# CHAPTER 1

## Deciphering the effect of *Litsea glutinosa* on the probable candidate genes involved in osteoporosis using Saos-2 cell line

## 3.0 INTRODUCTION

Osteoporosis is defined as systemic skeletal disease characterised by low bone mass and microarchitectural deterioration, consequently increases bone fragility and risk of fracture (Faienza et al., 2013). Osteoporosis has assumed major public health importance in recent years. As per reports, it has been projected that spinal compression fractures are associated with osteoporosis and 15 to 34% of all patients with hip fractures die from complications within 6 months (Shubhashree, 2018)

In normal process, activity of osteoblasts and osteoclasts are continuous, well balanced and harmonized process. Bone remodelling is the phenomena by which old bone gets resorbs and new bone forms. It is established since very old time that bone formation and bone resorption are sequentially coupled processes in which bone resorption is followed by bone formation. The amount of bone resorbed is quantitatively similar to the bone formed in young skeleton describing balance of the remodelling event (Sims et al., 2014). As mentioned, in normal bone remodelling process, balance between bone formation and bone resorption is tightly controlled and regulated process. It maintained to ensure that there should not be any major net change in bone mechanical strength or mass in mature bone post remodelling cycle. Regulation in bone remodelling process involves a series of well-coordinated signalling mechanisms. Nonetheless, if there is an imbalance between resorption and formation, it may invite certain pathological conditions like generation of abnormal bone and development of bone disorders (Feng, 2011).

Bone disorder mainly includes osteoporosis diseases. The pathogenesis of postmenopausal osteoporosis is caused primarily by the falloff in estrogen level which is associated with menopause. The main hypothesis was initially demonstrated in mid-20$^{th}$ century. This work reports that there is negative calcium balance in postmenopausal women with osteoporosis and this condition is reversed by estrogen replacement. (Feng, 2011; Tella et al., 2014). Later studies indicated that in case of estrogen deficiency, there will be increased activity of bone resorption rather than impaired bone formation (Garnero et al., 2009). As it was established that estrogen deficiency plays a

central role in osteoporosis, enormous efforts have been focused on exploring the mechanism of action of estrogen in past three decades. Besides the paucity of estrogens, multiple factors are responsible for the onset of osteoporosis such as alcoholism, smoking, prolonged premenopausal amenorrhea, low life-long calcium intake, impaired vision despite correction, inadequate physical activity etc. (Lin J. T, 2004; Ginaldi et al., 2005).

For the treatment of osteoporosis, there are many treatments and therapy available like hormonal replacement therapy, drug like bisphosphonate, Denosumab, recombinant PTH hormone etc. Majorly these drug are catagorised in two classes: 1. Anti-resorptive drugs (inhibiting osteoclasts) and 2. Bone building drugs (stimulating osteoblasts). Generally anti-resorptive drugs have been the drug of choice for osteoporosis. These drugs includes bisphosphonate, Selective Estrogen Receptor Modulators (SERMs), Tissue Selective Estrogen Modulator (TSECs) etc (Stapleton et al., 2017). However, serious adverse effects are associated with prolonged use of these drug (Barry et al., 2016). There are reports suggesting osteonecrosis of jaw, renal failure, arterial fibrillation and atypical subtrochanteric femoral fractures are associated with bisphosphonate use. It has also been reported that Etidronate, BP which decreases not only bone resorption but also calcification. Therefore its prolonged use enhances the risk of osteomalacia (Hoppé et al., 2012; Ukon et al., 2019). There are many short termed as well as long termed serious consequences associated with BP treatment. Short termed includes Upper GI adverse effects, acute phase reaction, severe musculoskeletal pain, hypocalcimia, esophageal cancer and ocular inflammation. Long termed consequences includes severe suppression of bone turnover along with the previously mentioned conditions (Kennel at el., 2009). This may be the prime reason behind decreasing the prescription for bone protecting mediations in western countries. Along with the prescription, there is also fall reported in admitting people for bone protecting medications (Khosla et al., 2016; Van et al., 2017). Because of these adverse conditions, new strategies are under investigations such as modifying bisphosphonates to target proteins, discovery of new molecules and targets (Barry et al., 2016; Mora et al., 2017). Because of these reasons, herbal medicines are gaining focus among scientific

community. In search of effective herbal based medicine and molecule, several plants and phytohormones are studied (Marie et al., 2011; Siu et al., 2013; Sun W. et al., 2014; CHEN et al., 2016; Wu et al., 2017; Yang Y. et al., 2017)

Traditionally many herbal plants and derived medicines have always been utilized as a cure against various critical diseases like dementia, hepatocellular carcinoma, vertigo in Asian countries (Chen K.-H. et al., 2017; So et al., 2015; Tsai et al., 2016, 2017). Globally several research groups have explored many herbals including *Herba epimedium, Salvia miltiorrhiza, Curcuma longa, Moringa olifera, Litsea glutinosa* and phytoactive molecules like Psoralen from *Psoralea corylifolia*, Poncirin from *Poncirus trifoliate*, Vanillic acid from *Sambucus williamsii* Hance, Osthole from *Fructus cnidii* and many more (Rangrez et al., 2011; Tang D.-Z. et al., 2011; Parikh & Rangrez, 2012; Patel et al., 2015; Lin J. et al., 2017).

*Herba epimedium* and its components have been evaluated *in-vivo* for its bone healing property. These components could prevent bone loss in ovariectomized (OVX) rats, which was identified by the suppression of BMD descent and the improvement of trabecular microarchitecture. Total Flavones of Epimedium (TFE) increased serum OCN and type I collagen protein in OVX rats (Xie et al., 2005). Besides this, TFE increased serum estrogen, expression of ERα and ERβ in hypothalamus and hippocampus and also inhibited level of IL-6, which was induced by OVX. TEF also enhanced the level of osteoprotegrin and recovered expression of Runx2 (Qian et al., 2006; Chen W.-F et al., 2011). Another species, *Epimedium brevicornum* Maxim has a long history of use in the treatment of estrogen deficiency-related diseases (Liu X. L et al., 2017). Icariin, an isolated compound from *E. brevicornum,* prevents bone loss in OVX rats by sustaining BMD and improving trabecular microarchitecture (Yang L. et al., 2013). Administration of Icariin has proved to alter the expression of marker proteins (Runx2, OCN, BMP2, BMP4, MAPK) and calcium signalling (Xue et al., 2016) Various constituents of the herb have been used for the treatment of cardiovascular disease, infertility,

impotence, amnesia, lumbago, arthritis, and numbness and weakness of the limbs (Ma et al., 2011).

