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Evaluation of Pinus Plant Species for Osteoporosis

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Abstract: *Background: Pinus plant species are found in most of Himalayan states of which Pinus roxburghii and Pinus wallichiana are used traditionally as a paste to treat bone related disorders and fractures. Osteoporosis is a skeletal disorder associated with low bone mass and strength leading to bone fractures. It is highly prevalent especially in geriatric populations. For several years estrogen therapy was used clinically for treatment and management of osteoporosis, However their prolong use are associated with various side effects including breast cancer and cardiovascular diseases. The aim of the present study has been designed to evaluate the pharmacological activity of stem bark of Pinus plants extracts and fractions in surgically induced osteoporosis in female rats. The bioactive fraction was further developed into a tablet formulation.*

Methods: The plant extracts and fractions are subjected for the phytochemical screening. The presence of gallic acid, quercetin, ascorbic acid, tannic acid, caffeic acid and catechin was quantified in plant extracts and in potent bioactive fraction using HPLC and HPTLC study. The three Pinus extracts Pinus roxburghii, Pinus wallichiana, Pinus gerardiana and further their four fractions prepared by using the solvents of graded polarity was subjected for antioxidant activity using DPPH, nitric oxide and hydrogen peroxide radicals assays. Anti-inflammatory activity was carried out using albumin denaturation and HRBC membrane stabilization assays. In-vitro osteoblastic proliferation was assessed on UMR106 cell lines. Acute toxicity study of prepared extracts was conducted as per OECD423 guidelines. In vivo antiosteoporotic activity was done on ovariectomized female rats. The most active fraction was then formulated into tablet.

Results: The qualitative phytochemical analysis of hydro-alcoholic stem bark extracts of three plant species revealed the presence of various biochemical compounds such as alkaloids, flavonoids, glycosides, triterpenoids and saponins. Quantitative phytochemical analysis of plant extracts showed the presence of phenolics, flavonoids, tannins. Plant extracts of three Pinus species showed significant antioxidant activity against DPPH, nitric oxide and H2O2 radicals. In in- vitro anti - inflammatory investigation P. roxburghii and P. gerardiana exhibited with percentage protection 86.54±1.85; 82.03±2.67.In in-vivo acute toxicity study studies Pinus plant extract revealed no sign of toxicity or mortality in rodents on. Administration of Pinus extracts and bioactive fractions prevented bone loss and showed marked efficacy against osteoporosis condition by modulating parameters, body weight change, biochemical parameters; serum phosphorus, calcium, estradiol, ALP and hydroxyproline levels. Biomechanical bone breaking strength for the tibia, 4th lumber, and femur bones, BMD, Pinus extract and bioactive fractions reversed the increased mRNA expression of RANKL, cathepsin K and decreased OPG expression in tibia bone tissue. Bone tissue histology showed the restoration of the disrupted trabecular network. The presence of gallic acid, quercetin and ascorbic acid in HPLC and HPTLC study signify their contribution to treating osteoporosis. The presence of phytoconstituents may be inhibiting bone loss, oxidative stress and suppress the release of inflammatory mediators. These may be responsible for the protective effect of extracts and fraction against the experimental model of osteoporosis. The most active n butanolic fraction of P.gerardiana was formulated into tablet and evaluation was done by testing the hardness, friability, disintegration time.

Conclusion: The findings from present investigation may conclude that the Pinus plant species has efficacy to ameliorate the pathological state of osteoporosis via modulation of estrogen, RANK and

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cathepsin signaling. This effect may be due to the presence of quercetin, gallic acid, ascorbic acid, catechin, caffeic acid and tannic acid phytoconstituents...

Keywords: Osteoporosis, *Pinus roxburghii*, *Pinus wallichiana*, *Pinus gerardiana,* Bone, HPLC, HPTLC.

I. INTRODUCTION

1.1 Osteoporosis

Osteoporosis is defined as a skeletal disease characterized by structural deterioration of bone tissue, low bone mass, and skeletal strength. It is recognized as a metabolic and hormonal disease and occurs due to an imbalance in estrogen and in micronutrients eg. calcium, magnesium, and phosphorus (Johnell et al., 2005; Das et al., 2013; Queally et al., 2013). The risk of osteoporotic fractures worldwide in women is reported 30-50% whereas in men up to 15-30%. Osteoporosis accounts for more than 8.9 million cases of fractures annually thus is a substantial economic burden on health care and major health hazard (Johnell, 2006; Ke et al., 1997). Whereas the prevalence of osteoporotic hip fracture was hypothesized 6.3 million by 2050 as it was reported 1.7 million in 1990 (Copper et al., 2008). At present in India, life expectancy is ~67 yrs, expected to increase to 71 yrs by 2025 and 77 yrs by 2050 (Khadilkar, 2015). Thus, a greater proportion of the Indian population with increasing longevity over the age of 50 yrs are likely to be affected by osteoporosis. Moreover, the peak incidence of osteoporosis in Indian women is more than western women (Mithal et al., 2013). In India, it is reported that above the age of 50 yrs 42.5% women and 24.6% men suffer from osteoporosis (Marwaha et al., 2011; Thulkar et al., 2015). The prevalence of vitamin D deficiency with low calcium intake accelerates the pre menopausal bone loss resulting in osteoporosis. It is becoming a major public health problem, especially in Indian women (Khadilkar, 2015).

In advancing age, the symptoms of bone fragility climb steeply due to nutritional & metabolic deficiencies, postmenopausal state (in women), and disease exposure in childhood which often predispose to osteoporosis in later life. The leading cause of osteoporosis includes lack of estrogen in females and androgen in males. Estrogen deficiency or menopause state in women reduces the cancellous metaphyseal bone, which results in a fall in bone mineral density (BMD) in human (Kolios et al., 2010).

Bone is an integral part of the body which is composed of compact structures of collagen embedded with micronutrients mainly calcium, phosphorus, and magnesium. Bone remodeling refers to the balance between the process of bone formation by osteoblast and bone decay by osteoclast cells (Ha et al., 2006; Yang et al., 2011).

The skeletal physiology emphasizes the role of estrogen in the closure of epiphyseal growth plates resulting in growth and maturation of bone. Estrogen reduces the differentiation of osteoclasts in active remodeling units in postmenopausal women and in animals underwent to ovariectomy. Receptor activator of nuclear factor kappa (RANK), a tumor necrosis factor belongs to cytokine family, is expressed by osteoclasts and stromal cells which interacts with Receptor activator of nuclear factor kappa B ligand (RANKL). The activation of osteoclast causes a massive release of cytokines; interleukin-6 (IL-6), Tumor necrosis factor-α (TNF-α) and growth factors (GF) which further potentiates the osteoclasts function and affect bone decay (Hofbauer et al., 2000). The resorbing osteoclasts secrete tartrate-resistant acid phosphatase (TRAP), cathepsin K, matrix metalloproteinase (MMP-9), and gelatinase from cytoplasmic lysosomes to digest bone matrix made of type I collagen forming saucer-shaped lacunae on the trabecular bone surface and in this way the resorption tunnels in bone (Nakashima and Takayanagi, 2011). The role of RANKL has been established in animal models. The removal of RANKL genetically leads to an osteoporotic phenotype with a complete absence of osteoclasts (Kong et al., 1999). Osteoprotegrin (OPG) is secreted by osteoblasts which is a “decoy receptor”, usurp RANKL, prevents its binding to

RANK and thus moderating osteoclastogenesis and bone resorption (Takayanagi, 2007; Nakashima and Takayanagi, 2009). The role of estrogen inactivation of GH/insulin-like growth factor 1 (IGF-1) axis and production of Tumor growth factor beta (TGF-β) and procollagen by osteoblast precursor cells in bone formation are also emphasized.

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Estrogen is also reported to antagonize the effect of cytokines like IL-6 and TNF-α in the bone remodeling process (Wattel et al., 2003; Raggatt et al., 2010).

The condition of bone porosity and bone loss is managed using a recommended treatment which includes hormone replacement therapy (HT) using estrogen hormone; selective estrogen receptor modulators e.g. Raloxifene; Bisphosphonates e.g. Risedronate by reducing bone resorption and human monoclonal antibody e.g. Denosumab as RANKL inhibitor. But the long- term use of these medication causes serious side effects on health such as the risk of breast cancer (by HT); hot flashes, breathing trouble, chest pain (by raloxifene), stomach upset, inflammation in esophagus, atrial fibrillation in women (by bisphosphonates) and joint and muscle pain, hypocalcemia, hypersensitivity reactions, hypercholesterolemia (by Denosumab). Thus, the emergence of treating the fatal state of osteoporosis is sought and may be resolved using herbal drugs (Kurt, 2009; Lewiecki, 2011).

In recent years, the use of herbal based medicines and supplements are high in demand in developed and developing countries (Pathare, 2012). Indian systems of complementary and alternative medicine carry age-old therapeutic basis for use of that medication because of their diverse biological activities, higher safety, and lesser costs. According to the Indian system of medicine, Ayurveda, osteoporosis is defined as a Vata disorder. Vata is an element of a Tridosha (Vata, Pitta and Kapha) which is condensed from an element, space. In India, indigenous remedies have been used in the treatment of diseases since the time of Charak and Sushruta (6th century BC). Out of 2500 species, around 150 species are used commercially on a large scale. Thus India is called a botanical garden of the world (Modak et al., 2007). The criticism in acceptance and efficacy of herbal medicines over synthetic drugs may suitably be addressed with an extensive scientific investigation (Pan et al., 2013). World Health organization (WHO) estimates that 80% of earth inhabitants rely on traditional medicines. The Indian Himalayan region, the birthplace of Ayurveda and alternative therapies, covers about 18% of Indian land and extended to landscape more than 2,800 km in length and 220-300 km wide with altitudes of 200-8000 m. This region provides a very large proportion of medicinal plants about 80% of Ayurvedic medicine, 46% of Unani drugs and 33% of allopathic drugs (Samant et al., 2007; Sharma et al., 2011; Chauhan, 2014). The unique climatic conditions enable a rich array of growth of various medicinally useful plants in the region (Chauhan, 2014).

Pinus species are important forest primarily used for timber purpose and as a source of gum oleoresins. Pinus species have a rich history of utilization as folklore medicine by ethnic groups in the hilly region. Traditionally, the paste of Pinus wallichiana, and Pinus roxburghii resin and bark are applied as a plaster for healing bone fractures (Shuaib et al., 2013). Three species of Pinus : Pinus roxburghii, Pinus wallichiana and Pinus gerardiana belong to the family: Pinaceae are found abundantly in this region. P. roxburghii Sarg, commonly called as chir pine, is a tall tree with spreading crown, found at altitude 450-2400 m from Kashmir to Bhutan and Siwalik hills (Shuaib et al., 2013). P. wallichiana also was known as blue pine, is found at an altitude 2000-3500 m whereas P. gerardiana, commonly called as Chilgoza is found at of 1600- 3000 m in Kinnaur district of Himachal Pradesh.

P. roxburghii is also reported to contain terpenes: alpha-pinene, flavonoids: rutin and quercetin; steroid: beta-sitosterol. P. wallichiana is also reported to contain betasitosterol. Quercetin, a flavonoid is reported for treating osteoporosis in the rat by improving bone mineral density and by affecting osteoblast-osteoclast proliferation (Derakhshanian, 2013) Gallic acid, a phenolic acid is reported to inhibit osteoclastogenesis induced by RANKL in RAW 264.7 cell lines in-vitro (Lin, 2009).

It is reported for antioxidant and anti-inflammatory activities (Franziska, 2007). P. roxburghii is reported for its anti-inflammatory activity and its one of the formulations, Rumalaya gel is used in orthopedic ailments. Thus, Pinus species may be the rich source of flavonoid, phenolic and tannins phytoconstituents which can be helpful in designing newer therapeutic treatment for bone disorders (Rastogi, 2004; Sharma, 2005). The mixture of flavonol constituents extracted from French maritime pine bark: Pinus pinaster Pycnogenol® (PYC), is an antioxidant and reported to affect bone remodeling by inhibiting matrix metalloproteinase expressed on osteoclast and bone matrix including collagen type I (Huang et al., 2015). The present research focuses on the evaluation of pharmacological efficacy of Pinus plant extract/bioactive fraction in postmenopausal cases of osteoporosis with mechanistic elucidation.

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1.2 Aim and Objectives

1.2.1 Aim

The aim of the present study has been designed to evaluate the pharmacological activity of stem bark of Pinus plant species in surgically induced osteoporosis in female rats.

1.2.2 Objectives

1. Phytochemical evaluation of Pinus plant species: Pinus roxburghii, Pinus wallichiana and Pinus gerardiana stem bark.
2. Evaluation for antioxidant, anti-inflammatory and immunomodulatory activities of plant extract / fractions.
3. Evaluation of in-vitro cellular proliferation by Pinus extract and fractions on UMR- osteoblastic cell lines.
4. Evaluation of in-vivo anti-osteoporotic activity of Pinus plant extract / fraction using in-vivo surgical ovariectomy induced osteoporosis in female rats.
5. Development of oral formulation of active plant fraction of Pinus for Osteoporosis.

