INTRODUCTION

1.1 INTRODUCTION TO BONE ANATOMY AND OSTEOPOROSIS

Bone is complex and dynamic tissue of the human body. It serves many important functions including providing strength to body, locomotion, ion storages, soft tissue protections and harbouring of bone marrow (Robling et al., 2006; H. K. Datta et al., 2008). Bone contains separated cells which are surrounded by extracellular matrix. This extracellular matrix is made up of 15% water, 30% collagen fibers and 55% crystalized mineral salts. Most abundant mineral salts include calcium phosphate which combines with calcium hydroxide resulting in formation of hydroxyapatite. Hydroxyapatite will combine with more salts like calcium carbonate and ions like magnesium, potassium, fluoride, sulphate. As a results, there will be formation of framework by collagen fibres of extracellular matrix and hence, hardening of bone. This process is known as Calcification, which is initiated by bone building osteoblasts, one of the bone cells (Tortora, 2017).

Bone cells are majorly of 4 types: osteoprogenitor cells, osteoblast, osteoclasts and osteocytes (Figure In- 1) (Florencio et al., 2015; Tortora, 2017). **Osteoprogenitor cells** are unspecialized bone cells which are derived from mesenchyme tissue. These cells undergo cell division, resulting cells develop into osteoblasts. **Osteoblasts** are key bone building cells, which synthesize and secrete organic components and collagen protein needed to build extracellular matrix of bone and calcification process. Upon secreting extracellular matrix material, these cells will entrap themselves into it and will be converted into osteocytes. **Osteocytes**, mature bone cells, are main cells which are present in bone tissue and responsible for daily metabolism like exchange of nutrients and waste with blood. These cells are last differentiated cells. Reports suggest that these cells can serve as orchestrators and mechanosensors in bone remodelling process (Clarke, 2008; Karsenty et al., 2009; Bonewald, 2011; Teitelbaum, 2007). **Osteoclasts** are the only bone resorpting cells, which are huge in size, multinucleated and formed by fusion of as many as 50 monocytes. These cells exhibit ruffled plasma membrane,

which is deeply folded on the side of the cell that faces the bone surface. Osteoclasts secret powerful lysosomal enzymes and acids which will digest the protein and solubilise mineral components at underlying bone surface (Tortora, 2017). Besides these cells, another cells, known as **bone lining cells**, are also found on bone surface. Bone lining cells are flat, slender and long in morphology. Unlike conventional reports, it is now established that osteoblasts do not differentiate to osteocytes or undergo apoptosis but become bone lining cells (Karsdal et al., 2002; Khosla et al., 2008; Matsuo, 2008).

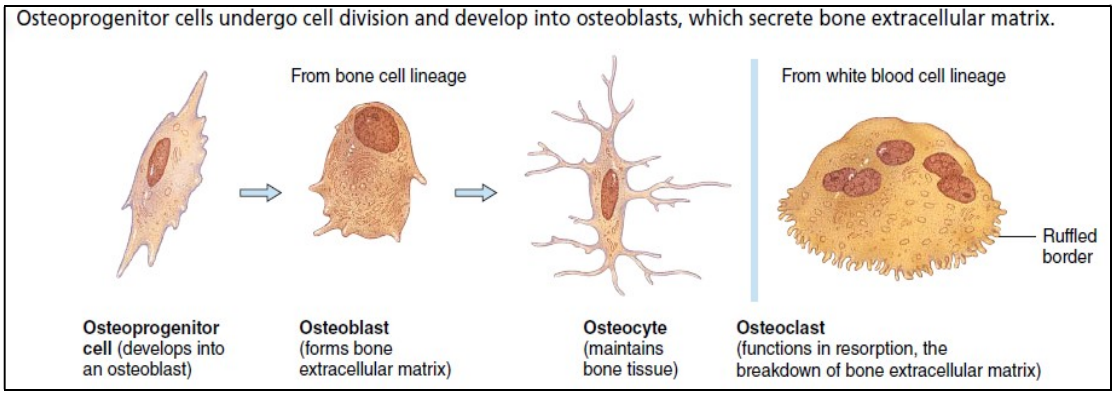


Figure In- 1: Type of bone cells

Figure In- 1 represents different types of bone cells and their progression. Osteoprogenitor cells are differentiated into osteoblast cells. Osteoblasts is mainly responsible for forming extracellular matrix. Osteoblasts will be ultimately differentiated into osteocytes. Osteoclasts are multinucleated cells, responsible for bone resorption activity (Tortora, 2017).

Despite of its inert appearance, they continuously undergo a process known as Bone remodelling, which makes bone a highly dynamic tissue. This process occurs as a result of resorption activity of osteoclasts and bone formation by osteoblasts. Synchronization of these processes plays a key role in preservation of Ca^{++} homeostasis and maintaining normal bone strength and growth (Leung, 2013; Suvarna et al., 2018). These process is tightly controlled and regulated which results in formation of new and healthy bone (Prentice et al., 2006). Bone Remodelling process includes series of activities, including 1) activation of osteoclast precursor cells by various hormones and cytokines to get converted into mature multinucleated osteoclasts, 2) bone

cavity formation by the activity of osteoclasts, 3) signal for reversal of bone resorption and 4) new bone formation by osteoblasts which will fill bone cavity (Cappariello et al., 2014; Chen L. R. et al., 2019; Ponzetti, 2019).

Upon unbalance of activity of the cells, there will be either hardening of bones, condition known as osteopetrosis or weakening of bone, condition known as osteoporosis (Stark et al., 2009; Chen S, 2013;). Bone weakening condition mainly includes osteoporosis and osteopenia (Lin et al., 2014). Osteoporosis is basically categorised into two stages. i) Primary osteoporosis: it is initial condition stage, which happens due to decreased Estradiol level and Vitamin D deficiency in post-menopausal women and men respectively; ii) Secondary osteoporosis: it is a group of bone disorders which happens due to different medical conditions of certain disorders. It is explained as low bone mineral density with greater risk of fracture (Jules et al., 2010; Flegar et al., 2015). As osteoporosis involves decline of bone density, it necessitates artificial enhancement of bone mineral density (BMD) with the aid of various therapeutic strategies which may constitute drugs or natural products.