*Salvia miltiorrhiza* is the herb whose roots have been well explored for treating postmenopausal syndrome. Various compounds like tanshinone IIA, cryptotanshinone, tanshinone I, 15, 16-dihydrotanshinone I, ferruginol, salvianic acid and falvanoids are the compounds which have been extracted from *Salvia miltiorrhiza*, which have been used for its properties against various diseases like menstrual disorders, blood stasis, and rheumatism (Lee S.-Y. et al., 2005; Guo et al., 2014;). *In-vivo* experiments by Chea group in 2004 has demonstrated *in-vivo* that aqueous fraction of this herb could enhance bone strength and prevent trabecular bone resorption in OVX Sprague-Dawley rats (Chae et al., 2004). Along with aqueous fraction, individual component tanshinone decrease bone loss in trabecular bone thickness and its microarchitecture. It also partially prevented a decrease in trabecular bone volume and trabecular number in the tibia (Cui et al., 2004). Moreover, *in-vitro* studies have also proved anti-osteoporotic property of *Salvia miltiorrhiza*. It was found that it increased osteoblast number and inhibited osteoclastogenesis process. Mechanism of its action in the osteogenesis process involves modulation of OPG/RANKL ratio, action through ERK signalling pathway; inhibition of osteoclast formation through inhibition of expression of c-fos, NFATc1, RANKL; reduction of osteoclasts through suppression of RANK activated AKT, NF-κB, and MAPKs signal transduction (Kim H.-H. et al., 2004; Kwak et al., 2006; Nicolin et al., 2010; Xu et al., 2014; Lin J et al., 2017).

Besides these, there are many other active components and phytochemicals from various herbs have been identified, known to have anti-osteoporotic properties. These components includes Saikosaponins, Echinacoside, Sweroside, Poncirin, Vanillic acid, sulfuretin, Genistein, Diosgenin, etc. Saikosaponins, major bioactive compounds isolated from Radix Bupleuri, have exhibited anti-inflammatory, antimycotic, and immuno-regulatory pharmacological properties. *In-vitro* studies have demonstrated saikosaponin A suppresses osteoclastogenesis in mice bone marrow monocytes and its

mediated osteoclast differentiation inhibiting RANKL-induced p38, ERK, JNK, and NF-κB activation in murine RAW264.7 cells (Chen J et al., 2015). Echinacoside, one of the major component of Herba Cistanches, is natural polyphenolic compound which exhibited neuroprotective, immunomodulatory, hormonal balancing, anti-fatigue, anti-inflammatory, hepatoprotection, anti-oxidative, anti-bacterial, anti-viral, and anti-tumor effects, etc (Fu et al., 2018). It prevents bone loss via increasing OPG/RANKL ratio, which reveals its potential of developing into a novel drug molecule for treatment in postmenopausal osteoporotic women (Yang et al., 2013). Herbal *Fructus Corni*, a traditional medicine, has a long history of treating osteoporosis in elderly men or postmenopausal women in Asia. Sweroside, bioactive herbal ingredient isolated from *Fructus Corni*, has the similar functions to Echinacoside like vasorelaxation, antihepatitis, antiinflammatory, and antiallergic effect. Sweroside was found to effectively induce proliferation and inhibit apoptosis in human osteosarcoma cell line MG-63 and Wistar rat osteoblastic cells (Sun H et al., 2013). Poncirin is isolated from *Poncirus trifoliata* and possesses anti-bacterial and anti-inflammatory activities. Reports suggests Poncirin could enhance osteoblast differentiation. In murine bone marrow stem cells poncirin enhanced the expression of OCN, ALP and Runx2 (Yoon et al., 2011). Vanillic acid is a phenolic acid isolated from the bioactive fraction of *Sambucus williamsii* Hance. Vanillic acid stimulates UMR106 cell proliferation and its ALP activity. It also increased expression of various genes responsible for osteoblast functions like OCN, Runx2 and ration of OPG/RANKL. However, its mode of action is not clear but it is believed to be mediated through ER and MAPK signalling pathways (Xiao H.-H et al., 2014). Collectively, number of herbals have been explored for their mode of action and the genes and proteins which are involved in their signalling cascade.

One such herbal medicinal plant is *Litsea glutinosa* (LG). Its bark is reported to contain properties of arousing sexual power, relieving pain and producing a soothing effect on the body. It is considered good for body, mildly astringent, used in diarrhea and dysentery. LG is being studied for its various beneficial effects like anti-inflammatory, antipyretic, hypotensive, chemoprotective

activities, antinociceptive properties and anti-osteoporotic properties (Sukhdev, 2006; Kshirsagar et al., 2009; Devi et al., 2010; Rangrez et al., 2011; Choudhury et al., 2013; Bhowmick et al., 2015; Unnikrishnan, 2016; Rahman et al., 2017). Its methanolic extract has shown antibacterial activity (Pattanayak, 2016). In the past, the development of herbal anti-osteoporosis formulations was pursued mainly by scientists in Asian countries, including China, Japan and Korea (Xie et al., 2005). For the treatment of osteoporosis, dry bark powder of the LG is used in formulations or prescribed directly. Many herbal formulations which are used for the prevention of osteoporosis are having Maida Lakri as their main herb (Sukhdev, 2006). Great deals of studies concerning the phytochemical and pharmacological aspects of the genus *Litsea* have been carried out. More than 200 chemical ingredients, covering flavonoids, terpenoids, alkaloids, butanolides and butenolactones, lignans, amides, steroids, fatty acids, megastigmanes, etc., have till now been isolated from these plant.

There are many phytohormones and phytochemicals are studied which work via different pathways involving MAPK/ERK, estrogen receptor, GPCR (PTH receptor), RUNX2 etc. As per current evidences, LG shows its anti-osteoporotic properties by improving bone health by improving microarchitecture. It increases the bone formation marker serum ALP and decreases TRAcP. LG also reduced $Ca^{++}$ in excretion. It is established that LG improves bone health *in-vitro* as well as *in-vivo* (Parikh, 2009; Rangrez et al., 2011). Therefore it was hypothesized that there might be some involvement of these genes in the action of LG also because LG contains some phytohormones as one of its constituents. They also may work through estrogen receptor and PTH receptor because these plant have similar phenotypic characteristics with LG.