II. REVIEW OF LITERATURE

2.1 Osteoporosis

Osteoporosis is a skeletal disorder associated with low bone mass and strength leading to bone fractures (Liang et al., 2011). It is highly prevalent especially in geriatric populations (Fouda et al., 2017). Osteoporosis is classified as primary and secondary osteoporosis. Primary osteoporosis is further classified as Type I: postmenopausal osteoporosis caused due to estrogen deficiency after menopause and type II age-related or senile osteoporosis. Secondary osteoporosis refers to the bone disorder that is the consequence of various other chronic medical condition like anorexia nervosa, hyperparathyroidism, thyrotoxicosis, cystic fibrosis, osteogenesis imperfecta, diabetes mellitus type I, gastrectomy, inflammatory bowel disease, rheumatoid arthritis and may be an adverse outcome of therapeutic interventions (Lerner et al., 2006; Feng et al., 2011).

Menopausal state in women is observed as a sign of decline in fertility and a severe decline in estrogen level has been noted after menopause. In postmenopausal osteoporosis, there is an increase of 90% in bone resorption whereas 45% observed in bone formation in women thus resulting in the net bone loss with each remodeling cycle (Garnero et al., 1996). An initial shorter phase lasting 3-5yrs with trabecular bone loss mainly and a second longer phase lasting 10-20 yrs occurs in women and men (agerelated bone loss) involving loss to both cortical (the outer shell) and trabecular compartments (Manolagas et al., 2013). Thus, the incidence of bone fracture in men increases about a decade later than in women. Osteoporosis is a treatable condition, but the number of people diagnosed with the condition after the event of a fracture. This silent disease may often lower the patient's quality of life and increase total healthcare costs unexpectedly.

Osteoporotic fractures of the hip, vertebral body, and distal forearm have long been regarded as the typical bone fractures. The fracture incidence in women over the age of 45 yrs rises steeply, twice those in men. The overall incidence of vertebral fractures was 5.7/1000 person- yrs for men and 10.7/1000 person-yrs for women. Most wrist fractures occur in women about 50% over 65 yrs old. The incidence of wrist fracture in men is low and does not rise much with aging (Cole et al., 2009).

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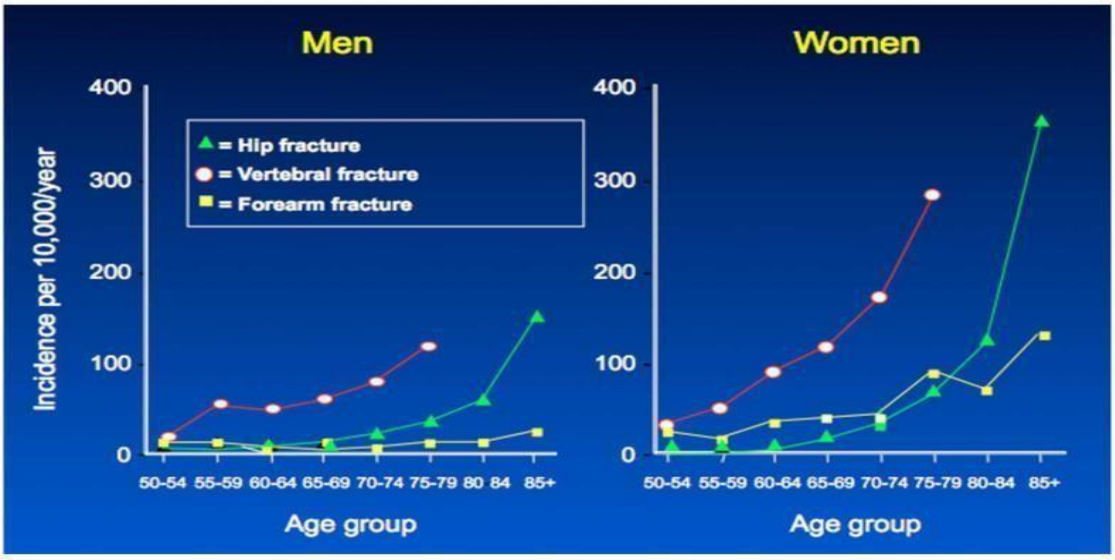

Figure 2.1 Fracture incidence with increasing age, the incidence of hip, vertebral and forearm in women and men (Cole et al., 2009)

The present review presents the updated literature for the process of bone remodeling with insights about the newer molecular targets modulating the process of bone remodeling. The impact of hormones and dietary factors for different types of osteoporotic states have also been emphasized.

2.2 Bone cells

Bone is a composite part, made up of crystals of mineral bound to protein. The mineral phase of bone consists of small crystals containing calcium and phosphorus, called hydroxyapatite. The minerals are bound in an orderly manner to a matrix which is made up largely of a single protein, collagen (Feng et al., 2011). Bone provides a structural framework in both light and strong forms. The outermost dense shell called as cortical bone or compact bone which protects and provides a mechanical function (Martin et al., 1988) whereas inner part of the cortical shell is a fine network of connecting plates and rods called as trabecular bone or cancellous or spongy bone. The trabecular bone provides strength and is metabolically more active than cortical bone in the bone remodeling process. Due to higher metabolic activity, trabecular bone is the main site for bone remodeling process and hence, it is the main site for occurrence of various bone diseases, including osteoporosis (Feng et al., 2011).

Bone remodeling is a physiological process in order to renew a fractured bone and to preserve mineral homeostasis (Teitelbaum, 2000). The spongy bone is the site for bone formation and the cortical bone for bone decay and an imbalance results in bone porosity. Three major types of bone cells osteocytes, osteoclasts, and osteoblasts which closely collaborate in the remodeling process maintaining and restoring bone structures (Feng et al., 2011; Arnett et al., 2016). Bone remodeling occurs in bone remodeling units and is a lifelong process by which old bone is replaced by osteoclast (called bone resorption) and new bone formed by osteoblasts (called bone formation) to maintain the integrity of skeleton (Eriksen et al., 2010).

2.2.1 Osteocyte

The mature osteocytes are former osteoblasts, stellate-shaped cells and enclosed by the unmineralized matrix. They are cocooned in fluid-filled cavities (lacunae) and have long dendrite-like processes that extend throughout canaliculi (tunnels) within the mineralized matrix. They interact with other osteocytes and also with osteoblasts on the bone

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surface (Raggat et al., 2010). They are the sensors for mechanosensation and mechanotransduction for the changes in mechanical loading and to local bone damage (Goldring, 2015).

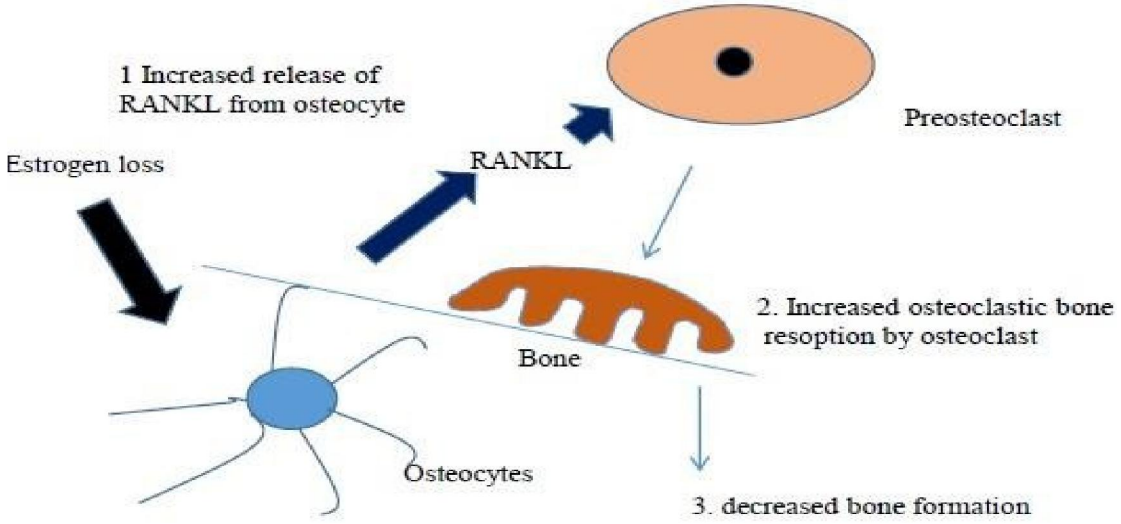

Figure 2.2 Initiation of bone remodeling cycle.

In the remodeling process, at the sites of micro damage, osteocytes undergo apoptosis, in response to stressors like estrogen loss, glucocorticoids, and immobilization. The adjacent osteocytes release products, including RANKL, vascular endothelial growth factor (VEGF), sphingosine-1-phosphate, dentin matrix protein 1 (DMP1), osteopontin (OPN), phosphate- regulating neutral endopeptidase on chromosome X (PHEX), osteocalcin (OCN), matrix extracellular phosphoglycoprotein (MEP), sclerostin, fibroblast growth factor 23 (FGF-23), and osteoprotegerin (OPG) that activate endothelial cells and recruit bone cell precursors, involved in mineralisation of bone (Schaffler, 2012).

2.2.2 Osteoblast

Osteoblasts are cuboidal mononuclear, specialized bone-building cells which are derived from mesenchymal stem cells (Boyle et al., 2003; Asagiri, 2007; Capulli et al., 2014). They form tight junctions with adjacent osteoblasts, working as a team to synthesize and secrete collagen fibers and another organic matrix for formation, deposition, and mineralization of bone. In order to form bone, osteoblasts mineralize the collagen with a form of calcium phosphate (hydroxyapatite), a composite material (Arnett et al., 2016). The hydroxyapatite makes the bone hard and resistant to compression. The collagen holds it together and prevents brittleness (Boyce et al., 2009).

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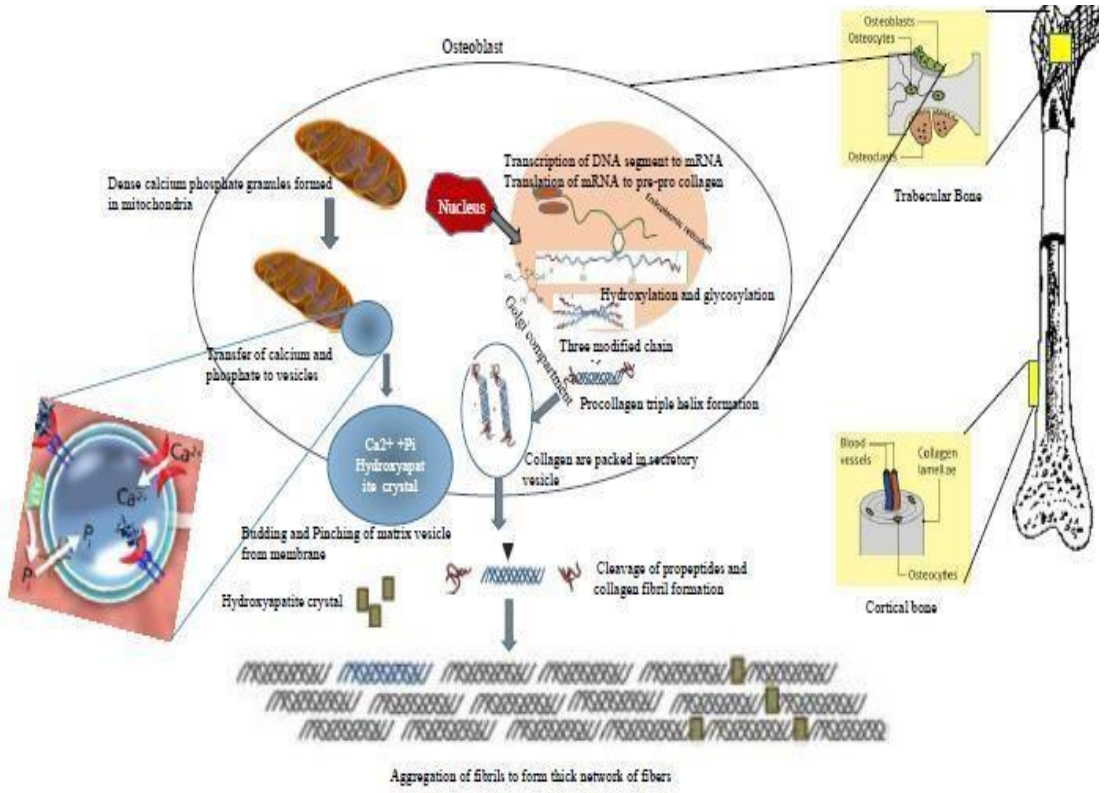

Figure 2.3 Role of osteoblast in the formation of hydroxyapatite templet

The bone collagen is a triple helical protein in extracellular space, initially synthesized in the nucleus of osteoblasts and also fibroblasts, where a particular segment of DNA is transformed into messenger ribonucleic acid (mRNA). It is then moved into the cytoplasm, where it is translated into polypeptide chains, known as pre-pro-collagen. Each chain has characteristic amino propeptides and carboxy propeptides on either side.

