



Original Research Article

Molecular insights for an anti-osteoporotic properties of *Litsea glutinosa* on Saos-2 cells: An *in-vitro* approach

Hitarth Changani, Pragna Parikh*

Department of Zoology, The M. S. University of Baroda, Vadodara, 390020, India



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ABSTRACT

Osteoporosis is a skeletal disease that is identified by the deterioration of micro-architecture of bone tissue, leading to enhanced bone brittleness and a consequential increase in fracture threat. There are many treatments available for osteoporosis such as bisphosphonate therapy, hormonal replacement therapy, herbal therapy etc. For decades, there are several herbs that are attributed to have anti-osteoporotic effects however the candidate genes involved in it remained unknown. In line with this, the present study is focused to elucidate the anti-osteoporotic property of *Litsea glutinosa* (LG). To understand the proliferative effect and identify involved players, gene expression was studied on the Saos-2 osteocytes *in-vitro*. The expression profile of candidate genes involved in different signaling pathways such as Egr-2, RUNX2, MAPK3, NFATc1, CREB, ERβ, along with proliferation and apoptotic markers in osteoporosis were selected for the study. The gene expression profile demonstrated a significant up-regulation of Egr-2, RUNX2, MAPK3, CREB, ERβ in the range of 1.5–2.2 folds, whereas NFATc1 was found to be down-regulated up to 0.4 times compared to control when treated with 250 μg/mL of LG. Besides this, anti-apoptosis effect of LG was also supported by flow cytometry results which also proved that LG induces proliferation and inhibits apoptosis, suggesting the proliferative role of LG. In conclusion, the present study gathers the potency of LG extract for its proliferative and anti-apoptotic effect on Saos-2 osteocytes and opens a new avenue for detailing the mechanistic actions of it on mitigating the pathophysiology of osteoporosis.

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1. Introduction

Osteoporosis is a frequently manifesting age-related disorder characterized by a systemic impairment of bone mass and altered micro architecture that results in fragility fractures [1]. It is a multifactorial disease that shows etiopathogenetic mechanisms which overlap vastly. Osteoporosis can be categorized in Primary (post-menopausal and senile) and secondary osteoporosis (caused by various drugs and pathologies) based on its cause [2–4]. The prevalence of osteoporosis in the world is very high, it affects around 200 million people and it becomes a major determinant of morbidity, mortality and disability of older people [5]. Considerable progress has been made toward treating the osteoporosis worldwide which includes drugs that target bone resorption, such as

bisphosphonate, calcitonin, Denosumab, estrogen and hormone replacement therapy [6]. Out of these, bisphosphonate is the first line of treatment clinically [7]. However, the major concern with the prolonged use of bisphosphonate and Denosumab is, it results in a greater risk of osteonecrosis of the jaw and atypical femoral fracture [6,8]. Ample studies have reported a potential risk of breast cancer, heart attacks, stroke, and blood clot formation associated with HRT (Hormone Replacement Therapy). These conditions have promoted USFDA to issue safety notice. Hence HRT is no longer recommended as first-line therapy for osteoporosis [9–11].

Due to the drawbacks with current medicines, the identification of new drugs to treat osteoporosis is a global necessity. Herbal medicines have unique advantages in the treatment or prevention of osteoporosis and have created a center of attention among scientists globally [12]. Many herbal compounds have been studied and confirmed their anti-osteoporotic properties such as Genistein [13–15], Sulfuretin [16], Diosgenin, [17]. In addition to these, isolated and purified molecules such as Salvianolic acid B (Sal B),

* Corresponding author.

E-mail: php59@yahoo.co.in

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Ugonin K, Kireanol have proved to possess anti-osteoporotic potential [13,18]. One such herb is *Litsea glutinosa* (LG), it has been reported to have anti-inflammatory, and wound healing activities [19], it also has been identified as cure of chronic cough [20] and has been used in drug development for cardiac diseases [21]. The *Charakasamhita* (~900 B.C.) is an ancient literature which is first recorded treatise entirely dedicated to the conception and practice of Ayurveda. In this literature, LG is classified as *Jivaniya* (Promoting longevity) and has been reported to have antibacterial, antioxidant, hypotensive, antipyretic and chemoprotective properties [22]. As per Ayurveda, it has proved to possess bone protecting outcomes. LG is commonly known as "*Maida Lakri/Lakdi*" (traditional name) and is known to be one of the most potent plants for the treatment of osteoporosis [23]. Further, experimental evidences have also confirmed its osteoprotective role and its ameliorating effect on osteoporosis in OVX mice [23,24], by reducing serum TRAcP (Tartrate-resistant acid phosphatase) levels, restoring ALP (alkaline phosphatase) and reducing the rate of Ca^{++} excretion in dose dependent and time dependent manner. Furthermore, the same group of researchers has validated the qualitative results through histological studies.

Till date, studies enlisting responsible genes in the mode of actions of medicinal plants are restricted to few herbs. In the consideration of LG, there is a knowledge gap that demands a focus on candidate genes that may be responsible for the plant's osteoprotective role, established *in-vivo* [23,24]. Therefore it was hypothesized that LG might have some proliferative effects and/or anti-apoptotic effects on osteocytes. To study the expression profile and role of the candidate genes, the present study has considered some marker genes of osteoblasts such as RUNX2 (Runt-related transcription factor 2), Egr-2 (Early Growth Response 2), NFATc1 (Nuclear Factor of Activated T Cells cytoplasmic); some genes involved in signalling pathways such as MAPK3, CREB (c-AMP Response Element Binding Protein), adenylate cyclase and ER β ; proliferative and apoptotic markers such as PCNA, osteocalcin, FasL, Caspase 3 and cytochrome C for gene expression studies.

Egr-2 and RUNX2 genes are involved in the process of osteoblastogenesis and proliferation of osteoblasts [25,26]. Egr2 is a zinc finger transcription factor that has been demonstrated to be responsible for the negative regulation and inhibition of RANKL mediated osteoclast differentiation [25,27]. Data of several studies suggest the importance of RUNX2 transcription factor in regulating osteoporosis [28–30]. RUNX2, an osteogenesis-promoting transcription factor, promotes the proliferation of osteoblast progenitors and their commitment to osteoblasts lineage cells. An upregulation of RUNX2 thus suggests that some degree of osteogenesis had occurred in Saos-2 cells [30]. NFATc1 is considered to be a master transcription factor required for differentiation of osteoclasts. Inhibition of calcineurin through genetic deletion in osteoblasts results in decreased dephosphorylation of NFATc1 and thus its translocation to nucleus ultimately leading to osteoblast differentiation [31,32]. MAPK3, ER β , CREB and adenylate cyclase are another set of genes which were considered in the present study. Out of these MAPK3, CREB and adenylate cyclase are important members of pathways such as MAPK3-RAS/RAF and GPCR (G protein coupled receptor) mediated signalling [33–35]. ER β is one of the receptors for estrogen which are expressed on osteoblasts [36].

