GENERAL CONSIDERATIONS

Bone is a dynamic tissue that is remodeled throughout our life allowing us to be one of the most adaptive species. The integrity and activity of bone is controlled by variety of factors, both genetic and environmental, and its dynamicity varies accordingly. Genetic factors govern variety of physiological processes, which control the bone tissue, including various hormones, cytokines and growth factors. Apart from this bone health also depends on the environmental factors, especially that of the serum. Any alteration in these factors like lower serum calcium or phosphate or higher glucose in the blood may lead to altered bone metabolism. Findings of this thesis enlighten this unsolved mystery and prove that hyperglycemia increases bone turn over and it has negative implications on bone health. Though, established previously (Gopalkrishnan et al., 2006), our study puts an insight into this mechanism and shows that hyperglycemia diminishes bone formation while aggravates osteoclastic bone resorption (Chapter I). Furthermore, we also explored MO for its effects on hyperglycemia induced bone loss (Chapter II). But in this study we could not achieve desired results and hence, we decided to continue ongoing studies in our lab where we try to explore few herbals for their osteoprotective effects. Hence, major part of this thesis focuses on exploring MO, for its osteoprotective effect both in vivo and in vitro. We successfully established that MO flower and fruit are having positive effect on bone and they prevented ovariectomy induced bone loss, but leaf, though reported to have an array of pharmacological properties, did not have osteoprotective effect (Chapter III). Exploration of MO components for their phytochemical constituents revealed that this plant is rich in variety of phytochemicals, including anti oxidants and phytoestrogens, which contribute towards its osteoprotective effect (Chapter IV). After establishing the osteoprotective effect of MO in vivo, to understand the cellular mechanism behind its osteoprotective effect we have conducted our studies in vitro, on osteoblastic cells and osteoclastic cells, which are the sole players of bone metabolism. Our findings revealed that MO fruit is having more potent effect in inhibiting osteoclastic cells compared to fruit, while leaf extract had no effect in inhibiting osteoclastic cells (Chapter V). Further, both MO fruit and flower were found to be having positive effect on bone forming cells. However, their mechanism of action was different, where flower extract stimulated osteoblast proliferation whereas fruit extract stimulated their differentiation and bone formation (Chapter VI).
Bone is made up of organic and inorganic materials; organic is chiefly collagen while inorganic is hydroxyapatite mineral. The major constituent is the calcium and phosphate; inorganic ions through which the bone is remodeled, i.e. when the serum runs low on calcium, bone is dissolved to release this mineral in blood and when the serum is having excess calcium, new bone is synthesized, which removes excess calcium. Apart from this primary job of serving as a reservoir for minerals, bone also dose variety of functions, including providing sites for erythropoeisis.

Though serving as a mechanical framework, bone is one of the most dynamic tissues of our body and its dynamicity is attributed to its bone remodeling units (BMU), which enables it to remodel as per the demand of the body. There are three distinctly recognizable forms of bone cells in BMU, namely, osteoblasts, osteocytes and osteoclasts: each has a specific function in the highly ordered sequence of formation, maintenance and removal of bone mineral. Osteocytes are considered as specialized and fully differentiated osteoblasts (Ducy et al., 2000), osteoblasts are described as differentiated fibroblasts. It is the multi-potent mesenchymal stem cells that give rise to fibroblasts, osteoblasts, adipocytes and osteocytes (Aubin, 1998). Compared to these, osteoclasts are hematopoietic in origin and they develop from monocytic cells in the bone marrow (Fujikawa et al., 1996).

Osteoblastic cells are the edifice squad of the bone. They not only form and synthesize the matrix, but also regulate the mineralization and development of mature bone. Bone homeostasis is an orchestral phenomenon dependent on variety of intrinsic and extrinsic factors such as important proteins integrins, connexins and cadherins responsible for cell to cell contact or cell-matrix contact. Other then these varieties of receptors and hormones; cytokines and growth factors are also equally important. This entire assembly controls the function of osteoblasts and regulates the mineralization; maintaining the bone homeostasis (Lecanda et al., 1998; Ferrari et al., 2000). After a long process of this bone synthesis, 90% of the osteoblasts undergo apoptosis, while remaining gets trapped inside the calcified matrix and change their phenotype into osteocytes. Compared to normal osteoblasts they reduce their organelle load and stops bone synthesis. They remain connected with other osteocytes and the osteoblasts which are lining the periostium, creating an extensive network of cells for intercellular communication. This communication is the one that plays a key role in bone regeneration and healing.
Osteoclasts are multinucleated cells, characterized by their ability to resorb fully mineralized bone. Osteoclasts develop from hematopoietic stem cells and they are highly migratory, multinucleated and polarized cells. They carry an armory of acids and enzymes which bring about the resorption of bone (Teitelbaum, 2000). They are unique compared to hematopoietic cells as they carry pleomorphic mitochondria, large number of lysosomes, filled with acids and hydrolytic enzymes and vacuoles (Walker, 1972). At the apical membrane they form tight junction with the calcified bone at the site of resorption; also called as Howship’s lacunae. At this site lytic enzymes are secreted, with lowering of pH by the action of proton pumps, which continuously pump H+ ions the resorption pit. This leads to acidic pH between the range of 2 – 4 and activation of acidic hydrolytic enzymes such as Tartrate resistant acid phophatase (Blair et al., 1989).