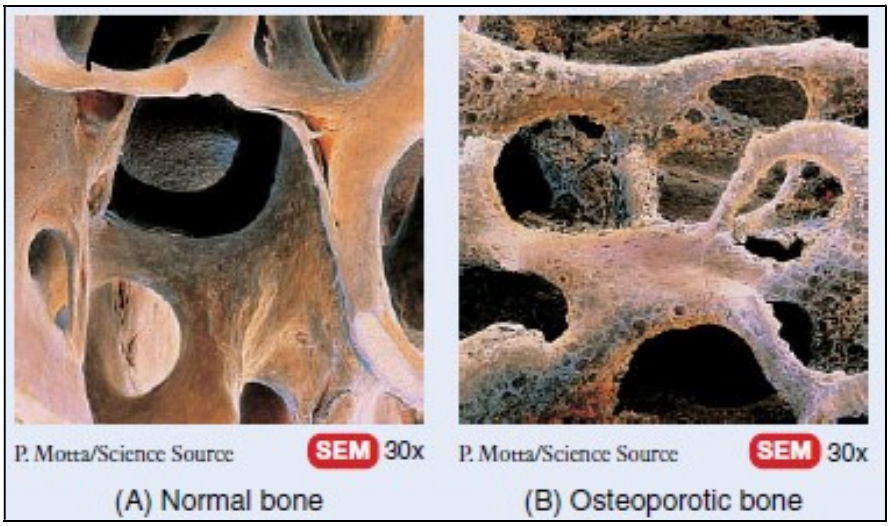


Figure In- 2: Normal Bone vs Osteoporotic spongy bone tissue

Figure In- 2 represents comparison of spongy bone tissue from healthy individual and the person with osteoporosis (Tortora, 2017).

Bone mineral density (BMD) is the amount of minerals in bone tissue. It is determined with the help of dual energy X-ray absorptiometry (DXA)

technique, which evaluates BMD in the form of T-score. As per WHO definition, healthy bone exhibits T-score between -1 to +1 standard deviation (SD). When this score is between -1 to -2.5 SD, it indicates osteopenia/low bone mass condition and the score below -2.5 SD indicates osteoporosis (Stepan, 2002; NIH, 2018). It has been studied that approximately 30% of postmenopausal women are suffering from osteoporosis, specifically at the age of 65 years (Gourlay et al., 2015). Globally, around 7,40,000 people lost their lives and 200 million people are suffering from due to osteoporosis (Sozen et al., 2017; Girgis et al., 2020). In current scenario, its etiology is attributed to diverse risk factors including age, sex, dementia, poor health or frailty, low body weight, cigarette smoking, estrogen deficiency and early menopause, prolonged premenopausal amenorrhea, low life-long calcium intake, impaired vision despite correction, alcoholism, inadequate physical activity etc. (Lane, 2004; Ginaldi et al., 2005).

Osteoporosis is the diseases which affects various system of the body including Immune System. Different cells of immune system also play crucial role in growth, proliferation and differentiation of osteoblasts and osteoclasts. This crosstalk play very important role in bone remodelling and maintenance of the bone tissue. The discovery of the involvement and participation of immune cells in various activities of bone has resulted in developing a new interdisciplinary field of osteoimmunology which is focused on the understanding of the crosstalk between the immune and bone systems (Gruber, 2010; Flegar et al., 2015). This field shows potential to deliver novel therapies against bone related diseases.

As mentioned, bone remodelling is very well controlled and balanced process between osteoclasts and osteoblasts cells. Bone remodelling process is regulated by various chemicals like hormones, cytokines and biochemical stimuli (Redlich et al., 2012; Emkey, 2014). Bone remodelling process also includes genesis of bone cells. Osteoblasts arise from mesenchymal stem cells. This process known as osteoblastogenesis. This process includes proliferation and differentiation of osteoblast progenitors through series of maturational stages to functional, bone matrix-secreting osteoblasts. Osteoblast play active

role in nurturing, differentiation and activity of osteoclasts by secreting receptor activator of nuclear factor-kB ligand (RANKL), osteoprotegerin (OPG) and macrophage colony-stimulating factor (M-CSF) (Baum et al., 2014; Šućur *et al.,* 2014). Osteoclastogenesis begins when mononuclear cells are generated from stimulated hematopoietic stem cells. Mononuclear cells then become preosteoclasts and they will enter into the blood stream. It is well established that this step is mainly driven by the ETS-family transcription factor PU.1 and macrophage colony stimulating factor (M-CSF) (Bar 2007; Russo et al. 2019). Osteoclast progenitor cells, mononuclear cells, express receptors for M-CSF (CD115, cFms) and receptor activator of nuclear factor-kB (RANK; also known as tumour necrosis factor receptor superfamily, member 11, TNFRSF11A), which are responsible for maturation and differentiation of osteoclasts (Walsh et al., 2014).

RANK/RANKL/OPG axis plays a central role in the process of bone remodelling. These three components cumulatively comprises paracrine system, which regulates activity and development of osteoclasts (Kohli 2011). RANKL, expressed as transmembrane surface protein by stromal cells, osteoblasts, osteocytes, effectively stimulates formation and activity of osteoclasts. RANK, active receptor of RANKL, is expressed by osteoclast progenitors and mature osteoclasts. The expression of RANK is strongly enhanced by M-CSF, which is secreted by osteoblasts. RANK/RANKL binding is essential for the trigger of osteoclast differentiation and proliferation (Faccio *et al.,* 2011). OPG is soluble receptor, produced by stromal cells and expressed by B lymphocytes and dendritic cells. OPG acts as decoy receptor of RANK, inhibiting osteoclast differentiation through prevention of RANK/RANKL binding. This report was supported by the study reporting severe osteoporosis in OPG knockout mice (Boyce et al., 2008; Faccio *et al.,* 2011).

There are many immune cells are known to be involved in bone physiology like T lymphocytes, dendritic cells, neutrophils, B lymphocytes etc (Rivollier *et al.,* 2004; Hajishengallis *et al.,* 2016; Mortaz *et al.,* 2018). Th17 cells, subcategory of $CD4^+$ T helper cells, has been anticipated to be most

influencing cells on osteoclastogenesis process. These cells can induce M-CSF and RANKL expression in osteoblasts and stromal cells, production of RANKL and TNF-α, and with parallel to this, increasing RANK expression in osteoclast precursors (Adamopoulos *et al.,* 2010; Adamopoulos et al., 2008). B lymphocyte development is reliant on several factors like RANKL, OPG, IL-7 which are secreted by bone marrow stromal cells and osteoblasts (Nagasawa, 2006). In turn, evidences of expression of RANKL by B lymphocytes strongly advocates the influence of B lymphocytes in osteoclastogenesis. It has also been reported that purified B lymphocytes can be differentiated into osteoclasts if they are treated with RANKL. It can be stated that they might act as a source of osteoclast progenitors *in vitro* (Manabe *et al.,* 2001; Pugliese *et al.,* 2012). However, because of lacking support of *in-vivo* studies, it is hard to conclude them as a source of osteoclast progenitor cells (Fujiwara et al., 2016).