***To scrutinize the hypothesis, it was decided to investigate the candidate genes like Egr2, Runx2, NFATc1, and genes of other proteins involved in various pathways like ERβ, MAPK3, CREB and adenylate cyclase were examined. To establish the experimental dosage range of LG methanolic extract using which osteoblastic cell line Saos-2 can be treated, MTT assay***

***was conducted. Post dosage determination, Saos-2 cells were treated with these doses and then gene expression study was performed using real time PCR. Expression of Egr2 was also validated using western blot studies.***

## 3.1 MATERIAL AND METHODS:

### 3.1.1 PREPARATION OF LG EXTRACT:

Bark powder of *Litsea glutinosa* was purchased from the local market. 50 g powder was weighed and resuspended in 400 mL HPLC grade methanol (SA8SF68037, Merck, USA) and volume was made up 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. Upon drying, remaining solid extract was collected, weighed and stored at -20°c for further processing.

### 3.1.2 CULTURING OF SAOS-2 CELLS:

Saos-2 cell line was procured from National Center for Cellular Science Institute (NCCS, Pune, India). Cells were maintained throughout in maccoy's 5A (16600082, Gibco, USA) + 10 % FBS media (RM9955-100ML, Himedia, India) at 37°c in $CO_2$ humidified chamber. For passaging, cells were washed with 1x PBS, followed by trypsinization (TCL099, Himedia, India). After detachment of cells, trypsin was diluted approx. (minimum 1:10) with media. These cells were counted using hemocytometer and were seeded to the fresh flask at a seeding density of 0.4 M/mL.

### 3.1.3 LG TREATMENT:

The methanolic extract was weighed 250 mg and it was dissolved in the final volume of 1 mL of DMSO (D8418, Sigma, USA), generating 250 mg/mL stock solution. Cells were seeded a day before treatment in T25 flask and serial dilutions of LG extract were prepared in cell culture media (maccoy'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 hrs.

### **3.1.4** 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 $5 \times 10^3$ cells/well/200 µL in 6 replicates. Along with control and vehicle control, test flasks 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 hr. Post 96 hours, the cells were washed with PBS. Further, 100 µL of culture media and 20 µL of MTT solution were added to each well. After that, cells were incubated in a humidified atmosphere of 5% $CO_2$ 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 570 nm using plate reader (n=6).

### **3.1.5** TRANSCRIPT ANALYSIS:

#### **3.1.5.1** 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.

**3.1.5.2** 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 C1. 2) were used for transcript analysis using PowerUP Sybr® green master mix (A25742, Thermo, USA). A standard manual PCR conditions (Table C1. 1) were used for amplification. The experiment was performed in triplicates (n=3).

**Table C1. 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 C1. 1 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 C1. 2: 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' CATCAAGAAGATCAGCCCCT 3' |
| **2** | RUNX2 | NM_001015051.4 | 67 | Forward | 5' CCTAAATCACTGAGGCGGTC 3' |
| | | | 64 | Reverse | 5' CAGTAGATGGACCTCGGGAA 3' |
| **3** | ESR2 | NM_001437.3 | 64 | Forward | 5' ACCAAAGCATCGGTCACG 3' |
| | | | 62 | Reverse | 5' CATGATCCTGCTCAATTCCA 3' |
| **4** | ADCY1 | NM_001281768.2 | 64 | Forward | 5' CGACACGCAGTAGTAGCA 3' |
| | | | 65 | Reverse | 5' ATGAGCTCTTCGGCAAGTTC 3' |
| **5** | EGR-2 | NM_001136178.2 | 64 | Forward | 5' AGCAAAGCTGCTGGGATAT 3' |
| | | | 64 | Reverse | 5' TTGACCAGATGAACGGAGTG 3' |
| **6** | NFATC1 | NM_001278675.2 | 65 | Forward | 5' CACCTCAATCCGAAGCTCAT 3' |
| | | | 65 | Reverse | 5' CCTGTCCCCTACGTCCTA 3' |
| **7** | CREB1 | NM_134442.5 | 66 | Forward | 5' GCTGGGCTTGAACTGTCATT 3' |
| | | | 68 | Reverse | 5' GTGACGGAGGAGCTTGTACC 3' |
| **8** | ACTB | NM_001101.5 | 66 | Forward | 5' GCAACGGAACCGCTCATT 3' |
| | | | 67 | Reverse | 5' AGCTGAGAGGGAAATTGTGCG 3' |

*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 ΔΔCT and thereby RQ (relative quantitation).ΔΔ$C_T$ is calculated from Δ$C_T$ mean (*

*Equation* ***1****). (MAPK3: MAP kinase 3/ERK1; RUNX2: Runx2; ESR2: Estrogen receptor β; ADCY1: adenylate cyclase; EGR-2: Egr-2; NFATC1: Nfatc1; CREB1: CREB; ACTB: β-actin)*

**Equation 1: Relative quantitation formula**

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

Where: $\Delta\Delta C_T$= ($\Delta C_T$ of test sample - $\Delta C_T$ of experiment control)

$\Delta C_T$ = (Test gene $C_T$ - Endogenous gene $C_T$)

### 3.1.6 WESTERN BLOT:

Saos-2 cells were treated with LG Methanolic extract for 96 hr along with untreated 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 protein was then quantified by Nanodrop (Thermo, NanodropC) at 280nm. After that, each sample was normalized and mixed with 6x non-reducing gel loading dye for PAGE (26616, Thermo, USA), samples were boiled for 10 min in boiling water bath. An equal amount of protein was loaded in well 4-20% gradient gel was run. After completion of gel, protein was electronically transferred to Nitrocellulose membrane (S80209, Pall, USA), It was followed by treatment of primary (anti-Egr-2) (AB108399, abcam, UK) and secondary (anti-rabbit HRP conjugate (A0545, Sigma, USA) antibody. For internal control, β-actin was (A1978, Sigma, USA) used. After completion of secondary treatment, membrane was developed using TMB substrate of HRP in dark environment. The experiment was performed in triplicates (n=3).

### 3.1.7 STATISTICAL ANALYSIS

All the data were statically 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 to obtain p-value each sample compared to control to understand the variance within the experimental groups and significance was noted at $p<0.05$.

## 3.2 RESULTS:

### 3.2.1 EXTRACTION:

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

### 3.2.2 MTT ASSAY:

In the MTT 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 (Figure C1. 1). The highest viability observed was at 250 µg/mL concentration. However, an insignificant declining trend was observed at 300 µg/mL dose, and at 400 µg/mL was found to be less than control values. Hence for further experiments, 3 doses were selected (50 µg/mL, 100 µg/mL and 250 µg/mL). Figure C1. 1 indicates % cell viability values obtained in each dosage of methanolic extract of LG.