In endoplasmic reticulum, there is hydroxylation of proline and lysine residues with the help of ascorbic acid, which will further aid in cross-linking of peptide chains resulting in triple helix structure (two α1 chains and one α2 chain in type I collagen). After achieving the helical conformation, they move from the ER. In Golgi apparatus, there is a formation of procollagen triple helix structure then they get packed in secretory vesicles or vacuoles (Wittkowske et al., 2016). Once the cell releases procollagen, the collagen fibers start to deposit directly. There is the formation of long-range assemblies, like in tendon and ligaments, there is the formation of parallel bundles and interlocking weaves in bone (Kadler et al., 2008).

Osteoblast cell undergoes a process of maturation, and formation which entails differentiation from progenitors into proliferating preosteoblasts and then mature osteoblast, the process is known as osteoblastogenesis (Eriksen et al., 2010). During osteoblast differentiation, there is activation of transcription factors Runt-related transcription factor 2 (RUNX2), Osterix transcription factor, activating factor 4 (ATF4) along with WNT signaling and sympathetic signaling, essential for osteoblast differentiation (Nakashima et al., 2002). In the proliferation phase, osteoblast progenitors show alkaline phosphatase (ALP) activity and are considered as preosteoblasts (Capulli et al., 2014). The transition of pre-osteoblasts to mature osteoblasts is triggered by an increase in the expression of osteocalcin (OCN), osteopontin and osteonectin, bone sialoprotein (BSP) I/II, and collagen type I. Conversely mature osteoblasts are capable of secreting pro-osteoclastogenic cytokines, including RANKL, macrophage-colony stimulating factor, IL-1, IL-6, IL-11, leukemia inhibitory factor and Oncostatin M (Atkins et al., 2000) altogether these factors regulate

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osteoclast activity(Teitelbaum et al., 2006). Osteoblasts also produce antiosteoclastogenic factors such as OPG, granulocyte–macrophage- colonystimulating factor, IL-3, IL-12, and IL-18 and appear to play a central part in bone remodeling thus creates balance in bone resorption and bone formation (Atkins et al., 2000; Takahashi et al., 2002.). The Canonical Wnt signaling described later plays a pivotal role in the formation of osteoblast, enhances osteoblastogenesis, and reduces osteoclastogenesis and bone resorption. At the end of their lifespan osteoblasts become entrenched more deeply into the bone and transform into osteocytes (Manolagas et al., 2000).

2.2.2.1 Runt-related transcription factor 2 (Runx2)

Runx2 is expressed in testes and thymus and most abundantly in bone and calcified tissues. Osteoblast differentiation and bone formation are controlled by gene RUNX2 also known as CBFA1 (core-binding factor A1) (Ducy et al., 1997; Olsen et al., 2000; Karsenty, 2002). Runx2 regulates expression of genes encoding osteocalcin, VEGF, RANKL, sclerostin, and dentin matrix protein 1 [DMP1]. Indeed, in Runx2 null mice cartilaginous skeleton, the lack of mineralized tissue was observed due to the arrest of osteoblast. The overexpression of Runx2 leads to osteopenia, indicating inhibition of the process of osteoblast maturation (Liu et al., 2001). Runx2 is a pleiotropic regulator of skeletogenesis beyond osteoblast differentiation playing a major role in hypertrophic chondrocyte differentiation and vascular invasion (Liu et al., 2001).

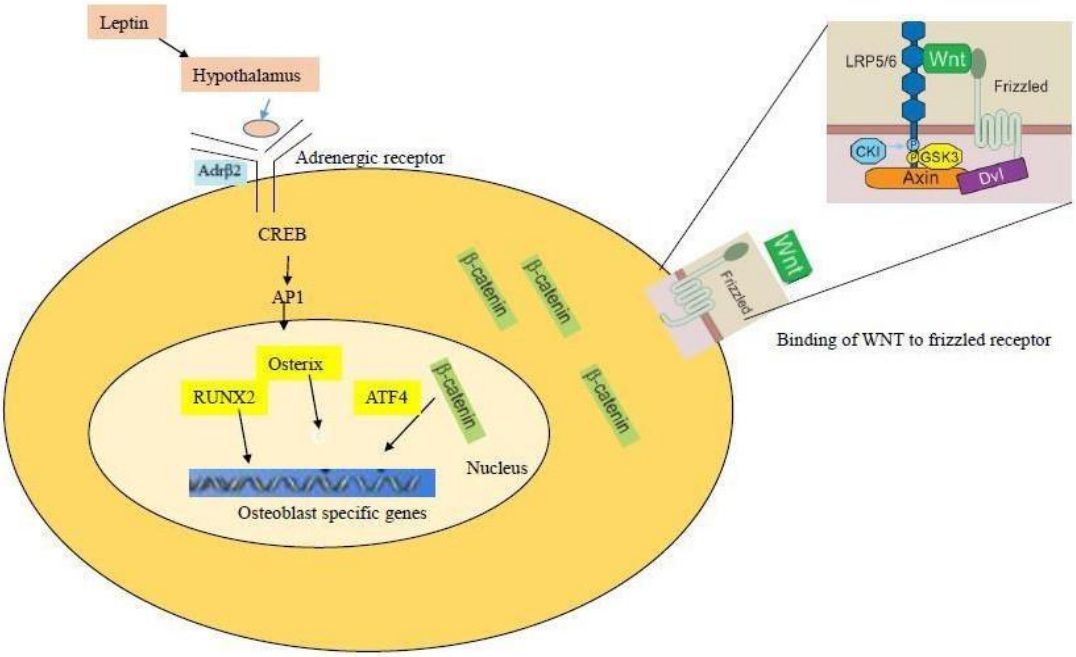

Figure 2.4 Molecular signaling in osteoblast differentiation

2.2.2.2 Osterix (Osx)

It is another transcription factor expressed in osteoblasts and regulates the repertoire of genes during final stages of bone tissue formation. Studies have shown that Osx is intensely expressed during regeneration of bone cells, and it is necessary for the activation of bone- specific genes that are involved in bone formation. Osx null mice die at birth as bones formed by intramembranous ossification are entirely non-mineralized, indicating the functions of Osx in mineral homeostasis osteoblasts. (Jensen et al., 2010)

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2.2.2.3 Wnt signaling

Wnts, are family of secreted glycoproteins with multiple inhibitors, for 7-membranespanning frizzled (FZD) receptors. In cellular biology, the Wnt family is involved, from the cell fate determination, polarity and differentiation to migration, proliferation, and function. Wnt proteins are divided into two classes. The first involves the activation of canonical Wnt signaling pathway, resulting in the formation of a complex between Wnt proteins, FZD, and low-density lipoprotein (LDL) receptor-related protein 5 (LRP5) or LRP6 receptors (Tamai et al., 2000; He et al., 2004). In noncanonical, pathway Wnt5a binds FZD proteins, resulting in the activation of heterotrimeric G proteins, and intracellular calcium concentration increases resulting in the change of actin cytoskeleton via protein kinase C dependent mechanisms or induces Rho- or c-Jun Nterminal kinase (JNK) (Tamai et al., 2000; He et al., 2004).

The canonical WNT/β-catenin signaling is a pivotal pathway for the development and differentiation of osteoblast. Activation of this signaling pathway occurs with the association of Wnt protein released from or presented on the surface of signaling cells and act on its receptor Frizzled (FZD) and low-density lipoprotein (LDL) receptorrelated proteins (LRPs) either LRP-5 or LRP-6 (Bryan et al., 2009). Signals are generated through the proteins Disheveled, Axin, and Frat-1 results in disruption of the protein complex and frequent inhibition of glycogen synthase kinase (GSK)-3β (Bryan et al., 2009) causing hypophosphorylation of its substrate. ß-catenin translocates into the nucleus to promote the transcription of genes responsible for osteoblastogenesis (Bryan et al., 2009) (Fakhry et al., 2013). Mesenchymal stem cells lacking β-catenin do not differentiate into osteoblasts, indicating that expression of β-catenin is critical for bone mass in humans and vertebrates. (Clevers, 2006 and 2012). Canonical Wnt signaling not only enhances osteoblastogenesis and bone formation but it also reduces osteoclastogenesis and bone resorption.

2.2.2.4 Activating transcription factor 4

Activating transcription factor 4 (ATF4) is a transcription factor, also known as cAMPresponse element protein 2 required for osteoblast differentiation, and for promoting osteocalcin expression. Moreover, the synthesis of type I collagen is regulated by ATF4. ATF4 is an important factor for bone homeostasis and its disruption contribute to reduced bone mass and bone formation rate (Yang et al., 2004). Osteoblast differentiation and bone formation induced by parathyroid hormone (PTH) are also regulated by ATF4 (Yu et al., 2008 and 2009).

2.2.2.5 Sympathetic signaling

The communication between the sympathetic nervous system (SNS) and the skeleton through the adrenergic receptor pathway is noted. An anatomical link between the central nervous system (CNS) and skeleton show a wide range of connections. Using the pseudorabies virus, retrograde tracing experiments revealed the spinal connections to the femur at dorsal root ganglia between Lumbar 2 and Lumbar 5. Some specific regions of the CNS, including the brain stem, hypothalamus, and cortex, which are connected via a neuronal circuit, imply a much deeper relationship between bone and CNS. In osteoblast β2 adrenergic receptor (β2AR) is the central mediator of SNS signaling. The activation of the SNS causes the release of catecholamines in bone, which leads to the activation of osteoblastic β2AR (Elefteriou et al., 2014). Immunolabelling studies reveal an association between glutamate, catecholamine, or peptide-containing nerve fibers with osteoblasts and osteoclasts in the endosteum (Takeda et al., 2002). Blockade of glutamate receptors was reported to reduce the DNA binding activity and expression of Runx2 in cultured osteoblasts (Hinoi et al., 2003).

The results of Fu et al. (2005) suggests that signaling by β2AR first activates the transcription factor CREB which in turn stimulates the expression of clock genes and further causes proliferation of osteoblasts.

2.2.3 Osteoclast

Osteoclasts constitute for the 1–2% mass of all bone cells. They are highly specialized, multinucleated polarized cells derived from hematopoietic stem cells, specifically derived from monocyte-macrophage lineage (Boyle et al., 2003; Asagiri et al., 2007). They are identified to remove mineralized bone matrix and minerals (Raggatt et al., 2010). The cells have resorptive activity and mostly found in contact with a calcified bone surface and within a lacuna (Howship's

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lacunae). Osteoclasts have abundant Golgi complexes, mitochondria, and transport vesicles loaded with lysosomal enzymes (Vaananen et al., 2000). These cells are responsible for bone resorption and the activity increases with common bone diseases, including post-menopausal osteoporosis (Itzstein et al., 2011). Myeloid progenitors' response to signaling induced by several transcription factors, including PU.1, Microphthalmia- associated transcription factor (MITF) and Tfe3 to differentiate osteoclast progenitors (OCPs). PU.1 and MITF activate expression of the M-CSF receptor (M-CSFR) (Mellis et al., 2011). The regulation of bone resorptive activity by osteoclast undergoes is stimulation of RANKL, M-CSF, and co-stimulatory immune-mediated signaling.

2.2.3.1 RANK-RANKL pathway

The osteoclast express RANK is a member of the tumour necrosis factor family. The interaction of RANK with its ligand (RANKL released from osteoblast is a common pathway through which bone resorption is regulated (Hofbauer et al., 2000). This interaction leads to the recruitment of TNF receptor-associated factor (TRAF) adaptor proteins including TRAFs1, 2, 3, 5, and 6 (Darnay et al., 1998; Walsh et al., 2003.). Whereas, TRAF6 is found crucial for the osteoclast formation and function. TRAF6 deficient mice have shown impaired osteoclast differentiation and bone resorption osteopetrosis (Lomaga et al., 1999; Naito et al., 1999). TRAF6 mediates in RANKL/RANK signaling to downstream targets such as nuclear factor kappa B (NFkB), c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), p38, Akt and NFATc1. RANKL also activates the PI3K/Akt pathway through TRAF6 which may also dependent on Src kinase activity in osteoclast (Wong et al., 1999). NF- κB is one of the important transcription factors for osteoclast differentiation (Mizukami et al., 2002; Hauer et al 2005). NF-κB signaling executes with the family of dimeric transcription factors residing in the cytoplasm in non-stimulated conditions. However, after stimulation RANKL enter into the nucleus and regulate transcription of several genes (Takayanagi et al., 2000; Boyle et al., 2003). It has been demonstrated that NFkB upregulates the expression of nuclear factor of activated T cells (NFATc1) another key molecule of osteoclast differentiation (Li et al., 2004; Takatsuna et al., 2005).

2.2.3.2 M-CSF-C-FMS signaling

The signals generated by the binding of M-CSF to its cognate receptor c-FMS results in the transphosphorylation of tyrosine residues resulting in the formation of a complex with c-Src (Takayanagi,2007;Yavropoulou,2008).This complex leads to the recruitment of phosphatidylinositol 3 kinases (PI3K) and CBI complex activating Akt pathway and causing proliferation and survival of osteoclast. The role of transcription factor complex AP-1which is which is composed of the c-Fos, c-Jun and ATF proteins. In particular, c Fos is specifically induced by RANK for osteoclastogenesis. The pivotal role of M-CSF in osteoclast differentiation was revealed by studies in the knock out cFos rats developing severe osteoporotic bone phenotypes due to a complete absence of osteoclasts (Wang et al., 1992).

