A molecular insight into anti-osteoporotic property of *Litseaglutinosa* on Bone cells: An *In-vitro* study

Research Synopsis for Ph. D

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1. Introduction

Osteoporosis is defined as a "systemic skeletal disease characterised by low bone mass and micro-architectural deterioration of bone tissue leading to enhanced bone fragility and a consequent increase in fracture risk" (Binkley , 2006). Basically it is a progressive skeletal disease characterized by low bone mineral density (BMD) with a consequent increase in bone fragility and susceptibility to fracture of the hip, spinal vertebrae, and wrist (Papaioannou, *et al*., 2010). This is a condition often seen in postmenopausal women with a rate of one in three over the age of 50 years worldwide affected (Kanis*et al*., 2008).

There are multiple factors involved in bone loss. This risk is enhanced by various factors like aging, menopausal status, injury, diet, drugs and chronic inflammatory diseases like rheumatoid Arthritis. Osteoporosis starts when the regular processes of bone formation and resorption become unbalanced culminating in net bone loss. Fractures can occur as a result of decreased bone mass and its strength. Vertebra, hips and wrists are the most common sites where osteoporotic fractures occur (Kelsey *et al*., 2009, Smith *et al*., 2000).

Estrogen plays crucial role in bone remodelling process. Estrogen is, thus, responsible for maintaining bone density by stimulating osteoblasts to form new bone. The hormone is produced by the ovaries before menopause. In post-menopausal women, the ovaries stop producing estrogen but the adrenal gland secretes small amounts of androgens and these are converted to estrogen by the enzymes aromatase. As a result of reduced estrogen levels in post-menopausal women, osteoblasts become less active resulting in decreased bone mass (Demontiero*et al*., 2012). Estrogen receptors, ERα and ERβ are generally present on osteoclasts. Binding of estrogen to its receptors will promote apoptosis in osteoclasts and synthesis of inhibitory proteins for osteoclast formation. However, their role is affected when estrogen production is deficient in post-menopause women, resulting in a large pool of osteoclast progenitors (Feng and McDonald 2013). It has been demonstrated that osteoclast formation is inhibited by estrogen*in vitro* when osteoclast precursors are co-cultured with osteoblastic cells. However, estrogen did not directly change precursors of osteoclast when they were cultured alone. The outcome of this study strongly indicated that estrogen regulation of osteoclast activity is controlled by osteoblasts (Martin *et al.*, 2015).

As a medication, to prevent the condition of osteoporosis in post-menopausal women, Hormone Replacement Therapy (HRT) is playing very promising role. HRT has been an established regime for prevention of postmenopausal bone loss (Gambacciani *et al.,* 2014). The aim in treating patients with osteoporosis is to provide hormonal dose to inhibit and/or restrict the activity of osteoclasts, the enhancement of osteoblast activity. Low-dose HRT is safe and effective for prevention of postmenopausal bone loss. Low-dose HRT has also been shown to be effective for reducing the number and severity of hot flushes, improving vaginal atrophy, and inducing favourable changes in lipids, lipoproteins and hemostatic factors. Moreover, low-dose regimens of CEE (Conjugated Equine Estrogen) and MPA (Medroxy-Progesterone Acetate) result in higher rates of amenorrhea and endometrial protection compared with the conventional dose of HRT (Xiangyan*et al.,* 2014).

Recent evidence indicates that long-term use of HRT is accompanied by many side effects, such as the increased risk of breast, ovarian and endometrial cancer (Fournier*et al.,* 2014; Shagafand Williamson, 2015; Michael 2016). In one study of 16 years, it has been observed that HRT increases risk of venous thromboembolism and gall bladder disease (Shagafand Williamson, 2015). Thus, alternative means of proven efficacy and safety should be developed for prevention and treatment of postmenopausal osteoporosis.

As prophylaxis of HRT is limited, herbal medicine is one of the potent candidates for the treatment of variety of diseases, including osteoporosis (Mahalakshmi *et al.*, 2014). In the past few years extensive research using animal models has provided convincing data to indicate a significant improvement in bone mass. Although these herbal medicines are seen as cost-effective alternatives by their traditional users, their international acceptance as a major regimen for prevention and treatment of diseases includingosteoporosis would require extensive research using modern science. There are many plants described in Ayurveda for the treatment of myriad of diseases. Ayurveda mentions a number of plants with anti-inflammatory and osteoprotective effect. However, the scientific base behind their osteoprotective effect is still not clear.

