GENERAL CONSIDERATION

GENERAL CONSIDERATION

Bone is a dynamic tissue. Throughout the life it is continually broken down and re-built allowing the skeleton to adapt to the changing stresses of life. Beside from its structural role, bone is also a reservoir for calcium, a crucial cell signalling ion, and the home for bone marrow which has critical roles in haematopoiesis and in the immune system. Bone metabolism is tightly regulated by hormones, cytokines and growth factors. Hormonal imbalance and/or chronic changes in cytokine or growth factor activity as a result of life-stage or lifestyle can drastically affect bone integrity ultimately leading to fracture (Cooper *et al.*, 1992).

Bone is a composite of organic and inorganic phases. Water accounts for approximately 20% of the wet weight of bone whilst about 75% of the dry weight is organic material. Bone is 75% solid and 25% fluid (Tate, 2003). The solid phase consists of an organic matrix comprised mainly of type-1 collagen on to which calcium, phosphate and trace amounts of other minerals are deposited. The majority of the calcium and phosphate in bone is in the form of a crystalline product known as hydroxyapatite [$Ca_5(PO4)_3(OH)$] (Marks and Hermay 1996). Several non-collagenous proteins are also present in the bone matrix such as osteonectin and osteocalcin. The function of these proteins is poorly understood however, they may be involved in the process of mineralization. There are three distinctly recognisable forms of bone cells, osteoblasts, osteocytes and osteoclasts: each has a specific function in the highly ordered sequence of formation, maintenance and removal of bone mineral.

During childhood and adolescence, bone increases in length as well as density by a process known as bone modelling. Long bone growth ceases in adolescence however, small increases in bone density can occur during early adulthood. Bone density peaks in approximately the third decade of life (Riggs, 2002) then begins to decline at an estimated rate of 3 % per decade for cortical bone and 7-11 % per decade for trabecular (O'Flaherty, 2000). In women the rate of bone loss accelerates to an estimated 9-12% per decade for cortical bone and 13% per decade for trabecular at the time of menopause (Seeman, 2003).

Bone remodelling is the method by which bone is continually turned over or renewed. It occurs throughout the lifecycle and involves the sequential and coupled resorption of a small area of bone tissue followed by replacement of this tissue with new bone (Pead *et al.*, 2005). In humans, an estimated 10% of bone is remodelled each year (Theill *et al.*, 2002), the vast majority of which is trabecular bone (Manolagas, 1999).

Bone remodelling takes place at discrete sites within the skeleton known as Bone Remodelling Units (BRU). Osteocytes, which are cells embedded within bone tissue identify the sites within bone which require remodelling and signal the need for establishment of a new BRU. In humans, bone resorption at a BRU takes approximately 2-3 weeks and is carried out by specialized macrophage-like cells known as osteoclasts. The replacement of resorbed bone with newly synthesized bony tissue requires 3-6 months and is accomplished by a third type of bone cell known as the osteoblast (Watkins *et al.,* 1996; Macdonald *et al.,* 2004).

Following new bone formation, some osteoblasts are killed by apoptosis whereas others become embedded within the lacunae in the bone tissue, subsequently transforming into osteocytes (Bonewald, 2004). Matrix metalloproteinases (MMPs) are involved in preventing osteoblast apoptosis possibly by degrading pro-apoptotic extracellular signalling molecules as well as by activating latent TGF -p enabling osteoblast differentiation into osteocytes (Karsdal *et al.,* 2002). Part of the osteocyte transformation process involves the production of long, dendritic processes by transforming osteoblasts which extend through the canaliculi in bone and connect to the processes from existing osteocytes (Bonewald, 2004). The end result is a 3-dimensional network, referred to as the osteocytic membrane or synctium, which not only connects bone cells but also defines a common fluid space (Knothe, 2003). This synctium also includes osteoblasts and to a lesser extent, osteoclasts, residing on the bone surface, allowing communication between osteocytes in different locations within bone as well as between the three cell types (Knothe, 2003).

Osteocytes can be viewed as highly specialized and fully differentiated osteoblasts. They are the principal cell in adult bone and as stated by Sommerfeldt and Rubin (2001) responsible for orchestrating the spatial and temporal recruitment of the cells that form and resorb bone. Functionally, osteoblasts are the cells within bone that lay down the extracellular matrix and regulate its mineralization. Osteoblasts have recently been described as sophisticated fibroblasts (Ducy *et al.,* 2000). They are highly anchorage dependent and rely on extensive cell-matrix and cell-cell contacts via a variety of transmembranous proteins (integrins, connexins, cadherins) and specific receptors (for cytokines, hormones, growth factors) to maintain cellular function and responsiveness to metabolic and mechanical stimuli (Ferrari *et al.,* 2001; Lecanda *et al.,* 1998). Osteoclasts, the multinucleated hematopoietic cells are responsible for breaking down calcified tissue (Kruger *et al.,* 1995). An activated osteoclast is able to resorb 200,000 μm3/day an amount of bone formed by seven to ten generations of osteoblasts with an average lifespan of 15–20 days (Albright and Skinner, 1987).

