GENERAL CONSIDERATION

6.0 GENERAL CONSIDERATIONS

Bone is the most dynamic and ever-changing tissue apart from blood. It is a very integrative tissue of human body which interacts with multiple system like immune system, circulatory system, endocrine system, body strength etc (Gunturet al., 2012; Pietschmann et al., 2016). Earlier it was considered to be a static and inert tissue, but with time and technology advancement, it was established that bone is active tissue of human body which continuously undergoes resorption and formation process by specialized bone cells known as osteoblasts and osteoclasts (Florencio et al., 2015). These rapid continuous changes are required for the control of calcium metabolism and to maintain the healthy state of the bone. Build-up and breakdown are kept at a harmonized speed, so that there is good balance between these two mechanisms remains in dynamic state. The balance is affected most commonly when the breakdown mechanism supersedes the restoration process and also other causes like nutritional, vitamin D deficiency (no sunlight), disuse, aging, and other disease conditions. Bone serves some essential functions like to provide strength and support to body, locomotion, protection of soft tissue, harbouring of bone marrow and Calcium and Phosphate storage, role in glucose and lipid metabolism (Robling et al., 2006; Datta H. K et al., 2008). Irregularities in the activities of these cells may summon disease conditions like osteoporosis, osteopetrosis etc (Stark et al., 2009; Alma et al., 2013). Osteoporosis is the most common metabolic bone disorder characterized by decreased bone strength. It is a condition in which bone health deteriorates and bone becomes more porous in nature, resulting high risk of fracture (Practitioners, 2017). Currently, osteoporosis is key health problem which is categorised as bone health affecting metabolic skeletal disease, globally. Osteoporosis is commonly observed in aged population and is considered as modern day epidemic. Increasing chances of bone fractures, osteoporosis comes with trivial trauma. Fractures lead to pain and permanent disability (Practitioners, 2017).

Osteoporosis has gained the attention due to its serious health consequences worldwide. As it draws patient's attention only when it causes fracture, it is thus considered as "Silent" (Khadilkar et al., 2013). Currently, one of the major challenges is to diagnose patient with osteoporosis who has high fracture risk (Nuti et al., 2019). Consequently, osteoporosis has become the prime reason of mortality and morbidity in Indian population due to increased longevity of life compared to western countries (Mithal et al., 2014). According to a forecasting report, osteoporosis is expected to curb 22% of population by 2025 and 33% in 2050 (Mithal et al., 2014). Osteoporosis prevalence is greater in women compared to men. Estrogen plays an important role in coordinating the activities of the bone-forming osteoblasts and bone-resorbing osteoclasts in bone homeostasis. Ovarian hormonal deficiency is one of the most important factors leading to postmenopausal osteoporosis, which has made hormonal replacement therapy popular in the early days for the prevention of bone loss in postmenopausal women (Sunyecz, 2008).

Challenges associated with osteoporosis includes the search for new promising and specific cell targets, its effective drug, combined or sequential treatment and personalised therapeutic protocols of treatments (Nuti et al., 2019). Currently there are various treatments available for osteoporosis. These treatments are either anti-resorptive, which inhibits osteoclasts or bone forming, which will promote osteoblasts (Harvey et al., 2017; Cotts et al., 2018). Most of the current therapies in the prevention of osteoporosis and fractures are designed to decrease bone resorption and they are known as antiresorptive agents. They include estrogen; bisphosphonates such as alendronate, risedronate, ibandronate, and zoledronic acid; SERM raloxifene; human monoclonal antibody against RANKL – denosumab; and strontium ranelate. SERMs either trigger apoptosis in osteoclasts through farnesyl pyrophosphate synthase inhibition (bisphosphonates) or inhibits osteoclasts recruitment through imitating estrogen activity (Kanis et al., 2012; Martinkovich et al., 2014; Sozen et al., 2017). Besides these bisphosphonate based treatments, hormonal/estrogen replacement therapies (HRT) are also among the preferred treatments of osteoporosis. HRT involved treatment of specified doses of estrogen in single or with combination of progesterone

hormone, which compensate the deficiency of estrogen (Gambacciani et al., 2014).