Osteoclasts are so energetic in bone resorption that they resorb bone 200,000 more potently then osteoblastic bone synthesis (Sommerfeldt and Rubin, 2001). Hence, bone formed by 7 to 10 generations of osteoblasts is resorbed by one single osteoclast; in its limited life span of 15 – 20 days (Albright and skinner, 1987).

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 (Bonedwald, 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 (Bonedwald, 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-Tate, 2003). This syncitium 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).

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 trans-membranous 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 signaling 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 E2 (PGE2), interleukins, and tumor necrosis factor (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).

Ultimately, the pathologies inherent in bone diseases are reflected by this physiologic remodeling 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. According to Dr. Susan Brown of the Osteoporosis Education Project,
osteoporosis can be seen as a consequence of chronic metabolic acidosis, which robs us of our
mineral reserves and impairs effort to rebuild the bone matrix. Emerging clinical and molecular
evidence suggests 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).

Estrogen 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 of TNF-α is also increased with Estrogen deficiency due to both an
increase in T-cell number and activation (Weitzmann and Pacifici, 2005). Estrogen 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
etiology (Teitelbaum, 2004; Pacifici et al., 1987).

Diabetes is the world’s largest disease involving metabolic disorders of carbohydrate, protein
and fats. According to WHO projections, the number of diabetes if patients is expected to be
doubled to 300 million in the year 2025 (Boyle et al., 2001). Diabetes affects the functioning of many tissues including bone. The mechanism of bone loss in type 1 diabetes is still unknown, although several theories exist based on animal and cellular models. Insulin-like growth factors and other cytokines may influence diabetic bone metabolism (Bullion, 1991). Among many systemic and local factors involved in the regulation of bone metabolism, insulin and insulin-like growth factor-I (IGF-I) are known to play important anabolic roles (Canalis, 1993; Thomas et al., 1997). Patients with insulin deficiency as exemplified by type 1 diabetes are associated with osteoporosis (Krakauer et al., 1997; Piepkorn et al., 1997); those with Laron syndrome caused by IGF-I deficiency also exhibit this condition (Laron et al., 1999). A reduction in IGF-I is also implicated as an important factor in the etiology of involutional osteoporosis, especially of age-related bone loss (Nicolas et al., 1994; Rosen, 1994; Reed et al., 1995; Canalis, 1997). Insulin and IGF-1 signaling is the most important factors in diabetes, but is also important regulators in bone metabolism. Insulin and IGF-1 have anabolic effects on osteoblasts in vitro and an association of diabetes with abnormal bone metabolism has been reported.

Osteoporosis is a bone condition defined by low bone mass, increased fragility, decreased bone quality, and an increased fracture risk (WHO, 1994). Osteoporosis is affected by many factors including genetic and environment. It is also affected by prevalence of other diseases such as diabetes, hypertension and obesity. Although osteoporosis traditionally has not been listed as a complication of diabetes, patients with either type 1 or type 2 diabetes are among those at increased risk for this disease. Type 1 diabetes is an autoimmune disorder resulting in loss of pancreatic insulin-producing β-cells that presents in childhood or early adulthood. Along with increased risk of complications including retinopathy, nephropathy, neuropathy, and cardiovascular events, adults with type 1 diabetes have decreased bone mineral density (BMD) compared with control subjects (Christensen et al., 1999; Kemink et al., 2000). In fact, osteoporosis is the most significant metabolic bone disease in individuals with diabetes (Inzerillo and Epstein, 2004). Patients with Type 1 diabetes have long been associated with low bone density. However, it was unclear until recently whether this translated into increased fracture rates.
The mechanism of bone loss in type 1 diabetes is still unknown, although several theories exist based on animal and cellular models (Meyerovitch et al., 1991; Rossini et al., 1995; Buschard, 1996; Brownlee, 2001; Apfel 2006; Jaiswal et al., 2010). Diabetes had been shown to have significant effect on the onset of osteopenia and osteoporosis. This study was established in both animal and human models. In a study by Locatto and his colleagues (1993) it was reported that bone volume of trabeculae gets affected in alloxan induced diabetic rats. Further, Bain and co-workers (1997) reported that this decrease in the bone volume directly correlates with the increase in glycemic levels. Later it was established that Diabetes not only affects bone volume or quality of bone, but it also decrease bone mineral content and is much more worsened with the progression of glycemic conditions (Verhaeghe et al., 2000). Among many systemic and local factors involved in the regulation of bone metabolism are insulin, insulin-like growth factor-I (IGF-I) and other cytokines (Canalis, 1993; Thomas et al., 1997; Yenush and White 1997; Withers et al., 1999; Kulkarni et al., 1999; Pessin and Saltiel 2000; Rui et al., 2001).