2. Materials and method

2.1. Preparation of LG extract

Bark powder of LG was purchased from the local market (Mewar Impex, ASIN: B08QV618V6; Item number: MI-MAIDALAKP-100-

20). LG powder (50 gm) was weighed and resuspended in 400 mL HPLC grade methanol (SA8SF68037, Merck, USA) and volume was made upto 500 mL. The solution was incubated at RT (room temperature) on magnetic stirrer (REMI, 1 MLH, India) overnight. After incubation, the solution was filtered through Whatman® filter paper (MN615A, MN, Germany) to remove insoluble particles. The methanolic extract was allowed to air dry by keeping it in waterbath at 60 °C for 48 hours. Upon drying, solid residue was collected, weighed and stored at –20 °C till further processing.

2.2. Culturing of Saos-2 cells

For all the experiments, well established Saos-2 cell line was used which was procured from National Centre for Cell Science Institute (NCCS, Pune, India). Throughout the experimentation, these cells were maintained in T75 and T25 flasks in McCoy's 5A (16600082, Gibco, USA) + 10% FBS media (RM9955-100 ML, Himedia, India) at 37 °C in 5–8% CO₂ humidified incubator. During routine culture of Saos-2 cells, cells were washed with 1x PBS, followed by trypsin (TCL099, Himedia, India) treatment. Post trypsinisation, these cells were counted using hemocytometer and were seeded in the fresh flask at a seeding density of 0.4 Million cells per mL. Throughout all the experiments, cells from lower passages (i.e., 4–8) were considered for the study.

2.3. LG treatment

The methanolic extract residue was weighed 250 mg. The methanolic extract residue was then dissolved in the DMSO (D8418, Sigma, USA), generating 250 mg/mL stock solution. One day prior to the treatment, cells were seeded in T25 flask and serial dilutions of LG extract were prepared in cell culture media (McCoy's 5A (16600082, Gibco, USA) + 10% FBS (10270106, Gibco, USA)) from stock solution to achieve 250 µg/mL, 100 µg/mL & 50 µg/mL working solutions and was filtered by 0.2 µ filter prior to treatment. Tests flasks, control (untreated cells) and vehicle control flasks were incubated at the same conditions for 96 hours.

2.4. MTT assay

MTT assay was performed to identify the effective concentration of LG methanolic extract. This was measured by an 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay (M2128, Sigma, USA). Saos-2 cells were plated in 96-well plates at a density of 5×10^3 cells per well per 200 µL in 6 replicates. Along with host control and vehicle control, all the replicates were treated with different concentration (500 ng/mL, 10 µg/mL, 50 µg/mL, 100 µg/mL, 150 µg/mL, 200 µg/mL, 250 µg/mL, 300 µg/mL, 350 µg/mL and 400 µg/mL) of LG for 96 hours. For DMSO control, 0.2% (v/v) DMSO concentration was considered. Post 96 hours, the cells were washed with PBS. Further, 120 µL of 500 µg/mL MTT solution (prepared in McCoy's 5A) was added to each well and cells were incubated in a humidified atmosphere of 5% CO₂ at 37 °C for 4 hours. Subsequently, 100 µL of 10% SDS-solution was mixed (10% SDS (L3771, Sigma, USA) in 0.01N HCl (320331, Sigma, USA)) and was added to each well and mixed thoroughly and the cells were incubated at room temperature for 10 minutes. The absorbance of each well was measured at OD_{570nm} using plate reader (n = 6). Obtained OD for all the samples were converted to % cell viability by considering host control OD as 100% cell viability.

Table 1
Primer details.

	Gene Name	RefSeq ID	TM value (°C)	Primer Type	Sequence
1	MAPK3	NM_001109891.2	62	Forward	5' GGATGCCGATGACATTCTC 3'
			64	Reverse	5' CATCAAGAAGATCAGCCCT 3'
2	PCNA	NM_182649.2	65	Forward	5' AAGAGAGTGGAGTGGCTTTTG 3'
			64	Reverse	5' TGTCGATAAAGAGGAGGAAGC 3'
3	CASP3	NM_001354783.2	62	Forward	5' TCGCTTCCATGTATGATCTTTG 3'
			63	Reverse	5' CTGCCCTTCCCCCATTCT 3'
4	FASLG	NM_000639.3	65	Forward	5' CAGAGGCATGGACCTTGAGT 3'
			65	Reverse	5' GTCTACCAGCCAGATGCACA 3'
5	RUNX2	NM_001015051.4	67	Forward	5' CCTAAATCACTGAGCGGTC 3'
			64	Reverse	5' CAGTAGATGGACCTCGGAA 3'
6	ESR2	NM_001437.3	64	Forward	5' ACCAAAGCATCGGTCACG 3'
			62	Reverse	5' CATGATCCTGCTCAATTCCA 3'
7	ADCY1	NM_001281768.2	64	Forward	5' CGACACGCAGTAGTAGCA 3'
			65	Reverse	5' ATGAGCTCTTCGGCAAGTTC 3'
8	BGLAP	NM_199173.6	63	Forward	5' CCTGGGTCTTCTACTA 3'
			65	Reverse	5' TCACACTCTCGCCCTATT 3'
9	CYCS	NM_018947.6	65	Forward	5' TGCCTTTCTCAACATCACCC 3'
			65	Reverse	5' GCGGTGTCCTTGACTTAGA 3'
10	EGR-2	NM_001136178.2	64	Forward	5' AGCAAAGCTGCTGGGATAT 3'
			64	Reverse	5' TTGACCAGATGAACGGAGTG 3'
11	NFATC1	NM_001278675.2	65	Forward	5' CACCTCAATCCGAAGCTCAT 3'
			65	Reverse	5' CCTGTCCCTACGTCCTA 3'
12	CREB1	NM_134442.5	66	Forward	5' GCTGGGCTTGAAGTGCATT 3'
			68	Reverse	5' GTGACGGAGGAGCTTGTACC 3'
13	ACTB	NM_001101.5	66	Forward	5' GC AACGGAACCGCTCATT 3'
			67	Reverse	5' AGCTGAGAGGGAAATTGTGCG 3'

Table represents the list of the all the primers of the targeted genes along with its RefSeq ID number and melting temperature. Table represents the list of the all the primers of the targeted genes along with its RefSeq ID number and melting temperature. MAPK3: MAP Kinase 3; ESR2: Estrogen Receptor β; ADCY1: Adenylate cyclase; RUNX2: Runx2; EGR-2: Egr-2; NFATC1: NFATc1; CREB1: CREB; PCNA: PCNA; BGLAP: Osteocalcin; FASLG: Fas Ligand; CYCS: Cytochrome C; CAP3: Caspase 3; ACTB: β-actin.