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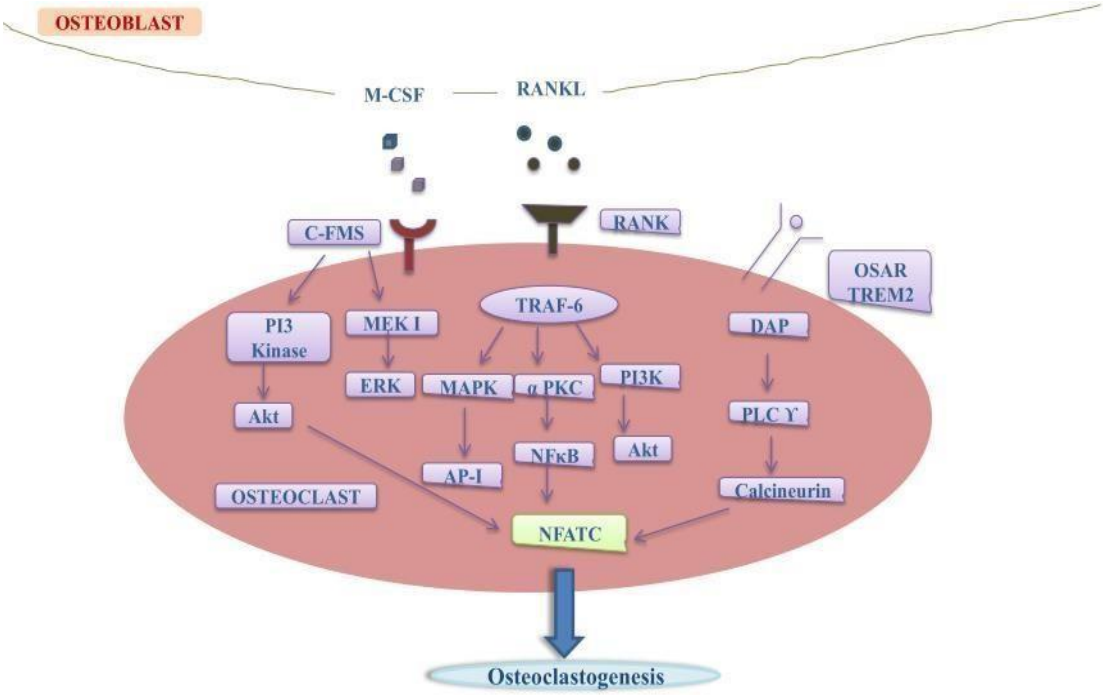

Figure 2.5 Osteoclastogenesis by M-CSF, RANKL, and costimulatory signaling c) Co- stimulating immune-mediated signaling

2.2.3.3 Co-stimulatory signalling

The RANK-RANKL signaling does not seem to directly initiate osteoclastic cascade and can only induce a partial activation of NFATc1 in osteoclast precursor cells (Huang et al., 2013). The osteoclast formation is also regulated by so-called co-stimulatory immune-mediated signaling through receptors, including osteoclast-associated receptor (OSCAR) and triggering receptor expressed in myeloid cells-2 (TREM 2) (Chabadel et al., 2007). These receptors activate immune receptor tyrosine-based activation motifs (ITAMs) in adaptor molecules such as Fc receptor common γ subunit (FcRγ) and DNAX-activating protein 12 (DAP12) in osteoclast progenitors mediate calcium signaling leads to activation of PLCγ. It mobilizes intracellular calcium, activating the calmodulin-dependent phosphatase calcineurin. Calcineurin dephosphorylates serine residues in NFATc1 for its rapid translocation into the nucleus and subsequent activation and thus regulating osteoclastogenesis (Shinohora et al., 2008).

2.3 Bone remodeling

The bone remodeling occurs in basic multicellular units (BMU) by the coordination of three bone cells osteocytes, osteoclast, and osteoblast. It is covered with bone lining cells, which in turn are connected with osteocytes (Hauge et al., 2001; Andersen et al., 2009). It is reported in human also in the mouse that the bone lining cells and specific macrophages (osteomas) respectively form a canopy structure on osteoblast at sites of bone formation (Pettit et al., 2008). The unique spatial arrangement of cells is important for bone remodeling process involving sequential highly regulated steps like activation, resorption, coupling and formation, and termination, which are discussed below.

2.3.1 Activation stage

The first phenomenon that occurs in bone remodeling involves detection of an initiating remodeling which usually occurs due to an influence of hormone e.g. estrogen or PTH on bone cells in response to more systemic changes in the

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body (Hauge et al., 2001). The signal sensed by the osteocytes causes the release of osteotropic growth factors and cytokines including IGF 1 TNF, PTH, and IL-6. These factors are attractant towards the for blood vessel activating lining cells for osteogenesis. The calciotropic hormone also is known as PTH is secreted by parathyroid glands in response to reduced serum calcium level and acts peripherally on kidneys and also on bone, The effects of PTH are mediated by PTH/PTH-related protein receptor, a G protein-coupled receptor activates the cAMP-dependent protein kinase (PK) and calcium- dependent PK. The activation of calcium intracellular signaling pathways in these cells also induce the recruitment of osteoclast precursors, induce osteoclast differentiation and activation, and establish a process of bone resorption (Ragatt et al., 2010).

2.3.2 Resorption phase

Osteoblasts respond to the signals generated by osteocytes or to the direct endocrinerelated activation signals discussed earlier and then recruit osteoclast precursors to the remodeling site. The signals for the activation of RANKL and M-CSF for activation of osteoclast likely to come from osteoblast lineage, endothelial cells and also from osteocytes. The local factors that released for osteoclast formation are IL-6, (Gao et al.1998) IL-17 (Kotake et al., 1999), TGF β. The osteoclasts travel to the site and attach through agile cytoskeleton (Teitelbaum 2000, Bonewald, 2008).

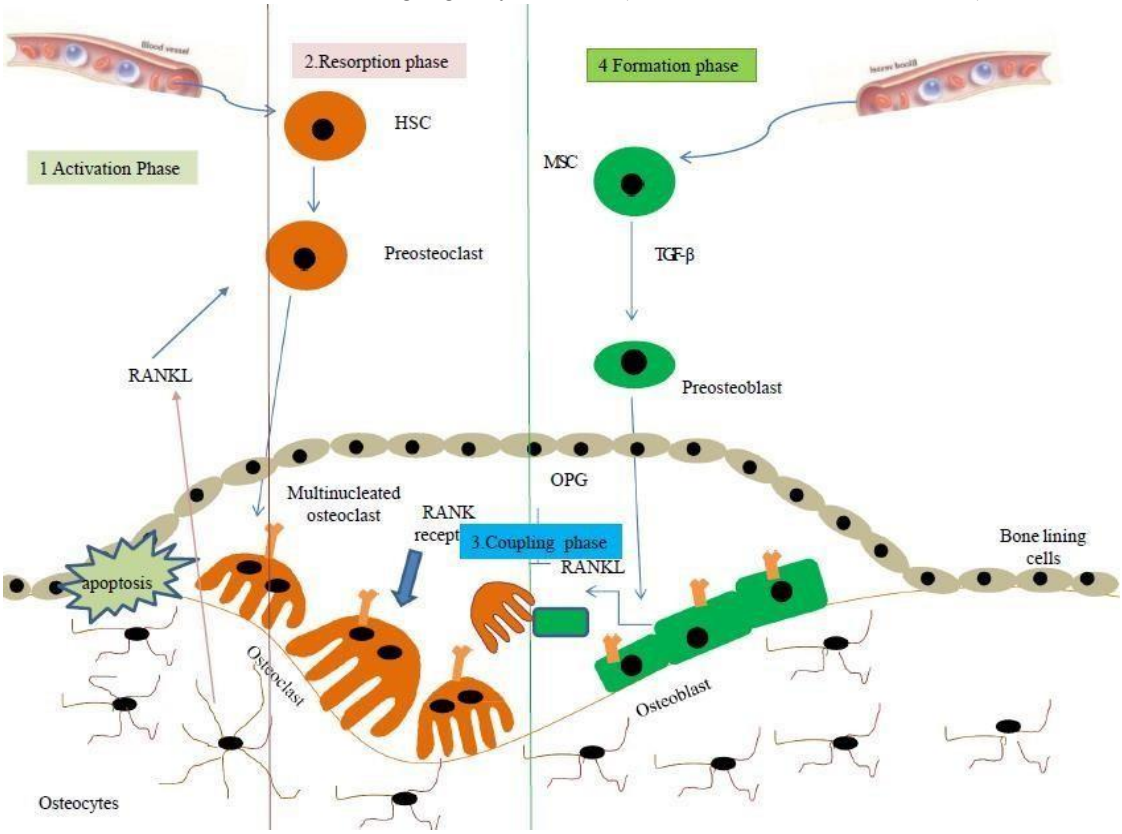

Figure 2.6 Phases of bone remodeling: 1) Activation phase; Bone remodeling begins with osteocyte apoptosis and RANKL mediated pre-osteoclast recruitment; 2) Resorption phase; osteoclasts resorb bone mineral matrix; 3) Coupling phase; recruit osteoblasts in the reabsorbed area; 4) Formation phase ;osteoblasts generate newly synthesized matrix and the differentiation of some osteoblasts into osteocytes completes the remodeling cycle.

The osteoclast first becomes polarized and its membrane is reorganized into four different domains: the sealing zone (SZ) that is tightly bound to the matrix; the membrane-rich ruffled border (RB) the basolateral domain (BD); and the

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functional secretory domain (FSD) at distal to the matrix (Coxon et al., 2008). The bone-resorbing surface develops a ruffled border which mediates for the attachment of the osteoclast podosomes to the bone surface. This attachment is mediated by actin cloud composed of $\alpha v\beta 3$-integrin, vinculin, paxillin linking this domain to the ECM. Podosome cores is associated with protein such as cortactin, and the transmembrane receptor CD44 increases overall adhesion of these cells (Saltel, 2004; Chabadel et al., 2007; Luxenburg, et al., 2007; Georgess et al., 2014). αvβ3 binds to a number of noncollagenous bone matrix containing-RGD sequence tri-amino acid sequence, arginine- glycine-aspartate including fibronectin, osteopontin, vitronectin, fibrinogen, and bone sialoprotein establishing a peripheric sealing. This attachment seals off a resorption zone which provide a highly enriched acidic microenvironment (Chabadel et al., 2007). The activated osteoclasts, begin the process of bone demineralisation, secrete hydrogen ions using a polarized vacuolar proton ATPase pump, H+–adenosine triphosphatase, which ultimately leads to the maintenance of a low acidic pH in the resorption lacunae.

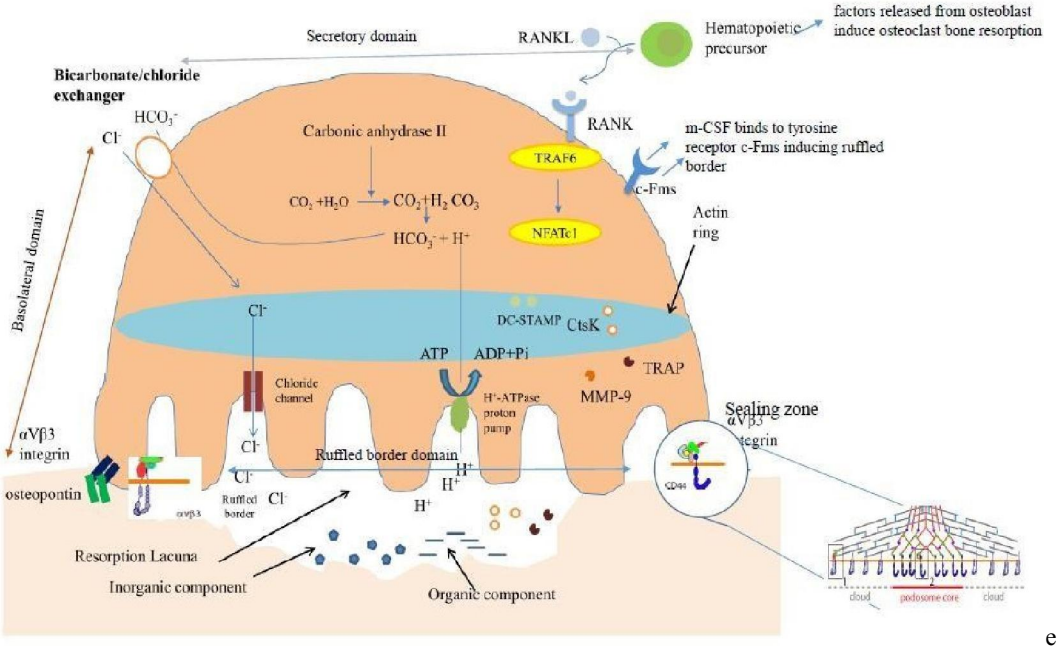

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Figure 2.7 Molecular signaling for bone resorption by osteoclast

The low acidic pH environment causes, dissolution of both ne mineral matrix (hydroxyapatite crystals). The resorbing osteoclasts secrete tartrate-resistant acid phosphatase (TRAP), cathepsin K, matrixmetalloproteinase-9 (MMP-9), and gelatinase from cytoplasmic lysosomes to digest the bone matrix, containing collagenous proteins (90%) mainly type I collagen. This results in the formation of saucer-shaped Howship' lacunae on the surface of the trabecular bone (Silva et al., 2015). An imbalance in osteoclastic activity or aggravation of bone resorption results in bone deformity and porosity respectively as found in severe bone diseases.