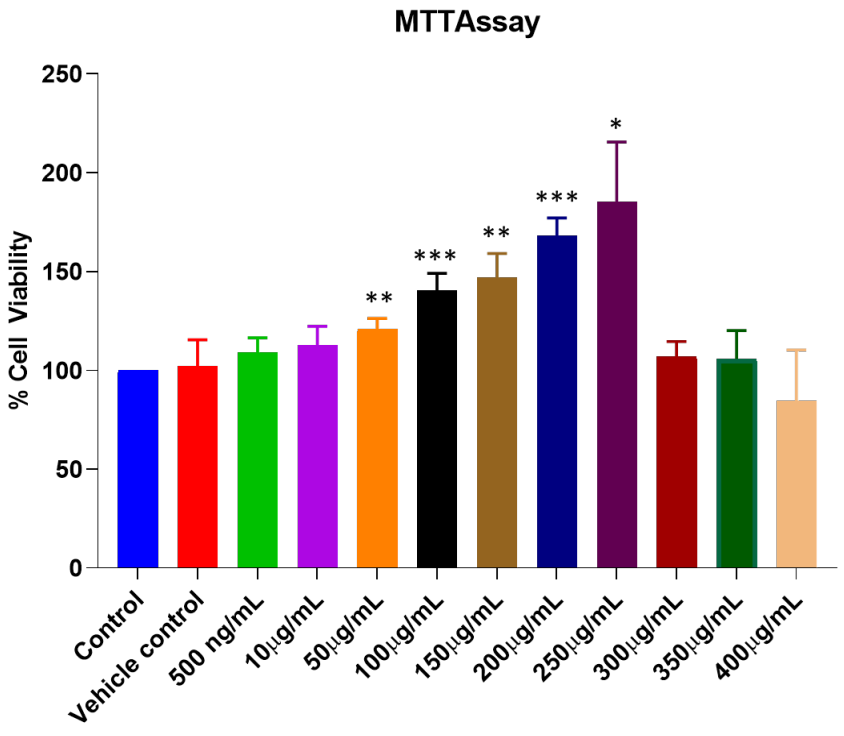



**Figure C1. 1: Graph showing results of MTT assay**

*Figure C1. 1 indicates that maximum effective concentration observed was 250 µg/mL. 400 µg/mL concentration was found to be*

*toxic. Data are presented as mean ±S.E. and are representative of six independent experiments. ***, p<0.001compared with the control group; **, p < 0.01 compared with control group; *, p < 0.05 compared with the control group (n=6).*

**Table C1. 3: Results of % cell viability by MTT assay**

| Dosage | % cell viability |
|---|---|
| **Control** | 100.00 |
| **Vehicle control** | 102.21 ± 13.22 |
| **500 ng/mL** | 108.87 ± 7.62 |
| **10 µg/mL** | 112.80 ± 9.38 |
| **50 µg/mL** | 120.68 ± 5.53 |
| **100 µg/mL** | 140.53 ± 8.53 |
| **150 µg/mL** | 146.81 ± 12.28 |
| **200 µg/mL** | 168.19 ± 8.84 |
| **250 µg/mL** | 185.13 ± 30.38 |
| **300 µg/mL** | 107.08 ± 7.47 |
| **350 µg/mL** | 108.62 ± 16.91 |
| **400 µg/mL** | 84.55 ± 25.68 |

*Table C1. 3 indicates mean values of % cell viability ± SD by MTT assay of Saos-2 upon treatment with LG methanolic extract. It can be observed that there is gradual increase in % cell viability with increase in dose from 500 ng/mL to 250 µg/mL significantly compared to control. Decline trend in % cell viability can be observed from 300 µg/mL to 400 µg/mL. (n=6).*

**Table C1. 4: Standard error and Significance value of % cell viability by MTT assay**

| Dosage | % cell viability |
|---|---|
| **Control** | 0.0 |
| **Vehicle control** | 5.4 |
| **500 ng/mL** | 3.11 |
| **10 µg/mL** | 3.83 |

| | |
|---|---|
| **50 µg/mL** | 2.26 ** |
| **100 µg/mL** | 3.48 *** |
| **150 µg/mL** | 5.01 ** |
| **200 µg/mL** | 3.61 *** |
| **250 µg/mL** | 12.4 * |
| **300 µg/mL** | 3.05 |
| **350 µg/mL** | 5.89 |
| **400 µg/mL** | 10.5 |

*Table C1. 4 indicates standard error with significance of % viability by MTT assay. *- p< 0.05; **- p<0.01; ***- p<0.001; ns: non-significant. All the data are presented as mean ±S.E. and are representative of three independent experiments. (n=3).*

In MTT assay % cell viability was increasing till 250 µg/mL then after it gradually decreased. Therefore results from 0.5 µg/mL to 250 µg/mL were selected for regression analysis and $R^2$ value was found to be 0.988 (Figure C1. 2). Based on regression analysis low (50 µg/mL), mid (100 µg/mL) and high (250 µg/mL) concentrations were selected for further experimentations.