In recent decades, Chinese medical herbal extracts have been extensively investigated for their effects on proliferation and differentiation of osteoclasts (OCs) and osteoblasts (OBs) *in vitro*, and/or therapeutic potency in OP *in vivo* (e.g., *Anoectochilusformosanus* (Masuda *et al.,* 2008), *Acanthopanaxsenticosus* (Hwang *et al.,* 2009), HerbaEpimedii (Mok *et al.,* 2010) and

Curcuma longa (Kim *et al.,* 2012; Wright *et al.,* 2010; Folwarczna *et al.,* 2010 and Rangrez *et al.,* 2011). **Curcumin** is one of such medicines. Its history goes back over 5000 years, to the heyday of Ayurveda. One of such plant is *Curcuma aromatica,* commonly known as 'JangliHaldi' belonging to genus curcuma, consisting about 70 species of rhizomatous herbs. It is widely used as a flavouring agent, condiment and a source of yellow dye (Wealth of India, 1995). Sulfuretin, major flavonoid of *Rhusverniciflua,* has been proved to promote osteoblastic differentiation. **Sulfuretin** works through various pathways involving Mitogen Activated Protein Kinase (MAPK), Akt, mTOR, BMP2 (Q-Schick *et al*., 2016).

DrynolCibotinis, botanical combination of eight botanicals, shows impact on osteoporosis via promoting cell growth, Ca^{++} uptake and collagen production (Barbara *et al*., 2010). **Diosgenin**, extracted from the root of wild yam (*Dioscoreavillosa*), has been reported to have anti-osteoporotic property. Diosgenin induces the expression of vascular endothelial growth factor (VEGF-A) and increases the activity of Hypoxia inducible factor-1 (HIF-1) to promote angiogenesis in osteoblasts during bone formation. It exerts its effect via phosphatidylinositol 3-kinase (PI3K)/Akt and MAPK signalling pathways (Men Luh*et al*, 2005).

Another compound has been found is **Genistein**, a major phytoestrogen of soy. It is well known for its potency against osteoporosis. It has very similar structure as estrogen, works via estrogen receptor (Alice *et al*., 2003). Recently it was found that Genistein also works via PTH receptor against Post-Menopausal Osteoporosis (Qing 2012).

Moringaolifera and *Litseaglutinosa* (LG) is described in Ayurveda for its bone protecting effect and used in traditional medicine in healing the fractures. LG is commonly known as "Maida Lakri" and said to be one of the most potent plants for treatment of osteoporosis, (Sukh*et al*., 2006). LG belongs to the family *Lauraceae* and many of its members are believed to have osteoprotective effect. Bark of the LG is used for the preparation of the dried bark powder (Parikh *et al*., 2009). This bark powder is prescribed directly or used in the formulation for the treatment of osteoporosis.

Earlier work done in our lab has provedLGto bequite promisingosteoprotectant, where LG showed ameliorating effect on Osteoporosis in OVX mice(Rangrez*et al.*, 2009, 2010 and 2011), by reduced serum TRAcP (Tartrate-resistant acid phosphatase) levels in dose dependent

and time dependent manner. TRAcP activity, which is regarded as an important cytochemical marker of osteoclasts, its concentration in serum, is utilized as a biochemical marker of osteoclast function and degree of bone resorption. Furthermore, LGtreatment also restored reduced level of ALP, which is osteogenic marker for osteoblasts along with the reduced rate of Ca^{++} excretion. To verify the efficacy of the plant, histological studies were also conducted, which resulted into a notable improvement in the quality and microarchitecture of bone in dose dependent manner.Further, the extended *In vitro* studies also proved the dual role of LG as osteoblast stimulant and osteoclast inhibitor on SaOS2 cell line. However due to time constrain, the final molecular mechanism of LG could not be elucidated. Conclusively, LG effect on osteoporosis wasextensively studied phenotypically mostly*in vivo*conditions and preliminary work on SaOS2 cell line till date has proved osteoprotective nature of LG, but the exact cellular mechanism by which it is exerting its effects in osteogenic cells is yet to be explored.

Therefore, the present study has been designed to systemically evaluate these obscure area, which will help us in understanding the osteoprotective role of L.glutinosaat molecular level and which might prove as one of the promising therapeutic potential against osteoporosis..

2. Objectives:

1. To explore the molecular mechanism of*Litseaglutinosa* extract on SaOS2 cell line.
    a. By checking the effect of *Litseaglutinosa*extract on homeostatic markers in SaOS2 cell line like Egr-2, NFATc1 and protein expression study by western blot.
    b. By checking the expression profile of MAPK, RUNX2, ERβ, Adenylate cyclase, CREB genes.
2. To study the proliferative effect of *Litseaglutinosa*extract on SaOS2 cell line.
    a. To check the rate of proliferation, its numbers and viability.
    b. To check the expression of proliferative markers like PCNA, osteocalcin.
3. To study the apoptotic effect of *Litseaglutinosa*extract on SaOS2 cell line.
    a. To check the expression profile of apoptotic markers like cytochrome C, caspase 3, Fas ligand.