2.3.3 Coupling

During the bone remodeling cycle, there is strict coordination called coupling with the formation component to fill up the resorption unit (Sims, 2014). It is direct and indirect communication between bone cells to work upon by releasing various factors such as platelet- derived growth factor (PDGF), (IGFs), TGF- , and BMPs, Fibroblast growth factor (FGF) to stimulate osteoblastic activity (Howard et al., 1981; Sims, 2015). The various signalling pathways involved in coupling includes Ephrin signalling, semaphorins signalling, collagen triple helix repeat containing 1 (Cthrc1) signalling, complement component 3a (C3a) signalling.

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2.3.3.1 Ephrin signalling

It is bidirectional signaling for coupling between osteoclast and osteoblast which is shown by ephrin (EPH) (Matsuo, 2010). Osteoblasts express multiple EPH ligands and receptors where EPH receptor B4 (EphB4) is the largest class of receptor tyrosine kinases seems to function as a receptor that transduces reverse signaling from osteoclastic ephrinB2. Some studies have shown that these ligands have been implicated in coupling and the interaction activates bidirectional signaling in both the cells receptor-expressing as well ligand-expressing cells. In forward signaling, osteoclast expressing EFNB2 binds to EPNBB4, found in the plasma membrane of osteoblasts. The ephrinB2/ephrinB4 binding transduces bidirectional signals which promote osteoblast differentiation whereas the reverse signaling (ephrinB4/ephrinB2) inhibits osteoclastogenesis (Matsuo, 2010). The result of the bidirectional signaling might affect the switch from bone resorption to bone formation.

2.3.3.2 Semaphorins

Semaphorins are involved in the function of bone remodeling found in bone resorption and formation (Negishi-Koga et al., 2012). It is shown to be involved in diverse biological processes such as regulation of immune response (Kikutani et al., 2007) angiogenesis (Conrotto et al., 2005) tumor progression (Negishi-Koga et al., 2011; Suzuki et al., 2008.). Semaphorin 4D (Sema 4D) expressed on osteoclast inhibits bone formation by acting on to its receptor, Plexin B1 found on osteoblast inhibiting osteoblast differentiation (Negishi-Koga et al., 2011). The inhibition of bone formation initially is required, in order to completely remove the aged or damaged bone. Sema 4D inhibits IGF-1 pathway that is involved in osteoblast differentiation. Conversely, Sema 3A expressed on osteoblast which bound to Nrp1 receptor which is expressed by osteoclast precursors and then suppress bone resorption (Hayashi et al., 2012). A study reported that Sema3A activates the canonical Wnt/β-catenin pathway in the process of osteoblast differentiation (Zhensia et al., 2017)

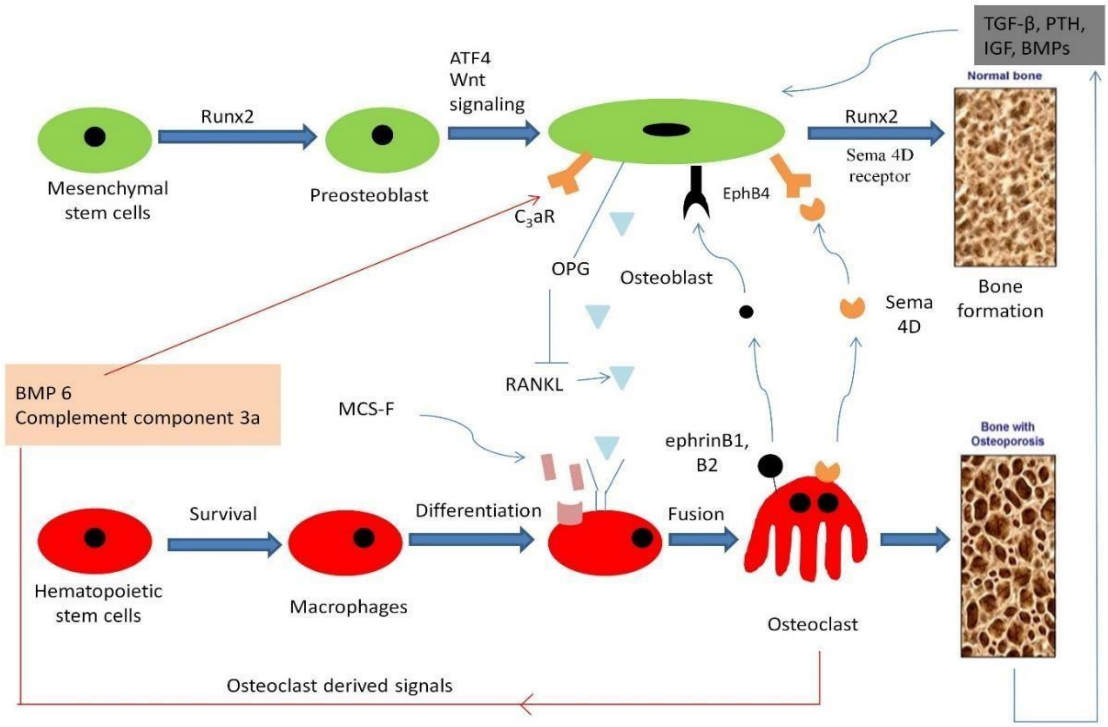

Figure 2.8 Coupling in osteoclast and osteoblast function

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2.3.3.3 Collagen triple helix repeat containing 1 (CTHRC1)

CTHRC1 is a protein involved in bone formation vascular remodeling and morphogenesis. Its expression was found to be increased in osteoclast only when the cells were localized to the mineralized matrix during bone resorption (Ikeda, 2014). The signaling pathway involved is the binding of osteoclast-derived CTHRC1 to receptor Wnt activated inhibitory factor 1(WAIF1) encoding trophoblast glycoprotein gene in stromal osteoblast cells. The activation of PKC δ - ERK osteoblastogenic signaling thus serves as a link between bone resorption and formation, whereas Wnt pathway has an anabolic action in bone formation (Matsuoka et al., 2018).

2.3.3.5 Complement component 3a (C3a)

In osteoclast when C3 gene expression was knocked down, the stimulation of osteoblast differentiation was noted by the abrogation of ALP activity. C3a acts by binding to a seven transmembrane, C3aR, G protein-coupled receptor which is expressed in osteoblast lineage cells (Ikeda, 2014). A recent study reported a distinct role of C3a inactivation of the process of bone formation relayed from bone resorption augmenting coupling between both cells. Its expression was also noted in ovariectomy-induced high bone turnover rate in bone and also in bone loss (Matsouka, 2014).

2.3.3.5 Other factors

Osteoblast differentiation is stimulated by a number of factors secreted by osteoclast such as Wnt 10b, sphingolipid, Bone morphogenetic protein 6, sphingosine-1phosphate (Pederson, 2008).

2.3.4 Bone formation

Osteoblasts are anabolic in action synthesize majority of extracellular matrix components and control the mineralization. It increases bone mass by secreting collagen proteins, mainly type I collagen and noncollagenous proteins includes osteocalcin, bone sialoprotein II, and osteoadherin osteonectin, osteopontin and proteoglycan, resulting in the formation of the organic matrix. During osteoblastic differentiation, the cells regulate matrix calcification by releasing matrix vesicles (MV), which have a variable diameter ranging from 100 to 400 nm. MVs are released from apical membrane domain of the osteoblasts promoting deposition of hydroxyapatite (Ca10(PO4)6(OH)2) (Kirsch et al., 2000; Wu et al., 2003). Osteoblasts secrete enzyme ALP causes the release of calcium from mitochondria and crosses through calcium channels on matrix vesicle with inorganic phosphate (Pi) (through phosphohydrolases) (Glimcher, 1998). Thus, the phosphate and calcium ions nucleate to form calcium phosphate and by pinching off or budding releases the MVs from apical microvilli into the ECM. The hydroxyapatite crystals so formed in an extracellular matrix (ECM) thus propagating for mineralization (Kirsch et al., 2000). The MVs released so far execute for the essential function of mineralization also possess lipids and proteins (Silva et al., 2015). The newly recruited osteoblasts then appear as a single layer of cuboidal bodies having rough endoplasmic reticulum and golgi complex. At the end of their lifespan, osteoblasts become entrenched more deeply into the bone and transform into osteocytes (Manolagas et al., 2000).

2.4 Essential metabolic elements in Bone remodeling

2.4.1 Estrogen

Estrogen belongs to the class of steroid hormones and regulates the development and function of the male and female reproductive organ (Birkhauser, 1996). In the ovary, theca cells begin estrogen and androgen synthesis and conversion of androgens to estrogens take place by the enzyme aromatase in granulosa cells. In male gonad, Leydig cells, Sertoli cells, and mature spermatocytes synthesize estrogens (Hess et al., 1997). Estrogen enters passively into the cells and bind to the estrogen receptors, and then regulate the transcription of downstream estrogen- responsive genes. There are three physiological forms of estrogen in females identified estrone (E1), Estradiol (E2), and estriol (E3).

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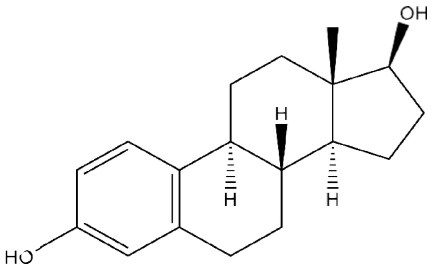

Figure 2.9 Chemical structure estradiol

Estrogens are female sex hormones important for the development of secondary female sexual characteristics and enabling success-full reproduction. Estrogens are female sex hormones important for the development of secondary female sexual characteristics and enabling success-full reproduction. In estrogen biosynthesis, cholesterol is converted into sex steroid by series of reactions. The first step involves cholesterol to pregnenolone, which is stimulated by adrenocorticotropin hormone (ACTH) in the adrenals and by luteinizing hormone (LH) in the ovaries. It is catalysed by the enzyme C20-22-lyase. Once pregnenolone is formed, it leads to the formation of progesterone, androgens, estrogens, and corticosteroids. Therefore, the pregnenolone is sometimes referred to as the "mother" steroid. E2 is a major product from the whole biosynthesis process and is the most potent estrogen in a woman's life. It plays a major role in the regulation of reproductive systems such as pubertal onset fertility, and estrous cycle (Zarate et al. 2012; Zhang et al., 2012).The brain also synthesizes estrogen using cholesterol as a precursor molecule. The presence of enzymes needed for E2 synthesis, P450scc, has been observed in hippocampus, hypothalamus, amygdala, caudate nucleus, thalamus, cerebral cortex, cerebellum, and some limbic areas of the brain which mediates the conversion of cholesterol to pregnenolone. In addition, the expression of aromatase, the enzyme responsible for the synthesis of E2, was described in hypothalamic and limbic areas (Naftolin et al., 1971; Azcoitia et al., 2011). Based on in situ hybridization, immunohistochemistry, and activity assays aromatase is localized to astrocytes and neurons in brain areas. (Gatson et al., 2011). Among different forms of estrogens, 17β-estradiol (estradiol) is the most common and potent form of estrogen and is also produced in a number of extragonadal organs including adrenal glands, brain, adipose tissue, skin and pancreas (Simpson 1999 and 2003; Nelson, 2001; Barakat et al., 2016). In human bone, aromatase expression has been demonstrated in osteoblasts, chondrocytes, and fibroblasts where they convert circulating androgens into estrogens (Sasano et al., 1997). In skin, aromatase expression occurs mainly in hair follicles and sebaceous glands (Cenci et al., 2000). In adrenal gland, adrenocortical cell shows the presence Cyp19mRNA, Cyp. 19 protein required for the synthesis of E2 (Nicol et al., 2009; Robic et al., 2016). Simpson, (2003) reported that when ovaries of postmenopausal women cease to produce estrogen, number of extragonadal sites, mesenchymal cells of adipose tissue including that of the breast, osteoblasts and chondrocytes of bone, the vascular endothelium and aortic smooth muscle cells, and numerous sites in the brain produces estrogen locally and fulfils the demand (Barakat et al., 2016)

Deficiency of estrogen contributes to the pathogenesis of osteoporosis. Skeletal activities are based upon estrogen receptors on osteoblasts and osteoclasts (Eriksen et al., 2010; Khosla et al., 2012) There are two types of estrogen receptors ERα and ERβ, The former is widely distributed and expressed in both osteoblasts and osteoclasts whereas ERβ expressed mainly in epithelial and mesenchymal tissues including osteoblasts (Lerner, 2006).