However, several drawbacks have been reported which are associated with these treatments. Despite of the reduction of fracture risk, bisphosphonate and HRT causes serious side effects to body which include stroke, venous thromboembolism, breast cancer, osteonecrosis of jaw, endometrial cancer (Solomon et al., 2009; Khajuria et al., 2011). Besides hormones, some chemicals like strontium ranelate have also antiresoptive and osteoanabolic properties. But due to serious outcomes like cardiovascular, hepatic and cutaneous side effects it is withdrawn from market. Beside this, some biological and immunological molecules are developed for osteoporosis treatment like anti RANKL antibody, Denosumab and many others are under development (Lewiecki, 2009; Cheng et al., 2020). Currently, because of such serious consequences, available treatment or therapy are less prescribed. Recent study by Zankar and co-workers (2019) has revealed a 50% fall in the prescription of biphosphonate and HRT (Zanker et al., 2019). ***As a consequence of these flaws of the available treatments, research community has started to explore traditional approach to develop medicines from herbal plants which exhibits few benefits over therapies and treatments like less side effects, cost effective, easy availability*** (Kenakin et al., 2013; Fouda et al, 2017)**.**

Natural products could be considered as a natural heritage from Mother Nature as a source of medicine. Herbals are traditionally being used as remedy to various disease conditions. The progression in the field of Phytomedicine has inspired scientists to make use of herbal plants for the development of medicine for the treatment of chronic diseases including osteoporosis (Zhang N.-D et al., 2016; Sucuoglu et al., 2017; Wu et al., 2017). Of the many herbs reported to be useful for osteoporosis, few of them are vigorously studied which include, *Passiflora foetida* (Tasadduq et al., 2017), *Rhizoma alismatis* (Wang L et al., 2017), *Hemidesmus indicus* (L). R. Br (Ahmad et al., 2017), *Curculiginis rhizoma* (Desai et al., 2017), *Dalbergia sissoo* (Jia et al., 2012), *Cissus quadrangularis* (Karvande et al., 2017), *Herba epimedium, Eucommia*

ulmoides, *Lepidium meyenii*, *Moringa oleifera*, *Litsea glutinosa* (Evelyn et al., 2019). These plants have been demonstrated to contain antiosteoporotic effects in *in-vitro* as well as *in-vivo* studies (Augustine et al., 2013; Zhang N.-D et al., 2016; Zhang L.-L et al., 2017).

Among these, *Herba epimedium* has been evaluated for its anti-osteoporotic properties *in-vivo*. Some of the reported outcomes of Total Flavones of Epimedium (TFE) include increase in serum proteins like type I collagen protein, OCN and hormones like estrogen, increase in expression of ERβ and ERα in hypothalamus and hippocampus, inhibition of IL-6 expression in OVX rats (Xie et al., 2005). It 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 exhibits extensive history of use in the treatment of estrogen deficiency-related diseases (Li *et al*., 2017). Icariin, which is an isolated compound from *E. brevicornum,* prevents bone loss in ovariectomized (OVX) rats by sustaining BMD and improving trabecular microarchitecture (Yang L et al., 2013). It modulates the expression of some known marker proteins like Runx2, OCN, BMP2, BMP4, MAPK and calcium signalling (Xue et al., 2016).

Another known herb is *Salvia miltiorrhiza* whose roots have been well explored for treating postmenopausal syndrome. It is known to contain various compounds like cryptotanshinone, tanshinone IIA, tanshinone I, 15, 16-dihydrotanshinone I, salvianic acid, ferruginol and falvanoids. These components have been used for its properties against various diseases like blood stasis, menstrual disorders, and rheumatism (Lee S.-Y et al., 2005; Guo et al., 2014). Moreover, *in-vitro* studies have established that there is some of *Salvia miltiorrhiza* in enhancing the number of osteoblasts and inhibition of osteoclasts. It works through ERK signalling pathway which results in modulation of OPG/RANKL ration. Another reports also suggest that it inhibits the expression of c-fos, NFATc1 and RANKL, consequentially there will be inhibition of osteoclasts (Kim H H et al., 2004; Kwak et al., 2006; Nicolin et al., 2010; Xu et al., 2014; Lin J et al., 2017). Many other herbs like Genistein, Sulfuretin, *Dioscorea villossa* are also explored with work through

many different pathways like ERK/JNK pathway, PTH receptor pathway etc (Miao et al., 2012; Auh et al., 2016; Alcantara et al., 2011).

Litsea glutinosa (LG) is the herb which has been recognized in Charaksanhita and ayureveda. *Litsea glutinosa* is a deciduous or evergreen plant. 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). 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; Unnikrishnan, 2016; Rahman et al., 2017). In *In-vivo* studies in OVX rates Rangrez and co-workers have proved bone healing properties of methanolic extract of LG (Parikh, 2009; Rangrez et al., 2011) and have established that it ameliorates excretion rate of Ca^{++} and bone microarchitecture as well as an increases serum bone formation marker ALP. It also increases bone formation marker proteins like serum ALP. Further, Phytochemical analysis of LG using GC/MS, TLC, IR techniques has indicated the presence of alkaloids, flavonoids, phytoestrogens, oleic acids etc (Parikh et al., 2012).