2.5. Transcript analysis

2.5.1. Total RNA isolation & cDNA synthesis

Total three test groups (50 µg/mL, 100 µg/mL and 250 µg/mL) along with host and vehicle control were treated with LG. Post completion of the LG treatment duration of 96 hours, cells were trypsinized and counted using hemocytometer. Total 2.5–3 million cells were collected in fresh tube and 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 OD_{260nm}. First strand of cDNA was synthesized using Thermo cDNA synthesis kit using 5 µg of total RNA. cDNA was converted using oligoDT primers using manual protocol (K1612, Thermo, USA). Details of reaction mix for cDNA synthesis have been provided in Supplementary table 1. Once cDNA was synthesized, it was used neat as a template for transcript analysis of desired genes.

2.5.2. Quantitative PCR

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

$$\text{Relative quantification} = 2^{-\Delta\Delta C_T}$$

where: a) $\Delta\Delta C_T = (\Delta C_T \text{ of test sample} - \Delta C_T \text{ of experiment control})$.
b) $\Delta C_T = (\text{Test gene } C_T - \text{Endogenous gene } C_T)$.

2.6. Western blot

Saos-2 cells were treated with LG Methanolic extract for 96 hours along with host and vehicle control. After completion of treatment, cells were harvested by trypsinization, followed by resuspension in TRIzol®. TRIzol® protocol was followed for isolation and initial purification of total proteins. Total proteins were then quantified by Nanodrop (Thermo, NanodropC) at OD_{280nm}. For sample preparation, 30 µg of protein was transferred to fresh tube. Each sample volume was normalized with autoclaved distilled water by adding remaining volume of water along with 6x sample loading dye (non-reducing) to make up final volume of 30 µL. Then samples were boiled for 10 min in boiling water bath. Whole sample volume was loaded in well of 4–20% gradient gel. Gel was run at 25 mA constant current till dye front elution.

After completion of gel, proteins were electronically wet-transferred to Nitrocellulose membrane (S80209, Pall, USA) at 200 mA of constant current for 2 hours in the presence of wet ice surrounding. After completion of transfer, membrane was carefully detached from gel and immediately dipped in blocking solution (5%

Table 2
Real time PCR conditions.

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

skim milk (GRM1254-500G, Himedia, India) in 1x PBS solution). Membrane was incubated for 2 hours on shaking platform. After 3 washes with 30 mL of 1x PBST (0.1% Tween20 (P1379, Sigma, USA) in 1x PBS solution), antibody treatments were given. 1:10k dilution was used for each primary antibody [anti-Egr-2 (AB108399, abcam, UK); anti- β -actin antibody (A1978, Sigma, USA)] and 1:100k and 1:50k dilution were used for secondary [anti-rabbit HRP conjugate (A0545, Sigma, USA); anti-mouse HRP conjugate antibody (AP127P, Sigma, USA)] respectively. After completion of secondary treatment, membrane was developed using TMB substrate of HRP in dark environment. The experiment was performed in triplicates ($n = 3$).

2.7. Cell viability determination by flow cytometry

Saos-2 cells were treated with LG Methanolic extract in concentration of 250 $\mu\text{g}/\text{mL}$, 100 $\mu\text{g}/\text{mL}$ & 50 $\mu\text{g}/\text{mL}$ for 96 hours along with untreated control. After completion of treatment, cells were harvested by trypsinization. These cells were then stained with CalceinAM (C3099, Thermo,USA) and Propidium iodide (PI) (P1304MP, Thermo,USA) stains using dye manual. 5 μM of Calcein AM and 3 μM of PI was used as working concentration for the staining. After that, cells were washed and analyzed in BD FACS-verse™ 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.

2.8. Statistical analysis

All the data were statistically analyzed. Dunnett's multiple comparisons test was used for statistical analysis. The statistical analysis was performed using one-way ANOVA using GraphPad Prism 8.3.1 Software (GraphPad Software, San Diego, USA) to obtain p-value. Each sample was compared to control to understand the variance within the experimental groups and significance was noted at $p < 0.05$.

3. Results

3.1. Extraction

To get the LG methanolic extract solution for further experimentation, LG bark powder was resuspended in the organic solvent and paste was obtained. The total yield obtained from LG methanolic extraction was 4.42 gm, which corresponds to ~8.8% total yield.

3.2. LG exhibits cellular proliferation – MTT assay

In the earlier lab study, it was observed that LG improves Saos-2 cells viability and health. To reproduce these results, MTT assay was conducted by treating Saos-2 cells with different concentrations of LG. In this assay, it was observed that Saos-2 cells were getting proliferated under the LG extract. Dose dependent significant ($p < 0.001$ and $p < 0.01$) increase in cell viability was observed (Supplementary Table 3). The highest viability observed was at 250 $\mu\text{g}/\text{mL}$ concentration. However, an insignificant declining trend was observed at 300 $\mu\text{g}/\text{mL}$ dose, and at 400 $\mu\text{g}/\text{mL}$, % cell viability was found to be less than the control (Fig. 1). To perform regression analysis, up trended results from 0.5 $\mu\text{g}/\text{mL}$ to 250 $\mu\text{g}/\text{mL}$ were selected, for which R^2 value was found to be 0.988 (Fig. 2). Hence for further experiments, 3 doses were selected (50 $\mu\text{g}/\text{mL}$, 100 $\mu\text{g}/\text{mL}$ and 250 $\mu\text{g}/\text{mL}$). These MTT assay results provided the direction of cell nourishing ability of LG on Saos-2 cells, but need further investigations in detail.

3.3. LG improves cell health and density

To obtain the phenotypic impact of LG in the Saos-2 cells, cell morphology study was conducted. In this study post 72 hours of the treatment, cells were examined microscopically to study cell architecture and growth. It was observed that cell health was very much improved and cell number was increased (visually) (Fig. 3) in a dose dependent manner compared to control and vehicle control.