In human patients femoral osteoporosis has often been reported as a complication of diabetes. This is particularly true for type I diabetes mellitus and an even higher frequency is likely if metabolic compensation is poor. Patients with diabetes are at risk for osteoporosis and its complications, including hip fracture (Forsen, 1999 and Nicodemus and Folsom 2001). Femur neck fractures are significantly more common in diabetics than in normal subjects, while histomorphological studies have shown a significant reduction in their bone trabecular volume and number of marrow capillaries. It is felt that vascular factors, especially augmented capillary permeability caused by the subendothelial alterations associated with diabetes, are the main cause of osteopenia. Work on the rat, however, has suggested that diminished bone mass is more a result of metabolic changes rather than of microangiopathy. A negative calcium balance has also been reported in the diabetes of recent origin. This is the outcome of both enhanced urinary calcium loss and depressed intestinal absorption, itself perhaps a consequence of reduced renal synthesis of 1, 25-dihydroxy vitamin D3. By contrast, the calcium balance becomes positive and bone turnover is reduced in the chronically diabetic rat, despite hypercalciuria and hyperphosphaturia. (Hough et al., 1982; Hoskins and Scott, 1984; Anwana and Garland, 1990; Ponde et al., 1990; Riccardi et al., 1995; Al-Quadreh et al., 1996; Hebert et al., 1997; Ward et al., 1998; Betul et al., 1999; Isidro et al., 2010; Steele et al., 2012)
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 \textit{et al.}, 2004; Yin \textit{et al.}, 2004; Vali \textit{et al.}, 2007).

Phytoestrogens are plant compounds which have an 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 an 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 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 Estrogen receptor (Setchell, 1998). Apart from this phytoestrogens are also known to have antioxidant effects and may reduce inflammation markers (Ibarreta \textit{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 \textit{et al.}, 2003; Cotter \textit{et al.}, 2003; De Wilde \textit{et al.}, 2004; Pan \textit{et al.}, 2005a, b; Ge \textit{et al.},
India is sacred with a habitat that harbors variety of botanicals with myriad of biological activities that needs to be explored (Sukh Dev, 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 knowledge and epidemiological evidences, one can hypothesize the effects of hyperglycemia on bone loss. Keeping this hypothesis in mind we explored effects of hyperglycemia on bone health (Chapter I). Further, as it is already reported that MO is having established antidiabetic effect, we explored this plant for its effect on hyperglycemia induced bone loss (Chapter II). As we did not find any desired results, we decided to explore MO for its osteoprotective effect and we explored MO for its osteoprotective effect in vivo (Chapter III). Further to understand the reason behind its osteoprotective effect, we studied this plant for its phytochemical constituents (chapter IV). Once establishing this plant for its potent osteoprotective effect, for understanding cellular means behind its osteoprotective effect, we explored this plant for its in vitro effect, on both osteoclastic cells (Chapter V) and osteoblastic cells (Chapter VI).

Induction of bone loss via ovariectomy in rats has been an established model for studying bone loss and associated osteoporosis and it has been validated by various studies. We further extended this protocol by inducing hyperglycemia in ovariectomized wistar rats. It is a well known fact that diabetes associated with estrogen deficiency may enhance the bone loss. However, the exact mechanism behind this loss is still not clearly understood. The low bone turn over during diabetes and high bone metabolism during menopausal condition may have a contrasting effect on the health of bone during post menopausal diabetic condition. Hence, to understand the complexity of diabetes on bone health, with special reference to post menopausal bone loss, this study was conducted, revealing the effects of hyperglycemia on bone loss.

One of the important features of diabetes is sudden decrease in the body weight. Contrary to this, ovarian function loss leads to weight gain. Similar conditions were observed in our study, where we observed decrease in the body weight of diabetic animals while the ovariectomized animals showed weight gain. However, when the both the effects were combined, the animals showed increase in the body weight. During diabetic complications, there is an increased possibility of
interference with urea and creatinin excretion, the bi-products of amino acid metabolism. It has been very well established that during diabetes there is increase in the serum urea and creatinin levels. Our study further confirmed these reports and also revealed that during post menopausal conditions there are no significant alterations in these parameters. Hence, to further confirm about our results, we carried out SGOT/SGPT and ALP activity. Serum SGOT/SGPT activity showed that diabetes and osteoporosis are two different diseases having unexplainable overlapping effect. It has been very well documented that diabetes affects bone health; however, very less data is available about the effects of estrogen loss on the severity of diabetes. Our study showed that estrogen loss was having very less effect on the onset of diabetic conditions. Further to confirm, we carried out these biochemical parameters in liver tissues, where we observed no significant correlations between the estrogen loss and diabetic complications.