3. Material and methods

Objective 1

Preparation of LG Methanolic extract:

Bark powder of *Litsea glutinosa* were purchased from local market. 50 g powder was weighed and resuspended in 400 mL HPLC grade methanol and volume was made up 500 mL. Solution was incubated at RT on magnetic stirrer overnight. After incubation, solution was filtered through Whatman® filter paper to remove insoluble particles. Methanolic extract was allowed to air dry. Upon drying, remaining solid extract was collected. This was weighed and stored at -20°C.

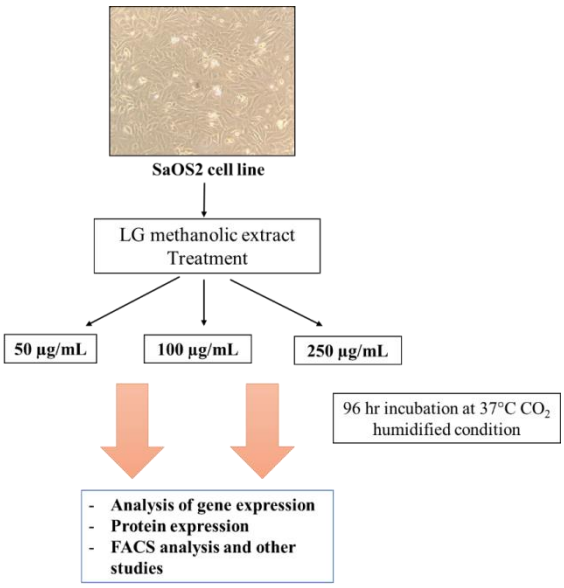
Culturing of SaOS2 cells:

SaOS2 cell line was procured from national center for cellular science institute (NCCS, Pune, India). Cells were throughout maintained in Maccoy's 5A + 10 % FBS media at 37°c in CO_2 humidified chamber. For passaging, cells were washed with 1x PBS, followed by trypsinization. After detachment of cells, trypsin was diluted (minimum 1:10) with media. These cells were counted using cell viability analyser (Beckman coulter). Again cells were seeded to fresh flask at seeding density of 0.4 M/mL.

LG treatment:

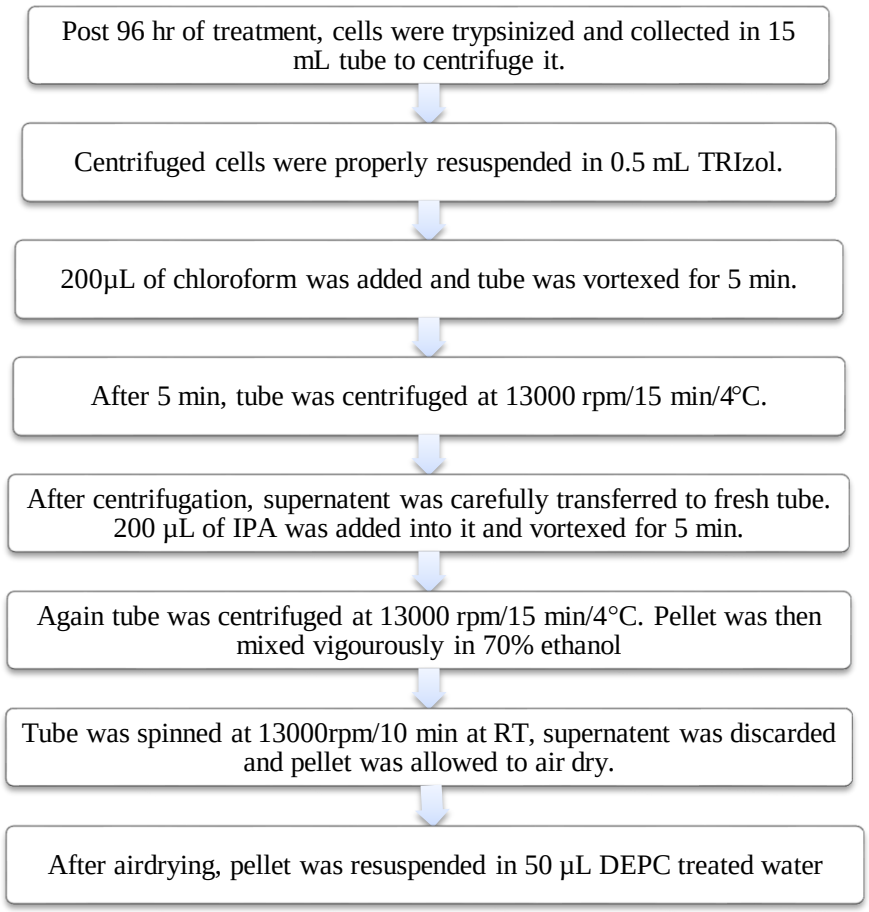
Methanolic extract was weighed 250 mg and it was dissolved in final volume of 1 mL of DMSO, generating 250 mg/mL concentration. The solution was diluted further in culture media (Maccoy's 5A+ 10% FBS) to treat the cells.