Cytokines play crucial role in interconnecting the bone physiology and immunology. There are many cytokines like IL-17, IL-22, IL-26, IL-27, IL-6, IL-11, IFN-γ, demonstrated role in regulating the activity of different bone cells (Schroder et al., 2004; De Benedetti et al., 2006; Adamopoulos et al., 2008; Adamopoulos et al., 2010; Wojdasiewicz et al., 2014; Shukla et al., 2017). IFN-γ is secreted by T lymphocytes, B lymphocytes, NK cells, macrophages and dendritic cells. Along with the key role in regulation of inflammation, specifically in bone, it affects both, osteoblasts as well as osteoclasts (Schroder et al., 2004; Tang M. *et al.,* 2018). IFN-γ plays positive role, evidenced by *in-vivo* study also, in differentiation of osteoblasts by increasing the expression of Runt related transcription factor 2 (Runx2), Alkaline Phosphatase (ALP), Osterix and Osteocalcin (Duque et al., 2011; Maruhashi et al., 2015; Rolph et al., 2020). IL-27 has also been studied thoroughly to demonstrate constructive role on bone formation activities. IL-27 involved in various pathways and activities which work in direction of bone formation. Studies have shown that T lymphocytes act as a major contributors to estrogen deficiency-induced bone loss (Weitzmann, 2006). Studies have shown that IL-27 suppresses osteoclastogenesis by means of various ways like with the assistance of IFN-γ, by abrogating induction of

NFATc1 (Nuclear Factor of Activated T Cells cytoplasmic) and suppressing proximal RANK signalling (Kalliolias et al., 2010; Park et al., 2012). *In-vivo* study shows that IL-27 has role in suppression of Th1, Th2, and Th17 cell functions (Adamopoulos et al., 2013). By induction of IL-10 from Tr1 (type 1 regulatory T cells), subset of T cells, IL-27 shows anti-inflammatory actions. For inducing IL-10, IL-27 uses transcription factors like STAT-1, STAT-3, Egr-2 (Early Growth Response-2) etc (Bosmann et al., 2013). Egr-2 is the transcription factor which is involved in number of activities like suppression of osteoclastogenesis, survival of osteoblasts, suppression of osteoblast apoptosis, reducing osteoclasts functions and many other indirect impacts (Hyun et al., 2012; Chandra et al., 2013).

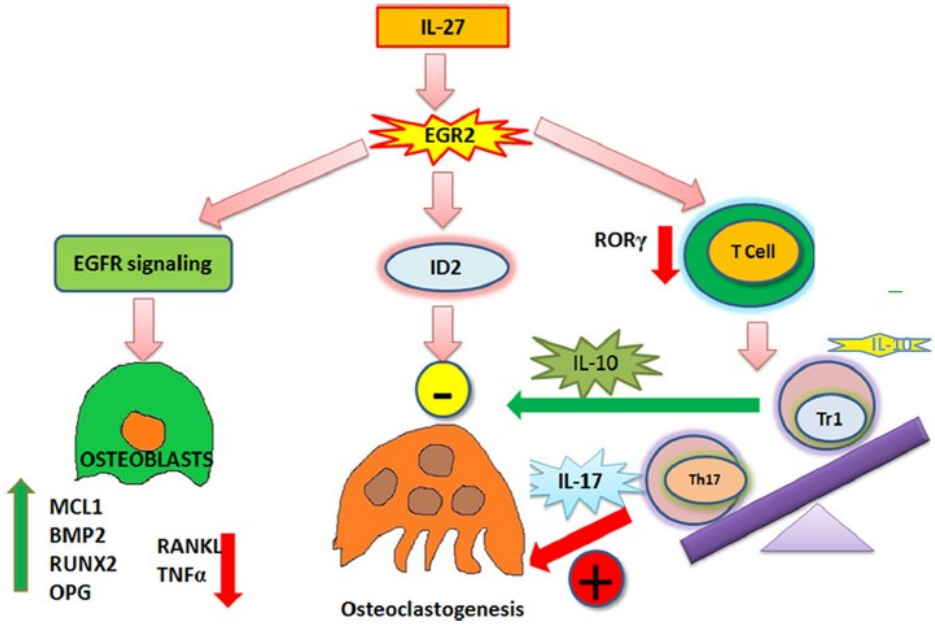


Figure In- 3: Schematic diagram of mode of Action of Egr-2 transcription factor

Diagram depicts mode of action of transcriptional factor Egr-2. Egr-2 is involved in enhancing proliferation of osteoblasts and suppressing osteoclastogenesis via upregulation of genes like MCL1, BMP2, RUNX2, OPG and downregulation of RANKL, TNFα, RORγ thorugh different mechanisms (Shukla et al., 2017).

RUNX2 is the transcription factor which belongs to the Runx family, which is homologous to Drosophila protein, Runt. This protein is essential for the differentiation of osteoblasts and subsequent bone mineralisation (Franceschi

et al., 2003). Runx2 directly stimulates the expression of osteoblasts related genes like Osteocalcin (OCN), type I Collagen, collagenase 3 by binding with specific enhancer region in DNA (Kern et al., 2001). As per earlier reports Runx2 plays a role in execution of PTH signal in osteoblasts. In the PTH action, PTH stimulates target promoters by phosphorylation of Runx2 and upregulation of c-Fos and c-Jun through phosphorylation of CREB, another transcription factor in GPCR signalling pathway (Franceschi et al., 2003). Besides the PTH signalling, Runx2 also participates in FGF2 signalling cascade. FGF2 is important regulator of bone growth and mineralisation. Studies suggest FGF2 acts as local regulator of bone formation, through proliferation of osteoblast and production of OCN. Runx2 protein acquires enhanced DNA binding ability and greater protein stability after dimerization with the protein called Cbfβ (Core binding factor) (Kundu et al., 2002; Komori, 2017). FGF2 binds with its different receptors FGFR1 to FGFR4. This binding induces receptor dimerization and subsequent phosphorylation. Further this receptor signal through MAPK pathway and protein kinase C (Kawane et al., 2018). There are three best studied group of MAPKs in osteoblasts: ERK, p38 and JNK. ERK has two isoforms ERK1 and ERK2 and both of which are expressed in osteoblast cells. ERK pathway plays significant role in regulation of Runx2 and ATF4, an important mediator of procollagen gene transcription during the later stages of osteoblast differentiation (Yang et al., 2004). The relationship between Runx2 and the ERK pathway was demonstrated directly *in-vivo* by Xiao and Ge research groups (Xiao et al., 2002; Ge et al., 2009). Runx2 is phosphorylated and activated by ERK1, which in turn gets associated with ERK1 and assists its binding to the promoter of OCN and bone sialoprotein (Bsp) in osteoblasts (Li et al., 2010). Extent of different transcription factors regulated by ERK, including key mediators of early- and late-stage osteoblast differentiation, RUNX2 and ATF4, suggests that the overall role of ERK in osteoblasts may vary substantially based on the stage of osteoblast differentiation and the anatomic location considered.

Another such transcription factor is NFATc1, which intensely inhibits differentiation and function of osteoblast. NFATc1 belongs to NFAT family of

transcription factors. This family consists of five proteins (NFATc 1 – 4). These proteins are known for their role in T-cell activation (Choo, 2009). Study suggests that disruption of Calcineurin, activator of NFAT pathway, and NFATc1 genes leads to enhanced proliferation of osteoblasts and bone formation activity in animal model (Yeo et al., 2007). Besides this, NFATc1 regulates osteoclastogenesis process as well. Cytokines like TNF and IL-23 regulates activation of NFATc1 and calcium signalling (Adamopoulos, 2018). Calcium signalling is known to activate calcineurin. Activated calcineurin will dephosphorylate NFAT to translocate to nucleus where it will get activated. In nucleus, it regulates many genes responsible for osteoclast function as well as numerous genes non-essential to osteoclast function (Aliprantis et al., 2008; Charles et al., 2012). According to study, it was observed that overexpressed constitutively active (nuclear) NFATc1 inhibited MC3T3 osteoblast differentiation *in vitro* and reduced expression of osteocalcin as a result of inhibited TCF/LEF transcriptional activity, which was due to sustained recruitment of HDAC3 and decreased histone acetylation at the proximal osteocalcin promoter (Jensen et al., 2010). Conclusively, all these reports suggest that NFATc1 transcription factor works in the direction of promoting osteoclastogenesis and suppressing the differentiation and proliferation of osteoblasts.