In the recent years, advanced techniques for quantitative high-throughput analysis of genes and proteins have been developed and are applied into the study various diseases conditions. Such methods have helped the scientists to detect even low copy number changes in the gene expression. At the mRNA level, gene expression profiling is attainable through the introduction of cDNA (Song J Y et al., 2015) and oligonucleotide microarrays, which permit simultaneous analysis of thousands of genes. In continuation with the present day demand for understanding the molecular mechanism of any herbal quantification is a pre requisite.

As far as the molecular mechanism of LG is concerned in ***our view there is a lacuna. Hence, to understand the mode of action of LG with reference to the role played by proteins (MAPK3, Adenylate cyclase and ERβ) and transcription factors (Egr-2, NFATc1, RUNX2 and CREB) for its mode of action, the present study is an attempt to investigate the role of candidate***

genes in exhibiting its response by exposure of the LG extract on Saos-2 cell line.

LG bark powder was purchased from local market. Methanolic extract was prepared and the yield was found to be 8.8%. It was further, was dissolved in DMSO and was used for further experimentation. Dose range of LG methanolic extract was determined using MTT assay. In MTT assay % cell viability was increasing till 250 µg/m, then after it gradually decreased. Thus results from 0.5 µg/mL to 250 µg/mL were selected for regression analysis and R^2 value was derived which was found to be 0.988. Based on regression analysis low (50 µg/mL), mid (100 µg/mL) and high (250 µg/mL) concentrations were selected for further experimentations. Gene expression and western blot were then performed. Study demonstrated modulation of some crucial genes (Egr-2, RUNX2, and NFATc1) involved in specific osteoblast proliferation signalling cascades (Shukla et al., 2017). LG exposure resulted into a dose dependent alteration with a significant upregulation of Egr-2 & RUNX2 and downregulation of NFATc1. RUNX2, an osteogenesis-promoting transcription factor regulates the proliferation of osteoblast progenitors, their commitment to osteoblasts lineage cells. An upregulation of RUNX2 thus suggest that some degree of osteogenesis had occurred in Saos-2 cells. Egr-2 is known to suppress osteoclastogenesis (Hyun et al., 2012) and apoptosis of osteoblasts (Shukla et al., 2017). Presented resulted of significant increase of Egr-2 is in agreement with the earlier reported upregulation of Egr-2 in osteoblasts differentiation and proliferation (Gabet et al., 2010). Egr-2 gene expression was also validated by western blot studies. Overall, up regulation of Egr-2 and RUNX2 genes indicates its involvement in osteoblasts proliferation, suggesting the mechanism of LG on Saos-2 cell line.

NFATc1, is considered to be a master transcription factor required for osteoclast differentiation, which controls the expression of osteoclast function-related genes and proteins such as TRAP/acp5, CTK, MMP, OSCAR, ATP6v0d2 and CAII. Inhibition of calcineurin in osteoblasts by genetic deletion decreased dephosphorylation of NFATc1 and thus its

translocation to nucleus which resulted in osteoblast differentiation (Yeo et al., 2007). In addition, it has also been reported that it upregulates various pro-apoptotic genes like FasL, TNF-α, etc (Mognol et al., 2016). In the present study a significant downregulation of NFATc1 indicates that LG may have role in suppression of osteoclastogenic activity through osteoblasts, and suppression of NFATc1 activity towards decreasing osteoblasts proliferation and differentitation (Choo, 2009). 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 will be helpful to identify the proteins which are responsible for transmitting the signal inside the cell.

To understand the probable signalling cascade of LG specific genes (MAPK3, adenylate cyclase, CREB and receptor like ERβ) were studied. Osteoblast growth, proliferation and differentiation is influenced by various hormones (PTH & Estrogen) due to the presence of receptors on them (Lombardi et al., 2011; Almeida et al., 2013; Van, 2014). PTH receptor, being a GPCR, signals through activation of adenylate cyclase and CREB proteins (Datta et al., 2009). In the present study LG treatment evoked a significant up regulation of adenylate cyclase and CREB implying its influence and its probable role in molecular mechanism of LG.

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 (Alma et al., 2013). Another group of scientists has reported a similar role of RUNX2 and MAPK3 in osteoblast (Greenblatt et al., 2013). 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).