Although it was suggested for many years that crosstalk between osteoblasts and osteoclasts must exist to coordinate processes of bone formation and resorption, it was not until 1997 that a molecular basis for this paradigm was discovered in the form of osteoprotegerin (OPG) and, shortly after, its cognate ligand OPG-L, a transmembranous receptor expressed on osteoblasts and immune cells. Both of these molecules can bind to RANK (receptor/activator of NF-κB), a transmembranous receptor expressed on osteoclast precursor cells. Interaction between OPG-L and RANK initiates a signalling and gene expression cascade resulting in the promotion of osteoclast formation from the precursor pool. In this setting, OPG, which is secreted by osteoblasts, but also expressed in many other tissues, acts as a soluble competitive binding partner for RANK-L, which inhibits osteoclast formation and, consequentially, bone resorption (Hofbauer *et al.,* 2000). This crosstalk mechanism also appears to be an endpoint for the action of several calciotropic hormones and cytokines such as 1,25(OH)2D3, parathyroid hormone (PTH), estrogen, prostaglandin E_2 (PGE_2), interleukins, and tumor necrosis factor α (TNF-α). As a result OPG has entered clinical trials as a promising therapeutic agent for osteoporosis (Aubin and Bonnelye, 2000; Hofbauer and Heufelder 2000).

Traditionally, "coupling agents" were believed to be involved in osteoclast/osteoblast cross-talk. Members of the TGF super family, particularly TGF-ß, as well as other growth factors such as IGF-l, IGF-2 and platelet-derived growth factor (PDGF), were proposed as candidate coupling agents (Mundy, 1995). These growth factors are synthesised by osteoblasts and secreted into the bone matrix during new bone formation. They remain embedded in the bone matrix until they are released during osteoclast-mediated bone resorption. The release of these growth factors was thought to stimulate the proliferation of osteoblast precursors and the attachment of new osteoblasts to the remodelling site hence ensuring the lacuna created by the osteoclast was re-filled (Mundy, 1995).

Under non-pathological conditions, osteoblasts accurately replace the volume of bone previously resorbed by osteoclasts and the overall effect of bone remodelling is no net change in actual bone mass. The mechanism triggering osteoblasts to stop synthesising new bone matrix once the lacuna is refilled however is poorly understood. Cross-talk between osteoclasts and osteoblasts is important for initiating both osteoblast and osteoclast formation, maturation and activity (Martin, 2004; Zhao *et al.,* 2006). It is also important for ensuring the timely and sequential recruitment and activity of osteoclasts and osteoblasts at a BRU.

In the adult skeleton, remodelling is by far the more active process, reflecting the process of "real-time" tissue replacement during bone turnover and repair. In some places, either as

bone-lining cells on a trabeculum or as infilling after an osteoclastic cutting cone in cortical bone, osteoblasts lay down new bone whereas in others, osteoclasts resorb the mineralized matrix. This cyclic sequence is also called the activation-resorption-reversal-formation (ARRF) sequence and takes about 3-6 months to be completed in humans. Remodelling is not only required to replace dead or damaged tissue, it also gives bone the capacity to adapt to changes in loading and to respond to nutritional and/or metabolic changes (Kruger *et al.*, 1999).

Ultimately, the pathologies inherent in bone diseases are reflected by this physiologic remodelling sequence, either by influencing bone formation (induction of osteoblast activity or inhibition of osteoclast activity) or resorption (induction of osteoclast activity or inhibition of osteoblast activity). According to this paradigm, osteoporosis is generally viewed as the inability of osteoblasts to fully repair the resorptive defects during normal osteoclast resorption, since mean wall thickness and porosity in cortical bone are generally elevated and trabecular spacing in cancellous bone is increased (Zietz *et al.*, 2003).

Osteoporosis is a condition of decreased bone density. It affects one in six women and one in eight men over the age of fifty and is most common among post-menopausal women. Osteoporosis is often the cause of many health complications, as it progresses silently and unnoticed for years. Only after years of bone loss do signs and symptoms appear, such as pain, spinal deformity, and fractures. In women the rate of bone loss accelerates to an estimated 9-12% per decade for cortical bone and 13% per decade for trabecular at the time of menopause (Kruger *et al.*, 1999). The consequences of osteoporosis are greater than simply an increased risk of bone fracture. Approximately one third of hip fracture patients die within the next year, usually as a result of cardiovascular disease. There is a strong, inverse relationship between degree of aortic calcification and bone density (Schulz *et al.*, 2004). Coincidentally, cardiovascular disease is linked with increased calcification of the arteries whereas osteoporosis results in decreased calcification of bone tissue (Klift *et al.*, 2002; Tanko *et al.*, 2003).