It is known that osteoporosis-associated fractures are increasingly prevalent in women after 55 years of age, as well as in men after 65 years of age, indicating the negative impact of aging on bone metabolism. Influence of hormones on both osteoclasts and osteoblasts are not limited to sex hormones but other hormones and vitamins are also impact on bone like PTH, Cortisol, IGF1, TSH, vit D etc (Cannarella et al., 2019). Estrogens have long been considered as bone stimulating hormone by promoting growth of osteoblasts and it is also known for hampering growth of osteoclasts (Shevde et al., 2000; Srivastava et al., 2001). It was studied *in-vivo* that ERβ knock out mice develops osteopenia, suggesting role of estrogen in preventing osteoporosis (Windahl et al., 2002). Besides estrogen, androgens also play role in bone metabolism directly as well as indirectly by binding to androgen receptor (AR) and acting on ER after their aromatisation. The AR has been identified to

promote cell proliferation and differentiation by inhibiting their apoptosis via IL-1 and FGF-mediated effects (Wiren et al., 2012). Androgens also seem to suppress osteoclast proliferation indirectly. Because it had been observed under hypotestosteronemia condition, there will be rise in RANKL secretion by osteoblast precursors, thus, in turn, stimulating osteoclast proliferation (Wiren et al., 2012). Besides these sex hormones, parathyroid hormone (PTH) also has significant impact on bone metabolism. *In-vivo* studies in mice and rat as well as results in human subjects suggest PTH has significant positive effect on bone formation when it is administered intermittently in post-menopausal women, elderly men, and women with glucocorticoid-induced osteoporosis (Zhang et al., 2011). PTH exerts its effect through PTH receptor which is GPCR. Although multiple intracellular signalling mechanisms are involved in mediating PTH function, the major downstream signaling pathway of PTH in bone cells involves cAMP, PKA, and CREB. Increasing activity of this pathway promotes osteoblast differentiation (Tyson et al., 1999; Swarthout et al., 2002).

1.2 OSTEOPOROSIS TREATMENTS AND FLAWS:

As mentioned, osteoporosis is mainly prevalent among postmenopausal women because there will be deficiency of estrogen, one of the two key hormones which are involved in the bone formation. Another hormone is PTH. These hormones play crucial role in osteoblast differentiation and proliferation (Pettway et al., 2008). These are the hormones which are employed during the treatment / therapy of osteoporosis. There are generally two types of medication available to treat osteoporosis: 1. Anti-resorptive drugs (inhibiting osteoclasts) and 2. Bone building drugs (stimulating osteoblasts).

Bone anti-resorptive drugs are most exploited treatment. This includes:

1) Bisphosphonate (BP) – a class of drugs which inhibit osteoclasts either by inducing apoptosis through farnesyl pyrophosphate synthase inhibition or inhibiting its recruitment through mimicking estrogen activity. They avidly bind to bone and will be internalized by osteoclasts. Fosamax®,

Actonel®, Boniva® and calcitonin are some of the known and marketed BP drugs (Tortora, 2017; Martinis et al., 2019).

2) Selective Estrogen Receptor Modulators (SERMs): Raloxifene, only SERM which is approved by FDA for the prevention and treatment of osteoporosis. SERM are non-steroidal chemicals which show tissue specific actions and induces osteoclast apoptosis. As per reports, Raloxifene reduces the risk of osteoporosis in the range of 30 – 55% (Chen L. R. et al., 2019). Strontium ranelate, the drug which comprises of divalent strontium and replaces Ca^{++} ions within bone. This mechanism decouples bone resorption from bone formation, promoting decrease in osteoclasts. However, Strontium ranelate is not approved by FDA and licenced for restricted use in Europe (Das et al., 2013). 3) Estrogen replacement therapy (ERT) / Hormone replacement therapy (HRT) - which replaces estrogen and estrogen & progesterone lost, condition during menopause respectively. These include Conjugated Estrogens/Bazedoxifen therapy, a combination of conjugated estrogen and Bazedoxifen (novel SERM); Estrogen-Progestin therapy; Testosterone therapy (Tu et al., 2018). In HRT, conjugated equine estrogen (CEE) and medroxyprogesterone acetate (MPA) are prescribed in combination. Based on the evidence and considering efficiency and treatment cost, HRT should be considered as first line therapy for prevention and treatment of osteoporosis in postmenopausal women. But due to serious side effects associated with normal doses, low and ultra-low doses use are in popularity (Gambacciani et al., 2014). Besides this, osteo-immunological drug has also been developed for the prevention and treatment of osteoporosis. Denosumab is the first fully human monoclonal antibody which targets Receptor Activator of RANKL, an essential mediator in bone resorption process. Denosumab has been reported to suppress bone resorption by 80-90% (Pavone et al., 2017; Ukon et al., 2019).

Bone building drugs mainly include Teriparatide (recombinant human Parathyroid hormone-rhPTH). It is an osteoanabolic drug which stimulates proliferation of osteoblasts and enhances bone formation. Teriparatide activates osteoblast by binding with PTH/PTHrP type1 receptor, initiating new

bone formation at various sites. In this way, Teriparatide increases the bone density which can be clearly observed in DXA scan (Pavone et al., 2017). Teriparatide is considered as promising mediator in combination and sequential therapy. These treatments employ anabolic and BP agents like PTH and Zoledronic Acid respectively. This combination demonstrated enhanced BMD gain compared to alone Zoledronic Acid. However this study was short termed and not powered with fracture reduction endpoints (Cheng et al., 2020). Teriparatide has also been studied in combination with Denosumab. DATA (Denosumab and Teriparatide Administration) study trial was conducted for this purpose, which showed significant improvement in lumbar spine, hip BMD compared to alone treatment of each drug. Again this study, however, was not powered with facture reduction endpoints (Cheng et al., 2020).

A major concern associated with prolonged use of the anti-resorptive drugs is serious adverse effects of drugs like osteonecrosis of jaws, enhanced risk of different cancers etc. Earlier we noticed that bone resorption and bone formation are the process which are well balanced and coupled. Hence by inhibiting bone resorption process using anti-reporting drugs, subsequently bone formation will also be affected (Cheng et al., 2020). There are ample articles suggesting almost all the treatments have serious side effects when administered upon prolonged use for treatment.