Polyphenols which act as SERM by upregulating the expression of ERβ, which is a positive sign of bone mineralisation (Setchell et al., 2003; Torre, 2017). A significant dose dependent upregulation of ERβ in the present study makes us to speculate that the polyphenol quercetin upregulates ERβ and hence bone mineralization. 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. ***Thus, putting together it can be concluded that LG exposure on Saos-2 cell line results into an upregulation of the candidate genes like MAPK3, adenylate cyclase, ERβ and transcription factors like Egr-2, CREB and RUNX2. It also exposes the probable signalling cascade through which LG is functioning. These study results can serve as a base to design further detailed studies. However further in-vivo investigations are recommended to validate these findings. (CHAPTER I).***

Natural herbs have been widely used in clinical practice in China and other countries since ancient times. An alternative therapy to HRT and bisphosphonates, herbals are now gaining popularity for the treatment of osteoporosis. No matter how many biologically active chemical components are contained in the herbs and how complex their mechanisms of action could be, the phytoestrogens in the herbal preparations, viz. certain flavones, isoflavones, flavanones, flavonols, coumestans, and lignans plays an important role in the ameliorating postmenopausal bone loss (Shuid et al., 2011; Folwarczna, 2013; Leung, 2013;). The effects of phytoestrogens have been reported in both laboratory studies and clinical trials. Studies in OVX rats have suggested that the known phytoestrogens such as coumestrol, genistein, and daidzein can reduce bone loss (Chitme et al., 2009; Wright et al., 2010; Parikh, 2012; Seif, 2014). Estrogenic compounds like phytoestrogens affect bone via promoting the production of calcitonin, lowering the sensitivity of bone mass to parathyroid hormone, reducing the

calcium excretion from the kidney, and accelerating intestinal calcium resorption. Further, estrogen can directly influence the bone, which, in turn, can inhibit bone resorption and increase bone density (Jia et al., 2012; Sungkamanee et al., 2014). Another mechanism is the anti-inflammatory and antioxidant effects of medicinal plants (Nadia et al., 2012). Phytoestrogens are able to suppress the production of proinflammatory cytokines like tumor necrosis factor-alpha (TNF-α), IL-1, IL-6, and IL-7 as well. This is why these proinflammatory cytokines are elevated, in addition, the antioxidant capacity of herbal drugs serves to scavenge free radicals, leading to the inhibition of cyclooxygenase-2 (COX-2) and TNF-α production and expression. These results in a decrease in the receptor activator of NF-κβ ligand (RANKL) expression leading to a decline in the osteoclast activity, which, finally, reduces bone loss (Sungkamanee et al., 2014).

The imbalances between bone resorption and formation are due to an extension of the working lifespan of the osteoclasts and shortening of the working lifespan of the osteoblasts. The amount of bone formed during each remodeling cycle decreases with age and increased by secondary hyperparathyroidism or by the continuing effect of estrogen deficiency. Further, Glucocorticoid excess has been reported to decrease intestinal calcium absorption and hypercalciuria due to defective vitamin D metabolism. These changes result in increased bone resorption, decreased osteoblast proliferation and biosynthetic activity, and sex-steroid deficiency, as well as hyperparathyroidism. Glucocorticoid excess has a suppressive effect on osteoblastogenesis in the bone marrow and also promotes the apoptosis of osteoblasts and osteocytes (Jia et al., 2012). The pharmacotherapy for osteoporosis is usually focused on accommodating the estrogen level or bone remodel. The mechanisms involves many aspects, such as stimulating parathyroid hormone (PTH) synthesizes; inducing the expression of OPG; decreasing IL-1, 4, 6, and M-CSF; increasing estrogens or like-estrogens; supplementing Ca, P in bones; to inhibit the proliferation of osteoclast and induce osteoclast apoptosis; and to enhance the proliferation and differentiation of osteoblasts (Zhang D.-W et al., 2008). Flavonoids, lignans, and coumarins, which are phyto-estrogenic constituents, modulate the bone

metabolism through estrogen receptor. Icariin, genistein, daidzein, kaempferol, and costunolide have been reported to decrease bone loss through increasing osteoblast proliferation and activity, via estrogen receptor. The phytochemicals with antioxidative capacity, such as kaempferol, quercetin, linarin, naringin, resveratrol, curcumin, tea polyphenols, curculigoside, and lycopene regulate bone metabolism through reducing the production of ROS and improving antioxidative capacity (Jia et al., 2012).

So far there are no reports with reference to LG and particularly with regards to rate of proliferation and apoptosis for bone renewal. Hence, after establishing the role of specific genes with their signalling cascade (Chapter I) our next goal was to have an insight for the proliferative and apoptotic effect of LG on Saos-2 cell line for which proliferative (PCNA and Osteocalcin) and apoptotic markers (FasL, Caspase 3 and Cytochrome C) were studied.