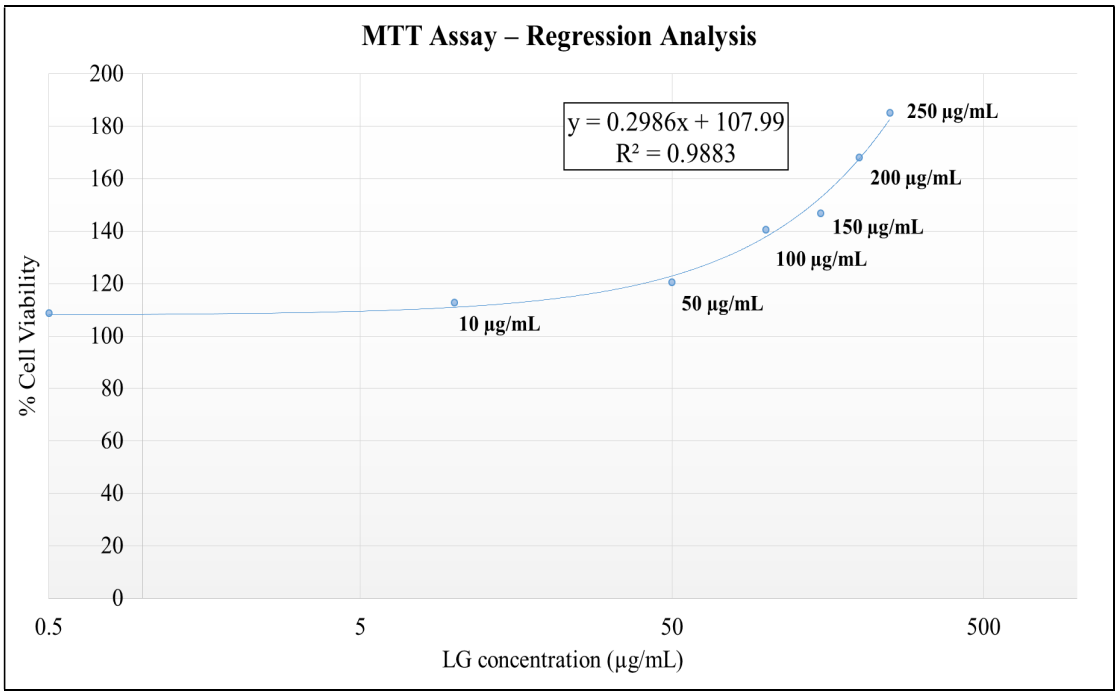



**Figure C1. 2: MTT assay regression analysis curve**

*Figure C1. 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 $R^2$ value was 0.988 with slope 107.99.*

### 3.2.3 QPCR:

The quantitative PCR results showed that expression of the Egr-2, CREB, Adenylate cyclase, MAPK3, RUNX2 & ERβ were getting significantly upregulated, whereas NFATc1 was significantly ($p<0.01$) getting down regulated in dose dependent manner after LG extract treatment.

In results, Egr-2, RUNX2 were upregulated significantly ($p<0.01$ for Egr-2 and $p<0.05$ for RUNX2) in the range of 1.8 – 2.2 folds compared to control upon treatment of 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 like adenylate cyclase, CREB, MAPK3 were also found to be increased significantly ($p<0.01$ for adenylate cyclase, $p<0.05$ for MAPK3) in the range of 1.56 – 2.18 compared to control when treated with 250 µg/mL concentration. Estrogen receptor ERβ was found to be

upregulated significantly ($p<0.01$) up to two times with compared to control at the same concentration (Table C1. 5; Figure C1. 1).

**Table C1. 5: Relative quantitation of genes using Real time PCR**

| RQ (Relative quantitation) | | | | | |
|---|---|---|---|---|---|
| | **Control** | **Vehicle control** | **50 µg/mL** | **100 µg/mL** | **250 µg/mL** |
| **Egr-2** | 1 | 1.03 ± 0.06 | 1.21 ± 0.08 | 1.55 ± 0.10 | 1.80 ± 0.15 |
| **RUNX2** | 1 | 1.02 ± 0.03 | 1.36 ± 0.07 | 1.85 ± 0.08 | 2.21 ± 0.11 |
| **NFATc1** | 1 | 1.03 ± 0.05 | 0.91 ± 0.08 | 0.85 ± 0.07 | 0.76 ± 0.10 |
| **CREB** | 1 | 0.99 ± 0.03 | 1.21 ± 0.05 | 1.32 ± 0.06 | 1.56 ± 0.11 |
| **Adenylate cyclase** | 1 | 1.00 ± 0.02 | 1.14 ± 0.10 | 1.62 ± 0.18 | 1.98 ± 0.03 |
| **MAPK3** | 1 | 1.02 ± 0.07 | 1.31 ± 0.09 | 1.51 ± 0.05 | 2.18 ± 0.15 |
| **ERβ** | 1 | 1.03 ± 0.06 | 0.91 ± 0.20 | 1.10 ± 0.15 | 2.01 ± 0.19 |

*Table C1. 5 indicates mean gene fold change ± SD value for all the genes. Values were calculated using Equation 1. Gene folds were calculated with respect to CONTROL. Hence value of CONTROL is 1 for all the genes.*

**Table C1. 6: Standard error and Significance value of Gene fold change**

| RQ (Relative quantitation) | | | | | |
|---|---|---|---|---|---|
| | **Control** | **Vehicle control** | **50 µg/mL** | **100 µg/mL** | **250 µg/mL** |
| **Egr-2** | 1.00 | 0.04 | 0.05* | 0.06*** | 0.09** |
| **RUNX2** | 1.00 | 0.02 | 0.04** | 0.05** | 0.06* |
| **NFATc1** | 1.00 | 0.03 | 0.01* | 0.01** | 0.02** |
| **CREB** | 1.00 | 0.01 | 0.03** | 0.03* | 0.06 |
| **Adenylate cyclase** | 1.00 | 0.01 | 0.06 | 0.10* | 0.02** |
| **MAPK3** | 1.00 | 0.04 | 0.05** | 0.03** | 0.09* |
| **ERβ** | 1.00 | 0.03 | 0.12 ns | 0.09 ns | 0.11** |

*Table C1. 6 indicates standard error and significance value of respective gene fold values. *- p< 0.05; **- p<0.01; ***- p<0.001; ns: non-significant. All the data are presented as mean ±S.E. and are representative of three independent experiments. (n=3).*