For the treatment, cells were seeded a day before treatment in T25 flask. Next day, serial dilutions were prepared to achieve 250 µg/mL, 100 µg/mL & 50 µg/mL concentrations and it was filtered by 0.2 µ filter. Spent media in flasks was replaced by these treated media. Along with treated flasks, two flasks were kept as control (host and vector control). Vector control was treated with 0.1% DMSO. In this way, all the flasks were incubated at same conditions for 96 hrs.



Transcript analysis:

RNA isolation:



After RNA isolation, it was quantified spectrophotometrically at 260nm using NanodropC.

cDNA synthesis:

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 (kit cat#: K1612, Thermo). Once cDNA was synthesized it was used for transcript analysis of desired genes.

Table 1: cDNA synthesis reaction mix

Reagent	Stock concentration	Volume
RNA	X ng/mL	25 µL
5x Buffer	5x	5 µL
Ribolock RNAse inhibitor	NA	2.5 µL
dNTPs	25mM	5 µL
MMLV Reverse trasncriptase	1 U/µL	5 µL
Oligo dT primers	10µM	2.5 µL
Total	**NA**	**50 µL**

After setting up reaction mix, samples were kept in thermocycler in following conditions:

Table 2: cDNA synthesis

Sr no	Stage	Temperature and Time
1	cDNA synthesis	37°C for 1 hr
2	Reaction stop	75°C for 5 min

Real time PCR:

Variable volumes was used as a sample for real time PCR to analyze expression level of various genes. Following sets of primers were used for transcript analysis using PowerUP Sybr® green master mix (cat#A25742, Thermo, USA). Standard manual protocol was used for amplification.

Table 3: real time PCR reaction mix

Reagents	Volume (µL)
2x SyBr green Mastermix	10
Fw Primer (10uM)	1
Re Primer (10uM)	1
cDNA	2
Total	**20**

Table 4: Real time PCR conditions

Step 1 (1 cycle)	Step 2 (1 cycle)	Step 3 (40 cycle)
50°C for5 min	95°C for min	95°C for 30 sec
		60°C for 30 sec
		72°C for 1 min

Table 5: Real time PCR primer sequences

	Protein Name	Gene Name	RefSeq ID	Primer Type	Sequence
1	MAPK3	MAPK3	NG_029936.1	Forward	5' GGATGCCGATGACATTCTC 3'
				Reverse	5' CATCAAGAAGATCAGCCCCT 3'
2	Runx2	RUNX2	NG_008020.2	Forward	5' CCTAAATCACTGAGGCGGTC 3'
				Reverse	5' CAGTAGATGGACCTCGGGAA 3'
3	ERb	ESR2	NG_011535.1	Forward	5' ACCAAAGCATCGGTCACG 3'
				Reverse	5' CATGATCCTGCTCAATTCCA 3'
4	Adenylate Cyclase	ADCY1	NG_034198.1	Forward	5' CCGACACGCAGTAGTAGCAG 3'
				Reverse	5' ATGAGCTCTTCGGCAAGTTC 3'
5	Egr2	EGR-2	NG_008936.2	Forward	5' AGCAAAGCTGCTGGGATATG 3'
				Reverse	5' TTGACCAGATGAACGGAGTG 3'
6	NFATc1	NFATC1	NG_029226.1	Forward	5' CACCTCAATCCGAAGCTCAT 3'
				Reverse	5' CCCTGTCCCCTACGTCCTAC 3'
7	CREB	CREB1	NG_023299.1	Forward	5' GCTGGGCTTGAACTGTCATT 3'
				Reverse	5' GTGACGGAGGAGCTTGTACC 3'
8	β-Actin	ACTB	NG_007992.1	Forward	5' GCAACGGAACCGCTCATT 3'
				Reverse	5' AGCTGAGAGGGAAATTGTGCG 3'
9	PCNA	PCNA	NG_047066.1	Forward	5' AAGAGAGTGGAGTGGCTTTTG 3'
				Reverse	5' TGTCGATAAAGAGGAGGAAGC 3'
10	Osteocalcin	BGLAP	NG_047015.1	Forward	5' CGCCTGGGTCTCTTCACTAC 3'
				Reverse	5' CTCACACTCCTCGCCCTATT 3'
11	Caspase 3	CASP3	836	Forward	5' TCGCTTCCATGTATGATCTTTG 3'
				Reverse	5' CTGCCTCTTCCCCCATTCT 3'
12	Fas Ligand	FASLG	NG_007269.1	Forward	5' CAGAGGCATGGACCTTGAGT 3'
				Reverse	5' GTCTACCAGCCAGATGCACA 3'
13	Cytochrome C	CYCS	NG_023438.1	Forward	5' TGCCTTTCTCAACATCACCC 3'
				Reverse	5' GGCGTGTCCTTGGACTTAGA 3'

β-Actin was taken as an endogenous control & vector control was taken as test control in real time PCR to calculate ΔΔCT and thereby RQ (relative quantitation).

