CHAPTER-5
DISCUSSION

The present study was aimed to study two commonly occurring plants *Lepedium sativum* and *Pinus roxburghii* for pharmacognostical, phytochemical and pharmacological screening of the extracts for antiosteoporotic activity in ovariectomized female Wistar rats.

Therapeutic efficacy of medicinal plants depends upon the quality and quantity of chemical constituents. Once raw material is converted to an extract, the morphological characters are lost and thus botanical and taxonomical identification becomes impossible. This leads to medicinal extracts being susceptible to adulteration and substitution. Therefore, in order to reduce such deliberate malpractices, phytoequivalence of extracts need to be maintained so that uniform therapeutic efficacy could be maintained. Pharmacognostic evaluation aims in identifying the botanical identity of the crude drugs and using methods such as HPTLC and HPLC to evaluate the phytochemical content of the extracts using marker compounds thereby defining the quality of raw material and extracts of crude drugs for medicinal use.

Pharmacognostic evaluation of the selected plants was carried out. Morphological studies confirmed the identity of the selected plants. The botanical identity of both the plants are well reported which were further confirmed by the microscopical characteristics (Fig. 4.1; Fig. 4.2). The physico-chemical quantification has been reported for the first time. The swelling index of the seeds of *L. sativum* was found to be almost equivalent to seeds of *Plantago ovata* (Isabghula) while the foaming index was found to be exceptionally high in the leaves of *P. roxburghii*. The other parameters assessed were ash values and extractive values of the plant parts to be used for further study (Table 4.1). These standardization parameters may provide useful information for future investigations. Preliminary phytochemical screening revealed the presence of flavanoids, phenolic compounds and saponins in the barks of *Pinus roxburghii* (Table 4.2). A resinous matter was obtained from the Pet.ether extract which upon distillation produced volatile oil. The needles contained high amount of saponins with a consistent froth for more than two days. The volatile oil was extracted from the fresh needles in the month of December by hydrodistillation was subjected to GC-MS analysis. The volatile principles were chiefly 3-carene, caryophyllene and α-pinene. Thunbergene also known as cembrene was reported for the first time in *P. roxburghii*,
although it has been well reported in various European species such as Pinus pinaster. Majority of the constituents were found to be monocyclic, bicyclic and tricyclic monoterpenoids (Fig 4.7; Table 4.8). Two pharmacologically relevant phytoconstituents of the Pet.ether and methanolic extracts of the seeds L.sativum were analysed quantitatively by HPTLC method. The Pet.ether extract contained 0.532% w/w of β-sitosterol and the methanolic extract contained 1.3% w/w of rutin respectively (Fig.4.5; Fig.4.6). Further, quercetin was isolated from the ethylacetate fraction of the methanolic extract of the bark of P. roxburghii (Fig.4.9-4.12). Presence of saponins caused high froth formation which made the isolation procedure difficult. The problem was solved by acetylation of the ethyl acetate extract with acetic anhydride and few drops of sulfuric acid. Acidic condition is used for aromatic compounds, while alkaline condition is used aliphatic compounds. Acetylation causes the otherwise polar hydroxyl group to convert into non polar acetate groups. Quercetin contains five hydroxyl groups and upon exhaustive acetylation converts into a non polar quercetin penta-acetate. Being non polar, when the end product is poured in ice cold water, quercetin penta-acetate separates out as white precipitates. Further purification was carried out by recrystalization in chloroform. Quercetin penta-acetate was conveniently converted into quercetin using ammonia and dil. HCl. Thus quercetin was isolated for the first time from the bark of P.roxburghii. The method employed for the purpose in an easy method to obtain large amount of high grade quercetin and could be further exploited commercially. Further HPLC analysis of methanolic extract of the bark of P. roxburghii revealed the presence of 25.936% of free quercetin thereby, revealing P. roxburghii as a potent medicinal plant less known till date.