Serum ALP and TRAcP are the most useful markers of studying bone formation and bone resorption, respectively (Alatalo et al., 2000; Janckila et al., 2001). It has been well documented that increase in serum TRAcP is associated with bone resorption and increased osteoclastic activity (Rico and Villa 1993; Nakasato et al. 1999; Halleen et al. 2003). Increased TRAcP levels has been well documented in the blood of patients with osteoporosis (Rico and Villa 1993; Halleen et al. 1996), bone metastases in cancer (Wada et al. 1999), hyperthyroid bone disease (Kraenzlin et al. 1990), and Paget’s disease (Rico and Villa 1993). Our findings supported the previous studies and showed increase in the TRAcP levels in ovariectomized rats. Parallel with these results ovariectomized hyperglycemic animals also showed the increase in the TRAcP activity, confirming the results of previous reporters who showed increased bone resorption due to hyperglycemia. This increased TRAcP indicates increased osteoclasts activity and increased bone resorption. These findings also confirmed the reports of previous workers who have established that ovariectomized rats exhibit increased TRAcP activity (Kawamoto et al. 2002; Manolagas et al. 2002; Gopalkrishnan et al., 2006).

When osteoblastic activity was taken into consideration, we observed increased osteoblastic activity in ovariectomized rats, as expressed by increased ALP activity. Our results are in accordance with previous studies conducted at our lab (Parikh et al., 2009; Rangrez et al., 2011) and with various other co workers who have established that ovariectomy leads to increase in
ALP activity. Interesting results were observed with ovariectomized hyperglycemic animals which exhibited lowered ALP activity; suggesting decreased bone formation. It has been previously reported that IGF-1 levels are reduced in absence of insulin, affecting many physiological processes. However, it was not established about proliferation of osteoblastic cells from pre-osteoblastic cells. Our study confirmed these results and showed that in diabetic condition, there is decreased osteoblastic activity, and associated decreased bone formation. Further confirmation to these findings was offered by bone calcium content measurement, which expressed lower mineral content in the bone of ovariectomized hyperglycemic animals.

In ovariectomized hyperglycemic animals, on one end there is increased osteoclastic activity while on the other hand there is decreased osteoblastic activity. Increased osteoclastic bone resorption may be attributed to increased production of various cytokines, as established by previous studies. In addition, it has already been established long back that STZ induced diabetic rats show decreased osteoblast and number and reduction in production of osteoid (Goodman and Hori 1984; Kawaguchi et al. 1994). Recent studies by Botolin and Mcabe (2007) proved that insulin absence leads to suppression of osteoblastic markers, with relative IG-1 deficiency, leading to poor bone mass and bone mineral density. Hence, on one end where the diabetic condition is leading to more vigorous osteoclasts which increase the bone resorption, at the other end, probably because of the absence of IGF-1 and other growth factors, osteoblastic cells are no more able to keep up with increased bone resorption. This imbalance in the osteoblast and osteoclasts activity leads to demolition of the bone. This is the probable reason why we observed worsen bone health in case of ovariectomized hyperglycemic animals, where the bone mineral content was also reduced.

Supporting these results were the findings, histological results which showed more damage to bone in ovariectomized hyperglycemic animals. Parallel with the previous findings we observed damage to liver and kidney in diabetic animals. In diabetic animals the kidneys were found to be exhibiting the phenomenon of glomerular congestion. Our study confirmed the findings of previous studies and showed that hyperglycemia affects glomerulus, damages the parenchyma and leads to vacuolar degeneration of tubular cells. Liver is an important site for metabolism and storage of glucose. Diabetes and associated hyperglycemia is established to have catastrophic
effects on liver health. In our study, we observed heptocellular necrosis in diabetic animals, along with leucocyte infiltration and hemorrhage. However, the histological observations of ovariectomized hyperglycemic individuals were not clear, as we observed cell vacuolation and disruption of cords of hepatic cells in these animals. Similarly in kidney glomerular congestion and associated degeneration of tubules were observed. In liver the ovariectomized hyperglycemic animals were having less intensity compared to diabetic group. However, kidney exhibited more damage in ovariectomized hyperglycemic group compared to diabetic group. These results were confusing and further studies are required to understand these changes and their correlation with hyperglycemia and estrogen absence.