2.4.1.1 Estrogen effect on osteoclast

Deletion of ERα on osteoclast has resulted decreased trabecular bone mass similar to the postmenopausal women (Nakamura et al., 2007; Martin-Millan et al., 2010). Estrogen suppresses RANKL and M-CSF induced osteoclast differentiation through decreased nuclear levels of the key osteoclastogenic transcription factors, c-Fos, AP-1, c-Jun activity mediated transcriptional activation (Huber et al., 2001; Srivastava et al., 2001).

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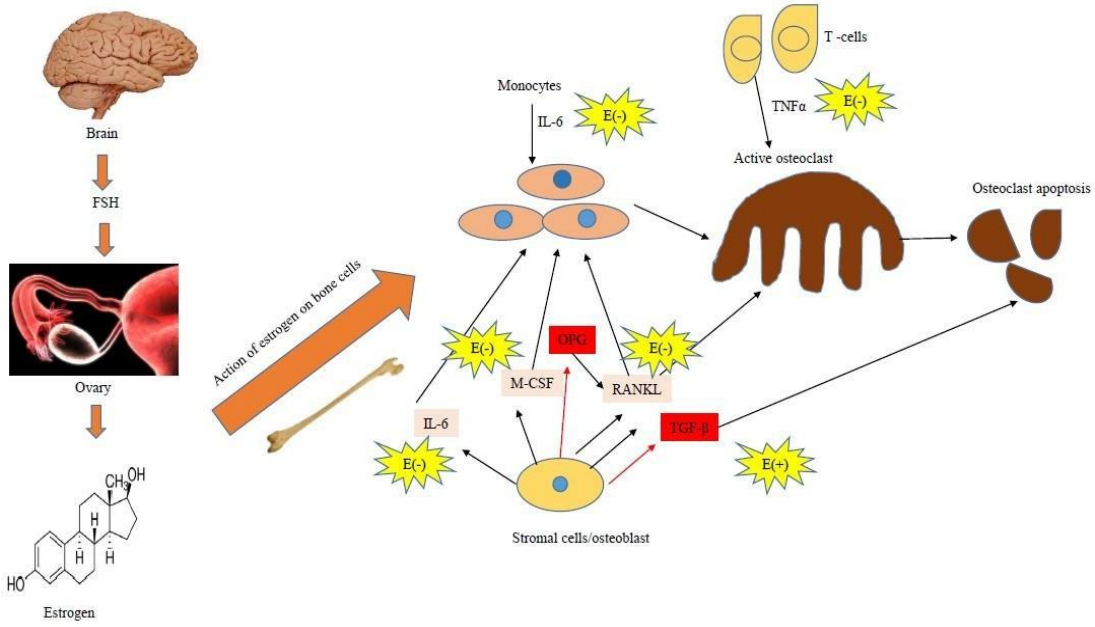

Figure 2.10 Effect of estrogen on bone cells

In monocytes, estrogen inhibits RANKL-stimulated osteoclastic differentiation, by the association of ERα binding with a scaffolding protein, BCAR1 (in rodents called p130C) which then sequesters TRAF6, leading to decreased nuclear translocation of NFκB and impaired RANKL-induced osteoclastogenesis (Robinson et al., 2009). Roggia et al. (2001) and Cenci et al. (2000) have shown that the loss of estrogen causes a rapid bone loss due to T cells mediated increase in TNF-α levels in the bone microenvironment and thereby indirectly enhancing osteoclastogenesis. A study by Bord et al. (2003) documented that low dose estrogen treatment in human osteoblasts responds with repression of RANKL while maintaining. OPG expression controlling osteoclast differentiation in human cells. At the cellular level, bone estrogen decreases the number of osteoclasts by inhibiting differentiation, probably mediated through some cytokines, IL-1 and IL-6 (Khosla et al., 2012).

2.4.1.2 Estrogen effect on osteoblast

OPG is secreted by osteoblasts which is a "decoy receptor", usurp RANKL, and prevents its binding to RANK preventing bone decay (Nakamura 2007; Nakashima 2009). 17 β estradiol upregulates OPG production in osteoblasts and decreases the production of IL-6 thus exerts anti-resorptive effects on bone (Bord et al., 2003). Estrogen increases the production of GH/IGF-1 axis and TGF-β &procollagen which are anti-resorptive in nature (Khosla et al., 2012). Extraskeletal estrogen deficiency is mainly based on increased renal calcium excretion and decreased intestinal calcium absorption. Estrogen has been shown to inhibit osteoblast apoptosis and increase osteoblast lifespan with downregulation of CREB, and c-Jun/cFos (Kousteni et al., 2001 and 2003). The treatment with estrogen to postmenopausal osteoporotic woman has been found to increase both serum Vitamin D levels and calcium (Sunyecz, 2008).

2.4.2 Parathyroid hormone

The chief cells in parathyroid gland secrete a parathyroid hormone known to regulate calcium homeostasis and bone remodeling (Qiu et al., 2015). PTH is secreted in blood to correct or maintain normal blood calcium levels. Calcium plays a pivotal role in physiological processes such as muscle contraction, blood coagulation, and synaptic activity (Senda et al., 2013) The glands detect the decrease calcium level in blood by membrane-bound calcium receptors

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ISSN: 2581-9429 Volume 6, Issue 1, June 2026 Impact Factor: 8.2

(CaR), which in turn modulates the secretion of PTH. Serum calcium, phosphorus, and vitamin D metabolites play a role in regulating PTH synthesis and secretion (Kumar et al., 2011).

In bone, PTH serves the dual effects, either a catabolic or anabolic hormone, depending on its administration. The continuous exposure to high levels of PTH is associated with catabolic effects, whereas intermittent exposure to low doses of PTH is primarily associated with anabolic action on bone i.e. bone formation (Kousteni et al., 2003). PTH mediates intracellular signaling by binding to the G-protein coupled type 1 PTH receptor (PTH1R). In bone, these receptors are present on osteoblasts and osteocytes and on tubular cells in the kidney (Fermor et al., 1995; Datta et al., 2009). The stimulation activates adenylate cyclase which in turn promotes cyclic AMP (cAMP) production with subsequent activation of protein kinase A (PKA) with alternative activation of protein kinase C (PKC). Gene expression profiling of PTH administration to rat bones suggests that PTH's dual effects the PKC pathway plays a limited role. The PKA pathway appears to predominate modulates key genes that control bone remodeling (Fu et al., 2002; Kramer et al., 2010). Finally, PTH binding to PTHR1 translocates β-arrestins to the cell membrane for subsequent activation of extracellularregulated kinases (ERK1/2) and also negative feedback mechanism involving PTHR1 desensitization thus inducing cAMP signaling. β-arrestins translocation contributes to the anabolic action of PTH on the bone (Gensure et al., 2005; Gesty et al., 2006; Silva et al., 2015).

2.4.2.1 Effect of PTH on osteoblast

PTH exposure stimulates cell proliferation of osteoblast by promoting expression of Wnt family members and Wnt signaling, and the suppression of Wnt antagonists Dickoppf 1 (DKK1) and secreted frizzled-related protein 1 (SFRP1) (Barnes et al., 2005; Guo et al., 2010; Kulkarni et al., 2005). This pathway is a major anabolic pathway for bone. PTH also stimulates the expression and activity of Runx2, expression of alkaline phosphatase and type I procollagen. During the time of bone remodelling, intermittent injection of PTH relocates blood vessels closer to sites of new bone formation expands both hematopoietic stem cells (HSC) and mesenchymal stem cells (MSC) thus facilitate bone remodeling (Calvi et al., 2003; Mendez-Ferrer et al., 2010; Prisby et al., 2011). Daily injections of PTH decrease osteoblast apoptosis observed in femoral and vertebral sections of mice by downregulation of the CARP-1 (Cell Cycle and Apoptosis Regulatory Protein) causing apoptosis and increasing the expression of Runx2 (Bellido et al., 2003; Zhao et al., 2012). However its prolonged exposure exert an attenuating effect on osteoblast it inhibits the synthesis of matrix proteins, including collagen I, osteocalcin, and alkaline phosphatase activity through a PKA-dependent pathway (Shimizu et al., 2007).

2.4.2.2 Effect of PTH on osteoclast

The in vitro and in vivo studies indicate that PTH enhances bone resorption through its indirect, through its actions on osteoblasts and osteocytes (Silva et al., 2015). Continuous administration of PTH affects the complex process of bone remodeling. During bone resorption, the formation of osteoclast is mediated by the production of the receptor activator of nuclear factor-κB ligand (RANKL and its soluble decoy receptor osteoprotegerin (OPG) (Lacey et al., 1998; Shiotani et al., 2002). PTH stimulates the mRNA encoding of RANKL and decreases the mRNA expression of OPG (Lee, 1999). Monocyte chemoattractant protein- 1 (MCP-1) has a major role in recruitment and differentiation of osteoclast precursors, PTH induced osteoblastic expression of MCP-1 favoring the increase in bone resorption over bone formation (Li et al., 2007).

2.4.3 Calcium

Calcium is a vital component of the bone architecture. It has a key role in various physiological functions including blood coagulation, neuromuscular activity, and cardiac function. The daily intake of calcium is 1200 mg from diet and supplements (Sunyecz, 2008). The demand of calcium may be exogenous forms of calcium using carbonate lactate, gluconate, phosphate, and citrate as well as hydroxyapatite, having elemental calcium (Chapuy et al. 1992). Calcium absorption has been reported in the distal and proximal part of the small intestine where the hormonal form of Vitamin

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International Open-Access, Double-Blind, Peer-Reviewed, Refereed, Multidisciplinary Online Journal
ISSN: 2581-9429 Volume 6, Issue 1, June 2026 Impact Factor: 8.2

D 1,25(OH)2 D3 by acting on vitamin D receptors (VDR) expressed in all segments of the small and large intestine (Veldurty 2016). 1,25(OH)2D3 regulates the expression of transient receptor potential vanilloid (TRPV) family, especially TRPV6 which transfers luminal calcium across the apical brush-border (Lieben et al., 2010). Intracellular calcium diffusion is facilitated by its binding to the calcium-binding proteins, calbindins (especially Calbindin-D9k), and finally, calcium is extruded across the basolateral membrane (Lieben et al., 2010). Few studies have shown that the systemic inactivation of VDR impairs intestinal calcium absorption resulting in hypokalemia, hypophosphatemia hyperparathyroidism resulting in rickets and osteomalacia. However, TRPV6 null mice were observed with increased bone turnover and impaired bone mineralization (Lieben et al., 2010). The overexpression of TRPV6 results in hypercalciuria, hypercalcemia, and soft tissue calcification, indicating a significant role for TRPV6 in intestinal calcium absorption (Cui et al., 2012). Estrogen regulate VDR by activating ER and TRPV6 or directly control intestinal calcium absorption similar to TRAF6 (Fleet et al., 2010). The efficiency of intestinal Calcium absorption is reduced to more than 75% in vitamin D deficiency (Pansu et al., 1983). Bone is a reservoir of calcium for maintaining the homeostasis and is an essential component for hydroxyapatite crystals which was explained earlier.

In conclusion, bone is an integral part of the body and composed of compact structures of collagen embedded in micronutrients calcium, phosphorus, and magnesium etc. Bone remodeling refers to the balance between bone formation by osteoblast and bone resorption by osteoclast cells. Bone homeostasis is under the control of local (e.g., growth factors and cytokines) and systemic (e.g., calcium, PTH and estrogens) factors. The transition of mesenchymal stem cells into bone forming cells and differentiation of osteoblast are controlled by signal transduction pathways Runx2, ATF4, Wnt signaling and sympathetic signaling. Osteoclast differentiation and the activity are controlled by M-CSF, RANKL, and costimulatory signaling. Moreover, these two functions are tightly coupled by ephrin and semaphorins signaling. In bone remodeling, RANK, a tumour necrosis factor cytokine family member, is expressed by osteoclasts and stromal cells which interacts with RANKL expressed on T cells. The resorbing osteoclasts secrete TRAP, cathepsin K, MMP-9 and gelatinase from cytoplasmic lysosomes to digest proteinaceous matrix of type I collagen and form saucer- shaped lacunae and resorption tunnels in trabecular bone. OPG prevents binding to RANK and thus moderating osteoclastogenesis. The role of estrogen in the growth and maturation of the bone and differentiation of osteoclasts has been established. It reduces the RANKL mediated osteoclastogenesis and increases osteoblast production by increasing the production of TGF β and IGF-1. Therefore, targeting one of the above signaling pathways may be an active target to prevent/manage postmenopausal osteoporosis. Thus, the emergence of treating the fatal state of osteoporosis is sought and may be resolved with the tool of modern herbal drug discovery.