Table In- 1: Summary of osteoporosis treatment with side effects

Agent	Mechanism of Action	Effect on Bone	Side effects
Calcitonin	Regulation of osteoclast function, Prevention of osteoclast precursors from maturing	Inhibition of bone resorption	- Gastrointestinal disorders, - Hypocalcemia, - Weak association between malignant tumour
SERMs	Interaction with RANK/RANKL/OPG system	Inhibition of bone resorption	- Thromboembolic events, - Pulmonary embolism, - Fatal strokes
Bis-	Induction of osteoclast	Inhibition of	- Gastrointestinal disorders,

phosphonates	apoptosis	bone resorption	- Osteonecrosis of the jaw, - Atypical femoral fractures, - Acute renal failure
Anti-RANKL antibody	Prevention of the RANKL/RANK system	Inhibition of bone resorption	- Osteonecrosis of the jaw, - Atypical fracture, - Hypocalcemia
PTH	Stimulation of osteoblast Differentiation	Activation of bone formation (intermittent PTH)	- Hypercalcemia, - Increasing risk of osteosarcoma

Error! Reference source not found. *indicates the summary of current treatment of osteoporosis along with its mode of action and plausible side effects (Ukon et al., 2019)*

Many studies in last 2 decades, indicate HRT to be associated with fatal strokes, thromboembolic events, pulmonary embolism, myocardial infarction, invasive breast cancer, deep vein phlebitis and cardiovascular diseases (Nelson et al., 2002; Chen L. R. et al., 2019; Ukon et al., 2019). Earlier reports suggest that HRT is linked with several harms like thromboembolic events, stroke, breast cancer and cholecystitis (Nelson et al., 2002). As per very recent nationwide population study, association has been found between the HRT and breast cancer. Risk of breast cancer increases proportionally with time duration (Yoo et al., 2020). Finally it can be stated that regardless of benefits against prevention and treatment of osteoporosis, HRT has long been held accountable consistently for its serious drawbacks since last couple of decades.

Reported adverse effects associated with BP osteonecrosis are osteonecrosis of jaw, renal failure, arterial fibrillation and atypical subtrochanteric femoral fractures. 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 et al., 2009). In recent retrospective cohort study, it was reported that there was increase in osteonecrosis of jaw incidences observed in the individuals with defined daily dose (DDD) of bisphosphonate exposure. Such reports provide strong correlation between risk of osteonecrosis of jaw and bisphosphonate exposure used during treatment of osteoporosis (Jung et al., 2018). Cumulatively, along with the positive impacts against osteoporosis, bisphosphonate has numerous side effects because of which it is less considered and new therapeutic targets and medicines have been proposed for the treatment (Iolascon et al., 2020). Likewise, a very similar adverse events have been observed with Denosumab also, like hypoglycaemia, femoral fracture, osteonecrosis of jaw (Selga et al., 2016; Cernes et al., 2017; Yoshimura et al., 2017). Qi et al had reported the risk of osteonecrosis of jaw through Denosumab in patients with cancer in a meta-analysis of seven randomized control trials. The overall frequency of osteonecrosis in patients with cancer receiving denosumab was 1.7 %. This analysis demonstrated that the use of denosumab was associated with an increased risk of developing ONJ when compared with BP treatment or a placebo; however, the increased risk was not statistically significant between denosumab and BP treatment (Qi et al., 2014). According to recent study, a great fall has been reported in the prescription of bone protective medication and treatment from 40 % to 21 % over last decade. Plausible reason behind would be the fear of adverse outcome of the treatments (Solomon et al., 2014; Van et al., 2017; Compston et al., 2019).

1.3 HERBALS IN OSTEOPOROSIS:

As we noted, there have been significant advancement of pharmacological interventions against osteoporosis. But these treatments exhibit serious adverse side effects and drawbacks like including nausea, joint aches, pain, or carcinoma, may increase the likelihood of non-adherence to treatment. In the view of these concerns, researchers have started exploring alternative scopes

for the treatment with fewer side effects to improve the therapeutic efficacy of osteoporosis. Compared to the chemically synthesized medicines, herbal medicines show less side effects with long term use and they are cost effect as well.

The World Health Organization acknowledged that the goal of "Health for All" cannot be accomplished without herbal medicines. In a broader scenario, demand for herbal medicines, medicinal plants, health products, etc is growing in all parts of the world, which indicates the popularity and belief of people in herbal medicines (Sen et al., 2015). Due to a shift in universal trend from synthetic to herbal medicine, growth has been recorded in plant extract market. In the current decade, many potent medicines have been derived from herbal formula, both a pure as well as crude (Maqbool et al., 2019).

The herbal medicines have always been recognised for the treatment against various diseases in Asian countries. Since last decades, herbs are used in many critical illnesses like dementia, hepatocellular carcinoma, vertigo (So et al., 2015; Tsai et al., 2016, 2017; Chen K.H et al., 2017). Globally, it is forecasted that among the estimated 250,000 – 400,000 species of plants, only 6% of species have been screened thoroughly and systematically for their biological activity and 15% have been investigated phytochemically (Sen et al., 2015). There are many herbal known to possess anti-osteoporotic properties and currently many research groups explore this area in the direction of finding of successful anti-osteoporotic formula or molecule. These group include herbal plants *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; Patel et al., 2015; Parikh et al., 2012; Lin J. et al., 2017).

Scientists have discovered the mode of actions as well as signalling cascade of many herbals like *Herba Epimedium*, *Salvia miltiorrhiza, Rhizoma Drynariae*, which can be serve as foundation of drug discovery for that

particular disease. Highlights and predicted pathways of these herbals are well illustrated in Figure In- 4.