In ovariectomized animals we observed thinning of bone trabecules and loss of bone marrow. Previous studies by Shaker and his colleagues (2005) have also shown similar results and proved that ovariectomy leads to bone loss and maximum damage is seen at the bone trabecules. It is now well established that estrogen plays its role in bone metabolism through secretion of osteoprotegrin. In absence of estrogen, there is aggravated osteoclastogenesis, leading to more bone resorption. Because of the lack of clear data, there is still no clear picture available regarding the possible mechanism behind the loss of bone in hyperglycemic individuals. Our study tries to justify the possible mechanism proposed behind the aggravated osteoporosis in diabetic individuals. Our study showed that hyperglycemia affects bone metabolism, reduces bone formation and promotes bone resorption, leading to net bone loss. However, on the counter side, loss of estrogen was not found to be having any significant effects on the markers of diabetes. Further studies are needed to understand the connection of these two diseases and develop strategies for their cure.

As it was not possible to continue the study of understanding the effects of these two diseases, we tried to find a cure for these diseases prevailing common individual. We used the model of hyperglycemic OVX rats. This study demonstrated that supplementing ovariectomized animals with MO flower and fruit extract of MO can have positive effect on bone health. In our study, MO flower and fruit had non-significant effect in reducing the weight gain. Maximum weight reduction was observed with leaf extract treatment, which may be due to presence of alkaloids in leaf of MO reported previously. Ovariectomy induced absence of sex hormone initially affects
the uterus, leading to uterine atrophy and decrease in the uterine weight. We also observed similar results where the uterine weight was decreased in ovariectomized animals. MO components treatment had no significant effects on the uterine weight, suggesting that constituents of this plant had no estrogenic potential. Our results we parallel with the results of previous scientists who reported that few osteoprotective plants do not stimulate uterus, but still have estrogen like effect on the bone (Roveri et al., 2000). In a similar study conducted by Xie and his co workers (2005) showed that plants may ameliorate ovariectomy induced changes on the bone, without affecting the uterus. Our results were also parallel where it was found that MO extract does not affect the uterus, but it potently inhibits the progression of osteoporosis.

Moreover, in this study, it was noted that following ovariectomy there is dramatic decrease in the serum calcium levels, while there is increase in the excretion of calcium. This is because of the excessive bone resorption as reported by Zhang and his team in 2006. Our study also showed similar results and further when excretory rate was considered, it was observed that calcium excretory rate is very high in ovariectomized animals. Probably this high excretory rate affects the overall calcium metabolism and negative calcium metabolism leads to further progression of the disease. MO plant ameliorated these high excretory rate, and these results were similar with Kim and his colleagues who proved that agents with osteoprotective effect are having positive effect on calcium metabolism. They also established that opposite to serum calcium profile there is increase in serum phosphorus, which is an indication of aggravated mineral metabolism. We also included this parameter in our investigation and showed that all MO components are having positive effect on mineral metabolism.

To investigate the mechanism of action, we evaluated enzyme profile as well. Previous studies in our lab have established that bone is maintained through coordinated activities of osteoblasts and osteoclasts and ovariectomy disrupts this balance, leading to over-activity of osteoclasts which is expressed by elevated TRAcP, followed by osteoblasts which express AIP (Parikh et al., 2009). After a lag phase, osteoblasts are not able to keep up with the pace of osteoclasts, leading to an imbalance, where the bone is resorbed much more then it can be synthesized. Shevde and co workers (2000) have proved that post ovariectomy rise in TRAcP is because of the disruption of the molecular mechanism of RANK, RANK L and osteoprotegrin pathway which governs the
osteoclasts recruitment and activity. And as the bone formation is directly in response to bone resorption, there is also associated increase in the ALP profile. We observed similar results and also observed that MO flower and fruit extract are having potent effect on both the markers. This parallel fall in both the markers clearly suggest that both flower and leaf ameliorate the damage caused by estrogen deficiency. To confirm that the elevation in serum ALP levels is because of bone remodelling only, we had estimated liver ALP levels also, which showed no significant variation between the plant treated group and control group. Further investigation in bone tissue profile showed parallel results with serum profile and showed that both fruit and flower of this plant are having potent effect on the changes induced by ovariectomy. Furthermore, histological observations also showed that ovariectomy induced damage to histoarchitecture of the bone and the damage was severe in cancellous bone. This may be because of the excessive bone resorption that is occurring due to aggressive osteoclastic cells which resorb the bone and on the counterpart the osteoblasts are not able to keep up with resorption. In flower and fruit extract treated animals, these changes were reversed, with a healthier bone trabecules and lesser sites for bone resorption. Though leaf extract also showed some effect in histology, from the overall results it was concluded that leaf extract is having moderate osteoprotective effect.