In many cases the standard 'causes' of osteoporosis do not occur. For instance, although sex hormone deficiency as a result of natural menopause may be the primary cause of osteoporosis in individual, age-related decreases in growth hormone production may also contribute to bone loss. Expression of RANKL by T cells also appears to increase with aging (Walsh *et al.*, 2006) which may result in increased osteoclastogenesis and contribute to bone mineral loss. McNamara and co-workers (2003) in their studies have inferred that a bone tissue material property gets altered during osteoporosis. Emerging clinical and molecular

evidence suggests that inflammation also exerts significant influence on bone turnover, inducing osteoporosis. Numerous pro-inflammatory cytokines have been implicated in the regulation of osteoblasts and osteoclasts, and a shift toward an activated immune profile has been hypothesized as an important risk factor. Chronic inflammation characteristic of aging, called "inflamm-aging," may be a determinant pathogenic factor (Ginaldi *et al.,* 2005). Interestingly, phenols are powerful phytonutrients that protect plants from oxidative damage and perform the same function for humans. The outstanding phytonutrient feature of phenols is their ability to block specific enzymes that cause inflammation (Hertog *et al.,* 1993).

Lean *et al.,* (2003) in their studies have suggested that estrogen deficiency causes bone loss by lowering thiol antioxidants in osteoclasts. This directly sensitizes osteoclasts to osteoclastogenic signals and entrains ROS-enhanced expression of cytokines that promote osteoclastic bone resorption. Zietz and co-workers (2003) opined that bone loss is a consequence of not only estrogen deficiency, but also of other situations in which reactive oxygen species are involved, such as aging and inflammation. This mechanism provides novel opportunities for the development of potential osteoporosis therapies. The use of antioxidants is associated with decreased levels of bone resorption. It is feasible that antioxidants may reduce the damaging effects of oxidative stress by reducing the up-regulated osteoclastic differentiation and enhancing the down-regulated osteblastic differentiation. Phytonutrients from greens, and fruits and vegetables of all colours, are among nature's most powerful and plentiful exogenous antioxidants (Khajuria *et al.,* 2008).

Oestrogen deficiency is associated with a gain in adipose tissue (Maeda *et al.,* 2002), and that adipose tissue may be a major determinant of circulating IL-6 levels (Pfeilschifter *et al.,* 2002). T-cell derived production o f TNF-α is also increased with oestrogen deficiency due to both an increase in T -cell number and activation (Weitzmann and Pacifici, 2005). Oestrogen deficiency is therefore associated with an increase in the overall degree of inflammation. As a result, postmenopausal osteoporosis is considered to have a strong inflammatory component in its aetiology (Teitelbaum, 2004; Pacifici *et al.,* 1987).

Due to lack of compliance in current pharmacological interventions targeting bone problems like postmenopausal osteoporosis, there is an urge for developing new alternative therapies for osteoporosis. In recent times, interest has been given to phytotherapy due their ease of availability and acquiescence. However, Due to the limited evidence accrued to date, the bone protective effect of these herbals and their constituents has not yet gained scientific acceptance in the medical community. A number of interventions have reported a beneficial

effect of various plant and plant derived products in osteoporosis (Bharti *et al.,* 2004; yin *et al.,* 2004; Putnam *et al.,* 2007; Vali *et al.,* 2007; Wang *et al.,* 2008; Zennaro *et al.,* 2008).

Phytoestrogens are plant compounds whose ability to mimic the action of estrogens has resulted in their usage for the treatment of menopausal symptoms. Despite uncertainties about the safety and effectiveness of phytoestrogens in humans, the use of market phytoestrogenic neutraceuticals and botanicals is on the increase. Positive epidemiological study findings couples to an entrenched belief in many societies about the superiority of what they view as "natural" remedies, as well as the reluctance of women to use the traditional hormone replacement therapy due to its association with detrimental health effects. The classical phytoestrogens, so far known, constitute a group of plant-derived compounds which include mainly isoflavones, lignans, coumestanes, stilbenes and the flavonoids quercetin and kaempherol. The discovery of many more novel estrogen-like compounds in the plant kingdom demonstrates that the spectrum of phytoestrogens in nature is expanding (Moutsatsou, 2007). Phytoestrogens have steroid-like structures and are highly stable due to the presence of Phenolic compounds at both ends of the molecule (Adlercreutz and Mazur 1997). The Phenolic ring allows binding to the oestrogen receptor (Setchell, 1998). Apart from this phytoestrogens are also known to have antioxidant effects and may reduce inflammation markers (Ibarreta *et al.,* 2001). The classical as well as the novel phytoestrogens show a complex mode of action via interaction with the nuclear estrogen receptor isoforms ERα and ERβ, exhibiting either estrogen-agonist or estrogen-antagonist effects. Their final biological activity, assessed by cell culture assay systems, animal studies and clinical trials, depends on multiple factors such as the chemical structure of the phytoestrogen, the kind of tissue and cell type, the intrinsic estrogenic status, the route of administration, the metabolism as well as the time and the level of exposure. They are characterized by high tissue specificity and dose-dependent activity (Chang *et al.,* 2003; Cotter *et al.,* 2003; De Wilde *et al.,* 2004; Pan *et al.,* 2005; Ge *et al.,* 2006). India is sacred with a habitat that harbours a variety of botanicals with myriad of biological activities that needs to be explored (Sukhdev, 2006). Investigation of these plants for their biological activity and their pharmaceutical role can develop new therapeutic drugs that are with enhanced potency and lesser side effects.