### **3.2.3.1** GRAPHICAL REPRESENTATION

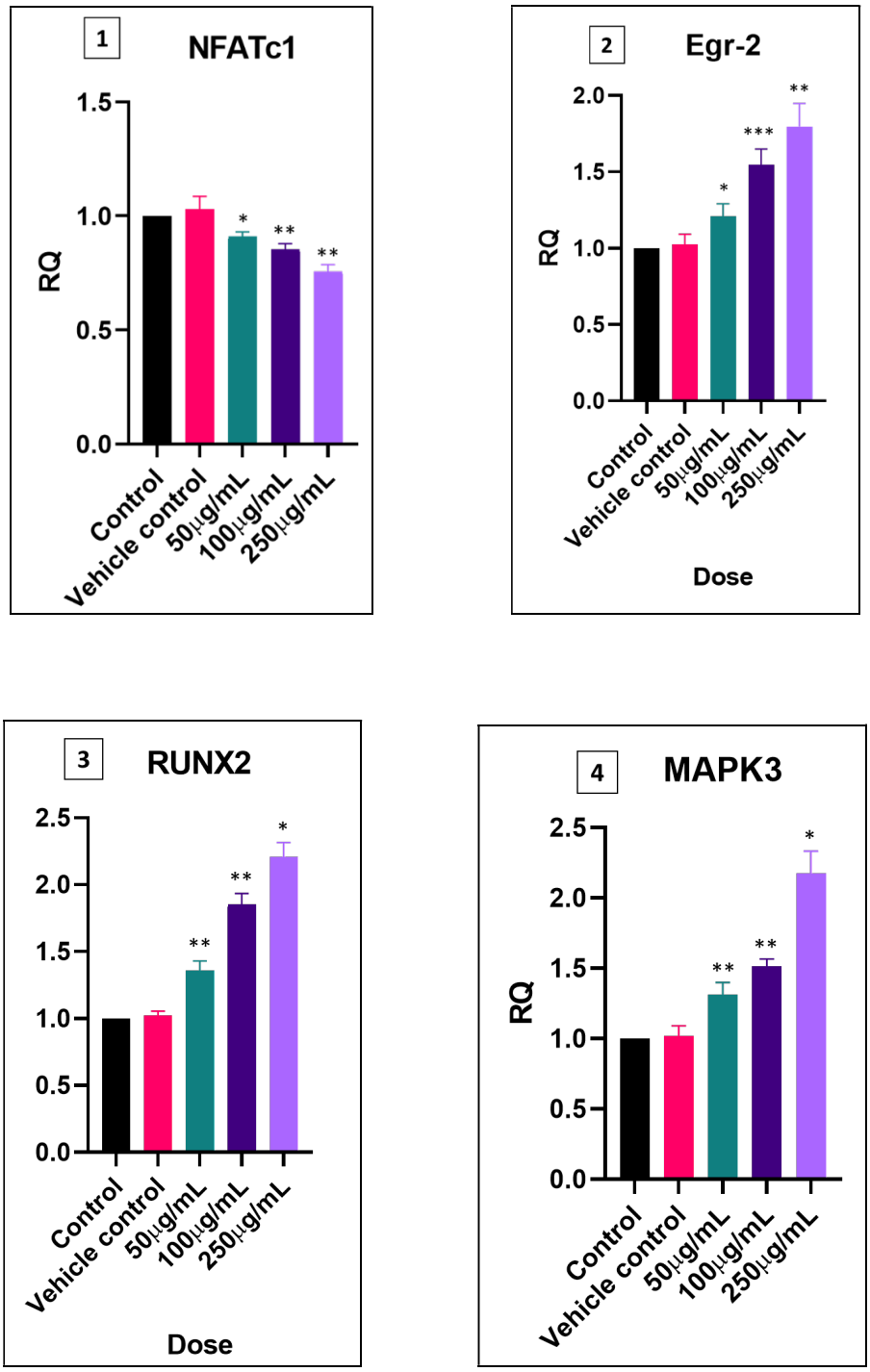



**Figure C1. 3: Gene expression profile (transcript analysis) of various genes**

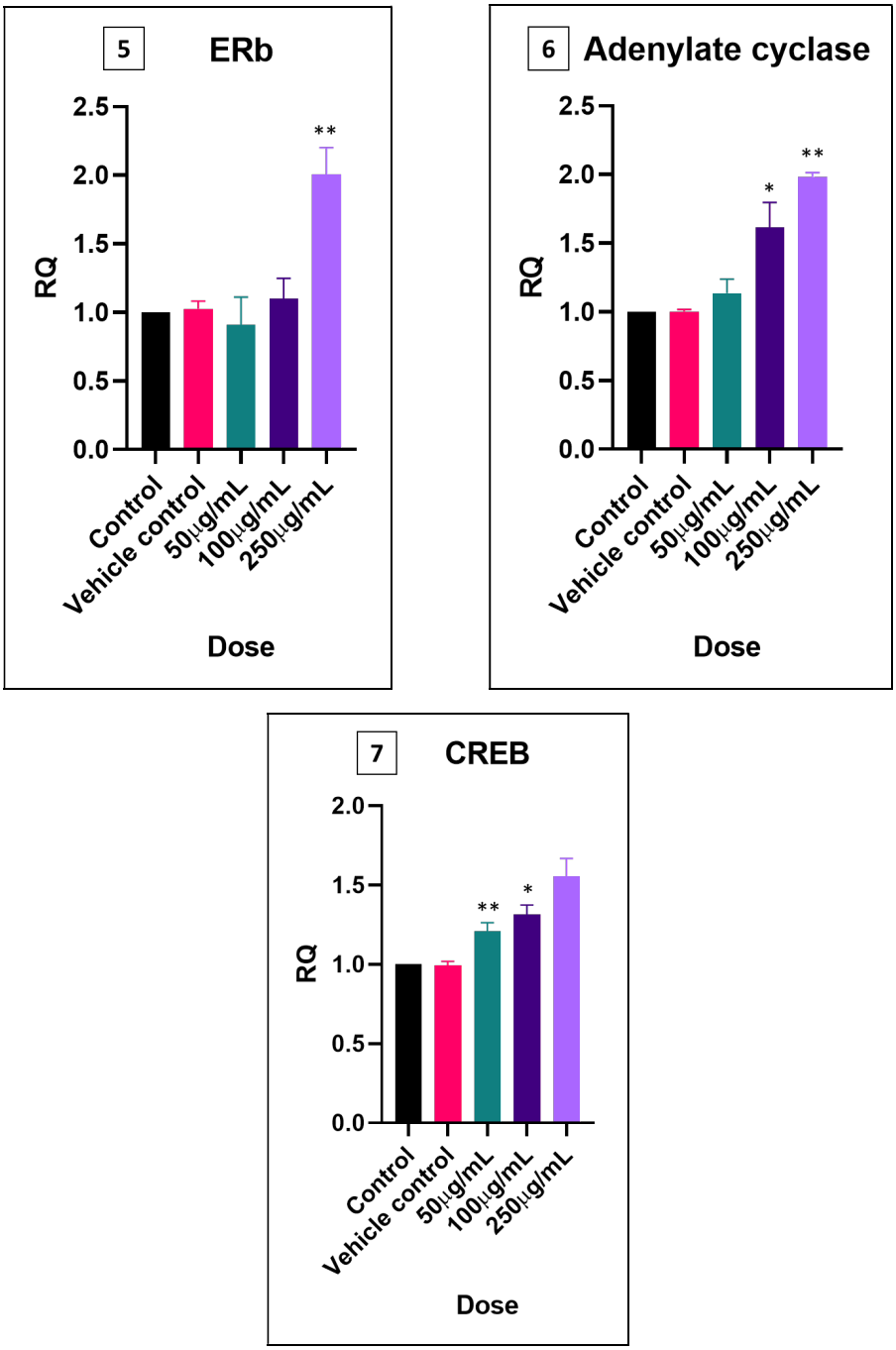



**Figure C1. 3: Gene expression profile (transcript analysis) of various genes**

*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). *- p< 0.05; **- p<0.01; ***- p<0.001; Calculated using Dunnett's multiple comparisons one-way ANOVA test. Data are presented as mean ±S.E. and are representative of three independent experiments (n=3).*

### 3.2.4 WESTERN BLOT:

To study further and verify results of qPCR, the expression of Egr-2 protein was examined via western blot. Post development, western blot bands were densitometrically analysed using ImageJ software. Peak area increases significantly ($p<0.001$ and $p<0.01$) and in dose dependent manner (*Figure C1. 4; Table C1. 7*). In other words, the expression of Egr-2 protein was found to be increased in dose dependent manner upon treatment with LG methanolic extract.