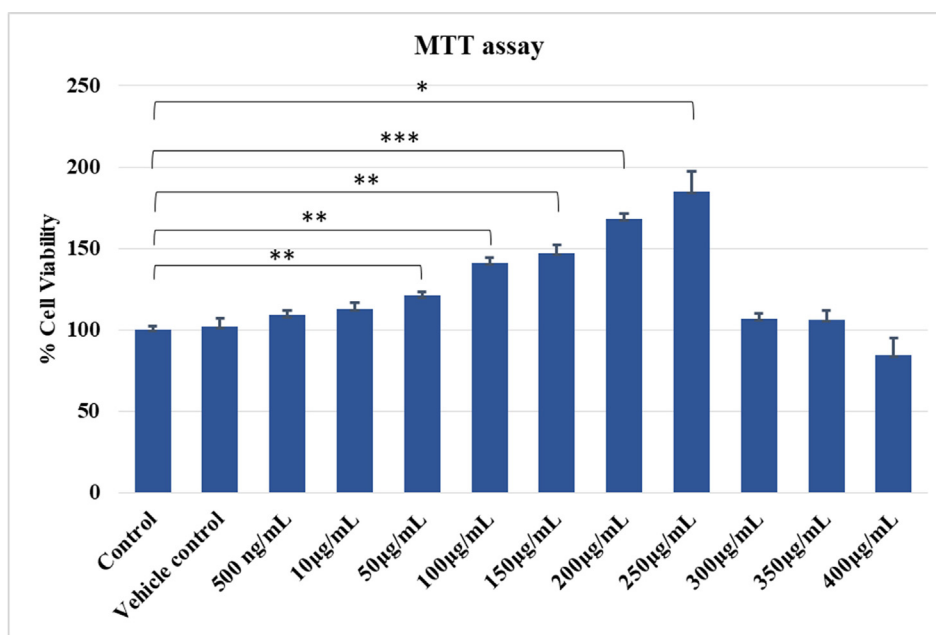


Fig. 1. Indicates that maximum effective concentration observed was 250 $\mu\text{g}/\text{mL}$. 400 $\mu\text{g}/\text{mL}$ concentration was found to be toxic. Data are presented as mean \pm S.E. and are representative of six independent experiments. ***, $p < 0.001$ compared with the control group; **, $p < 0.01$ compared with control group; *, $p < 0.05$ compared with the control group ($n = 6$).

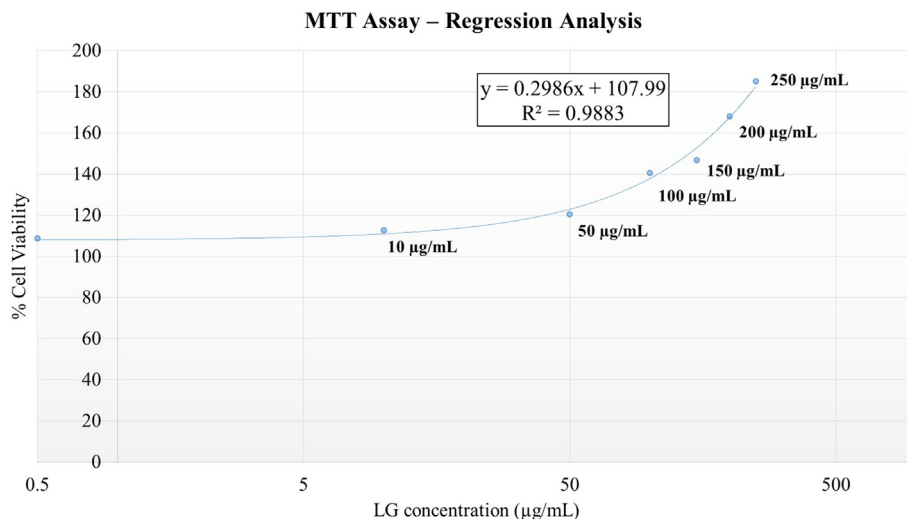


Fig. 2. Represents regression analysis of the MTT assay data from 0.5 µg/mL to 250 µg/mL. Linear trend line was plotted in semi-log graph, which reveals LG increases the % cell viability significantly till 250 µL/mL. Obtained R² value was 0.988 with slope 107.99.

Along with the cell morphology study, cells were counted after staining with trypan blue stain using hemocytometer to calculate the viability. It was clear indication that % viability and cell numbers are increasing in dose dependent manner (Fig. 4). These results supported the MTT observations. Hence to scrutinise these observations further, gene expression study was conducted.

3.4. Saos-2 cells treated with LG shows alteration in candidate gene expression

The quantitative PCR results showed that expression of the Egr-2, CREB, Adenylate cyclase, MAPK3, RUNX2 & ERβ were getting

upregulated, whereas NFATc1 was significantly (p < 0.01) down regulated in dose dependent manner after LG extract treatment (Supplementary Table 4; Fig. 5).

In results, Egr-2, RUNX2 were upregulated significantly (p < 0.01 for Egr-2 and p < 0.05 for RUNX2) (1.8–2.2 folds) compared to control upon treatment with 250 µg/mL concentration of LG extract. NFATc1 was downregulated up to 0.86 fold compared to control. Expression of the genes involved in the other pathways such as adenylate cyclase, CREB, MAPK3 were also increased significantly (p < 0.01 for adenylate cyclase, p < 0.05 for MAPK3) (1.56–2.18) compared to control when treated with 250 µg/mL concentration. Estrogen receptor ERβ was found to be upregulated

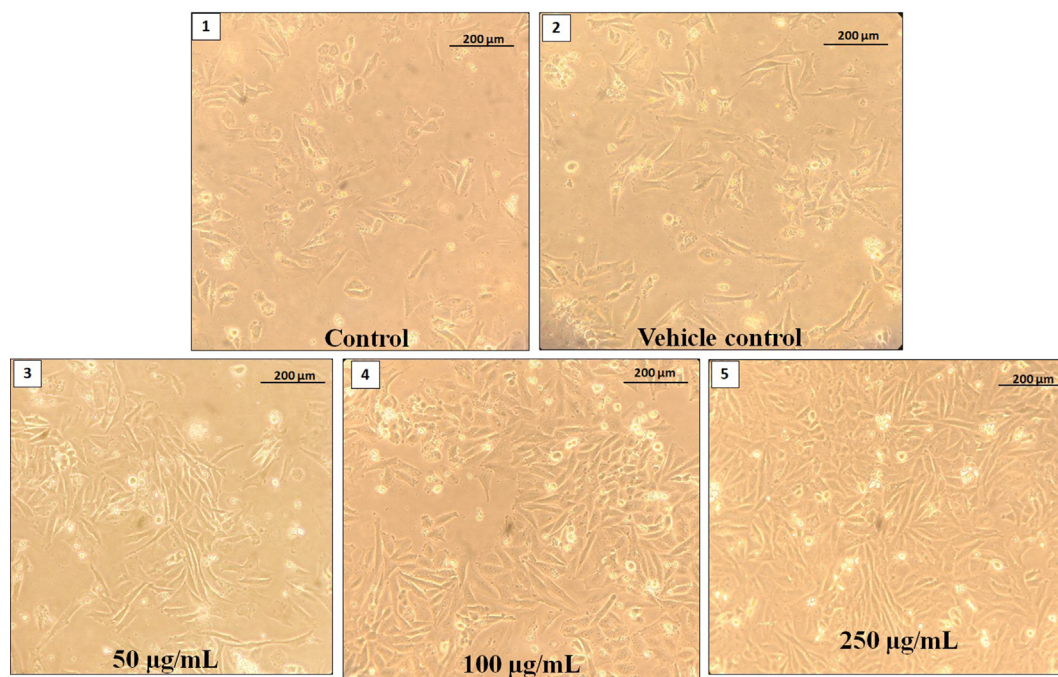


Fig. 3. Indicates that there is high cell density in 50 µg/mL compared to control and gradually getting increasing in 250 µg/mL which is in dose dependent manner. Along with the cell density, cell health is getting improved with dose. Each image is designated with respective dose concentrations; 'Control'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