Western blot:

To validate theformation of Egr-2 at protein level, western blot study was performed from treated cell.

- SaOS2 cells were treated with LG Methanolic extract for 96 hr along with untreated and vector 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. Protein was then quantified by Nanodrop at 280nm. After that, each sample was normalized and mixed with 6x non-reducing gel loading dye for PAGE, samples were boiled for 10 min in boiling water bath. Equal amount of protein was loaded in well 4-20% gradient gel was ran. After completion of gel, protein was electronically transferred to Nitrocellulose membrane. It was followed by treatment of primary (anti-Egr-2) (make: abcam; AB108399) and secondary (anti-rabbit HRP conjugate) antibody. For internal control, β-actin was used. After completion of secondary treatment, membrane was developed using TMB substrate of HRP in dark environment.

Results:

Objective 1

RNA isolation:

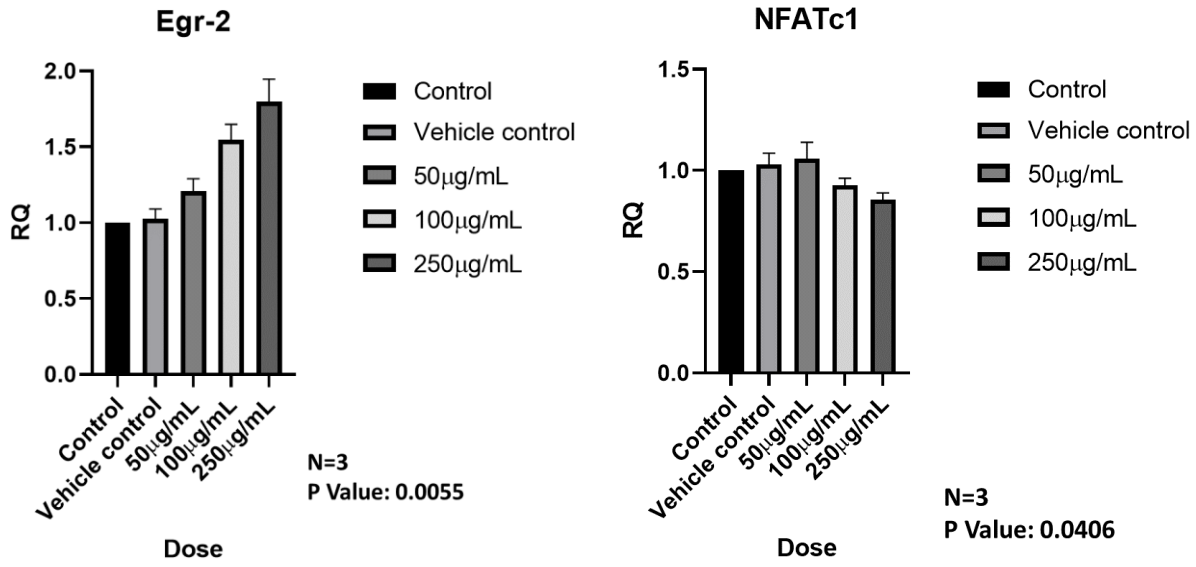
After isolation of RNA, they were quantified using Nanodrop C.

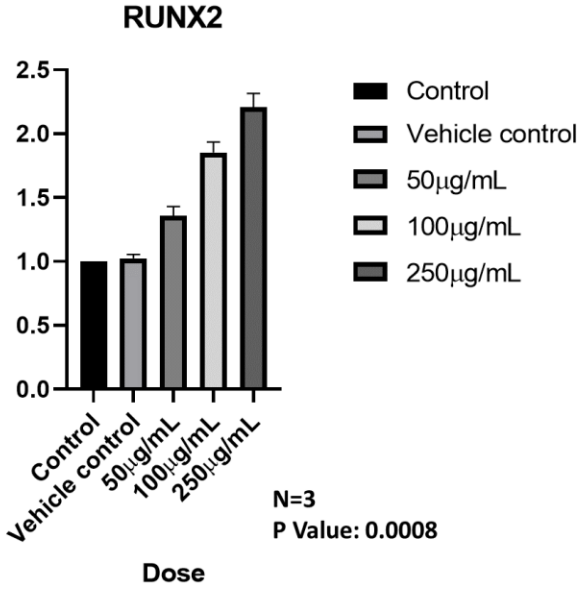
Table 6: RNA reading

Sample	ng/uL	A260/A280	A260/A230
Control	333.3	1.87	2.34
Vehicle control	357.5	1.93	2.32
50 µg/mL	347.4	1.88	2.33
100 µg/mL	359.8	1.89	2.34
250 µg/mL	332.7	1.88	2.36