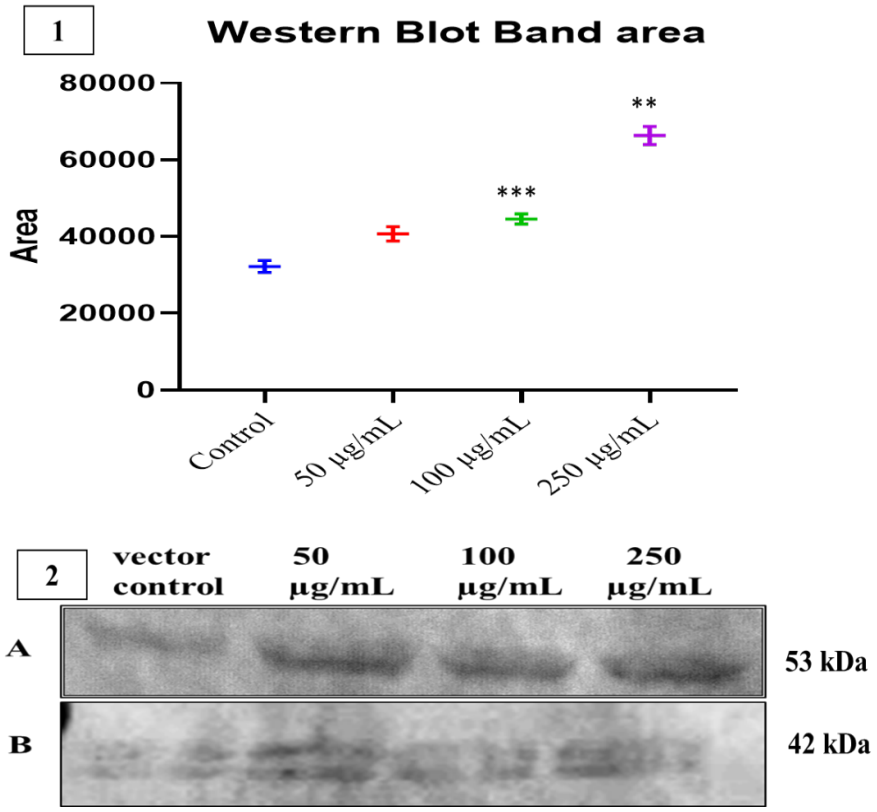



**Figure C1. 4: Western blot analysis of Egr-2 protein expression upon LG treatment**

*Figure C1. 4 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. (A) Egr-2 lane (B) internal control β-actin. **- p<0.01; ***- p<0.001. (n=3)*

**Table C1. 7: Densitometric analysis of western blot bands**

| Densitometric analysis of western blot bands | | | | |
|---|---|---|---|---|
| Sample | Control | 50 µg/mL | 100 µg/mL | 250 µg/mL |
| Peak Area | 32188.4 ± 900 | 40682.0 ± 1078 | 42548.2 ± 767 *** | 65040.1 ± 1362 ** |

*Table C1. 7 represents Densitometric analysis of western blot of Egr-2 gene upon LG treatment. Analysis was performed by ImageJ software (version: 1.52a). Total band area was calculated and then statistical analysis was done using GraphPad Prism 8.3.1 software, respective graph is shown in Figure C1. 4. Values were calculated using Dunnett's multiple comparisons one-way ANOVA test. Data are presented as mean ±S.E. and are representative of three independent experiments (n=3). **- p<0.01; ***- p<0.001. All the data are presented as mean ±S.E.*

## 3.3 DISCUSSION

Osteoporosis is a metabolic bone disease, which results due to an impairment and imbalance of osteoclasts and osteoblasts activity. Due to the serious drawbacks of currently available medicines and treatments, lots of research are being focused toward herbal molecules to identify potent candidate molecule or formula, which can suppress the condition of osteoporosis effectively (T. Wang et al., 2017). Many herbals are studied till clinical level for its anti-osteoporotic effects (Leung, 2016). With one such herbal LG, an *in-vitro* study was conducted to elucidate its effects on candidate proteins in the signaling pathway of the Saos-2 osteoblastic cell line. In brief, this chapter reports the modification of the genes under different doses of LG extract.

Herbal bioactive molecules and have been well explored concerning its effect on the genes involved in the signaling pathway. Like Poncirin, a flavonoid obtained from the fruit of *Poncirus trifoliate* which possess an anti-osteoporotic property by regulation of OPG/RANKL ratio, ERK/JNK/MAPK, estrogen receptor (ER); flavones of *Epimedium* has been proved to possess estrogenic effects through enhanced expression of RUNX2 in *in-vivo* study, it also improves maturation of osteoblast, Herb *Epimedium* activated extracellular-signal regulated kinase (ERK), p38, c-Jun N terminal kinase

(JNK)/MAPK pathways in rat (Jolly et al., 2018). Besides this, the same research group has shown that *Salvia miltiorrhiza* inhibits osteoclast formation by restraining the expression of c-Fos and NFATc1 in calvarial osteoblast co-culture system.