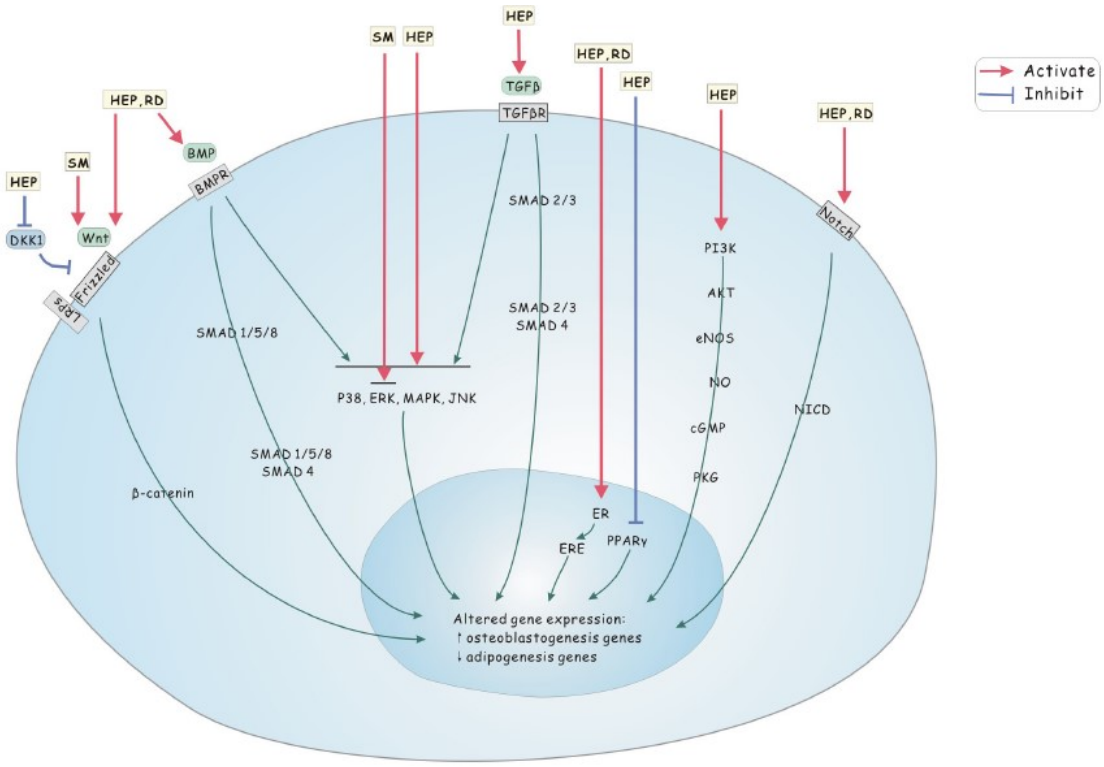
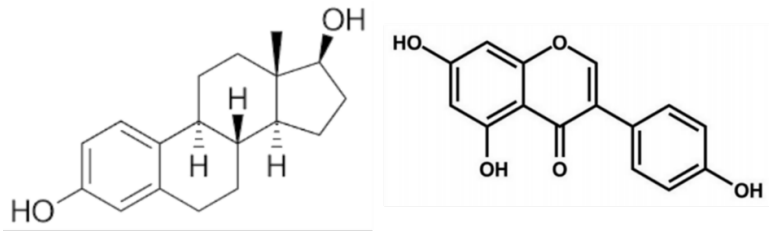


Figure In- 4: Mechanism of action of various herbs in Pre-osteoblasts

Figure In- 4 represents the interaction and mode of action via signalling pathway of various herbs in pre-osteoblasts: i) via BMP signaling pathway, ii) via Wnt/β-catenin pathway, iii) activate ERK, p38, JNK, MAPK pathways, iv) via up-regulation of TGF-β1 expression, v) through ER signal activation, vi) activate PI3K-AKT-eNOS-NO-cGMP-PKG signal pathway, vii) via Notch signaling pathway, and viii) reduce PPARγ mRNA and DKK1 protein to inhibit adipogenesis. (HEP, Herba Epimedium; SM, Salvia miltiorrhiza; RD, Rhizoma Drynariae; DKK1, dickkopf-related protein 1; LRPs, lipoprotein receptor-related proteins; BMP, bone morphogenetic protein; BMPR, BMP receptor; JNK, c-Jun N terminal kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular-signal-regulated kinase; TGFβ, transforming growth factor β; TGFβR, TGFβ receptor; ER, estrogen receptor; ERE, estrogen-response element; PI3K, phosphatidylinositol-3-kinase; AKT, protein kinase B; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; cGMP, cyclic guanosine monophosphate; PKG,

protein kinase G; NICD, Notch intracellular domain; PPARγ, peroxisome proliferator-activated receptor γ.) (Lin J. et al., 2017)

Although herbal medicines are supported by few studies with negative results, phytoestrogens have raised great interest since last decade. Phytoestrogen has gained increased attention due to its ample clinical benefits in estrogen dependent diseases (Levis et al., 2011). Phytoestrogen have similar structure to estrogen and therefore that can be used to overcome estrogen deficiency state (Figure In- 5). Phytoestrogen like Genistein is one of the extensively studied herbal molecule for its various properties. Genistein is natural flavonoid of *Leguminoseae* plants. It is mainly found from soybean plant and known to improve bone loss (Thent et al., 2019).



(A) Estrogen (B) Genistein

Figure In- 5: Chemical structures of Estrogen and Genistein

Besides mimicking estrogen like properties, Genistein has also been reported for exerting its effect via PTH receptor (PTHR1). It has been studied that Genistein treatment restored mRNA and protein level of PTHR1 in ovariectomized rat. Along with it, it also restored serum mineral profile and alleviating mineral excretion in urine (Miao et al., 2012).

Another similar phytochemical is Diosgenin. It is extracted from the root of wild yam (*Dioscorea villossa*) is claimed to have esteogenic property (Alcantara et al., 2011). Diosgenin exhibits estrogenic effect, anti-inflammatory effect and positive effect on maintenance of blood cholesterol level. It serves as the starting material for the synthesis of hormonal products, such as dehydroepiandrosterone (Scott et al., 2001). Diosgenin has been reported to induce upregulation of VEGF-A via of activation of HIF-1 by the

estrogen receptor-dependent PI3K/Akt and p38 MAPK signaling pathways in MC3T3-E1 osteogenic cell line (Figure In- 6) (Men et al., 2005).

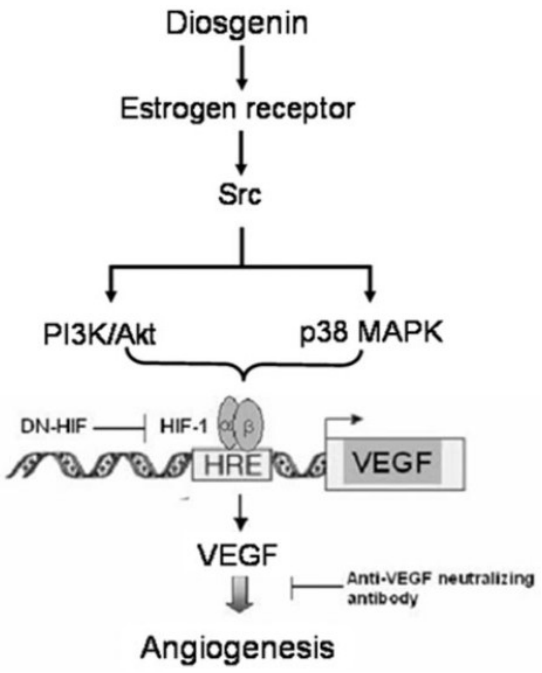


Figure In- 6: Angiogenic signaling pathway activated by Diosgenin in MC3T3-E1 cells.

Figure indicates signalling cascade of Diosgenin through estrogen receptor which works through involvement of PI3k and MAPK. Ultimately signal reaches to expression of various genes which are responsible for angiogenesis (Men et al., 2005).

Sulfuretin is major flavonoid of heartwood of *Rhus verniciflua* Stokes. It is traditional medicine in East Asia for the treatment of gastritis, stomach cancer, and arteriosclerosis (Nune et al., 2015). Sulfuretin is also known for its cytoprotective effects, anti-inflammatory effects. Besides all these, it is also reported to induce differentiation in osteoblast through TGF-β signalling activation in various cell lines like C3H10T1/2, MC3T3-E1 and primary bone marrow cells (Song et al., 2015). It has been established that Sulfuretin works using multiple pathways, one of which is BMP signalling pathway (Figure In-7). BMP pathway requires involvement of downstream participants like Smad 1/5/8 and Runx2. Other pathways include MAPK/ERK and JNK pathway, AKT/mTOR and Wnt/ β-catenin pathway. It is well known that MAPK

pathway is important for mineralisation and bone formation by osteogenic cells. Sulfuretin induces activation of ERK and JNK based pathway in osteoblasts, which in turn phosphorylates Runx2, activating it. In this way, Sulfulretin promotes differentiation and proliferation of osteoblasts by multiple signalling pathways (Auh et al., 2016).