In the light of considerable volume of *in vitro*, *in vivo* and epidemiological evidence in favourable health effects of herbals, the aim of our study was to perceive the efficacy of few of the botanicals, and benchmarking the activity of the most potent extract with enhanced osteogenic potency and efficacy against osteoporosis.

In order to achieve the above objectives one explored the osteoprotective effect of few botanicals on the experimentally induced osteoporotic animal model and tried to develop a scientific basis for their ethno-botanical usage in the treatment of osteoporosis. The ovariectomized (OVX) rat model is a scientifically accepted model of osteoporosis. Various pathological changes noticed in this model are similar to those found in humans. In both the species bone loss is most rapid after the onset of estrogen deficiency. This is characterized by a period of increased bone turn over during which resorption exceeds formation. In both species, bone loss from trabecular bone is greater than cortical bone (McCann *et al.,* 2005). These similarities make the OVX model best suited for studying the prevention and treatment of postmenopausal bone loss (Xiao *et al.,* 2002; Hidaka *et al.,* 2006). According to Dontas *et al.,* (2006) OVX is a consistent and reproducible model used in skeletal research. In the present study therefore, one used ovariectomy for inducing post menopausal osteoporosis like condition in female Wistar rats. Moreover, to reaffirm the potent osteoclastic resorption property of the selected botanical one used scientifically acceptable *in vitro* systems *viz.*, the co-culture system and SaOS 2 osteoblastic cells.

Initially screening of three botanicals *viz. Litsea glutinosa, Terminalia arjuna* and *Curcuma aromatica* was carried out (Chapter 1). *Litsea glutinosa* and *Curcuma aromatica* could effectively prevent high bone turnover and calcium loss caused by E_2 deficiency, without substantial effects on the uterus; but *Terminalia arjuna* had no significant effect on OVX induced changes. Although *Terminalia arjuna* did not show any osteoprotective role, it resulted in reducing the weight gain in experimental animals. *Terminalia arjuna* has been reported to be rich in tannins, which might decrease the food consumption (Sukhdev, 2006) and justifies its usage as anti obesity agent. Administration of *Curcuma aromatica* and *Litsea glutinosa* for four weeks ameliorated OVX induced osteoporosis. Both the plants reduced the upregulated AlP and TRAcP - the markers of bone formation and resorption respectively, suggesting their osteoprotective role. Regardless of its mechanism of action, the drastic decrease in rate of bone turnover provides a direct explanation for the observed increase in serum and bone calcium content.

Curcuma aromatica is known to be rich in curcumin, a potent anti inflammatory agent and a proved osteolysis inhibitor by inhibiting osteoclastogenesis (Bharti *et al.,* 2004). Curcumin is an established osteoprotective agent due to its osteclast inhibiting property which acts through NF κβ ligand signalling pathway (Bharti *et al.,* 2004). Folwarczna *et al.,* (2009) have shown that the curcumin is having osteoprotective effects in OVX rats. These reports justify the

osteoprotective effect of this plant observed in the current study. Hence, no further *in vivo* analysis was carried out for *Curcuma aromatica.*

While *L. glutinosa* has been widely used in India in various medicinal formulations in the treatment of bone diseases, no data was available to substantiate its beneficial effect on osteoporosis. Thus it was further evaluated for its osteoprotective effect and checked for its possible mechanism(s) of action and its harmful side effects. Further exploration of *Litsea* demonstrated that feeding the animals with *L. glutinosa* bark powder partially prevents bone loss caused by estrogen deficiency, without affecting the uterus.

There are now available extensive bodies of literature on the use of biochemical markers of bone turnover in the clinical development of drugs that affect bone metabolism. They are also found important in assessing disease activity in osteoporosis (Ramprasanth *et al.,* 2006). Biochemical markers of bone turnover fall into two categories namely, markers of bone formation and markers of bone resorption (degradation). A number of markers are used as bone turnover indices. Number of workers has shown that bone formation is broadly reduced whereas bone resorption is specifically increased during osteoporosis (Garnero *et al.,* 1999). Studies in chapter 2 are intended to look into the effects of the *Litsea glutinosa* on few markers of bone metabolism such as calcium, TRAcP and AlP.

Litsea glutinosa showed ameliorating effect on all the markers of the osteoporosis. The levels of TRAcP and AlP in bone were elevated in osteoporotic condition, which shows the altered turnover during the disease. These results support the previous reports on bone metabolism (Tamura *et al.,* 2002) where they have shown that the serum AlP and TRAcP were significantly altered. TRAcP is exocytosed from osteoclasts along with bone matrix products into the circulation where its activity reflects the bone resorption rate (Janckila *et al.,* 2002; Ramprasanth *et al.,* 2006). Further, to check its estrogenic potential uterine histology was done, being the primary estrogen target organ. It is widely known that estrogen treatment maintains uterine weight. In contrast, feeding with *L. glutinosa* powder led to a slight increase in uterine weight. Osteoprotective effects were similar to those of E_2 as reported in the present study as well as in studies by Schulz and Morris (1999). Despite the similarity of the effects in bone, our results showed that *Litsea glutinosa* extract did not mimic the effect of E_2 on body and uterus weight. The latter is of particular interest as it indicates that *Litsea glutinosa* extract exerts its beneficial effects on bone without inducing potentially harmful proliferative effects in reproductive tissues. Our results are in agreement with Xie and co-workers (2005) who have reported beneficial effect of *Herba epimedii* plant. A dose dependent and time dependent response was seen in the present study.

