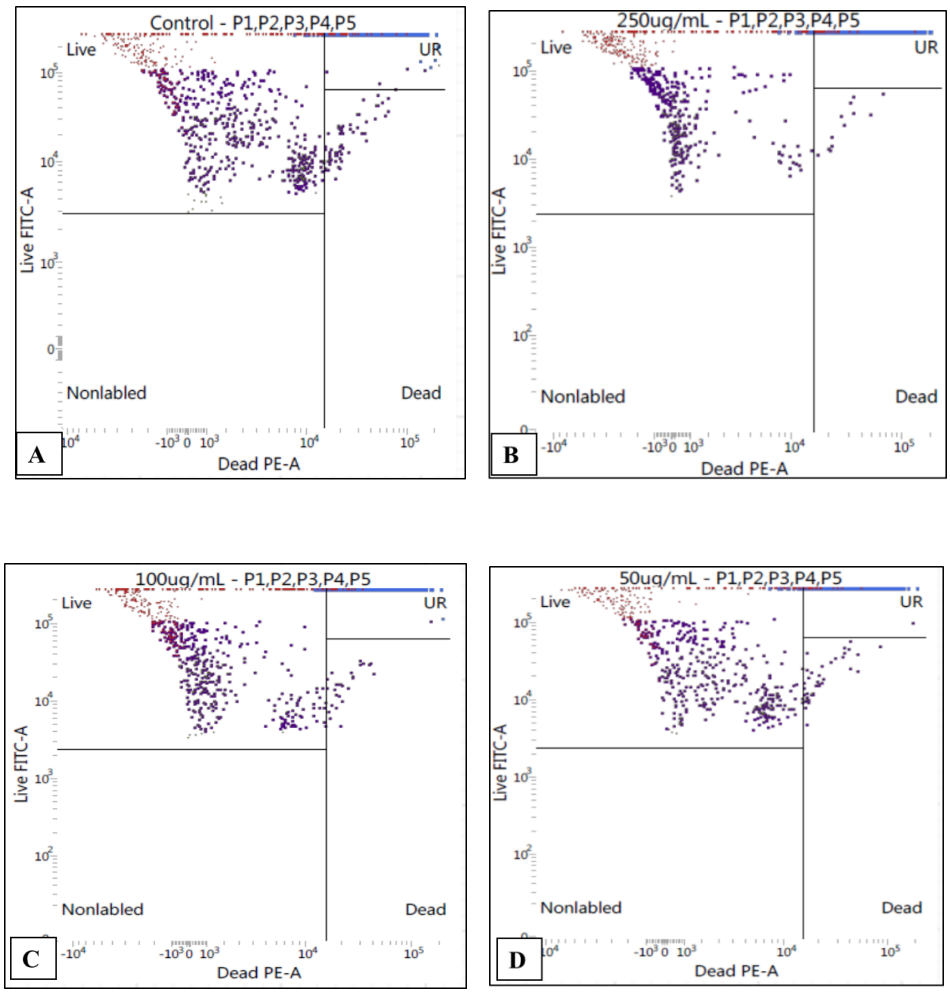


Figure C2. 3: Scattered plot diagram of cell viability assay analysed by BD FACSverse™.

Figure C2. 3 indicates FACS cell viability assay analysis of Saos-2 cells post LG methanolic extract treatment. Scattered plots for (A) Untreated control cells; (B) 250 µg/mL; (C) 100 µg/mL; (D) 50 µg/mL. It can be observed that number of dead cells are getting gradually decreased with increment in LG dose.