It has been established that phytochemical profile of MO leaf, flower and fruits are different and they exhibit different pharmacological property. Various studies have proved that both flower and fruit are having a milieu of various pharmacological agents which are having anti inflammatory activity (Aguwa and Nwanko, 1988; Ezeamuzie 1996). Proliferation and maturation of osteoclasts follows a pathway that involves variety of inflammatory cytokines. As the fruit and flower are having robust anti inflammatory activity, they might be directly affecting the inflammatory pathways of development of osteoclasts. In a recent study by Penolazzi and his team (2008) proposed that plants with potent anti inflammatory activity are having osteoprotective effect. Our results also supported his hypothesis and showed that MO flower and fruit are having osteoprotective effect. Furthermore, an array of studies have proved that both flower and fruit are rich of flavinoids and other phytochemicals which are having potent anti oxidant property (Bharali et al., 2003; Kumar and Pari, 2003; Bajpai et al., 2005; Pari et al., 2007; Owusu-Ansah et al., 2011). Osteoclastic bone resorption involves secretion of variety of digestion enzymes and acids. These antioxidants probably serenes the bone resorption process.
and adds to the osteoprotective effect of this plant. Findings of the chapter 3 revealed that of all the three components of MO, leaf, flower and fruit; both flower and fruit are having positive effect on the ovariectomy induced bone loss. As both these components are having an array of phytochemicals with anti inflammatory as well as antioxidant effect, consumption of this plant may be helpful in preventing postmenopausal osteoporosis.

Abalaka and co-workers (2012) in their study have proved the antibacterial potentials of *M. oleifera* leaves, and have opined that it could be probably due to the presence of the broad spectrum bioactive compounds. Further they have also suggested that the *M. oleifera* can be a promising natural anti microbial agent with potential applications in pharmaceutical industry. Our study is in support with these workers and showed that leaves of this plant are rich in quinoline derivatives (Bennette *et al.*, 2003). Rastogi and his co-workers (2009) reported the leaves of MO to be having anti helminthic activity, with reference to piperizine citrate. Chapter 4 justifies his finding and reveals that leaves are having piperizine, which are probably the reason behind its anti helminthic activity. Faizi and his team (1994) reported two new glycosides from this plant, which was in accordance with our findings. Apart from this, there were also reports of variety of anti oxidant phenols and glycosides which contribute for the anti inflammatory activity of this plant (P. Siddhuraju & K. Becker; 2003; Nikkon *et al.*, 2003). Our study was in accordance with these findings and showed that this plant was having the presence of various phenols and glycosides. In addition we have reported various adenosine derivatives in leaf extract, which play a key role as an intra cellular messenger as well as an instant source of energy (ref). Our study not only showed the presence of alkaloids, but also indicated the presence of a very important alkaloid, ergolin, which is having variety of anti oxidant effect including against the neuronal damage (Mizuno *et al.*, 2005).

It has been well documented that coumarin, cinnamic acid and other polyphenols have potent anti oxidant and anti cancer property (Atawodi *et al.*, 2010; Vijayalakshmi *et al.*, 2010). Our study supported these findings and showed that anti oxidant activity and anti tumor activity established previously is because of the presence of various polyphenols and anti oxidants present in it. Presence of coumarin in a botanical also favors its usage as a bone health promoting agent, justifying the use of this plant in improving bone health. In addition, our finding also
showed the presence of tetrahydroquinoline, a potent and proven antioxidant with variety of pharmaceutical activities. Apart from having varied pharmacological activity, recently, cinnamic acid had been proven to have osteoprotective effect as it stimulates bone formation and inhibits bone resorption in rat femoral tissues (Lai et al., 2006). As MO is rich in cinnamic acid, probably its osteoprotective efficacy is enhanced by the presence of cinnamic acid like constituents.

An informative historical account of research in the phytochemistry of *M. oleifera* is well documented by Saleem (1995). Ever since after that, the research has been expanded and refined, not only on the chemical structures of plant molecules, but also on their nutritional and medicinal properties. Of major medicinal interest are three structural classes of phytochemicals: glucosinolates, flavonoids, and phenolic acids (Saleem, 1995; Bennett et al., 2003; Lako et al., 2007; Manguro and Lemmen, 2007; Coppin, 2008; Amaglo et al., 2010; Kasolo et al., 2010). Our study supported the findings of these previous workers and added that components of MO are also rich in various sugars which also contribute towards the nutritional role of this plant. Furthermore, our study is the first to report the presence of quebrachamine from this tree and no previous reports are available reporting the presence of this alkaloid from this plant.

Flavonoids are widely distributed in plants fulfilling many functions. They have been shown to have antifungal activity in vitro (Galeotti et al., 2008). The potent antioxidant activity of flavonoids reveals their ability to scavenge hydroxyl radicals, superoxide anions and lipid peroxy radicals; this may be the most important function of flavonoids (Alan and Miller, 1996). Many recent studies have also established that flavonoids are also responsible for osteoprotective effect of many pharmacological agents. The presence of flavonoids at all stages of maturity of MO may be responsible for the medicinal qualities accorded the leaves, flower and fruit. Recent studies have also proved that flavonoids are having anti osteoclastic activity and their consumption may be helpful in preventing osteoporosis. As this plant is rich in flavinoids and other anti oxidant, it justifies our previous studies which showed in vivo osteoprotective effect of this plant (CHAPTER 2 and 3). A recurring explanation for the therapeutic actions of MO medication is the relatively high antioxidant activity of its leaves, flowers, and seeds (Chumarket et al., 2008; Sreelatha and Padma, 2009; Verma et al., 2009; Atawodi et al., 2010).