Osteocalcin (OCN) is the protein which is secreted solely by osteoblasts. Being a marker protein of osteoblast proliferation, it is reported to be involved in versatile functions like glucose metabolism, male fertility and neuronal development (Moser et al., 2019). Expression of osteocalcin is mainly expedited by MAPK3 protein and RUNX2 transcription factor (Liu L et al., 2019). In the present study exposure of LG extract on Saos-2 cell line resulted into a significant increase in OCN expression. OCN is known to be governed by the participation of RUN2 and MAPK3 proteins (Liu et al., 2019). Upregulation of RUNX2 and MAPK3 (**Chapter I**) thus is a self-explanatory rationalization for the increased expression of OCN. Further, our result are parallel with the earlier reported work of (Mukudai et al., 2014) who have monitored the OCN expression for demonstrating the proliferative effect of osteoblasts and have got a positive results with three herbals viz. *C. atratum*, *M. azedarach* and *C. Turtschaninovii*. In cell proliferation, FGF signaling plays important roles through RAS-MAPK, PI3K-AKT, and canonical Wnt signalling (Katoh et al., 2014; Kawane et al., 2018). Our results further confirmed previous findings by (Qiao et al., 2016) who have opined that expression of $Runx_2$, effectuates the expression of bone-specific genes, such as

Osx, Col1a1, osteocalcin and so on, by binding to the promoters of these genes. Hence, we can conclude that the increased proliferation of osteoblasts is mediated through this mechanism.

PCNA is a highly conservative nuclear protein of DNA polymerase-Δ, and is considered to be a useful marker to assess cell proliferation and progression (Ji et al., 2017). A dose dependent upregulation of PCNA was observed in the present study with the LG treatment on Saos-2 cell line. In addition alteration in the morphology was also observed, where the cells appeared to be healthier compared to control as well as there was a distinct increase in the number. Thus, upregulation of PCNA and OCN along with morphological observation clearly indicates the proliferative effects of LG. This results are in accordance with the previous studies on increased proliferation with number of herbals (Song L et al., 2013; Zhang et al., 2019).

Proliferation and apoptosis are the essential event for maintaining homeostasis. With regards to osteoporosis. It has been suggested that suppression of proliferation and maturation of osteoclasts with associated function of osteoblasts which needs to be increased and in no case should enter into apoptotic pathway. Upregulation of OCN and PCNA confirmed the Proliferative role of LG, however, its anti-osteoporotic property can be well understood by studying the apoptotic markers. Hence, gene expression study of Caspase 3, Cytochrome C and FasL which are established markers for apoptosis (Kim J et al., 2015; Lin J et al., 2018) were studied. LG treatment on Saos-2 cell line resulted into a significant down regulation of Caspase 3 and Cytochrome C, however FasL did not show any significant alterations. To confirm further, FACS was conducted to understand the level of apoptosis.

Flow cytometry results revealed a dose dependent alteration in the number of dead cells versus live cells of Saos-2 cell line with LG treatment. The result of flow cytometry was parallel with the down regulation of the apoptotic markers confirming the anti-apoptotic potential of LG. Our results are in line with the results reported by other scientists, studying different herbals like Icariin, DBT (Danggui Buxue Tang), *Acacia catechu* bark powder extract (Song L et al., 2013; Lakshmi et al., 2017; Gong et al., 2019). However, In-significant

alteration of FasL which is considered to be an initiator protein for apoptosis suggest that the osteoblasts cells are taking some other route for its initiation and it might be possible that LG has apoptosis suppressive properties which involves no direct role of FasL which needs to be validated.

Conclusively, by studying different proliferation markers like PCNA, OCN and apoptosis markers like Caspase3 and cytochrome C it can be stated that LG has proliferative as well as anti-apoptotic properties. Because of such effects, LG has role to play in increasing osteoblasts number and its viability. Together these data serves a strong basis of our earlier reported in-vivo studies where a significant increase in serum ALP was observed and thus validates the therapeutics potential of LG for osteoporosis. (Chapter II)

Phyto chemical analysis of LG has been reported earlier (Parikh, 2012), where they have confirmed the presence of various alkaloids, phenols, tannins, flavonoids, glycosides and saponins. Hence, after understanding the probable molecular mechanism of the LG it was thought to perform *In-silico* analysis which is a computer –aided drug design (CADD) method has proved to be important approach to identify the probable human target proteins. In biomedical field, computer-aided or in silico design which uses computational techniques in drug discovery process is being used to streamline and accelerate hit identification and hit-to-lead optimization process (Ekins et al., 2007). Methods employed in CADD can be broadly break down into two general categories: structure-based and ligand-based. When the target protein's structure is known, structure-based CADD is generally favored especially for soluble proteins that can readily be crystallized. However, when there is no information on the structure of the target, ligand-based CADD is applied based on information of known active and inactive compounds through chemical similarity searches or construction of predictive, quantitative structure-activity relationship (QSAR) models (Kapetanovic 2008; Katsila et al. 2016). Keeping in view the application and advantage of CADD an attempt was made to understand the targets of compounds like Androstane, crinamine, Gestonorone, Cinnamic acid, Cinnamolaurin, thiocoumarin and piperzines.