Rutin is the glycosidic form of the aglycone quercetin, a potent flavonoid. Both β-sitosterol and quercetin are known to be phytoestrogens. Phytoestrogens are defined functionally as substances derived from plant sources that promote estrogenic actions in mammals and are structurally similar to mammalian estrogen 17β-estradiol (E2). Phytoestrogens exhibit a diverse biological activity as estrogen agonists and as estrogen antagonist. As estrogen agonists, phytoestrogens mimic endogenous estrogens and cause estrogenic effects. As estrogen antagonists, they may block or alter estrogen receptors (ER) and prevent estrogenic activity, causing antiestrogenic effects (Brzezinski and Debi, 1999).

As estrogen agonists and antagonists, quercetin can also be classified as biological selective estrogen receptor modulators (SERMs). SERMs are non-steroidal chemicals with a similar structure to E2 and an affinity toward estrogen receptors (Riggs and Hatmann, 2003).
They are unique in that they can function as agonists or antagonists depending on the tissue, ER and concentration of circulating endogenous estrogens (Gruber et al., 2002). Tamoxifen and raloxifene are well-known synthetic SERMs. Tamoxifen has been used in clinical practice for breast cancer patients because it acts as an estrogen antagonist in breast tissue, slowing cancer cell proliferation and an estrogen agonist in bone tissue and in the cardiovascular system to prevent osteoporosis and heart disease. However, tamoxifen has shown estrogenic activity in the uterus and therefore may increase the risk of endometrial cancer (Poulet et al., 1997; Pukkala et al., 2002). Phytoestrogens are able to interact with enzymes and receptors, and because of their stable structure and low molecular weight they can pass through cell membranes (Adlercreutz, 1987). Mechanistically phytoestrogens have been shown to bind to two types of estrogen receptors: estrogen receptor α (ERα), which was cloned in 1986, and estrogen receptor β (ERβ) cloned in rats (Kuiper et al., 1996) and in humans (Mosselman et al., 1996). The two receptors differ in their tissue distribution and affinity to ligands, yet there is some overlap. In rats, ERα and ERβ both are clearly expressed in ovary and uterus tissue (Kuiper et al., 1997). ERβ has been shown to have ligand specificity toward phytoestrogens and is distributed in humans in ovary, spleen, testis and thymus tissue (Mosselman et al., 1996) and in rats in bladder, brain, lung, ovary, prostate, testis and uterus tissue (Kuiper et al., 1997). Phytoestrogens show a lower binding affinity than E2 and some show a higher binding affinity for ERβ than for ERα, which may suggest different pathways for their actions and explain tissue specific variability of phytoestrogenic action (Kuiper et al., 1998; Setchell, 1998). The complexity of phytoestrogens and ERs appears to be further compounded because different transcriptional activities in vitro are activated depending on the ligands, as well as the environment of the promoter region of specific genes for translated ERα and ERβ receptors (Paech et al., 1997).

Phytoestrogens stimulates osteoblastic activity through an estrogen receptor mediated action (Choi et al 2001) or by increasing the production of insulin-like growth factor-I (IGF-I) (Arjmandi et al 1998). It is known that IGF-I enhances osteoblastic activity (Sugimoto et al 1997) and positively affect bone mass in postmenopausal women (Boonen et al 1996). In continuation to the identification of the present phytoconstituents in the seeds of L. sativum and P. roxburghii, antiosteoporotic activity of the said plants were carried out in vivo in ovariectomized female Wistar rats. For the purpose of the study, petroleum and methanolic extracts of seeds of L.sativum and methanolic extracts of needles and bark of Pinus roxburghii were used for assessing the antiosteoporotic activity at dose levels of 100 and
200 mg/kg. The bioassay was conducted on female ovariectomized rats and results were evaluated by comparing the selected plant extracts against standard drug Tamoxifene (1mg/kg), a selective estrogen receptor modulator (SERM) used clinically in the management of post menopausal osteoporosis.

Osteoporosis is a disease characterized by low bone mass and microarchitectural deterioration of bone tissues, leading to enhanced fragility and consequent increase in fracture risk that result in fractures with minimal trauma. Both estrogen and dietary calcium deficiencies are important risk factors in the pathogenesis of osteoporosis. Post-menopausal osteoporosis is considered to result from ovarian exhaustion. Age related bone loss is greatly accelerated in women after the menopause and women lose approximately 30% of their cortical bone during their life time (Ettinger et al., 1985; Nuki, 1988).