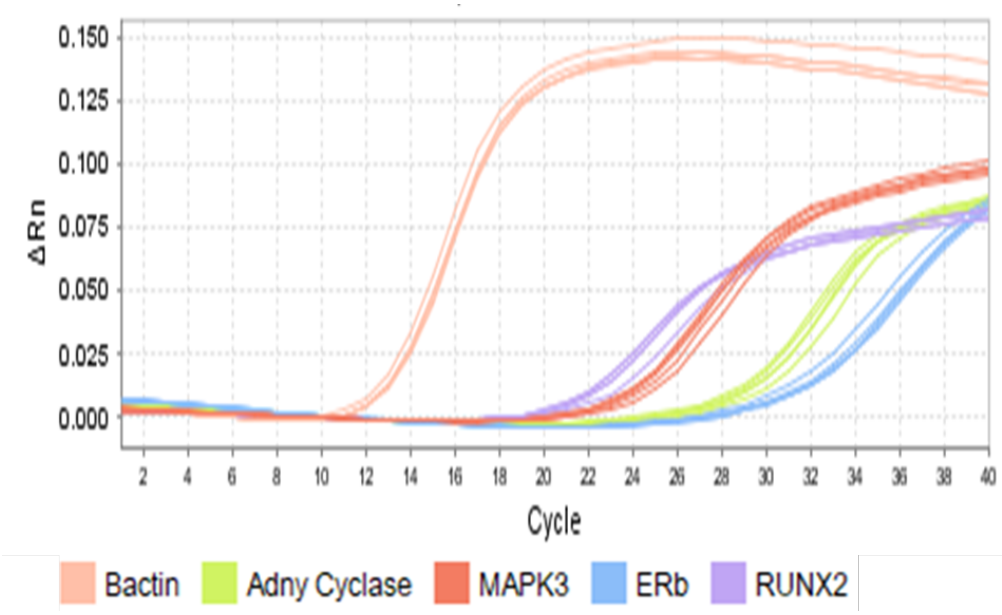
On Similar lines, these detailed gene expression studies revealed that LG methanolic extract treatment dose-dependently enhances gene expression of Egr-2, RUNX2 and downregulates NFATc1 significantly, all these genes play a crucial role in the regulation of osteoporosis which results from resorption and formation of bone cells (Shukla et al., 2017). Further, the western blot studies also supported enhanced gene expression of Egr-2. The upregulation of Egr-2 and RUNX2 genes 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 J et al., 2017). Further, the downregulation of NFATc1 by LG in the present study is in accordance with the earlier reported work by Hong group showing inhibition of osteoclastogenesis in OVX-Induced Osteoporosis (Hong et al., 2020). So summing up together, the alterations in the expression of the studied genes indicates that these candidate genes are modulated by LG, which are involved in osteoblasts proliferation. However, further supportive studies on the downstream functions of these candidate genes can be helpful to identify the proteins which are responsible for transmitting the information inside the cell.

It is well known that osteoblasts carry receptor for various hormones like PTH (Lombardi et al., 2011), 1,25 (OH)2D3 (Van, 2014) and estrogen (Almeida et al., 2013), which are mainly involved in osteoblasts differentiation and its other functions. PTH receptor, being a GPCR, signals through activation of adenylate cyclase, CREB proteins (Datta et al., 2009). In these results, an upregulation of adenylate cyclase and CREB suggests that these genes are affected upon LG treatment. Alma Y research group has shown that PTH is responsible for the expression of osteoblastic gene means of increasing expression and phosphorylation of RUNX2 and activation of MAPK3 and PI3K signaling which results in osteoblastogenesis and osteoblasts survival. Another group of scientists has reported a similar role of RUNX2 and MAPK3

in osteoblast. 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 (Alma et al., 2013). However, further studies are advisable to strengthen involvement of ERK pathway, which involves these genes and functions during early osteoblast differentiation, to identify possible involvement of this pathway in the mechanism of LG (Greenblatt et al., 2013). Reports suggest that polyphenols, specifically quercetin, act as selective estrogen receptor modulator by upregulating the expression of ERβ, which is a positive sign of bone mineralisation (Setchell et al., 2003; Torre, 2017). These findings are in line with presented results like upregulation of ERβ in dose dependent manner upon LG treatment. It can be speculated that there some phytochemicals present in LG methanolic extract which are responsible for the upregulation of ERβ. Collectively, it has been inferred that components present in the LG methanolic extract, can be responsible for upregulation of genes like MAPK3, RUNX2, ERβ and GPCR pathway candidate proteins. Study results can serve as a base to design further detailed studies which can solidify these outcomes.

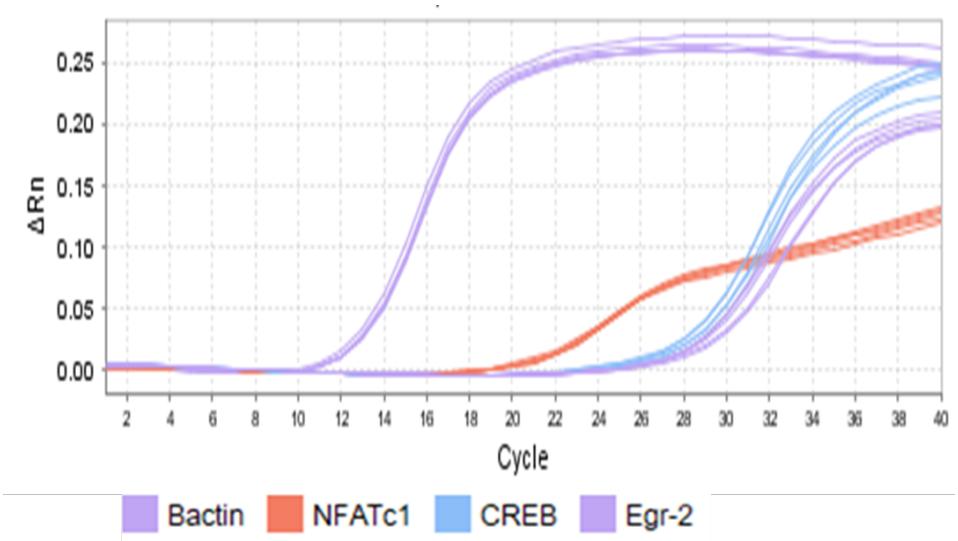
***In conclusion, LG treatment to Saos-2 cells has enlightened the involvement of various crucial genes in molecular mechanism of LG. Based on upregulation of transcription factors like Egr-2, RUNX2 and CREB, it can be speculated that they may have role in initiation of expression of the genes which are responsible for osteoblasts growth and proliferation. Upregulation of genes like adenylate cyclase, MAPK3 and ERβ indicates that these genes may be involved in signalling cascade of LG action. These chapter explores an attempt to find out the identification of the candidate genes through which LG exerts its effects.***

**APPENDIX A1.1 FIGURE**




*Appendix A1.1 figure represents the amplification curve of one of the triplicate run of the gene expression study of Adenylate cyclase, MAPK3, estrogen receptor β and RUNX2 transcription factor and endogenous control β-Actin. Curve is plotted by Applied Biosystems StepOne Software v2.3. 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.*

**APPENDIX A1.2 FIGURE**




*Appendix A1.1 figure represents the amplification curve of one of the triplicate run of the gene expression study of transcription factors like Egr-2, NFATc1 and CREB and endogenous control β-Actin. Curve is plotted by Applied Biosystems StepOne Software v2.3. 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.*