Serum TRAcP titre, an osteoclastic resorption indicator, showed a gradual increase with time in OVX animals. Bone formation gets triggered in response to bone resorption. Elevated ALP titre confirms the process of bone formation. *Litsea* treatment in OVX animals reduced the TRAcP levels, with a parallel reduction of AlP. Bone AlP and TRAcP levels were also similar to the serum profile thus suggesting osteoprotective effect of the plant. Uterine histology confirmed that *Litsea* does not have any estrogenic potential. Moreover, histology of the bone clearly indicated the osteoprotective effect of this plant with all the doses tested. Kim *et al.,* (2003) in their studies have also got similar observations. The present data provide the first ever direct *in vivo* evidence that *Litsea glutinosa* has a bone-protecting effect caused by estrogen deficiency, without undesirable side effects on the uterus. The beneficial effect is mediated, at least in part, by dual regulation of the enhancement of osteoblast function and induction of osteoclast apoptosis. Do *et al.,* (2007) examined the effect of the methanol extract from the fruit of *Rubus coreanus* (RCM) and He *et al.,* (2010) examined *n*-BuOH soluble fraction of the root of *Achyranthes bidentata* and have reported that they are effective at preventing bone loss in OVX rats and has recommended the plant as an alternative therapy for osteoporosis.

Dietary calcium intake has a major impact on bone mass as it is the main source of calcium for bone mineralisation. Factors influencing dietary calcium intake, intestinal calcium absorption and renal calcium reabsorption determine overall calcium balance in the body. Calcium is an important second messenger. Intracellular calcium concentration can rapidly increase by up to 100-fold as part of the calcium-signalling process (Brown *et al.,* 1996). Extracellular calcium concentration however is maintained within a very narrow range (1.1 - 1.3 mM). A specialised, G protein-coupled calcium-sensing receptor (CaR) expressed on cell membranes senses extracellular calcium concentration and modulates the synthesis and secretion of systemic hormones accordingly (Purroy and Spurr, 2002; Tfelt-Hansen and Brown 2005). Acute changes in calcium balance do not invoke bone remodelling. Such fluctuations are buffered by the "exchangeable calcium pool" present within bone fluid. Approximately 25% of bone is fluid and an estimated 1% of total body calcium ("the exchangeable calcium pool") is contained within bone fluid (Knothe, 2003). Bone fluid circulates within the canalicular system and is separated from plasma and extracellular fluid by the synctium (Knothe, 2003). The exchangeable calcium pool present in bone fluid is the primary source for replenishing extracellular calcium levels and the "sink" for excess extracellular calcium. The buffering effect of the exchangeable calcium pool means that small fluctuations in extracellular calcium concentration can be rectified without the need for inciting changes in bone cell activity. Chronic changes in extracellular calcium balance

however will trigger a hormonal cascade ultimately leading to a shift in the set-point of the bone resorption vis-à-vis formation balance (Mundy, 1995).

Estrogen and calcium deficiencies are important risk factors in the pathogenesis of osteoporosis. These changes are partly due to hyperparathyroidism secondary to calcium deficiency and exacerbated by estrogen deficiency (Riggs and Melton, 1986). Thus we decided to investigate the effects of the plant extract on the metabolism of Ca in the OVX rats. *Litsea glutinosa* was found to have beneficial effect on bone and serum calcium without affecting the uterus. Fall in serum calcium in OVX treated animals with *Litsea* is whether by increasing intestinal calcium absorption and suppressing PTH or by directly inhibiting osteoclastic cells and preventing the resorption is not explicable. Thus, a further exploration on the calcium metabolism and excretory rate was carried out on a long term study to see the osteoprotective effect of the plant. Study on calcium metabolism and excretory rate proved that the osteoprotective effect was direct by lowering the excretory rate and that it is not influenced by intestinal absorption. GC MS analysis of the plant revealed that this plant is rich in various phytoestrogens, suggesting that phytoestrogens of this plant does not have potent stimulatory effect on neither uterus nor intestine.

Fasting urinary calcium excretion could also be used as an important variable for estimating net bone resorption. Nyda and co-workers (1948) were the first one to observe that ovariectomy leads to decrease in serum calcium and increased urinary excretion of calcium. Our results indicated similar profile of serum and urinary calcium. We also observed that excretory rate of calcium was reduced with *Litsea* plant treatment in a dose dependent manner. This decrease in the excretory rate due to *Litsea* treatment suggests that this plant extract might be inducing the beneficial effect by directly acting on the bone cells. Ishida *et al.,* (1998) also suggested similar results and reported that ovariectomized animals had uterine atrophy and it could be prevented by estrogen but not with the phytoestrogens like genistin or diadizine, as they do not stimulate the estrogen receptors in uterus. Parallel with these results, phytochemicals observed in GC MS analysis also must be acting through similar pathway as they do not stimulate uterus or intestine.