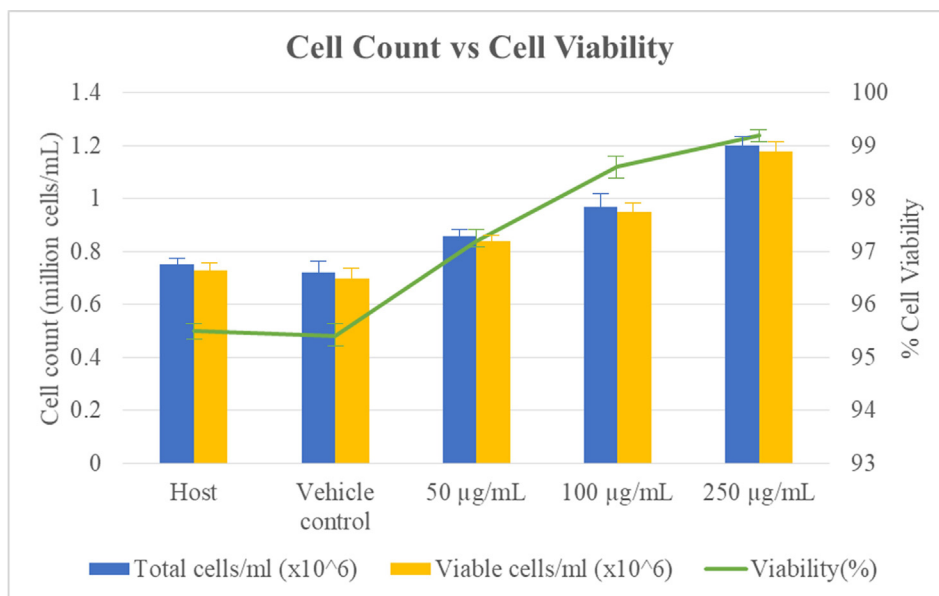


Fig. 4. Graph represents LG performance on cell viability and cell counts. Experiment was performed using Trypan blue stain to count the cells under Hemocytometer. It can be observed that there is increase in the cell count (million/mL) along with the %viability in dose dependent manner. 'Control' indicates untreated cells; 'vehicle control' indicates cells treated with DMSO (n = 3).

significantly ($p < 0.01$) up to two times compared to control at the same concentration (Fig. 5).

Proliferative markers such as PCNA, osteocalcin were also found to be upregulated significantly ($p < 0.01$ for each) in the range of 2.1–2.5 with compared to control. Whereas 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. There was no change in the expression of FasL upon LG treatment. Quantitative data are provided in [supplementary table 4](#) which shows the fold change (relative quantification) of candidate genes compared to untreated control and vehicle control. The expression of all the genes was following dose-dependent response except FasL (Fig. 5).

Gene expression study revealed that LG influences the gene expression of the candidate genes responsible for different pathways. However, to get stronger support, these results demand further protein expression studies and protein interaction studies. In addition to that, study on phosphorylation status of MAPK3 and RUNX2 are also advised.

3.5. LG enhances Egr-2 protein expression

After getting dose dependent increase in gene expression of Egr-2 gene, Egr-2 was studied at protein level. The expression of Egr-2 protein was examined via Western blot. Post development, it was visually observed that band density is increased compared to control, especially in 250 µg/mL concentration. To get quantitative numbers, Western blot bands were densitometrically analysed using ImageJ software (National Institute of Health, USA). Obtained peak areas were employed to calculate % relative protein content. It was observed that % relative protein content increases significantly ($p < 0.05$ and $p < 0.01$) and in dose dependent manner ([Supplementary Table 5](#); Fig. 6) compared to control sample. In other words, the expression of Egr-2 protein was found to be increased in dose dependent manner upon treatment with LG methanolic extract. Western blot results supported the enhanced gene expression of Egr-2 and confirmed that same is reflected at protein level also.

3.6. LG demonstrated to have proliferative action on Saos-2 cells by flow cytometry

Though LG has been demonstrated to have positive impact on osteoblastic cells at gene level, direct phenotypic presentation of proliferative action of LG is yet to be established. Hence we sought to study the proliferative action using flow cytometry. In this study, flow cytometry cell viability assay was conducted. It was observed that there was a gradual increase in the proportion of live cells in dose dependent manner ([Supplementary Table 6](#); Fig. 7, Fig. 8). These results formed strong basis of proliferative action and anti-apoptotic action of LG on osteoblastic cells.

4. Discussion

Osteoporosis is a metabolic bone disease, which results due to an impairment and imbalance of osteoclasts and osteoblasts activity. Due to the serious side effects of currently available medicines and treatments, research is focused toward herbal molecules to identify potential candidate molecules or formula, which can suppress the condition of osteoporosis effectively [37]. Many herbals are studied till the clinical level for their anti-osteoporotic effects [38]. The present study was conducted with one such herb, LG, to elucidate its effects on candidate genes and proteins involved in the signaling pathway of the Saos-2 osteoblastic cell line. Along with the gene expression study, MTT assay, increased cell count and %viability accompanied by the microscopic observation, supported the proliferative effect and enhanced cell growth. To the best of our knowledge, this is the first study to report gene alterations by LG extract in Saos-2 cells.