Herbal drugs have shown to possess many therapeutic properties. Plants have been known to constitute plethora of phytochemicals. Crude extracts contain such phytochemicals which provide amelioration of general health by the synergistic action. Treatment of chronic diseases may prove to be challenging as prolonged use of single constituent can prove to be toxic in due course of time.

The ovariectomized model in female Wistar rats share many common characteristics to post menopausal bone loss in humans, such as increased bone turnover, bone resorption exceeding bone turnover resulting in micro-architectural deterioration of bone mass. These similarities thus provide strong basis for ovariectomized rat as a suitable model for the study of prevention and treatment of post menopausal osteoporosis (Kalu, 1991). Though the molecular mechanistic basis of the role of estrogen has not yet been well elucidated, but its therapeutic role has been well documented as an inhibitor of bone resorption.

The end results of the study were assessed by three major parameters: biochemical, biomechanical and histopathological. The study was carried out for a period of one month. Healthy female albino Wistar rats in the age group of 70-90 days were selected. Extracts of both the plants: Petroleum ether extract of *L. sativum*, methanolic extract of *P. roxburghii* (needles and bark) were assessed for antiosteoporotic activity based on three parameters at doses of 100mg/kg and 200mg/kg. Acute toxicity studies were performed on the selected extracts of both plants and found to be safe with LD50 greater than 5000mg/kg. Since the experimental model is a chronic model for a time period of 30 days, hence the doses were restricted to 100 and 200mg/kg.
The antiosteoprotic potential of the selected extracts were evaluated in comparison with the standard drug Tamoxifene (1mg/kg) and control (ovariectomized untreated control and sham control) groups. A sham group was also incorporated in which the ovaries were exposed and placed back and sutured. The sham group represents experimental control. Animals that were subjected to surgical removal of ovaries showed marked changes in biochemical, biomechanical and histopathological parameters. In all the parameters that were assessed, the sham group exhibited similar effects compared to control group suggesting that sham operated animals did not produce osteoporotic condition after 30 days.

Biochemical assays for the skeletal metabolism hold great importance. The biochemical parameters reveal changes in bone metabolism much earlier, than the other methods. They are valuable tools to evaluate the risk of accelerated bone loss in postmenopausal subjects. Their potential in monitoring the changes in bone resorption and bone formation which would ultimately help in predicting risk of osteoporosis related fractures (Shukla et al., 2013). These tests are faster and more specific. Serum and urine parameters are the commonest tests employed in the investigation of osteoporosis. The biochemical tests were conducted in serum and urine samples collected 30 days post ovariectomy. Serum parameters included: Tartarate Resistant Acid Phosphatase (TRACP), Alkaline Phosphatase (ALP), Calcium (Ca) and Inorganic phosphate (IP), while hydroxyproline content was evaluated in urine.

The process of resorption occurs after the attachment of osteoclasts to the bone surface and follows the secretion of acid and enzymes into a space created between the osteoclast and the bone. The acidic environment is produced by the action of carbonic anhydrase and an H-ATPase proton pump. TRACP, one of the enzymes secreted into this space located in the adjacent osteoclast membrane (known as the ruffled border. The amount of ruffled border and the expression of enzymes, including TRACP, are increased when resorption is increased. Therefore, increase in TRACP in serum directly correlates with bone loss due to resorption (Raisz, 1992; Minkin, 1982). In the study the TRACP levels in the OVX group was found to be markedly elevated due to increased bone resorption. Conversely, in control and sham operated groups the TRACP levels remained significantly low compared to OVX group as in both the groups there no development of osteoporotic condition. SERMs have been clinically proved to reduce bone resorption which was well correlated with decreased serum TRACP levels in animals treated with Tamoxifene. The effect of serum TRACP on the plant extract treated animals showed decrease in serum TRACP levels owing
to decrease in bone resorption. The protective effect against bone resorption in ovariectomized rats treated with methanolic extract of *L. sativum* at 200mg/kg dose was similar to those treated with Tamoxifene, while reversal of ovariectomy induced bone resorption effects was observed in group treated with methanolic extract of *P. roxburghii* at 200mg/kg (Fig.4.14).