2.5 Pinus plant species

The Indian Himalayan region (IHR), covers about 18% of India and extends more than 2,800 km long and 220-300 km wide with altitudes of 200-8000 m. It is known as the birthplace of Ayurveda and alternative therapies, (Samant et al., 2007; Sharma et al., 2011; Chauhan et al., 2014). The State of Himachal Pradesh includes parts of Trans Himalayas including Ladakh (Jammu & Kashmir) and Lahaul & Spiti (H.P.) whereas Northwest Himalaya covers 9% of the IHR (Samant et al., 2007). Pinus is the most common genus belongs to the family Pinaceae, which in turn is the largest family within the coniferous. It is a large genus with over 110 species worldwide (Richardson et al., 2007). The genus is divided into two subgenera: Strobus (Haploxylon, soft pines) and Pinus (Diploxylon, hard pines). Five species of pines are indigenous to India viz. P. roxburghii (Chir pine), P. wallichiana (Blue pine), P. gerardiana (Chilgoza pine) found in Himachal Pradesh P. kesiya (Khasi pine), and P. Markus (Teriasserian pine) are indigenous to Assam and Burma.

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Volume 6, Issue 1, June 2026
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2.5 Pinus species from North western Himalayas

2.5.1 Pinus roxburghii

2.5.1.1 Habit and Habitat

P. roxburghii Sarg. belongs to the family Pinaceae is an older terrestrial ornamental plant. It is popular for the resin and timber is yielded from the species. It is found from North Western Himalayas especially in Himachal Pradesh, Kashmir and Uttaranchal in India (Puri et al., 2010). P. roxburghii grows at an altitude of 450-2400m from Kashmir to Bhutan and Siwalik hills (Shuaib et al., 2013).

2.5.1.2 Morphology

P. roxburghii is a large tree with spreading crown reaching 30-50 m with a trunk diameter up to 2 m (Khan, 2012). The stem bark is dark gray, often reddish, deeply fissured, rough and exfoliating longitudinally elongated plates. The leaves are found in clusters of three, 20-30 cm long, triquetrous, finely toothed, needle-like, light green and persisting for one year. Male flowers are about 1.5 cm long, arranged in the form of cones and female are solitary or 2-5 together, ovoid and 10- 20 cm 7.5 13 cm when ripe (Shuaib et al., 2013). A clear, transparent oleo-resin with the pungent and bitter taste is tapped from the pine stem.

Figure 2.11 P.roxburghii a) Cone b) Tree c) Needles d) Trunk

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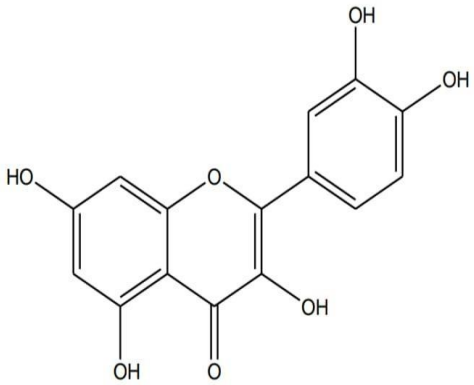
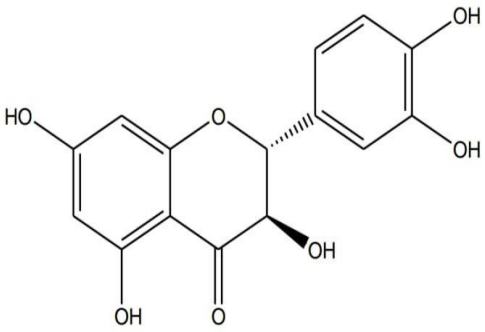
2.5.1.3 Phytochemical constituents

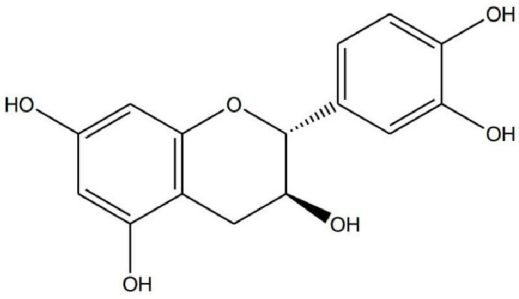
P. roxburghii is reported to contain terpenoids, flavonoids, tannins, and xanthones. Bark and needle extract have been reported for the presence of quercetin and its derivatives, resin acid (abietic acid, neoabietic acid), taxifolin, catechin, kaempferol, rhamnetin isorhamnetin, glucopyranoxanthone, friedelin, ceryl alcohol, b-sitosterol, 3,4-dihydroxybenzoic acid, 3,4- hydroxycinnamic acid, pinosylvin, pinoresinol, sterols, and tannins (Beri 1970; Swales, 1979; Coppen et al., 1988; Rawat et al, 2009; Willfor et al., 2009).



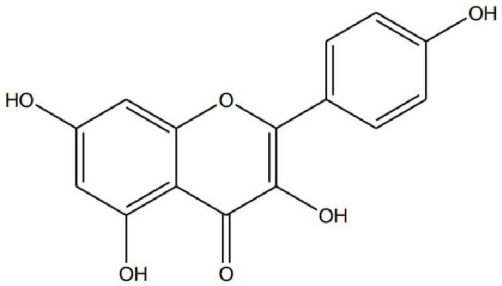
Quercetin



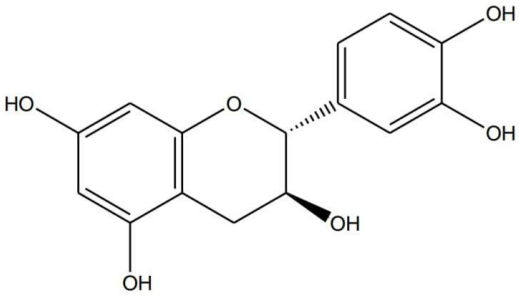
Taxifolin



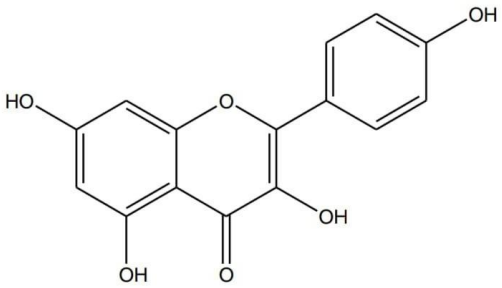
Catechin



Kaempferol



Catechin



Kaempferol

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ISSN: 2581-9429
Volume 6, Issue 1, June 2026
Impact Factor: 8.2

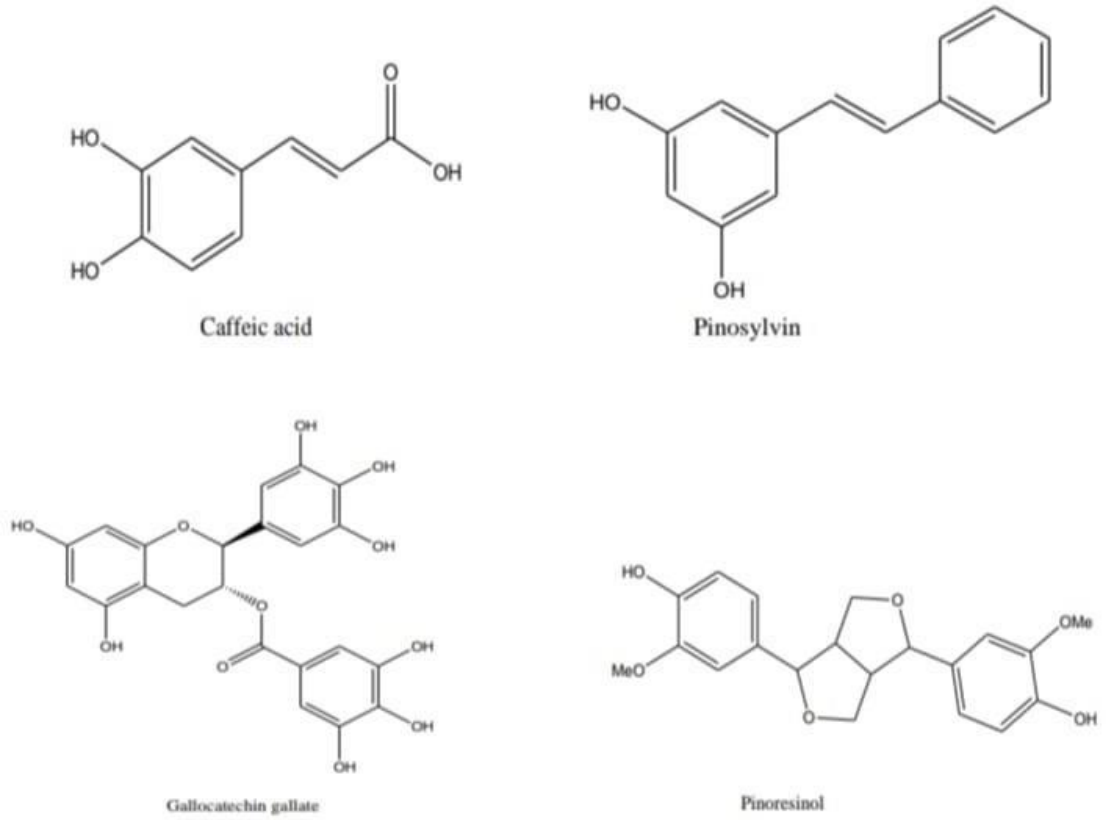

Figure 2.12 Chemical structure of chemical constituents from P. roxburghii bark

2.5.1.4 Ethnopharmacological uses

P. roxburghii has been used as a traditional remedy by the local tribes in various hilly parts of Northern India (Hussain et al., 2010). The volatile component of resin known as turpentine oil is included in the Indian Pharmaceutical Codex as Oleum terebinthinate for treatment of chronic bronchitis (Bajracharya, 1979). The resin (Biroja) is used for bangles, varnish, paints, polish industries, an ingredient of printing inks and batteries. The boiled resin (khaida or Leesa) are used to heal foot cracks. The ash or carbon collected from the burnt resinous wood (doi) of

P. roxburghii is mixed with mustard oil forming a paste (kajal) which is applied inside the lower eyelids to keep the eyes clean and attractive (Singh et al., 1990). The resin is also mixed with the ash of Betula utilis (Himalayan Birch), applied over sprains and also plastered on the fractured bone for a quick recovery, soften scar tissue. It is also consumed as a remedy in worm infection and gastric trouble (Uniyal et al., 2006). The oil obtained by the distillation of the needles is used in muscular pains and also as an expectorant (Bissa, 2008). Traditionally, the paste of resin and bark from P. roxburghii are applied as a plaster for healing bone fractures (Shuaib et al., 2013).

2.5.1.5 Commercial uses

P. roxburghii is a timber yielding plant and hence possess high commercial value. The heartwood of the plant is used in making furniture and building houses while the softwood is used in packaging cases and tea chest. The bark is rich in tannins and finds application in tanneries. The resin is commonly used to repair broken ceramic pottery. It is also used to prepare protective coatings, varnishes and printing ink. A commercially important component produced from Pinus is turpentine oil which is used to prepare varnishes, thinners, sealing wax, soaps, and disinfectants (Shuaib et al., 2013).

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ISSN: 2581-9429
Volume 6, Issue 1, June 2026
Impact Factor: 8.2

2.5.1.6 Pharmacological uses

P. roxburghii wood oil is reported as hepatoprotective due to antioxidant action at doses of 200, 300 and 400 mg/kg. It restored the elevated hepatic enzymes or markers enzymatic levels of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, lipid peroxidation and decreased level of reduced glutathione (GSH) and total protein (Khan et al., 2012). Few studies have shown that the medicinal plants possessing potential antioxidant activity are more effective for disease control in aging bone (Domazetovic et al., 2017).

P. roxburghii alcoholic bark extract at 100, 300, and 500 mg/kg has been reported as analgesic and anti-inflammatory due to polyphenolic compounds like rutin and quercetin (Kaushik et al., 2012).

2.5.1.7 Marketed formulations of P. roxburghii

The marketed formulation of P. roxburghii are polyherbal oil extracts (PHOE, Karachi, Pakistan) and Rumalaya gel (Himalaya Drug Company, Bangalore) for analgesic, antiinflammatory activities and also used in joint and bone pain. (Azmat et al., 2006; Rastogi et al., 2004; Sharma, 2005)

2.5.2 Pinus wallichiana

2.5.2.1 Habit and habitat

P. wallichiana commonly known as Bluepine/Kail is a coniferous evergreen tree native to Himalayas, Karakoram, and Hindu Kush mountains. It is also found in eastern Afghanistan, across northern Pakistan and India. The species grows naturally along the entire length of temperate Himalayas usually at altitudes range 2000 - 3,500 m (Shuaib et al., 2013).