Herbal bioactive molecules and have been well explored concerning their effects on the genes involved in the signaling pathway. Some examples include Porcirin, a flavonoid obtained from the fruit of *Poncirus trifoliata* and flavones of Epimedium. These compounds show anti-osteoporotic property through various signalling pathways and proteins such as OPG/RANKL ratio, ERK/JNK/MAPK, estrogen receptor (ER), RUNX2 and P38 protein [39,40]. Besides this, the same research group has shown that *Salvia*

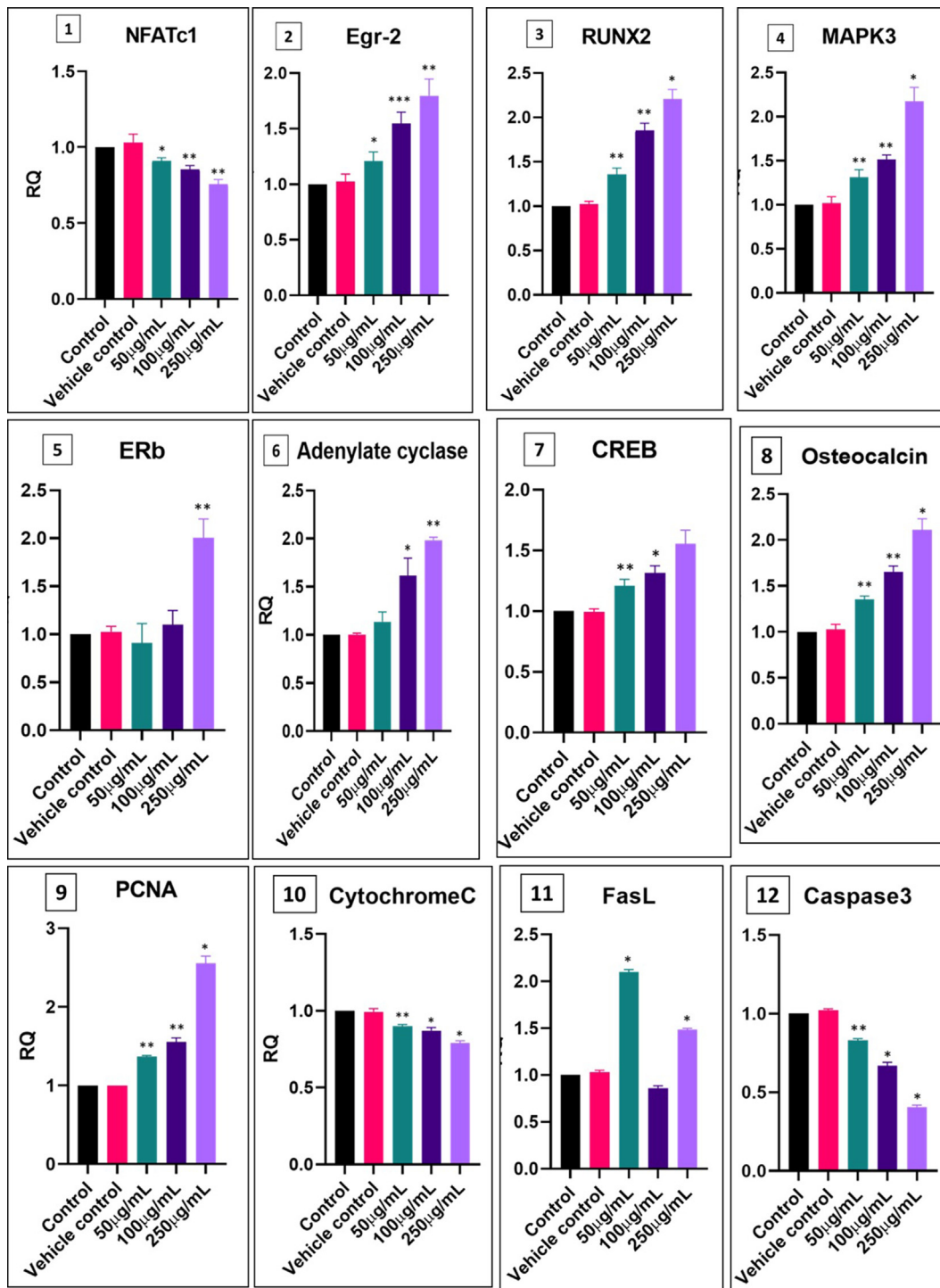


Fig. 5. Each graph is showing the expression profile of different genes ((1) NFATc1; (2) Egr-2; (3) RUNX2; (4) MAPK3; (5) ERβ; (6) Adenylate cyclase; (7) CREB; (8) Osteocalcin; (9) PCNA; (10) Cytochrome C; (11) FasL; (12) Caspase 3). *- p < 0.05; **- p < 0.01; ***- p < 0.001. p value Calculated using one-way ANOVA test followed by Post hoc test by Dunnett multiple comparison test. Data are presented as mean ± S.E. and are representative of three independent experiments (n = 3).

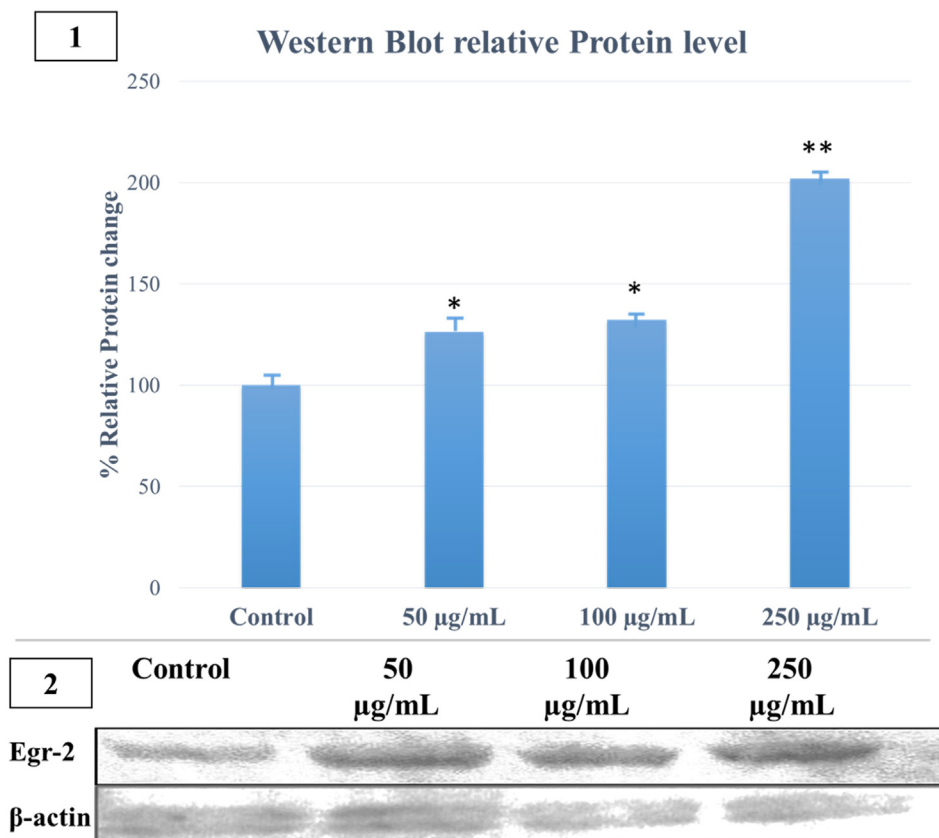


Fig. 6. Indicates: 1) Densitometric analysis of Western blot bands of *Egr-2* protein; 2) Western blot band profile; Vehicle control: whole cell lysate of vehicle control (DMSO treated); rest of 3 lanes are showing different concentrations used for treatment of LG substance. *- $p < 0.05$; **- $p < 0.01$. p value Calculated using one-way ANOVA test followed by Post hoc test by Dunnett multiple comparison test. (n = 3).

multiorrhiza inhibits osteoclast formation by restraining the expression of c-Fos and NFATc1 in calvarial osteoblast co-culture system.