Saponins were also detected in MO and they have been shown to possess both beneficial
(cholesterol lowering) and deleterious (cytotoxic; permeabilization of the intestine) properties (Price et al., 1987, Oakenful and Sidhu, 1989). Although some saponins have been shown to be highly toxic under experimental conditions, acute poisoning is relatively rare both in animals and man (Osagie, 1988). Studies have illustrated the beneficial effects on blood cholesterol levels, cancer, bone health and stimulation of the immune system. Hence, one can speculate that osteoprotective effect of MO, we have shown, is may be because of a set of useful phytochemicals this botanical harbors.

Steroid’s presence in any botanical is of great importance as they are of interest in pharmacy due to their relationship with such compounds as sex hormones. Steroids increase protein synthesis, promoting growth of muscles and bones. Our interest was more in the presence of steroids because lately various studies have established that plants with phytoestrogens are having positive effect on bone (Rangrez et al., 2011; Xang et al., 2002). Our study is the first to report androstan derivatives in the flower extract of this plant. Presence of these steroids in flower extract also supports our previous study which showed osteoprotective effect of flower extract. Further the plants components were found to be rich in various PUFA, which are recently shown to have beneficial effect on bone health (ref). Furthermore, the plant components were also found to be rich in polyphenols, which are long established as antioxidants and anticancer agents (Hollman, 2001).

In general, the presence of these Phytochemicals could account for the much touted medicinal properties of these leaves in various disease conditions such as atherosclerosis, arthritis, diabetes, nausea, asthma, skin antiseptic, diarrhea, dysentery, colitis and cancer. Because of the chemical complexity of the MO, one individual phytochemical cannot be given the credit for its pharmacological property. Some compounds may be collectively affecting broad aspects of physiology, detoxification mechanisms, reducing the stress and re-supplementing the lost hormones such as phytoestrogens. However, further research is needed to isolate pure compounds out of this botanical and explore their molecular mechanism behind their pharmaceutical property.

Furthermore, in chapter 5 we have shown that osteoprotective effect of MO presented previously is mainly attributed to its osteoclast inhibiting property. Of all the components of MO, maximum
effect was observed with fruit extracts. Osteoclastic bone resorption is thought to be mediated by two different processes: one is the formation of new osteoclasts, and the other is the resorption activity of osteoclasts. It was reported that bone resorbing agents such as VitD₃, PTH, and IL-1 markedly stimulated the formation of osteoclasts (Qin et al., 2003). Vit D₃ is thought to stimulate osteoclast formation by a common mode involving prostaglandin E₂ (PG-E₂), which is also found in osteoporosis (Akatsu et al., 1992). Hence, in this coculture system, conditions were mimicked to that of natural onset of osteoporosis and we tried to explore the osteoprotective effect of MO in this system.

Of all the fractions of MO, fruit extract reduced the osteoclastic resorption in a dose dependent manner and it was most significant in high doses after 48 hours of treatment. Parallel with these results were the results of calcium release; one of the important indicators of resorption. Fruit extract also inhibited calcium release, supporting its role as an anti-resorptive agent. Though flowers also showed some effectiveness in lowering these markers of resorption, its potency was quite low compared to fruits, which was evident from the statistical significance of the data. Development of osteoclasts also depends on various paracrine factors secreted from osteoblastic cells and they are also associated with bone formation. Hence, those agents who are exerting their effect on osteoblastic cells may affect the osteoclastic activity. Hence, to explore whether MO components are directly exerting their effect on osteoclastic cells or not, we carried out ALP activity as well, which is an established marker of osteoblast activity. ALP activity, showed no significant variation, with any of the components of MO, suggesting that MO components target osteoclastic cells.

Results of SEM analysis further boosted our hypothesis and showed that MO fruit has direct effect on osteoclastic resorption. MO potently inhibited the resorption and significantly reduced the area of resorption to only 6 % in high dose group. Histology and image analysis also confirmed these results, showing that the number and size of resorption pits formed were much less in MO fruit extract treated groups and least was found in 400 µg/ml plant treatment group. MO treatment not only reduced the number of osteoclastic resorption pit, but it also reduced the intensity of resorption. Results of the co-culture studies clearly proved that MO fruit is having
significant osteoclast inhibiting activity as it ameliorates the markers of osteoclastic resorption, as well as it reduces the bone resorption in a dose dependent manner.