Serum alkaline phosphatase (ALP) is one of the most widely used markers for osteoporosis. It is produced by osteoblasts to provide a high phosphate concentration at the osteoblast cell surface during bone mineralization and is a marker of bone formation. It is found in elevated levels in osteoporosis due to decreased bone formation and subsequent release in the blood. A significant high increase in the serum ALP in the ovariectomized rats absence of any treatment compared to control and sham operated is suggestive of development of osteoporotic condition characterized by increased bone turnover due to decreased osteoblastic activity. All the treated animals showed a significant decrease in bone turnover compared to OVX group. The protective effect of higher dose treatment with methanolic extract of *L. sativum* seeds and *P.roxburghii* bark was maximal with therapeutic efficacy significantly similar to Tamoxifene (Fig.4.15).

Maintaining serum calcium levels is a dynamic process. Free calcium is usually 50% of total calcium is the biologically active form. The calcium in the blood is filtered continuously through the kidneys. Bone is the main repository of calcium and its active resorption in osteoporosis cause increase in the serum calcium. However, homeostatic mechanisms regulated by calcitonin and parathyroid hormone (PTH) keeps serum concentration maintained in the serum by increasing its excretion in the kidneys. Paradoxically, low calcium intake in the diet may result in calcium compensation by the bones resulting in development of osteoporosis. In this study, the serum calcium levels did not change significantly. Similar finding was also observed in various other ovariectomized models where calcium homeostasis caused consistent levels of serum calcium in OVX, sham and treated groups (Lee et al., 2011) (Fig.4.16).

Calcium and Phosphorus are major minerals constituting bone mass. Phosphorus in the body is present in the form of phosphates. Both calcium and phosphate are deposited in the bone and are also resorbed together. The maintenance of phosphate levels in the body is also closely related to that of calcium. Phosphates act as major buffers maintaining physiological pH. Phosphate homeostasis is primarily controlled by renal excretion. In
ovariectomized rats, the phosphate economy is clearly visible in OVX and treatment groups. The results were correlated with other studies involving ovariectomized rats in the evaluation of antiosteoporotic activity of therapeutic agents (Lee et al., 2011) (Fig.4.17).

Bone resorption reflect osteoclastic activity or collagen degradation. Calcium and phosphates level in the serum are regulated by hormonal and renal excretion which maintain the homeostatic levels. However, hydroxyproline found mainly in collagen, a modified amino acid derived from proline by a post translation hydroxylalation occurring within the peptide chain. Free hydroxyproline is liberated from the breakdown of collagen and is not reutilized in the collagen biosynthesis. About 90% of the endogenous hydroxyproline is released by the breakdown of collagen during bone resorption gets degraded into free amino acids and cabolises into urea and carbon dioxide in liver. The remaining 10% pass through the glomerulus and gets excreted in the urine without any further metabolic alterations. Since collagen is majorly found in the bone, determination of hydroxyproline provides a useful parameter in the determination of bone resorption. Dietary factors (gelatin containing food) also influence hydroxyproline levels, therefore animals were put to overnight fasting to eliminate the effect of dietary hydroxyproline.

The urinary hydroxyproline levels in the untreated ovariectomized animals was found to be significantly elevated compared to sham operated group. All the treatment groups showed decrease in urinary hydroxyproline against OVX group. Methanolic extract of *L. sativum* and *P. roxburghii* (bark) at dose levels of 200 mg/kg effectively reduced bone resorption (in terms of hydroxyproline levels) and lowered hydroxyproline content comparable to Tamoxifene treated ovariectomized animals. The protective effect of methanolic extract of seeds of *L. sativum* was found to be more pronounced than pet.ether extract (Fig.4.19).