On Similar lines, our detailed gene expression study revealed that LG methanolic extract treatment enhances gene expression of *Egr-2*, *RUNX2* and downregulates *NFATc1* significantly in dose dependent manner. All these genes play a crucial role in the regulation of osteoporosis [25,41]. Further, the western blot data also supported enhanced protein expression of *Egr-2*. The upregulation of *Egr-2* and *RUNX2* genes and protein in the present study suggests that both the genes are getting affected by LG treatment. These results are in line with the work carried out by Lin et al., and Zaman et al., [40,42]. Further, the downregulation of *NFATc1* by LG in the present study is in accordance with the earlier reported work of Hong et al., [43]. In addition, it has also been reported that *NFATc1* upregulates various pro-apoptotic genes including *FasL*, *TNF- α* , etc [44]. Hence, downregulation of *NFATc1* indicates that LG may have a role in the suppression of osteoclastogenic activity through osteoblasts, and suppressing the *NFATc1* activity such as decreasing osteoblasts proliferation and differentiation [31]. In Summary, the alterations in the expression of the studied genes indicate that these candidate genes are modulated by LG, which are involved in osteoblasts proliferation. However, future studies on the downstream functions of these candidate genes will be required to identify the proteins which are responsible for transmitting the information inside the cell.

It is well known that osteoblasts carry receptors for various hormones such as PTH [45], 1,25 (OH)₂D₃ [46] and estrogen [47], which are mainly involved in osteoblasts differentiation and its

other functions. PTH receptor, being a G-protein coupled receptor, signals through activation of adenylate cyclase, CREB proteins [35]. In the present study, we have obtained significant upregulation of adenylate cyclase and CREB genes upon LG treatment. Upregulation of adenylate cyclase and CREB can be correlated with earlier reports, which suggest that upregulation of *RUNX2* is associated with the increased expression of CREB and works via PKA and CREB pathway [48]. Overall it can be stated that LG extract has some components which work via GPCR pathway, however further protein level studies and pathway elucidating studies are recommended to support these observations.

Alma et al., [49] has shown that PTH is responsible for the expression of osteoblastic gene by means of increasing the expression and phosphorylation of *RUNX2* and activation of ERK1/2 and PI3K signaling which results in osteoblastogenesis and osteoblasts survival. Another group of scientists has reported a similar role of *RUNX2* and ERK1/2 in osteoblast [33]. Relating these reports with the present study, significant dose dependent upregulation of both *RUNX2* and *MAPK3* strongly suggests that the expression of these genes is getting enhanced by LG treatment. However, further studies are advisable on ERK pathway, which involves these genes and functions during early osteoblast differentiation, to identify possible involvement of this pathway in the mechanism of LG [33]. Torre et al., has shown that polyphenols, specifically quercetin, act as selective estrogen receptor modulators by upregulating the expression of ER β , which is a positive sign of bone mineralisation [50,51]. These findings are in line with our results such as upregulation of ER β in dose dependent manner upon LG treatment. It can be speculated that some