Apart from calcium metabolism and excretory rate, it was also observed that plant have positive effect on the serum biochemical markers like AlP and TRAcP. Our results are in agreement with the work of Lee *et al* (2004) and Kim *et al.,* (2004). In OVX, one important phenomenon observed was that AlP levels remained elevated throughout the study period, while the TRAcP levels progressively increased, suggesting that osteoclastic activity is escalating slowly, while the osteoblastic activity is remaining stable. Treatment with *Litsea* in

OVX reduced the levels of serum AlP and TRAcP levels suggesting that the osteoclastic activity has reverted to the normal condition and is parallel with the noticeable reduction in the ALP activity. *Litsea glutinosa* significantly checked the TRAcP activity, indicating its effect on osteoclastic resorption. And as the osteoclastic activity is checked, osteogenesis, which is linked to resorption, is also reduced, which was observable in the form of reduced AlP activity.

The distal metaphyseal region of femur that contains both cortical and trabecular bone is very sensitive to estrogen deprivation and results in rapid and profound osteopenia (Westerlind *et al.,* 1997). To verify the sensitivity of the plant, histological studies were done. OVX group depicted severe damage in trabecular bone compared to the sham group and showed altered bone architecture. *Litsea* treatment showed a notable improvement in the quality and micro architecture of bone in a dose dependent manner. The result indicates that *Litsea glutinosa* is effective in preventing the OVX-induces profound alteration in the histomorphology of the bone.

Biochemical as well as the histological alterations thus ascertained the potential of the plant and enticed us to go for further analysis of the plant extract. Studies of Mandal *et al.,* (2000) have proved the antibacterial activity of the plant; however, very scanty data is available about the phytochemical constituents, and hence, the plant was screened for its phytochemical constituents to understand its biological activity. Antibacterial and antifungal activity of the plant was also checked. *Litsea glutinosa* bark showed the presence of various phytochemicals including alkaloids, steroids, triterpenoids, saponins, and tannins. We could successfully prove that alkaloids of *Litsea* are responsible for its antibacterial activity. These results were more profound when antifungal activity was carried out, indicating that alkaloids were responsible behind the antifungal property of *L. glutinosa.* This plant was found to contain oleic acid which is reported to have variety of biological effects, including hypotensive effect (Teres *et al.,* 2008). Our study suggested that *Litsea glutinosa* is rich in alkaloids, phytoestrogens which contribute towards its biological activity. *Litsea* was also found to be rich in various long chain poly unsaturated fatty acids (LCPUFAs) which have been proved to be enhancing the osteoprotective potential of phytoestrogens. Studies have reported a beneficial effect of n-3 LCPUFAs on bone mass in ovariectomised animal models (Kruger and Horbin, 1997; Watkins and Seiferts, 1996; Das, 2000). Poulsen *et al.*, (2007) from their studies have also concluded that LCPUFAs enhances the activity of phytoestrogens. Thus, *Litsea* with a multipotential and safe property suggest that consumption of this plant can be

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

In vivo studies of *Litsea* and *curcuma* established the osteopotency on OVX rats it was thought worth exploring the effect of these herbs *in vitro* to understand their cellular mechanism of action (Chapter 5). *In vivo* studies by Burali *et al.,* (2010) have proved the osteoprotective effect of *Moringa* on OVX Wistar rats. Hence, an *in vitro* study was carried out, comparing three different herbal extract (*Litsea, Moringa* and *Curcuma)* for their effect on the osteoclastic resorption using the co-culture system and SaOS 2 osteoblastic cells. Our *in vitro* study revealed that all three plants were inhibitors of the osteoclastic resorption. The osteoclast induced bone resorption is reported to be mediated by two different processes by the formation of new osteoclasts and the resorption activity of osteoclasts (Penolazzi *et al.,* 2006). The activity of *Curcuma* plant which is rich in curcumin –an osteoclastogenesis inhibitor (Bharti *et al.,* 2006) is thus self explanatory. Furthermore, the effect of *Litsea glutinosa* can be credited to the presence of various Phytoestrogens in it, as these Phytoestrogens stimulate osteoblasts to inhibit the osteoclastogenesis (Poulsen *et al.,* 2007). *Moringa oliefera* showed osteoclasts inhibiting property only at higher doses. Osteoclastic activity involves generation of various free radicals and H^+ ions. As *Moringa* is rich in various natural anti oxidants like qucertin, it may be suppressing the osteoclastic activity. In our study, Compared to *Moringa oliefera, Litsea glutinosa* and *Curcuma aromatica* were found highly potent in inhibiting the osteoclastic resorption and favouring the osteoblastic activity. Cold calcium release (Richard *et al.,* 1997) and TRAcP (Okazaki *et al.,* 1999; Qin *et al.,* 2003; Penolazzi *et al.,* 2008) are established markers of osteoclastic resorption. The analysis of these markers revealed that the *Moringa oliefera* impede the experimentally induced osteoclastic resorption in a dose dependent manner. *Curcuma aromatica* and *Litsea glutinosa* showed potent osteoclast inhibiting property suggesting their potent therapeutic role as an osteoprotective agent.