Bioinformatics results revealed that all the compounds of LG verified binding probability with human target proteins. Of all the molecules, Androstane, Gestonorone and Cinnamolaurine showed maximum binding probability whereas Cinnamic acid, Thiocaoumarin, Piperzines and others demonstrated very less probability which was below 0.3 out of 1, and hence only Androstane, Gestonorone and Cinnamolaurin were considered for further analysis. Androstane showed high binding probability with Androgen Receptor, Cytochrome P450 19A1, Nuclear receptor subfamily 1 group I member 3. The outcome of the Gene expression study on treating Saos-2 cells resulted into a significant up regulation of cytochrome P450 19A1 and androgen receptor. Cytochrome P450 19A1 (Aromatase) is the protein responsible for the conversion of androgens to estrogen. Estrogen modulates osteoblast-derived cytokines, resulting in decreased differentiation and maturation of osteoclasts from precursors and increased osteoclast apoptosis (programmed cell death). Estrogen may also induce direct effects on osteoblasts, further favoring bone formation over resorption. In concert, these cellular responses have the net effect of dampening bone turnover under estrogen-replete conditions (Hadji et al., 2017). Furthermore, RUNX2 transcription factor has been shown to interact directly with aromatase gene promoter and promote its expression (Jeong et al., 2010). Enhanced gene expression of aromatase and RUNX2 in the present study is axiomatic and thus corroborates the probable role of Androstane in enhancing the expression of aromatase, leading to local estrogen level surge. Thus our results are in accordance with the earlier reported work (Simpson, 2002; Perez et al., 2006; Shaheenah et al., 2008), where they have proved the importance of aromatase in bone health.

AR belongs to a member of the nuclear receptor superfamily of ligand-dependent transcriptional factors. In the absence of a ligand, AR is localized in the cytoplasm. Upon binding with androgens, AR translocates into the nucleus. (Verrijdt et al., 2006; Vicencio et al., 2011;). Following nuclear translocation, the AR homodimer binds to androgen responsive elements (AREs) in the enhancer regions of its target genes. In the present study treatment of LG extract on Saos-2 cell line resulted in an up regulation of the

AR Reports suggest androgen receptors are closely linked with osteoblastic differentiation, proliferation and bone mineralisation (Takeuchi et al., 1994; Notelovitz, 2002; Urano et al., 2009) OCN and ALP are the anabolic bone turnover markers and are very important in bone metabolism. LG treatment on Saos-2 cell line exhibited an upregulation of OCN and earlier in our lab in-vivo studies have also reported increased activity of ALP in OVX rats (Rangrez et al., 2011; Patel et al., 2015;). Along with the increased OCN, an increase in AR is also reported in the present study. Previous report has shown that androgens induce Akt-phosphorylation through AR (Yatsu et al., 2018), while other group of scientists have proved that the AKt signalling induces osteoblasts proliferation through increased OCN (Mukherjee et al., 2009). So putting together the increased OCN and AR in the present study confirms the role of the bio molecule androstane of LG in triggering Saos-2 proliferation. The response of NR1I3 on exposure of LG on Saos-2 cell line also resulted into an up regulation; however the response was not dose dependent I. Taking into consideration the binding probability which was 0.34 out of 1, which is indicative of its less binding probability with target proteins and also points to the fact that Androstane have very less binding with the receptor for modulating the expression of the NRI3. However, detailed investigation will validate the fact.

In-silico studies of Cinnamolaurine for its binding probability with the target proteins illustrated high binding probability with Dopamine D2 and D1 receptor, and Dopamine transporter. Dopamine which is an important neurotransmitter and exerts its effect by binding with five different types of dopamine receptors of which, receptors D2R and D1R are expressed on Osteoblasts (Cheong et al., 2018). Further, earlier studies also suggest that dopamine is involved in the proliferation of osteoblasts, bone formation and mineralisation (Bliziotes et al., 2002; Lee D J et al., 2015). LG treatment to Saos-2 and subsequent gene expression study revealed that there is upregulation of Dopamine related genes. D2R transduce its signal through G-protein by activating adenylate cyclase and by forming cAMP the signal to downstream pathway (Cheong et al., 2018; D. J. Lee et al., 2015). Correlating with the results of present study, significant upregulation of dopamine D2R

and dopamine transporter indicates that LG has role in these modulations. Moreover, the upregulation of adenylate cyclase (**Chapter I)** further supports the reasons for its upregulations.