In osteoporotic condition, persistent bone resorption due to osteoclastogenesis cause lowering of bone mass and consequently bone strength. Decrease in biomechanical properties lead to predisposition of fractures. In ovariectomized rat model microarchitectural alteration in bone is similar to those observed in postmenopausal and age dependent osteoporosis. In adult rats ovariectomy is followed by an increase in bone turnover associated with bone loss and a permanent deficit of bone mass at several skeletal sites including vertebral bodies, proximal femur and metaphysis of long bones such as the distal femur and proximal tibia.
Ovariectomy induces rapid loss of cancellous bone mass and consequently bone strength. The skeletal bone loss in ovariectomized rats mimics the features that occur in post menopausal women. Decrease in bone mass occurs in a less rapid rate and in a site specific fashion. Significant bone loss occurs in the tibial metaphysis, lumbar vertebral body and femoral neck. The progression of bone loss post ovariectomy develops from 14 days to 120 days and approximately 30 days post ovariectomy the status is similar to the post menopausal syndrome in humans. The biomechanical parameters were aimed in evaluating the effect on load stress in the tibia bone, femoral neck and 4\textsuperscript{th} lumbar vertebra in order to understand the susceptibility to fractures, a common feature in post menopausal osteoporosis.

Three point bending force was applied to the isolated tibial bone and bone strength was measured by force (weight) applied at the mid shaft till the bone fracture occurs. A marked decrease in the force was observed in the OVX untreated group compared to Sham operated animals indicating sharp decrease in bone strength resulting from effective bone resorption. All the treated groups exhibited significant higher tolerance to applied stress of which greater bone strength was observed in animals treated with methanolic extract of \textit{L. sativum} seeds than its Pet.ether extract at both the dose levels compared respectively. The methanolic extract of the bark of \textit{P. roxburghii} was found to exhibit greater protective effect than methanolic extract of the needles at respective dose levels. The tibial bone strength of the animals treated with methanolic extract of the bark of \textit{P. roxburghii} at 200 mg/kg was found to be significantly similar to the group treated standard drug Tamoxifene, indicating reversal of disease condition (Fig.4.20)

The compression of 4\textsuperscript{th} Lumbar vertebra was performed to evaluate the protective effect of the plant extract against the rate of bone loss from the spine, which is generally found to be greater than other sites in response to estrogen deficiency (Slemenda \textit{et al.}, 1997). Previous studies by Shirwaikar \textit{et al.}, 2003 have revealed the compression of 4\textsuperscript{th} Lumbar vertebra as a guiding parameter for the assessment of antiosteoporotic activity. The 4\textsuperscript{th} lumbar vertebra was isolated and subjected to an increasing force (weight in Kg) till compression occurred. A significant decrease in the compression force was observed in the untreated ovariectomized rats compared to sham operated animals suggesting considerable bone resorption from the vertebra and increased susceptibility to vertebral fractures. Oral administration of Tamoxifene and plant extracts (100 & 200mg/kg) showed a significant increase in the vertebral bone strength compared to OVX group. Treatment with methanolic extract of \textit{L. sativum} at a dose of 200mg/kg exhibited greater protective action against
vertebral bone resorption compared to the Pet.ether extract. A significant similar effect was observed in groups treated with higher doses of needle and bark extracts (methanolic) of *P. roxburghii* with that of Tamoxifene. Further, animals receiving Tamoxifene and methanolic extract of *L. sativum* and *P. roxburghii* (bark) at dose of 200 mg/kg respectively exhibited complete restoration of mechanical strength of the 4th lumbar vertebral bone comparable with sham operated group Fig.4.22).

The femoral neck is one of the areas prone to fracture in osteopenic condition due to its fragility and constant axial tension of the body weight. This was further confirmed by the significant decrease in the bone strength observed in the OVX group compared to the sham operated group. The significant increase in the femoral neck strength of the ovariectomized treatment groups compared to ovariectomized untreated group provides insight to amelioration of the condition on administration of Tamoxifene and plant extracts. All the groups treated with 200 mg/kg plant extract (*L. sativum* and *P. roxburghii*) showed decreased bone resorption at the site of femoral neck similar to Tamoxifene treated animals and was significantly concomitant with that of sham operated group Fig.4.12).