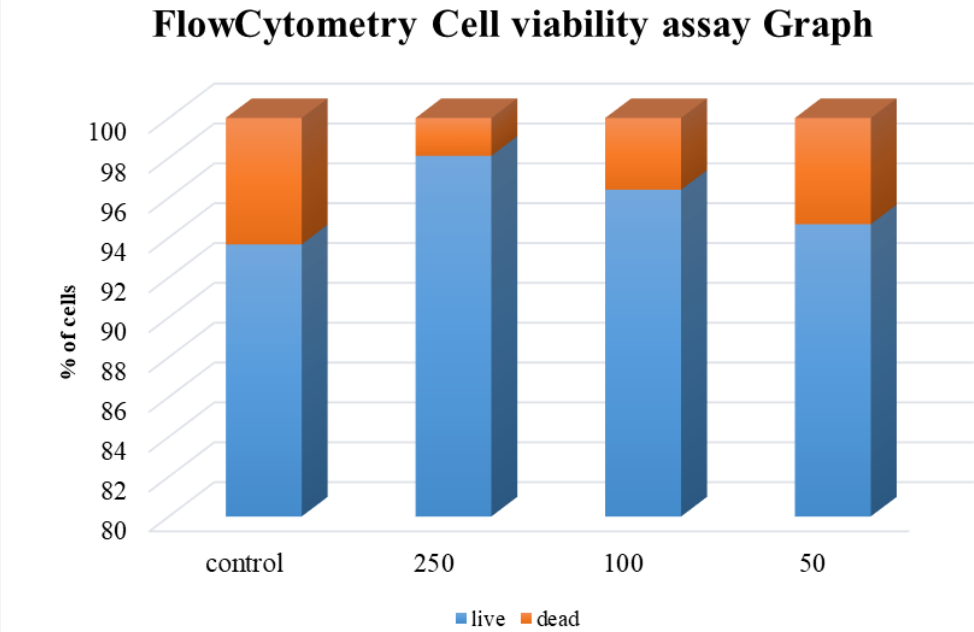


Figure C2. 4: Flow cytometry cell viability assay graph

Figure C2. 4 indicates the total proportion of % viable and % dead cells post LG treatment to the Saos-2 cells. It can be observed that % dead cells gets decreased gradually with dose indicating anti-apoptotic property of LG.

4.3 DISCUSSION

Osteoporosis is a frequent age-related disorder characterized by a systemic impairment of bone mass and altered micro architecture that results in fragility fractures (Pietschmann et al., 2016). It is a multi-factorial disease which shows etiopathogenetic mechanisms which overlap vastly. Primary (post-menopausal and senile) and secondary osteoporosis (caused by various drugs and pathologies) can be distinguished (Güler et al., 2018). Besides this, osteoporosis prevalence in world is very high, it affects around 200 million people and it becomes major determinant of morbidity, mortality and disability of older people (Martinis et al., 2019). There are many treatments and therapies available in the market. However, the major concern with the prolonged use of these therapies results in a greater risk of osteonecrosis of the jaw and atypical femoral fracture (Cheng et al., 2020). Besides this, there are ample studies showing potential risk of breast cancer, heart attacks, stroke, and blood clot formation associated with HRT. These conditions have promoted USFDA to issue safety notice. Hence HRT is no longer recommended as first-line therapy for osteoporosis (Stephenson, 2003; Chen L. R. et al., 2019).

In search of safer medicine from herbal sources, LG is being investigated for its anti-osteoporotic properties. In chapter 1, it was observed that LG treatment on Saos-2 cell line modulates various crucial transcription factors like RUNX2, ERG2, CREB, NFATc1 as well as some genes of important signalling pathways like adenylate cyclase, MAPK3 etc. Earlier the studies done *in-vivo* by Parikh group has already been observed that serum ALP levels are increasing upon LG treatment to OVX rates (Parikh, 2009). These results were correlated with many other herbals like *Drynaria fortune, Angelica sinensis, Cissus quadrangularis,* which are reported to have proliferative effects on osteoblasts (Dietz et al., 2016; Tasadduq et al., 2017; Sun X et al., 2020). To study the proliferative and apoptosis effects of LG on Saos-2 cells, respective markers were studied along with cell morphology and growth.

Osteocalcin (OCN) is the key player in bone endocrinology, which is secreted solely by osteoblasts. A recent article enlightens osteocalcin, being a marker

protein of osteoblast proliferation, is involved in versatile functions like glucose metabolism, male fertility and neuronal development (Moser et al., 2019). Expression of osteocalcin is mainly facilitated by MAPK3 protein, which binds to the promoter of osteocalcin through association with RUNX2 (Liu L et al., 2019). Hence it can be stated that a significant dose dependent upregulation of OCN, RUNX2 and ERK1 (MAP3) indicates that LG may be involved in the upregulation of osteocalcin via participation of RUNX2 and ERK1 proteins. Our results are parallel with the work of Liu group results (L. Liu et al., 2019). Another research group has also reported the similar results. They have studied three *C. atratum*, *M. azedarach* and *C. turtschaninovii* herbals for its proliferative effects on osteoblasts by monitoring osteocalcin level (Mukudai et al., 2014). These results also support presented outcomes of LG proliferative effects.

PCNA is very well known marker for studying proliferation in various types of cells. This is one of the proteins which are synthesized between G1 and S phase of cell cycle. (Strzalka et al., 2011; Juríková et al. 2016). Many researchers have demonstrated proliferation effects of herbal compounds on osteoblasts by means of expression of PCNA gene. Like icariin (Song L et al., 2013; Zhang et al., 2019). Therefore to check proliferative effects of LG, gene expression level of PCNA was also checked after LG treatment to Saos-2 cell line. It was also observed that PCNA is also getting significantly upregulated upon LG treatment. Besides these, cell morphology was studied during the treatment. It was observed that the cells were comparatively healthier having the intact morphology and extensions. The cells were found to be les stressed and were more in number when counted visually which was in an increasing trend in the dose dependent manner. Thus, high number of cells suggest that a significant role of LG which may have induce proliferation in the Osteoblast of Saos-2 cells.