In-silico studies of Gestonorone for its binding probability with the target proteins illustrated high binding probability with GCR, AR and Dopamine transporter. Glucocorticoid belongs to the class of steroid hormones and exerts its effect through GCR which are well distributed on the cells of most of the tissues. Glucocorticoids are known to have negative effects on osteoblast differentiation. It reduces expression of RUNX2, marker enzyme alkaline phosphatase, osteocalcin and bone mineralisation (Frenkel et al., 2015). Along with these, glucocorticoids has also been testified to increase apoptosis rate in osteoblasts by increasing and activating the expression of Caspase-3 (Weinstein et al., 1998) leading to overall bone loss. (O'Brien et al. 2004; Rauch et al., 2010; Hachemi et al. 2018). A significant down regulation of GCR on Saos2 cell line with the treatment of LG is suggestive of the probable involvement of Gestonorone in regulation of apoptosis which is well supported by a significant down regulation of caspase 3 (**Chapter II**) hence, playing role in suppressing bone loss activities.

In silico and *In vitro* studies jointly concludes the probable mechanistic pathways of LG crude extract. Gestonorone, Cinnamolaurine and Androstane are the molecules which may be involved in upregulation of the genes which are found to be common targets viz. AR for Androstane and Gestonorone as well as Dopamine transporter for Cinnamolaurine and Gestonorone which are working in concert and probably responsible for osteoblast proliferation. Thus, this finding elucidates the bioactive properties of LG extract and thus can be accounted for improving bone health.

All the in-silico and in-vitro study results makes the earlier picture clearer about the mechanism of action of LG. Studies outcomes provided the direction of probable involved pathways of LG which include genes like adenylate cyclase, RUNX2, Egr-2, CREB and receptors and transporter for dopamine and androgens. Considering these information, probable schematic model of

LG signalling cascade has been designed which presents excerpt of the present study (Figure GC- 1).