It is evident that increase in obesity in artificially induced and natural menopause associated with reduced ovarian function (Teede *et al.*, 2010). Several alterations in fat deposits occur with the advent of the menopause, leading to a change in the distribution of body fat. Hypoestrogenism has a negative effect on fat metabolism, favoring the appearance of central-body obesity (Douchi *et al.*, 2003). Physiological withdrawal of estrogen brings about changes in fat distribution (Dubnov-Raz *et al.*, 2007). Similar findings were observed in the study. A significant increase in the difference in body weight was observed in OVX animals compared to the Sham group indicating increased body weight due to ovarian exhaustion. Supplementation of plant extracts of *L. sativum* and *P. roxburghii* and Standard drug Tamoxifene showed significant decrease in body weight changes. Methanolic extract of *L. sativum* seeds and bark of *P. roburghii* were more effective in suppressing any change in body weight, similar to the effect produced by Tamoxifene. Increase in body weight is one of the prominent features that have been postulated to provide a partial protection against the development of osteoporosis in long bones (Roudebush *et al.*, 1993). A significant reduction in body weight was observed in the methanolic extract of *L. sativum* seeds and bark of *P. roburghii* at dose of 200mg/kg were more effective in suppressing any change in body weight, similar to the effect produced by Tamoxifene, thereby supporting the previous results of the presence of estrogen like activity in the reversal of osteoporotic condition (Fig.4.23).
In animals and humans, loss of ovarian function causes dramatic changes in bone mass, due to an imbalance between the amount of resorbed bone and that formed at each remodelling site. Internal microarchitecture and strength are also impaired, leading to increased bone fragility (Melton et al., 1988). Histopathological examination of the femur bone clearly demonstrates decreased ossification and mineralization in ovariectomized untreated group. The protective action of the Tamoxifene is evident of increased osteoblastic activity. Further, marked increase in bone restoration was observed in the group treated with plant extracts especially in the ovariectomized animals receiving methanolic extract of bark of *P. roxburghii* at a dose of 200mg/kg (Fig.4.25). The results of biochemical and biomechanical studies clearly correlate to the histopathological studies demonstrating a significant dose dependent protective effect of the plant extracts.

The most significant finding of the study is presence of high amount of quercetin in the bark of *P. roxburghii* along with potent antiosteoporotic activity. Quercetin is regularly consumed by humans and the major sources of quercetin are tea, wine, onions, broccolis, and apples. The average daily consumption of quercetin is about 30 mg per day (de Vries et al., 1997) and depends on dietary habits. The daily intake influences the plasmatic concentrations which have been evaluated in micromolar range, even if a weak intestinal absorption has been reported (Hollman et al., 1997). These plasmatic levels may be sufficient to exert biological effects. However, much higher concentrations should be achieved through the use of pharmaceutical preparations or dietary supplements. The possible mechanism involved in the exertion of antiosteoporotic activity of *P. roxburghii* could be attributed to the antiosteoporotic effects of quercetin.

Induction of osteoclastic differentiation by RANKL requires the activation of nuclear factor kB (NFkB) and activator protein 1 (AP-1) transcription factors via tumor necrosis associated factors (TRAF) family proteins from receptor activator of NFkB (RANK) (Darnay et al., 1998; Ye et al., 2002).

NFkB and AP-1 are two key transcription factors highly involved in osteoclastic differentiation by modulating the differentiation, survival and activation of osteoclasts (Grigoriadis et al., 1994; Iotsova et al., 1997; Jimi et al., 1998). Inhibitory effects of quercetin on NFkB and AP-1 activation have already been reported in cellular models, especially mesangial cells (Ishikawa et al., 1999; Ishikawa and Kitamura, 2000). Also
quercetin inhibits RANKL induced transcription factor activation in osteoclastic precursors. The inhibition of RANKL-induced NFkB and AP-1 activation by quercetin at low concentrations could be one of the cellular targets involved in the decrease of OCL formation.

The molecular mechanism whereby quercetin can decrease osteoclastogenesis remains to be specified. The best-described property of quercetin is its capacity to act as an antioxidant (Rice-Evans et al., 1995) and y demonstrated that quercetin keeps its antioxidant properties in mature osteoclastic cells (Wattel et al., 2003). Osteoclasts are cells known to produce high amounts of reactive oxygen species (ROS) and these free radicals play an important role not only in bone resorption (Bax et al., 1992), but also in the process of osteoclast differentiation (Suda et al., 1993), possibly involving activation of the transcription factor NFkB and AP-1 (Schreck et al., 1991; Lo et al., 1996).