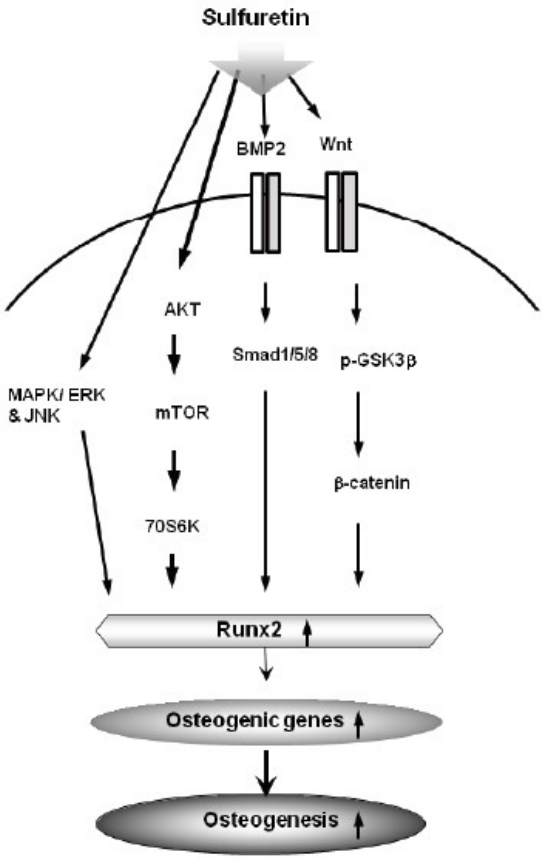


Figure In- 7: Schematic diagram illustrating mechanistic pathway of Sulfuretin

Figure represents the mechanistic action of Sulfuretin which act through different pathways including MMAPK, AKT, Smad proteins and Wnt pathways. Sulfuretin upregulates the expression of RUNX2 and which in turn enhance the osteogenic genes expression (Auh et al., 2016).

A recent report updates Indian Plant Extract market has witnessed an astonishing growth, as there has been a shift in universal trend from synthetic to herbal medicine. Cumulative annual growth rate (CAGR) of Indian Plant Extract market has been forecasted to grow 17% in year 2018-2023 (DUBLIN,

2019). As a result, strong demand and the new era has come for the identification and development of herbal based new medicine to overcome the drawbacks of other therapies and treatments.

1.4 STUDY SO FAR

As mentioned earlier, there are ample number of herbals that have been identified and studied for assessing the bio-activeness of phytochemical, and phytocompounds that show ameliorative disease properties including osteoprotective one. One such herbal is *Litsea glutinosa (LG)*, which is also known as Maida Lakdi. In charakasamhita it has been classified as *jivaniya*, *sukrajanana* and *snehopaga*. *Litsea glutinosa* is a deciduous or evergreen plant. It is found in India, South China, Australia, Malaysia and the western Pacific Islands. It is considered as a medicinal plant which is known as Indian *laural, soft/brown bollygum or beech/bolly beech, and sycamore* (Kumar et al., 2018). Traditionally, it is considered as medicine for vast conditions including longevity, semen generation, emollient, diarrhoea, dysentery and rheumatism. The mucilaginous leaves are considered emollient and antispasmodic. Besides these, bark paste, made by grinding bark with water, is used as a plaster in cases of bone fractures, bruises, sprain, wounds, inflammation, back pain, gouty and rheumatic joints, etc. (Devi et al., 2010). It is reported to have antibacterial, antioxidant, anti-inflammatory, antipyretic, hypotensive, analgesic, emollient, chemoprotective activities, antinociceptive properties and anti-osteoporotic properties (Sukhdev, 2006; Rangrez et al., 2011; Rahman et al., 2017; Unnikrishnan, 2016).

Figure In- 8: Illustration of Plant *Litsea glutinosa* showing the lead, fruit and flowers

The process of bone metabolism which includes bone formation, remodelling, and healing involves well-coordinated activities of various bone cells. As a results of technology evolution and understanding of biology of bone cells, it could possible to develop various *in-vitro* culture model of different cells from different origin. With parallel to that, it could also be possible to conduct intensive research in bone tissue engineering, which eased the use of these cell line in place of limited available primary human osteoblasts/other bone cells. There are some human cell lines available which are considered as role model for conducting bone cell related research, for example MC3T3-E1 (Quarles et al., 2009), MG-63 (Billiau et al., 1977) and Saos-2 (Rodan et al., 1987) cell lines. Most of these are malignant cell lines. Roden group had characterised Saos-2 cell line in 1987. It is the human osteosarcoma cell line, which was isolated from an 11-year old Caucasian female in 1975. These cells exhibit phenotypes of mature osteoblasts with high ALP activity. With compared to primary osteoblast cells, ALP activity of Saos-2 cells is comparable at early time points, but it is reported to be increased by 120 fold post 14 days (Saldaña et al., 2011). Interestingly, it has also been reported that Saos-2 cells form calcified matrix which is highly similar in pattern with bone (Rodan et al., 1987). Besides this, these cells secrete cytokine and other growth factors which is highly similar to primary human osteoblast cells (Asai et al., 2003; Burmester et al., 2014). It has been established that like primary osteoblast cells, Saos-2 cells express receptors for PTH hormone and 1, 25 (OH)2 D3 (Czekanska et al., 2012). Along with these, other benefits like world wide availability, good and well documented characterisation, less doubling time make cell line most preferred choice of researchers for bone research. Saos-2 has been widely used as *in-vitro* model in place of primary human osteoblasts to explore effects of many natural compounds like *Eucommia ulmoides* (Lee J. E. et al., 2016)*, Moringa olifera* (Patel, 2013)*, Cissus quandrangularis* (Zakłos et al., 2020). Collectively these results demonstrate the similarity of Saos-2 and normal osteoblast cells in response to various herbs. Hence in the present studies Saos-2 cell line was utilized in the present studies. However there are

some reports indicating phenotypic instability of Saos-2 cells upon long term culture (Hausser et al., 2005). Here in studies, cell line was used for experiments during initial passages only, no long term culture has been used.