Osteoblastic differentiation is a complex process of sequential expression of osteoblastic markers such as AlP, osteocalcin, type I collagen etc. The developmental sequence associated with SaOS 2 makes it a useful model for the *in vitro* study of osteogenic agents. In this study we determined the effect of *Litsea glutinosa* on the osteoblastic cells and checked it for its osteogenic potential. Our results demonstrated that SaOS 2 cells have osteogenic property and their phenotypic expression was dependent upon the duration of culture. During the initial phase of proliferation cells have typical fusiform appearance but as the treatment progresses, the cells assume a mosaic shape and are clustered together. Here too, *Litsea glutinosa* and

Curcuma aromatica were potent stimulators, while *Moringa oliefera* had no significant effect on osteoblastic cells. Previous studies reported that *Moringa oliefera* is having osteoprotective effect in OVX rats (Burali *et al.,* 2010). However, our results showed that *Moringa oliefera* had no significant effect on the SaOS 2 cells, signifying osteoprotective effect of the plant solely to its osteoclast inhibiting property. *Curcuma aromatica* showed reduction in cell viability while *Litsea* showed increase in the cell viability. Though *Litsea* showed dose dependent increase in the cell viability, higher doses of it showed toxicity and reduced the cell viability, suggesting that this plant is an osteoblastic stimulator but at higher doses, it is toxic to the osteoblastic cells.

AlP is one of the very important markers of osteoblastic function (Sajeda *et al.,* 1997; Parikh *et al.,* 2009). During SaOS 2 culture we observed that both *Curcuma aromatica* and *Litsea glutinosa* are potent stimulators of this marker. However, treatment using *Curcuma* indicated contradiction in MTT and AlP results, which can be explained by the hypothesis that this plant might be stimulating osteoblast differentiation rather than stimulating osteoblastic proliferation. Curcumin is a known anti tumor agent as well as bone promoter and as this cell line is a known sarcoma, so inhibitory activity of this plant on MTT and increased AlP levels might be attributed to curcumin (Sukhdev, 2006). *Litsea glutinosa* was found to be showing similar profile of AlP as that of MTT assay and it showed dose dependent increase in osteoblastic activity. Thus, it is possible that at lower doses *Litsea glutinosa* is stimulating osteoblastic proliferation, but at higher doses it induces their differentiation and leading to functionally activated osteoblast which secrete more AlP. It was noticed that *Litsea glutinosa* was more potent compared to *Curcuma aromatica* in stimulating the osteoblastic cells. *Litsea glutinosa* not only stimulated the proliferation of osteoblastic cells, it also increased AlP, suggesting that *Litsea glutinosa* stimulates both proliferation and differentiation of SaOS 2 cells. When the cells were stained using Acridine Orange - Ethidium Bromide, it was noticed that *Moringa oliefera* was non toxic, while *Curcuma aromatica* showed toxicity in higher doses. *Curcuma aromatica* was found to have more ethidium bromide stained cells, suggesting that this plant is more toxic to the osteoblastic cells compared to *Moringa oliefera* and *Litsea glutinosa. Moringa oliefera* plant was found to be least toxic to the SaOS 2 system supporting its wide nutritional usage.

From the *in vitro* study on osteoblasts and co-culture it was learned that osteoprotective effect of the *Litsea glutinosa* is due to its dual role both as osteoblast stimulant and osteoclasts inhibitor. It was learnt from the previous studies that antibacterial activity of this plant is due to its alkaloid content. Hence, we carried out a further study to explore the osteogenic

potential of the plant and see its effect in the proliferation and differentiation of SaOS 2 cells. This cell line (SaOS 2) is an established *in vitro* model for studying the osteogenic potential of various stimulants. They not only grow rapidly but also form matrix and develop different stages of differentiation (Vali *et al.,* 2007). More the differentiated cultures are, they express more and more AlP (Madunka *et al.,* 1993; Rao *et al.,* 1994; Rodan *et al.,* 1997) and thus it can be used as functional marker of osteoblastic bone formation. Based on different levels of AlP expression, they can qualitatively represent different stages of osteoblastic phenotype (Rao and Murray 2000; Vali *et al.,* 2007).

Phytochemical content of the bark of *Litsea glutinosa* was found to possess various constituents that include alkaloids, steroids, triterpenoids, saponins, and tannins. The most prominent phytochemical constituent of the bark of *L. glutinosa* was found to be Alkaloids that presumably is responsible for their antibacterial and antifungal activity. Bioactivity – guided fractionation and sub fractionation can throw much detailed information of the plant and enhance the perception of the osteoprotective constituent. To comprehend the precise component of the plant fraction responsible for its osteoprotective value a further detailed analysis on SaOS 2 was conducted. *L. glutinosa* aqueous (LG AQ) and *L. glutinosa* alkaloid (LG ALK) fractions showed an affirmative osteogenic property; comparatively LG ALK fractions were more potent then LG AQ. Sub fractions of LG ALK i.e. sub fraction D, E, F and G showed the confirmed presence of long chain poly unsaturated fatty acids further potentiating the osteogenic efficiency of the plant. Therefore, it is logistic to surmise that the phytoestrogens and LCPUFAs in alkaloid fractions could be the potential bioactive molecules responsible for the enhanced osteogenic activity.