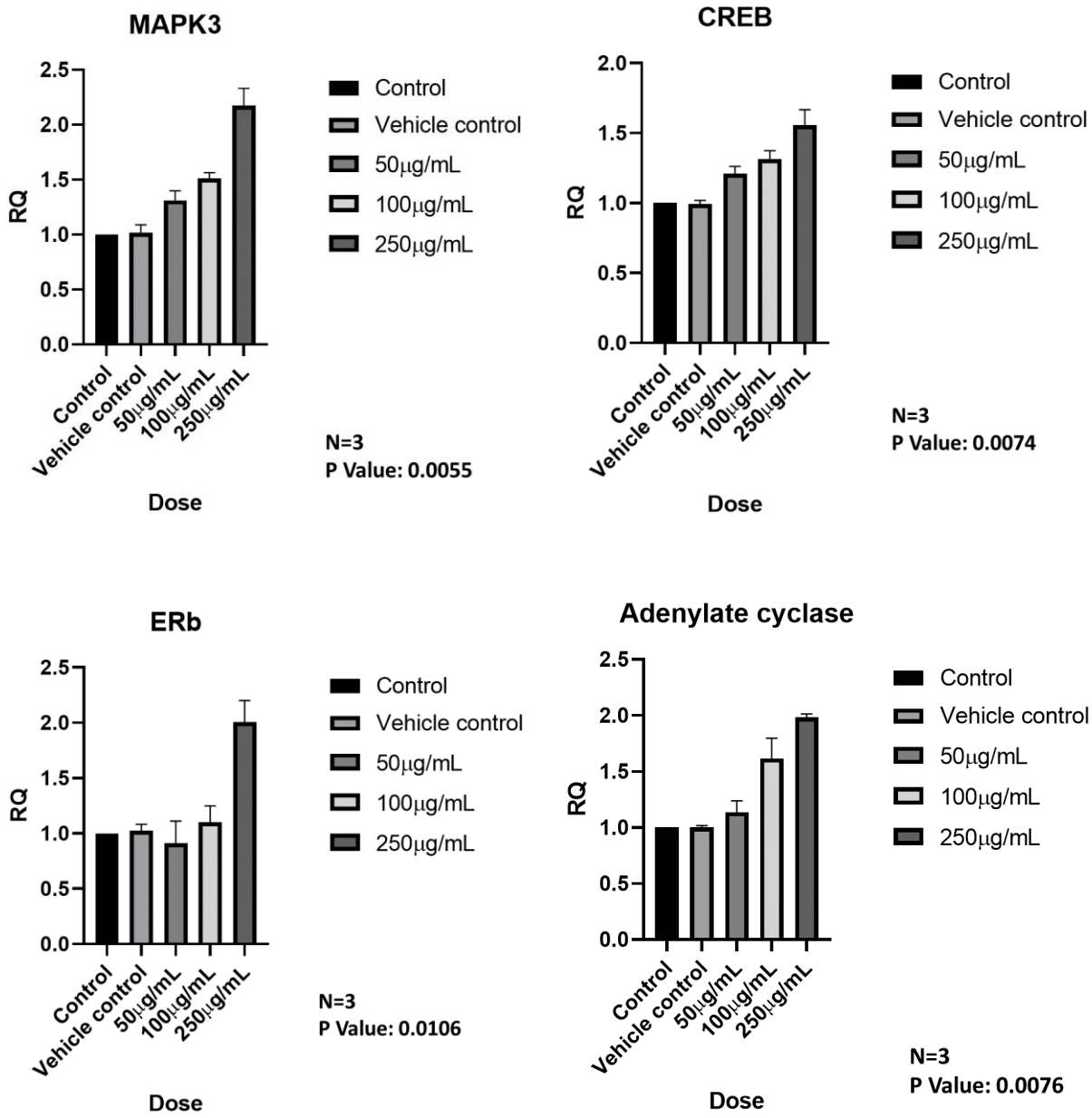
Quantitative PCR:

The quantitative PCR revealed that, expression of the Egr-2, CREB, Adenylate cyclase, MAPK3, RUNX2 & ERβ are getting upregulated, whereas NFATc1 is getting down regulated after LG extract treatment.