Previously in lab, it has been established that LG possesses anti-osteoporotic properties through various *in-vivo* studies. Bark powder of LG was fractionated as aqueous, non-aqueous and methanolic. All the fractions were tested in OVX rats whether it ameliorates OVX induced osteoporosis. It was observed that out of all the three fractions, methanolic fraction is most effective one. Post LG treatment in OVX rats, various parameters were measured to evaluate the fractions. Serum Ca^{++} and phosphate level, serum alkaline phosphatase (ALP), tartarate resistant acid phosphatase (TRAcP), bone microarchitecture, bone ALP, bone TRAcP, Ca^{++} excretion and uterus weight. It was observed that LG improved bone microarchitecture upon treatment. It also enhance level of serum ALP whereas decreases TRAcP significantly. Treatment also diminished Ca^{++} excretion rate. Along with all these observations, it is important to note that treatment didn't enhance the uterine weight, unlike estrogen control. This is indication of uterine atrophy. The observation indicates possibly these botanicals also may reduce the risk of breast and ovarian cancer associated with ERT/HRT. Overall these results demonstrate anti-osteoporotic properties of LG (Rangrez et al., 2011).

To elucidate the chemical composition of LG methanolic crude, TLC and GC-MS were performed. Results enlightened LG constitutes various phytochemicals like Flavonoids, Terpenoids, Phenols, Tanin, Saponin, Alkaloids. These phytocompounds include Piperzine carobnitrile, Androstan, Cinnamic Acid, Cinnamolaurine, Crinamine, Gestonorone, Thiocoumarin, Quinoline, cinnamon, Piperzine, oleic acid, Pregene derivative and Androsta-triones etc (Parikh et al., 2012). Oleic acid has been reported to contain hypotensive effects (Teres et al., 2008). There are many phytoestrogens also like Pregene derivative and Androsta-triones, which are proven osteoprotective agents (Yang X.-X. et al., 2006). Presence various phytoestrogens and other alkaloids suggest that consumption of this plant can

be helpful in treating osteoporosis and it can be worth exploring this plant for other pharmacological interventions.

As described, till date LG has been explored via *in-vivo* study and all the conclusions were derived from phenotypic observation and results. So far there is no study describing and explaining the molecular mechanism of LG through which it is exerting its osteoprotective effects. Therefore, to unravel the genotypic basis and to elucidate the genes and proteins involved in mechanistic pathway of LG, it was decided to perform gene expression studies of various genes (Figure In- 9).

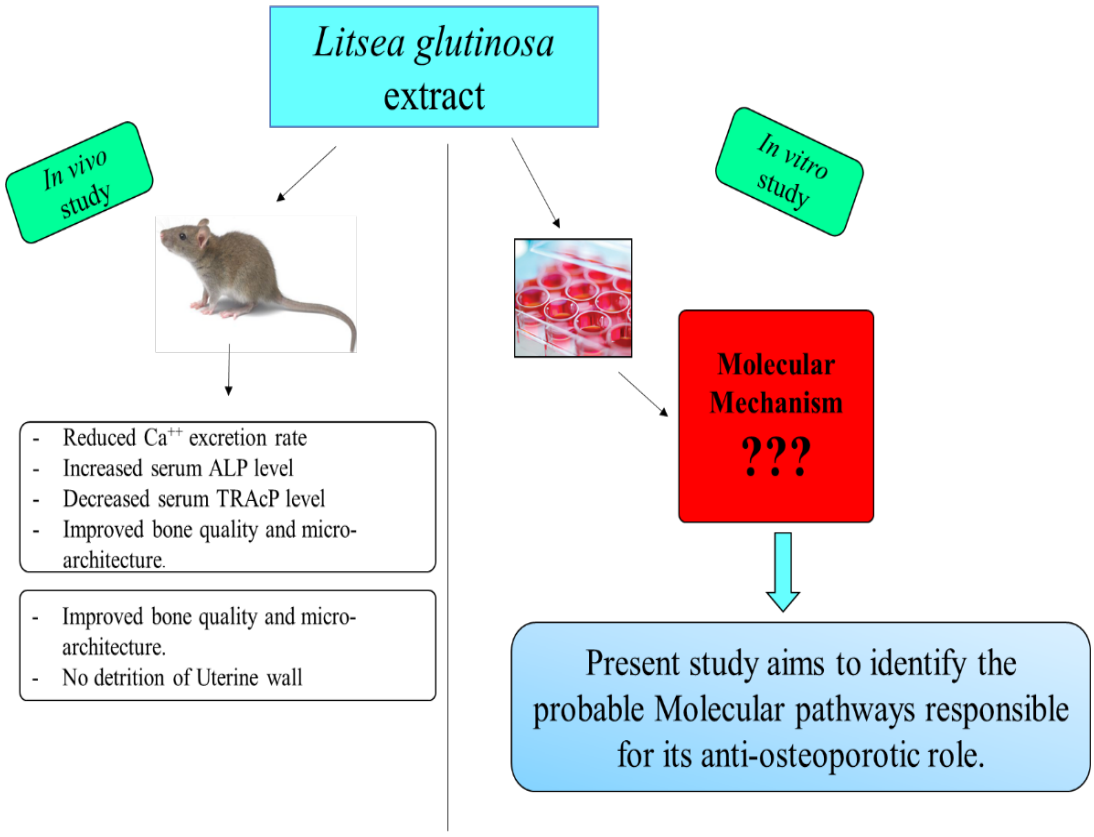


Figure In- 9: Schematic diagram of study proposal

Figure In- 9 represents the proposal of the study. LG methanolic extract has been shown to produce ameliorative effects in OVX rats like reduction of Ca++ excretion rate, increase in ALP level and improved bone quality and health. However molecular mechanism of the LG has still been poorly understood. Hence proposal figure explains the current gap of the study which has been attempted to fill in the present study.

Study was planned to examine expression profile of candidate genes and transcription factors which are key players in different pathways for osteoblastogenesis like Egr-2, Runx2, NFATc1. Along with them, others protein which are involved in multiple pathways like MAPK3 (ERK1), CREB, ERβ, adenylate cyclase. Therefore, the present study has been designed to systemically evaluate these obscure area, which will help us in understanding the osteoprotective role of LG at molecular level and which might prove as one of the promising therapeutic potential against osteoporosis. ***Hence, to discover the genotypic basis and elucidate the genes and proteins involved in mechanistic pathway of LG, it was decided to perform gene expression studies of various genes. Hence, we hypothesize, that LG might have some proliferative effects and anti-apoptosis effects on osteoblastic cells. To validate its proliferative property and to understand the mechanism of actions the study was undertaken on Saos-2 cell line.***

1.5 STUDY OBJECTIVES

1. **To explore the molecular mechanism of *Litsea glutinosa* extract on Saos-2 cell line.**
    a. By checking the effect of *Litsea glutinosa* extract on extract on homeostatic markers in Saos-2 cell line like Egr-2 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 and anti-apoptotic effect of *Litsea glutinosa* extract on Saos-2 cell line.**
    a. To check the rate of proliferation, its numbers and viability.
    b. To check the expression of proliferative and apoptotic markers like PCNA, osteocalcin, FasL, cytochrome C and Caspase 3.
3. **To identify the molecular targets of *Litsea glutinosa* extract: an *In-silico* and *in-vitro* approach.**
    a. To conduct target prediction study to identify molecular target of LG using bioinformatics software
    b. To study gene expression of identified targets in *in-silico* study