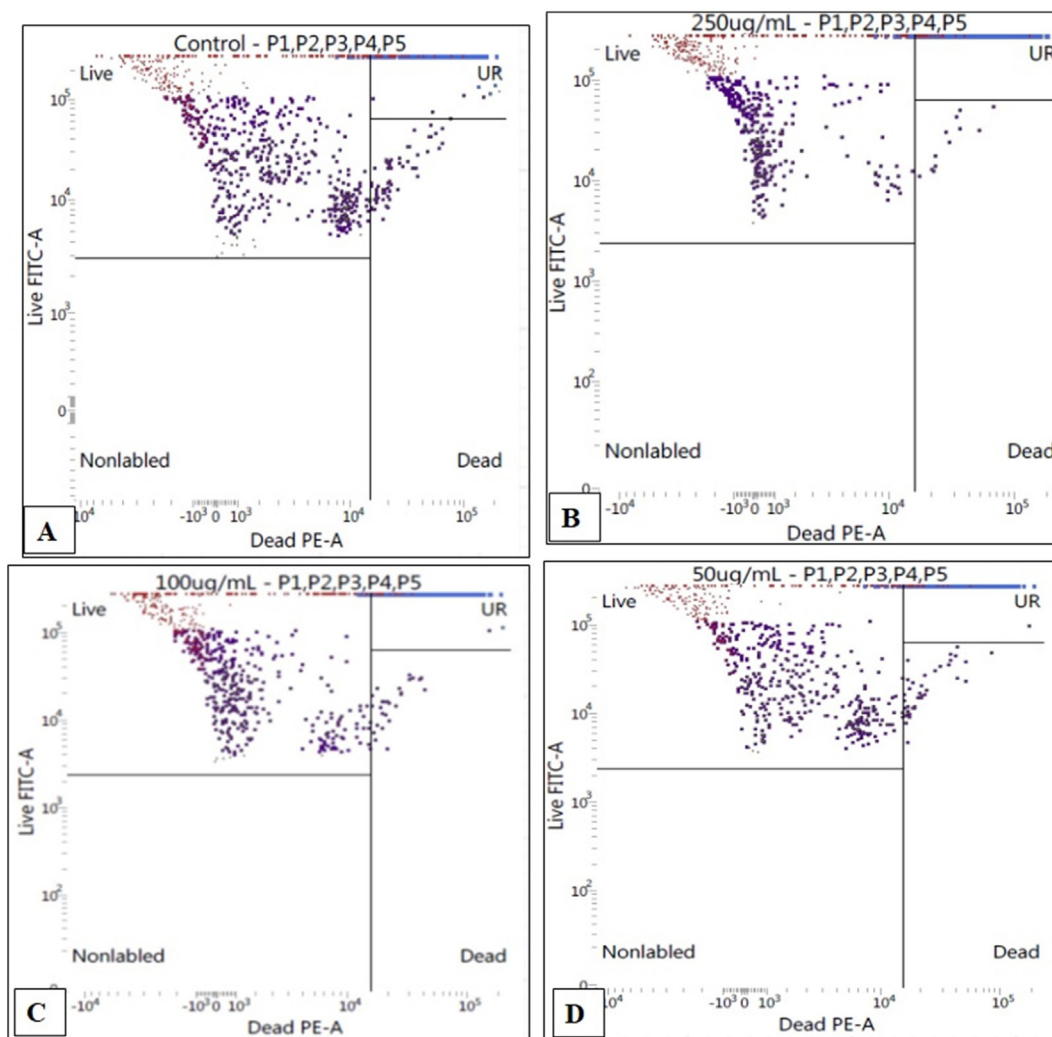


Fig. 7. Indicates Flow cytometry cell viability assay analysis of Saos-2 cells post LG methanolic extract treatment. Scattered plots for (A) Untreated control cells; (B) 250 µg/mL; (C) 100 µg/mL; (D) 50 µg/mL. It can be observed that number of dead cells are getting gradually decreased with increment in LG dose ($n = 3$).

phytochemicals present in LG methanolic extract which are responsible for the upregulation of ER β . Collectively, it has been inferred that components present in the LG methanolic extract, can be responsible for upregulation of genes such as MAPK3, RUNX2, CREB, ER β and GPCR pathway candidate proteins. Our results can serve as a base to design further detailed studies which can solidify these outcomes.

Osteocalcin (OCN) is the key player in bone endocrinology, which is secreted solely by osteoblasts. It has been recently reported that osteocalcin, being a marker protein of osteoblast proliferation, is involved in versatile functions such as glucose metabolism, male fertility and neuronal development [52]. Expression of osteocalcin is mainly facilitated by MAPK3 protein, which binds to the promoter of osteocalcin through association with RUNX2 [17,53]. In the present study, exposure of LG extract on Saos-2 cell line resulted in a significant increase in OCN expression. Thus it can be rationalized that LG may be involved in the upregulation of OCN via participation of RUNX2 and MAPK3 proteins. Our results are corroborating the work of Liu et al., [53]. Along with osteocalcin, a dose dependent upregulation of PCNA was also observed in the present study with the LG treatment on Saos-2 cell line. Additionally, alteration in the cell morphology was also observed, where the cells appeared to be healthier compared

to control as well as there was a visual increase in the cell number. Thus, upregulation of PCNA and OCN along with morphological observation clearly indicate the proliferative effects of LG. These results are in accordance with the previous studies on increased proliferation with number of herbs [54,55].

Study of apoptosis markers was also conducted to test our hypothesis. Expression profile of caspase 3, cytochrome C as an indicator of apoptosis has been reported earlier [56–58]. Downregulation of gene expression of cytochrome C and caspase 3 observed in the present study, suggests LG suppresses the expression of these apoptotic markers. Further suggesting LG may have an effect in decreasing the process of apoptosis, however FasL did not show any significant alterations upon LG treatment. To further validate the results, cell viability assay was performed using flow cytometry, results of which revealed a dose dependent increase in the number of live cells versus dead cells of Saos-2 cell line with LG treatment. Our results are similar to the results reported by other scientists, studying different herbs such as Icarin, DBT (Danggui Buxue Tang) and *Acacia catechu* bark powder extract [54,59,60]. However, insignificant alteration of FasL, an initiator protein for apoptosis, suggests that LG has apoptosis suppressive properties which involves no direct role of FasL. This observation needs to be validated.

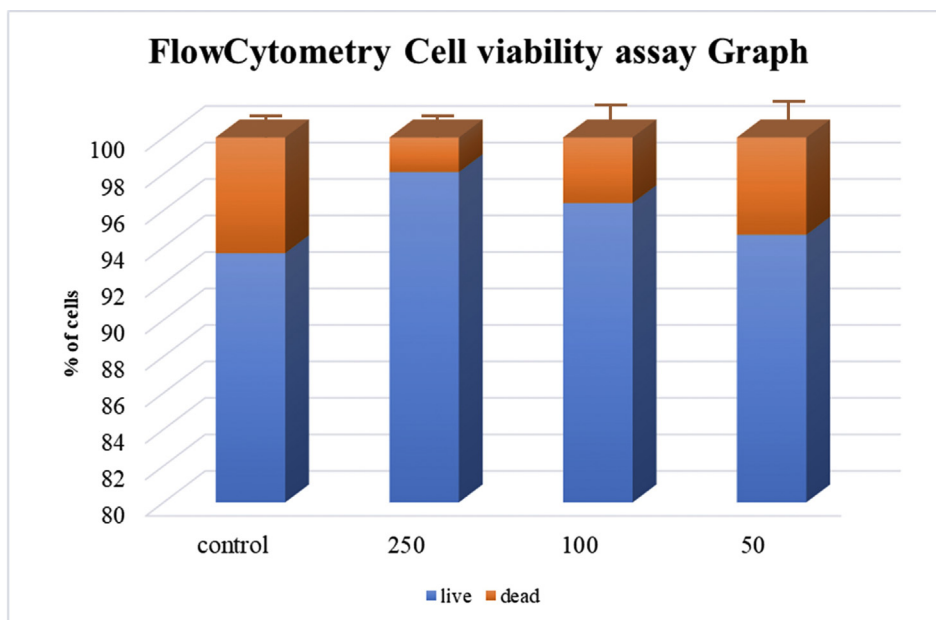


Fig. 8. Indicates Flow cytometry cell viability bar graph analysis. Bar graph evidently shows proportion of live cells are gradually increasing and similarly dead cell population is decreasing in dose dependent manner. These results are an indicative of Proliferative action of LG on Saos-2 cell line supporting earlier observations of MTT assay and gene expression study.

5. Conclusion

LG methanolic extract showed upregulation and down-regulation of various crucial genes that are involved in some decisive pathways of osteoblast proliferation and differentiation. LG also suppressed the genes which are involved in apoptosis in Saos-2 cells. Candidate genes such as MAPK3, adenylate cyclase, CREB which were getting upregulated upon LG treatment, gives a probable direction indicating involvement of these genes in signaling cascade of LG. However, further *in-vitro* as well as *in-vivo* protein expression studies of these genes and others such as SIRT1, Wnt, RANK, OPG, m-CSF are recommended to scrutinize the detailed mechanism.

Conflict of interest

None.

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Author contributions

Pragna Parikh: Conceptualization, Methodology, Writing - Review & Editing, Supervision, Project administration.

Hitarth Changani: Conceptualization, Methodology, Software, Validation, Writing - Original Draft, Visualization, Formal analysis, Investigation, Resources.

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Appendix A. Supplementary data

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