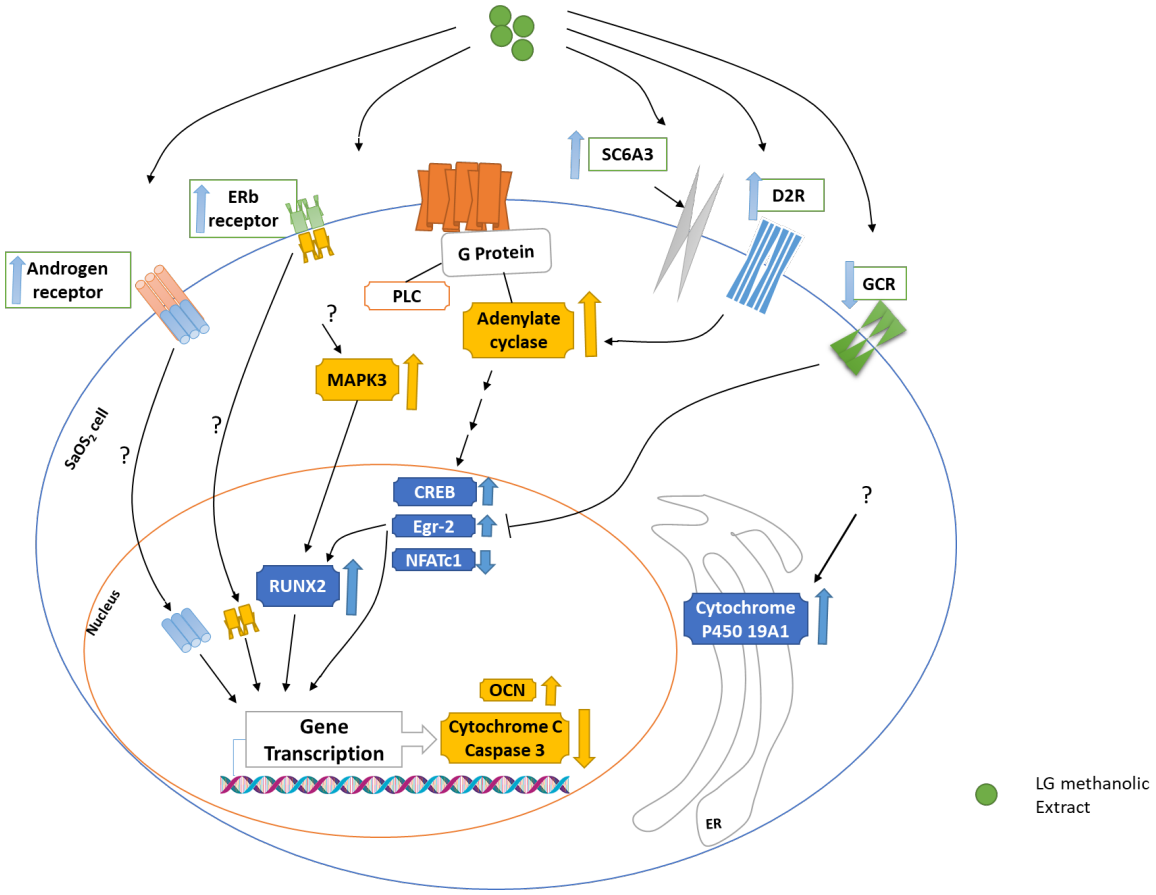

Figure GC- 1: Proposed Model of LG Signalling cascade

Proposed model indicates probable mode of action of LG. LG modulates many crucial genes including different transcription factors, kinases, receptors and transporters. All the affected proteins have been displayed at its native cellular location. Gene expression upon LG treatment has been indicated by arrow beside protein name. Arrow in upper direction represents upregulation of the gene and arrow in down direction represents downregulation of the gene. Proposed pathway or direction of domain to nucleus has been shown by arrow and inhibition has been indicated by T shaped arrow. ERb- Estrogen receptor β; SC6A3- Dopamine transporter; D2R- Dopamine receptor D2; OCN- Osteocalcin; MAPK3- mitogen activated protein kinase 3 (ERK1); GCR- Glucocorticoid Receptor; CREB- Cre binding protein; Egr-2- early response gene-2; NFATc1- Nuclear Factor of Activated T Cells cytoplasmic; RUNX2- Runt related transcription factor 2

Present study revealed that LG, which consists of many phyto-components, may work through multiple signalling pathways including from various receptors to many transcription factors. Along with this, it was also shown that LG contains proliferative and anti-apoptotic effects on osteoblastic cells. Proposed model can be presented as a conclusive remarks of this study. However this model seeks supports of orthogonal studies and further experiments.

6.1 HIGHLIGHTS OF THE STUDY

- The optimum dose was selected for the study was 50 ug/ml, 100ug/ml and 250 ug/ml was selected using MTT assay.
- Out of all the genes, the LG treatment resulted in the upregulation of RUNX2 and EGR2 while, NFATc1 was found to be downregulated.
- Adenylate Cyclase, CREB, ER-β and MAPK3 was upregulated upon LG treatment, thus illustrating its effect through this pathways.
- Similarly, Proliferation was found to be increased where PCNA, osteocalcin was upregulated whereas, the cell death was found to be decreased which showed the downregulation of apoptotic markers like Caspase3, Cytochrome C.
- *In-silico* analysis suggested that three bioactive molecules were found to be having binding probability namely;1) Androstane via Androgen receptor and Aromatase 2) Cinnamolaurine via Dopamine Receptor D2R, and Dopamine Transporter 3) Gestonorone via Dopamine transporter androgen receptor and Glucocorticoid Receptor.
- Validation of *In-silico* was done through Gene Expression where, Dopamine Receptor, Androgen Receptor, Dopamine Transporter and Aromatase was found to be upregulated while, Glucocorticoid Receptor was found to be downregulated.

FUTURE PROSPECTS

7.0 FUTURE PROSPECTS:

Presented study enlightens the involvement of many genes in the signalling cascade of LG and probed the proliferative and anti-apoptotic impact of LG on Saos-2 cells. However, this study can be more productive if it is validated in *in-vivo* platform and individual phyto-component is studied which is mainly unexplored.

- Expression of these genes should be validated at the protein level as well as in *in-vivo* conditions to prove its action which will connect the *in-vivo* and *in vitro* mechanism of action of LG.
- Present study had been conducted on Saos-2 osteoblastic cell line. To understand it more elaborately, cross talk between osteoblasts and other associated cells like osteoclasts, bone marrow stem cells can be elaborated in detail.
- This study was performed using LG methanolic extract, which is crude compound. Individual bioactive phyto-compound can be purified from crude extract and its combination and individual efficacy can be checked for its effect on these candidate genes in *in-vitro* as well as *in-vivo* study.