When the findings of the studies were summarized, it justified the previous reports (Burali et al., 2010) of osteoprotective activity of fruits of MO. Further, it gave first insight into the cellular mechanism behind the osteoprotective activity of this plant and showed that it is mainly attributed to its osteoclast inhibiting property. Though it had no osteoblast stimulating potential, it was safe to osteoblastic cells. This study offers a first understanding about the cellular means behind the osteoprotective effect of these plant components. MO fruit significant prevented the osteoclastic resorption, decreased the TrACP activity, cold calcium release and prevented the bone slices from induced resorption by osteoclast like cells. MO flower extract also exhibited similar profile, but it was not comparable to the efficacy of fruits. Leaves of the MO did not show any pharmacological effect in inhibiting or stimulating osteoclastic cells. These results confirm that MO fruit has potent osteoclast inhibiting property and supports its ethnobotanical usage as an osteoprotective nutritional plant. Contrary to this, MO had non-significant effects on osteoblastic cells.

As the counter side of bone resorption is bone formation, we explore that bone forming potential of MO on osteoblastic cells SaOS 2 in chapter 6. We have shown that MO flower and fruit extract are having osteoblast stimulating property. Of all the three components of MO, fruit and flower extract were having significant osteoblast proliferating and stimulating property. Our data provided first evidence about the cellular means of the osteoprotective effect of this plant observed in previous studies. It has already been established that MO is having positive effect on bone and it protects bone from ovariectomy induced bone loss (Burali et al., 2010). In a recent study by vali and coworkers (2007) it was shown that certain flavinoids can have positive effect on bone nodule formation in vitro. As MO is rich in certain flavinoids; they might be the one playing a key role in stimulating osteoblastic cells. In the previous studies (Burali et al., 2010) where MO fruit extract was shown to be having positive effect on bone loss; it was also shown to be having positive effect on calcium balance. Though various studies have established the role of MO fruit extract on different cell types; very few studies have been conducted till now on flower extract. In this study, we also discovered that MO flower is not only increasing the activity of
osteoblastic cells; it also promotes the osteoblastic cell division, making it a more potent osteoprotective agent. At higher doses where MO fruit extract showed lower activity, flower extract was found to be having dose dependent activity on all parameters considered for this study. To our knowledge, this study is the first to explore various components of MO for their osteoprotective effect \textit{in vitro} on osteoblastic cells SaOS 2.

Bone formation is a 3 staged process, namely; proliferation of osteoblasts; secretion of extract cellular matrix by osteoblasts and mineralization (Aubin, 1998). ALP activity is an established marker of osteoblast activity. We observed that MO flower and fruit extract treatment increases the ALP activity. This rise in the ALP activity with herbal treatment indicates that MO extract differentiates osteoblastic cells towards the differentiated bone forming phenotype. Of all the three components, leaf extract did not show any rise in ALP activity. Compared to leaf extract, both flower and fruit showed positive effect on this osteoblastic function marker. However, flower was found to be less potent in stimulating ALP activity compared to fruit. But when this data was considered with MTT assay, it showed contrasting results where fruit extract was found to be promoting osteoblast cell division. Hence, by combining this two results, one develops an understanding that MO fruit extract increases the number of osteoblastic cells, favoring their proliferation, while fruit extract not only increases the number but also stimulates them to undergo differentiation into mature bone forming cells. Hence, both the extracts, though favoring bone formation, their mechanism of action was different, as flower helps to increase the osteoblast number, while fruit increases its activity and mineralization as well.

It is an established fact that osteoblast number or ALP alone does not account for the bone formation. Hence, we used both calcium and hydroxyproline estimation to confirm the osteoblastic activity. Calcium is the chief constituent of the bone mineral hydroxyapatite, while hydroxyproline is an important amino acid required for the formation of collagen; constituent of the bone matrix. Our results, however, showed positive activity on both the parameters, suggesting that these plant components are having positive effect on bone osteoblastic cell growth, their activity, synthesis of bone matrix and its mineralization.
Our study showed that hyperglycemia escalates osteoclastic bone resorption and diminishes osteoblastic bone formation, resulting in net bone loss. Due to this, patients with diabetic complications may suffer from troubled bone health. Patients suffering from these both complications might have more severe osteoporosis. Consumption of MO during these complications might be beneficial to overall health of the patient. Further, MO fruit and flower are having positive effect on bone health and their consumption might be helpful to postmenopausal women as it contains an array of phytochemicals which not only decrease bone resorption, but also strengthens bone by increasing osteoblastic bone formation. This plant is having both nutraceutical and pharmaceutical potential on bone health as well as glycemic control and consumption of this plant may be helpful in fighting these complications. However, our study was pioneering in this applied field and further explorations are needed to understand the entire mechanism. Hence we suggest following recommendations for future study:

1. **Isolation and exploration of phytochemicals of MO flower and fruit, which have common effect as anti diabetic and anti osteoporotic agent.**
2. **Exploration of flavonoids of MO flower for their osteoblast stimulating potential.**
3. **Exploration of phytoestrogens of MO fruits for their effect on osteoclastagonesis and bone resorption.**