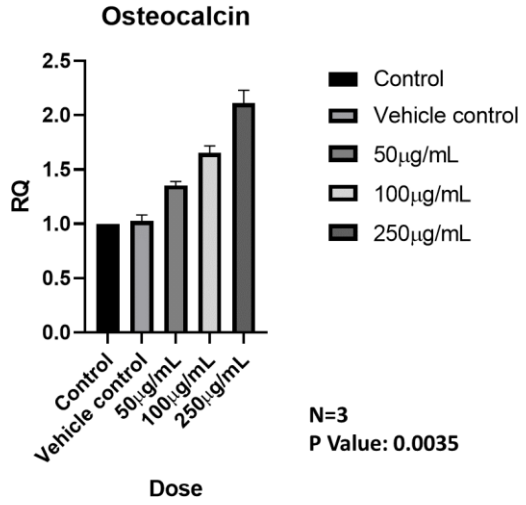


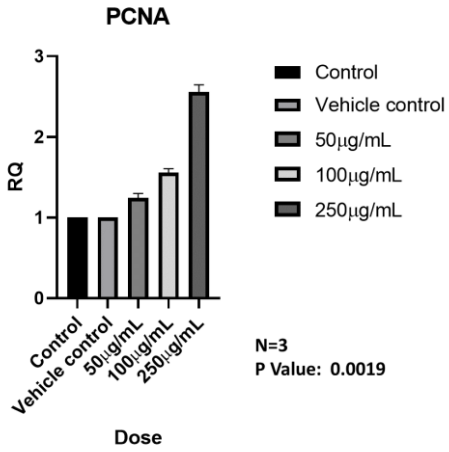


Egr-2, RUNX2, NFATc1 are the genes which play crucial role in osteoporosis.

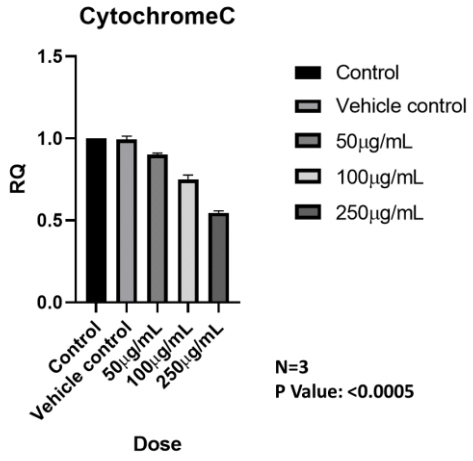

MAPK3
RQ
2.5
2.0
1.5
1.0
0.5
0.0
Control
Vehicle control
50µg/mL
100µg/mL
250µg/mL
Dose
N=3
P Value: 0.0055
CREB
RQ
2.0
1.5
1.0
0.5
0.0
Control
Vehicle control
50µg/mL
100µg/mL
250µg/mL
Dose
N=3
P Value: 0.0074
ERb
RQ
2.5
2.0
1.5
1.0
0.5
0.0
Control
Vehicle control
50µg/mL
100µg/mL
250µg/mL
Dose
N=3
P Value: 0.0106
Adenylate cyclase
RQ
2.5
2.0
1.5
1.0
0.5
0.0
Control
Vehicle control
50µg/mL
100µg/mL
250µg/mL
Dose
N=3
P Value: 0.0076

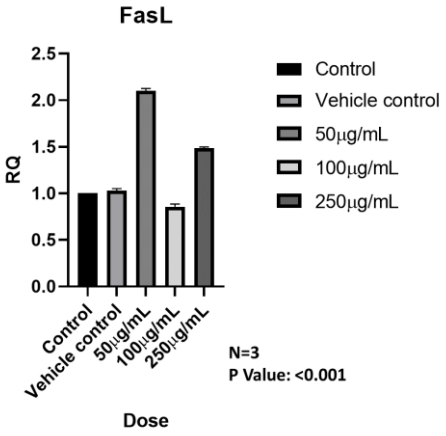
Proliferation Markers:

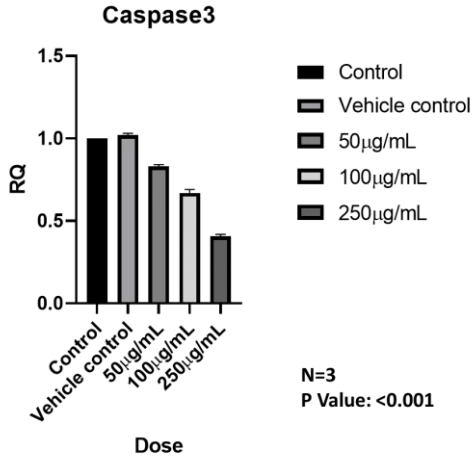




Apoptosis Markers:







Western blot results:

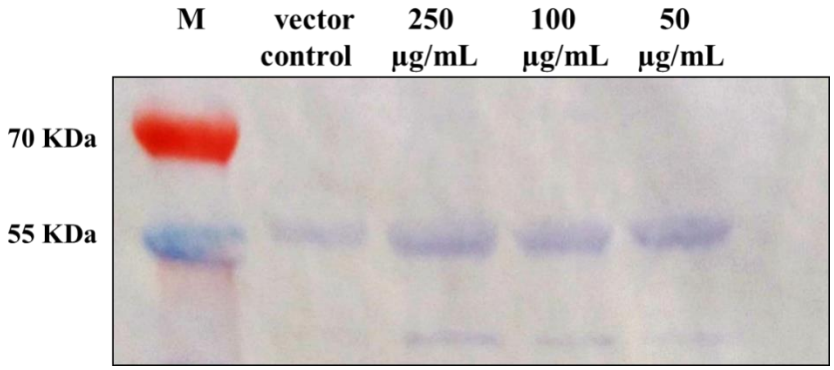


Figure 1: western blot showing protein expression of Egr-2 (~53.8 KDa). Lane M: pre-stained protein ladder (Thermo SM 26619); Vector control: whole cell lysate of vector control (DMSO treated); rest of 3 lanes are showing different concentrations used for treatment of LG substance. Here marker RED band corresponds to ~70 kDa & BLUE band corresponds to ~55 kDa molecular weight.