It has been established that LG promotes growth of Saos-2 cells. To scrutinize the rest part of the hypothesis, key markers for apoptosis were studied like caspase 3, cytochrome C, FasL. These markers are very well established and studied as an indicator of apoptosis (Kim J et al., 2015; Lin J et al., 2018). For

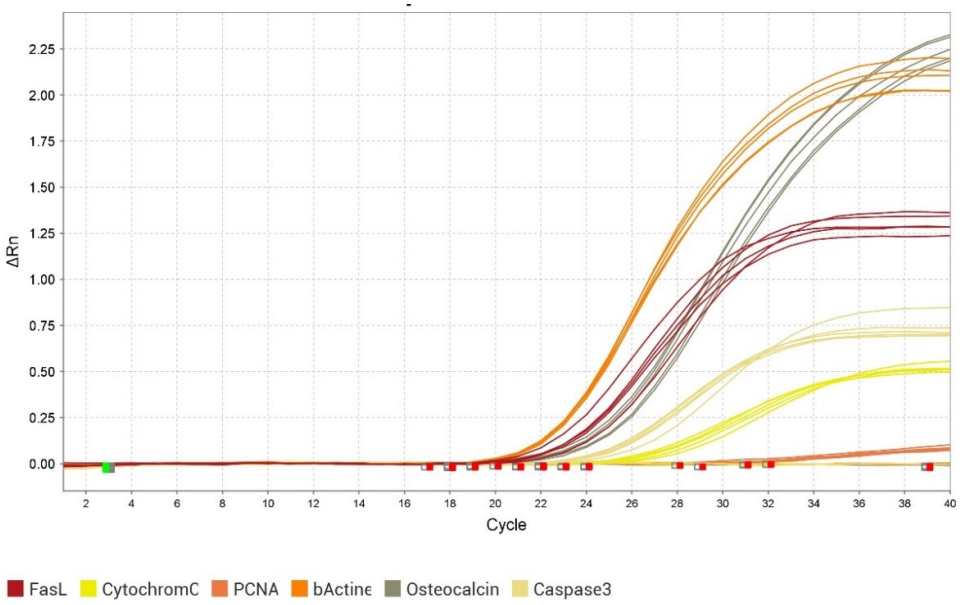
this, expression profile of these genes were studied. It was observed that Cytochrome C and Caspase 3 were getting significantly downregulated upon LG treatment in dose depended manner. This observation suggests LG suppresses the expression of these apoptotic markers. Further suggesting LG may have an effect in decreasing the process of apoptosis. These results are well consistent with the results reported by other scientists in studying effects of other compounds like Icariin, DBT (Danggui Buxue Tang), *Acacia catechu* bark powder extract (Song L et al., 2013; Lakshmi et al., 2017; Gong et al., 2019) Expression of FasL was inconsistent with the dose and was not significant. FasL is the protein which is monitored to study apoptosis. In contrast to that, there are reports which suggest FasL regulates apoptosis in osteoclasts in paracrine manner and some herbals enhances the FasL expression which may in turn promote the apoptosis in osteoclasts (Krum et al., 2008). Conversely, the expression profile in the present work directs that LG may have no impact on FasL expression. This may be due to it may exert its effect via other markers which in directly affects the FasL expression.

Proliferation was also validated using flow cytometry based cell viability assay. Reports suggests that some fluorescent dyes can be employed to analyse the cell cytotoxicity, viability, etc (Stoddart, 2011; Chan et al., 2012). Using such dyes like Calcein AM and propidium iodide, cell viability assay was run in flow cytometry to examine overall proportion of live vs dead cells in LG treated Saos-2 cells. Results revealed that there was a decrease in the population of dead cells in dose dependent manner. These results support earlier obtained results. It can be commented that LG is promoting the growth of Saos-2 cells and along with it also curtailing the apoptosis process which resulted in decrease in dead cell population. Our results are in parallel with the results reported by (Gillissen et al., 2016). This group had used the same assay to indicate the proportion of PMBC.

In conclusion, it can be stated that LG has proliferating as well as anti-apoptotic effect on osteocytes like cells, Saos-2. This effect may result in increase in the population of osteoblasts. This might be the reason behind its bone healing and anti-osteoporotic properties. However, these results can

further be supported by performing the protein expression markers and other enzyme activity assays to strengthen the presented results.

APPENDIX A2.1 FIGURE



Appendix A2.1 figure represents the amplification curve of one of the triplicate run of the gene expression study of proliferative and apoptosis markers and endogenous control β-Actin. Curve is plotted by Applied Biosystems QuantStudio™ Design and Analysis Software v1.5.1. Y axis represents Fluorescence collected by the detector and X axis represents no. of cycle. Each target gene represents different samples like host, vehicle control, test 50 µg/mL, 100 µg/mL and 250 µg/mL.