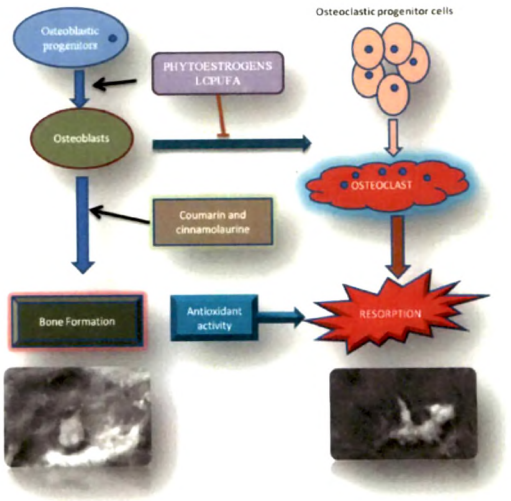
In addition, the anti-inflammatory activity of the phytoestrogen might help in minimizing the bone loss. Our data provided cellular evidences for the osteoprotective effect of this plant and suggested that osteoprotective effect of this plant is due to its alkaloids. Phytochemical analysis had shown that out of the eight fractions, fraction A was found to be rich in Phytoestrogens, fraction B was containing coumarin derivative and fraction C was having tetrahydroisoquinoline and cinnmol derivatives. Coumarin derivatives present in medicinal plants, including grasses, orchids, citrus fruits and legumes. Our phytochemical analysis revealed the presence of coumarin derivatives, which increase the osteogenesis by directly stimulating p38 and ERK dependent pathway (Tang *et al.,* 2008). *Litsea* was also found to contain thiocoumarin derivative in the alkaloid fraction. Due to this alkaloid, probably *Litsea* is having its osteogenic potential. Osthole, a coumarin derivative extracted from many medicinal plants, such as *Cnidium monnieri* and *Angelica pubescens*, has been shown to

exhibit estrogen-like effects and prevent postmenopausal osteoporosis in OVX rats (Li *et al.*, 2002). Kuo *et al.*, (2005) have reported that osthole induced AlP activity through BMP-2-dependent pathway in osteoblasts. As *Litsea* was rich in coumarin derivatives, it showed osteogenic potential in both *in vivo* and *in vitro* studies. Further, when the alkaloids were isolated from the plant, the alkaloid fraction was also found to contain the coumarin derivatives. The heightened osteogenic potential observed in the alkaloid fraction treated group reaffirming the fact that the osteogenic potential of *Litsea glutinosa* might be due to the coumarin present in it. Coumarin rich fraction B also showed osteogenic potential and increased the AlP activity, indicating that this fraction is promoting differentiation of osteoblasts.

Findings from the current study demonstrated that *Curcuma aromatica*, *Litsea glutinosa* and *Moringa oleifera* are potent osteoprotective agents, having positive effect on bone metabolism both *in vivo* and *in vitro*. Of all the three botanicals *L. glutinosa* is having unequivocal effect on bone remodelling *i.e.* it hampers osteoclastic cells as well as the process of resorption and favours osteoblast proliferation and differentiation. Phytochemical analysis suggested that twin effect of the plant in bone remodelling is due to various beneficial phytochemicals present in it. Phytoestrogens, LCPUFA and coumarin like compounds probably promote bone formation process and inhibits osteoclastogenesis, contributing to its osteogenic potential, while various antioxidants and alkaloids are most likely to be inhibiting the resorption process. As this plant had been described for various biological activities, including positive effect on bone remodelling, this thesis justifies its role as an osteoprotective agent, and suggests that intake of this plant may be helpful in preventing osteoporosis.

However, one needs to ponder further to gain insight on to the precise fraction(s) that elicit the desirable pharmacological property and also needs to elucidate the finer molecular mechanisms of action of osteoprotective property of the proposed botanicals before the said fractions *viz.*, phytoestrogens and LCPUFAs are conclusively recommended as therapeutic agent. These challenges were beyond the scope of the present study, nevertheless the following recommendations have been made as a guideline to address these pertinent questions in the time to come.

The probable mechanism behind the osteoprotective effect of *Litsea glutinosa* is thematically represented as follows:



Recommendations for the Future Research

1. Isolated sub fractions need a detailed analysis to understand their role in bone remodelling. Further, these phytochemicals can be explored for their bioactivity in the established OVX model from this study. Once confirmed *in vivo*, these phytochemicals can be explored for their cellular mechanism of action *in vitro* on SaOS2 and FLG 29.1 like cell lines.
2. Osteoblast-osteoclast cross talk is mediated by various paracrine factors like osteocalcin and osteonectin. Exploring the effect of phytomolecules on these factors will help in understanding the mechanism behind bioactivity of the molecule.
3. Determining the signal transduction pathway(s) (OPG-RANK) will give a clear understanding of how the phytochemical alters the maturation and functioning of osteoclastic cells.