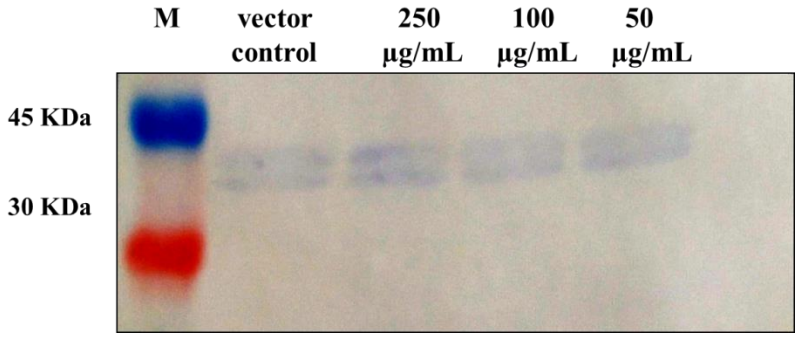


Figure 2: Western blot of endogenous gene β-actin control (Mol wt ~42 KDa)

4. Discussion:

Detailed gene expressions study revealed that LG methanolic extract treatment enhances gene expression of crucial genes like Egr-2, RUNX2 and downregulates NFATc1, all these genes are playing important role in osteoporosis.We also have shown dose dependent protein expression of Egr-2 using western blot studies.Mechanism of osteoporosis generation which consists of resorption and formation of bone cells is governed by these genes.Out of these, Egr-2 gene is involved in pathways like suppression of osteoclast formation, negative regulation of

RANKL mediated osteoclast differentiation and secretion of IL-10 which inhibits osteoclastogenesis (Hyun-Ju *et al.,* 2012) RUNX2 is corebinding factor and the essential regulator that guides the chondrocytematuration and the differentiation of osteoblasts (Chen and Pan, 2013) RUNX2 works downstream to Egr-2 protein. NFATc1 is the prime transcription factor which is mainly involved signalling of RANK-RANKL interaction responsible for differentiation of osteoclasts. It has also been established that NFATc1 suppresses the expression of osteocalcin during osteoblast differentiation (Choo, 2009). Upon LG treatment to SaOS2 cell line, osteocalcin also gets upregulated. Being bone formation marker, upon upregulation of osteocalcin, it can be stated that LG has some positive effects in osteocyte proliferation and bone formation.

Along with above mentioned genes, we also studied other genes like MAPK3, CREB, adenylae cyclase and ERβ, which are involved in different cell signalling pathway. There are many other herble and phytochemicals, which exerts its effect via these pathways like ERK1/MAPK3, GPCR etc (Men Luh *et al,* 2005). To have some more focus on signalling pathway of LG extract in SaOS2 cells, their expression study was performed. In the gene expression study, it was observed that these genes are getting upregulated in dose dependent manner. It is well established that PTH is showing its effect via PTH-CREB pathway, where CREB acts as transcriptional factor (Rongrong, 2011). Overexpression of CREB and adenylate cyclase indicate that LG may exert its effect via PTH / GPCR based cell signalling pathway further investigation is suggested.

As per Men et al., 2005 study, ERK1 has very important role in osteoblast. They has shown that in *Erk*$^{-/-}$mice, there are low bone mineral density. Their studies suggests that active ERK may bind tothe promoters of osteocalcin (Ocn) and bone sialoprotein (Bsp) in osteoblasts via the associationbetween ERK and RUNX2. This research paper supports our data of upregulation of these gene, establishing that LG is showing its effect via ERK1 and RUNX2 (earlier mentioned).

Besides these genes, study of proliferative markers and apoptotic markers explored that proliferative markers like PCNA and osteocalcin are getting upregulated upon LG treatment in SOS2 cell line. Whereas apoptotic markers like cytochrome C and caspase3 are getting

downregulated upon treatment. This indicates LG has proliferative and anti-apoptotic effects on SaOS2 cells.

5. Work to be carried out

To perform FACS analysis to study proliferation / anti-apoptotic effect of LG on SaOS2 cells.

6. Conclusion

From all the experiments and results, it can be concluded that LG methanolic extract is showing anti-osteoporotic effects via upregulation and downregulation of various crucial genes. It has also been observed that LG assists in proliferation and also suppresses apoptosis in SaOS2 cells.It can be stated that LG extract may work using multiple cell signalling pathways, like GPCR signalling pathway, ERK1/MAPK3pathway, but further studiesneeds to becarried out to establish.

7. References:

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