2.5.2.2 Morphology

The tree is 50 m tall with a straight trunk and short down curved branches. The branches are longer in solitary trees creating a dome-like crown. The bark is smooth and resinous in young stems, turning grey and corky with shallow fissures on mature trees with a height of 36 m. Bluish green leaves are needle-like and are in clusters of five, 10-20 cm long, cones are light brown, when ripe is very resinous and easily distinguishable from other pines. Female cones are found in groups of 1-6, 20-30 cm long, erect when young, bluish-green when young, maturing to light brown with pale brown apophyses (Sharma et al., 2018) It is the finest pines of the north-western Himalayan region and is well known for its commercial and ecological importance. The wood of the blue pine is considered to be the best and stands next to deodar in value (Aslam et al., 2010). The tree has been exploited mainly as a source of timber but is a good source of oleoresin too. The resin is used for the production of turpentine oil, rosin, needle oil and camphor (Rehman et al., 2017).

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ISSN: 2581-9429
Volume 6, Issue 1, June 2026
Impact Factor: 8.2

Figure 2.13 P. wallichiana a) Cone b) Tree c) Needles d) Trunk

2.5.2.3 Phytochemical constituents

The stem bark of P. wallichiana has been reported to contain Kaempferol, rhamnetin, myrcetin, isorhamnetin, and quercetin. (Naeem et al., 2010)

2.5.2.4 Traditional and ethnopharmacological uses

The bark of P. wallichiana is applied as a plaster on the fractured bone (Gaur et al., 1992). It is also used by many tribes for the treatment of diarrhoea, stomach problems, infectious diseases and in wounds healing (Joshi et al., 2016). A green stem bark decoction is used for abdominal/intestinal ailments and skeleto-muscular problems (Ahmed et al., 2015).

2.5.2.5 Commercial uses

In, India P. wallichiana is of commercial importance as its turpentine is of superior quality to that of the P. roxburghii, but due to exploitation its commercial use is limited (Peltier et al., 2009). It is of similar timber properties and quality to P. strobus and P. monticola in North America, with tall, straight trees producing wood of good strength.

2.5.2.6 Pharmacological activities

Insecticidal activity of hydroalcoholic extract of leaves and stem bark of P. wallichiana was reported (Rahman et al., 2017).

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International Open-Access, Double-Blind, Peer-Reviewed, Refereed, Multidisciplinary Online Journal
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Volume 6, Issue 1, June 2026
Impact Factor: 8.2

2.5.3 Pinus gerardiana

2.5.3.1 Habit and Habitat

P. gerardiana was known as Noosa, is an evergreen pine tree growing up to a height of 25 m. It is native to the north-western Himalayas. There are about 29 species of pine which produce edible nuts those are utilized by indigenous tribal cultures. In India, out of six species of pine,

P. gerardiana is an only species which produces edible and highly nutritious nuts. The species is distributed not only in India but also in Afghanistan, Tibet, Baluchistan (Pakistan) at an elevation range of 2000-3350 m.In India, it is distributed in Himachal Pradesh (Kinnaur and Chamba districts) and Jammu and Kashmir (Kumar et al., 2016).

2.5.3.2 Morphology

The branches are slightly ascending, and usually not whorled. The bark exfoliates in irregular thin flakes and has a gray color. The leaves are needle-like, stiff, dark green, and are arranged in clusters of three (Peltier 2009).

Figure 2.14 P. gerardiana a) cone b) tree c) needles d) trunk

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ISSN: 2581-9429
Volume 6, Issue 1, June 2026
Impact Factor: 8.2

Figure 2.15 Pinus gerardiana kernels

Male cones are long and female cones are oblong-ovoid with thick woody scales. Seeds are cylindrical, elongated, dark brown pointed at the tip, bear a rudimentary wing. P. gerardiana is well known for its edible seeds or nuts (Chilgoza). Chilgoza nuts have got immense social forestry importance because it is an income source for tribal people in Kinnaur district of Himachal Pradesh good source of nutrients (Peltier, 2009).

2.5.3.3 Phytochemical constituents

The nuts are considered to be a rich source of various nutrients including proteins, carbohydrates, fibers, minerals besides its higher amount of oil. The oil is free of cholesterol and is a rich source of fatty acids like stearic acid, linoleic acid (Omega-6), linolenic acid (Omega-3), oleic acid (Omega-9), arachidic acid, palmitic acid (Thakur et al 2015). The seeds with edible kernels obtained from ripe cones are credited with carminative stimulant and expectorant properties.

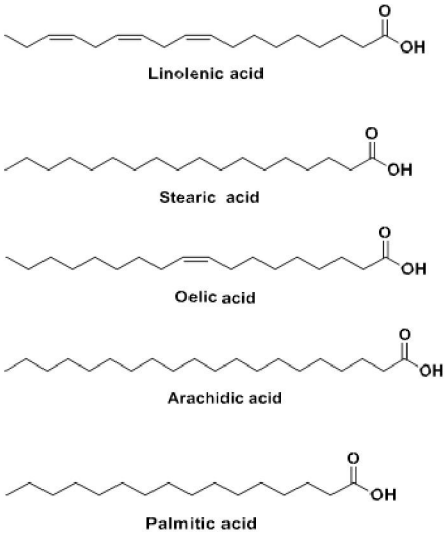

Figure 2.16 Chemical structure of chemical constituents from P. gerardiana

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ISSN: 2581-9429
Volume 6, Issue 1, June 2026
Impact Factor: 8.2

2.5.3.4 Ethnopharmacological and traditional uses

Fresh stem bark is fastened around the broken bone. The decoction of stem bark is given for abdominal ailments, resins are used for wound healing, wood-oil is given orally for intestinal warms, head warms, abdominal ailments, against a cough, better digestion Wood oil is also given at the start of summer to protect the livestock from the effect of change in weather (Ahmed et al., 2015)

2.5.3.5 Commercial uses

The main economic use of the plant is the use of oil-rich seeds as an edible product. The Chilgoza pine on tapping yield oleoresin but owing to its limited availability and avoidance of destruction of trees the species has not been exploited commercially for timber. Chilgoza nuts have become a source of income for the people of the region (Khurram et al., 2016). In traditional systems, enough cones are usually left on the tree to ensure that some seed is available for natural regeneration (Peltier, 2009).

2.5.3.6 Pharmacological uses

P. gerardiana nut oil is reported for the treatment of cardiovascular disorders and thrombo embolism (Rehman et al., 2017).

2.6 Potent phytochemicals for osteoporosis

2.6.1 Gallic acid

Gallic acid (3, 4, 5-trihydroxybenzoic acid), is a natural phenolic compound found in various plants and fruits (Haute et al., 2015). It has reported as antioxidant, anticarcinogenic, antifungal, anti-inflammatory, anti-malarial and anti-viral properties. It has been reported to inhibit osteoclastogenesis induced by RANKL in RAW 264.7 cell lines in-vitro (Reddy et al., 2012; Haute et al., 2015).

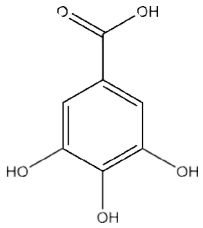

Figure 2.17 Chemical structure of gallic acid

2.6.2 Quercetin

Quercetin (3,3′ ,4′ ,5,7 – pentahydroxy-flavone) a flavonoid considered as a phytoestrogen (Wong, 2008). It is reported to have antioxidant and anti-inflammatory property and has a beneficial action in bone loss (Forte et al., 2016). It increases bone forming activities in the impaired microarchitecture of femurs in diabetic rats through improved antioxidant capacity (Liang et al., 2011). It also inhibits osteoclastic differentiation and bone resorption via inducing apoptosis and NF-kb pathway. In MG63 human osteoblasts, quercetin increased the ALP activity (Prouilleta et al., 2014). It was found to induce differentiation and proliferation of human adipose tissue-derived stromal cells (DSC) (Kim et al., 2006). The polyphenolic compounds quercetin and rutin are reported to be anti-inflammatory, antiasthmatic, analgesic anti-inflammatory, and antioxidant. Many plants containing flavonoids have been shown to have antispasmodic and anti-inflammatory actions (Kaushik et al., 2012). Flavonoids are well known to inhibit pain perception as well as anti-inflammatory properties due to their inhibitory effects on chemical mediator of inflammation (Kaushik et al., 2012). The ability of flavonoids to inhibit eicosanoid (prostaglandins), that involved in various immunological responses are reported. They are the end products of the cyclooxygenase and lipoxygenase pathways

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ISSN: 2581-9429
Volume 6, Issue 1, June 2026
Impact Factor: 8.2

involved in pain and inflammation (Sawadogo et al., 2006; Jothimanivannan et al., 2010). Moreover, flavonoids are able to inhibit neutrophils degranulation and thereby decrease the release of arachidonic acid (Hoult et al., 1994).

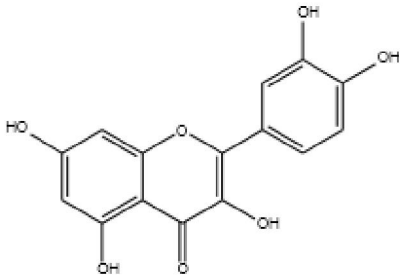

Figure 2.18 Chemical structure of quercetin

2.6.3 Ascorbic acid

Ascorbic acid also called vitamin C is an essential component involved in collagen formation (Simon, 2001). Its dietary intake is associated with bone mineral density among premenopausal women whereas its deficiency is associated with abnormal bone development. It is an important cofactor for many propyl and lysyl hydroxylases which actively participate in collagen maturation (Walmsley et al., 1999; Marini et al., 2007).

Ascorbic acid prevented loss of osteoblast differentiation markers (Osterix, osteocalcin, Runx2, BMP-2) and stimulated bone formation in OVX rats (Aghajanian et al., 2015). A depletion in the level of ascorbic acid results in increased ROS in postmenopausal osteoporosis which may lead to insufficient trabecular and cortical bone mass of the femur (Lai et al., 2017).

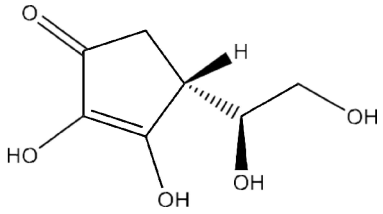

Figure 2.19 Chemical structure ascorbic acid

2.6.4 Tannic acid

Tannic acid is a plant-derived polyphenolic compound having antioxidant and free radical- scavenging properties and has health-promoting potential for humans (Xu et al., 2017). It is reported to increase the calcium content and provided protection on structure and function of bone (Tomaszewska et al., 2017).

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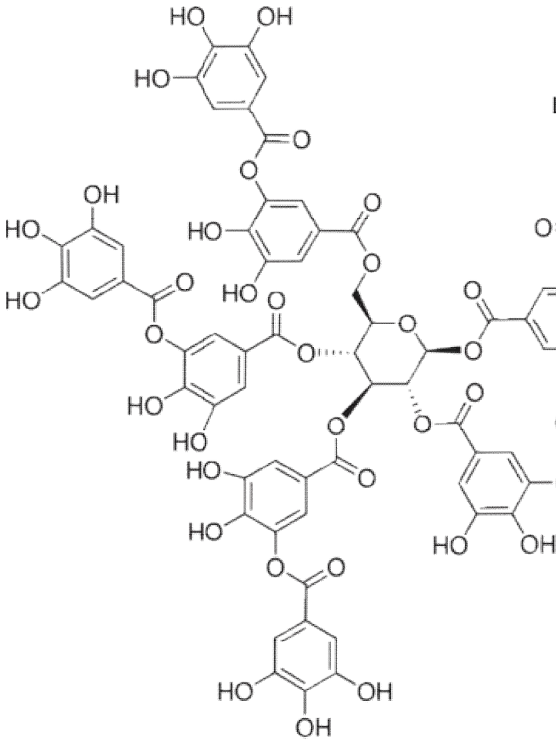

Figure 2.20 Chemical structure of tannic acid

On the basis of the literature survey, it is perceived that the Pinus species have got a rich repository of its traditional and local uses in various diseases and fractures. The scientific studies supporting their use for medicinal and health purposes found scanty. Moreover, the phytoconstituents like quercetin, gallic acid, ascorbic acid, and tannic acid have been studied in vitro or in vivo for their potential in the pathological state of oxidative stress, inflammation involved in bone dyshomeostasis.

2.6.5 Catechin

Catechins are known as tea polyphenols, mainly epigallocatechingallate, epicatechingallate, epicatechin, and epigallocatechin. They are considered to have potent antioxidant actions. In postmenopausal osteoporosis oxidative stress plays a significant role, the levels of lipid peroxides and H2O2 were increased and antioxidants superoxide dismutase, glutathione peroxidase, and glutathione S-transferase were decreased (Muthusami et al., 2005). Ferrario et al., 2011 had also reported that catechin are effective against osteoporosis by its anti- inflammatory action.

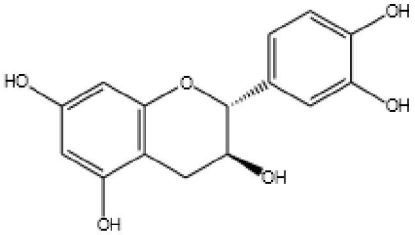

Figure 2.21 Chemical structure of catechin

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Volume 6, Issue 1, June 2026
Impact Factor: 8.2

2.6.6. Caffeic acid

Caffeic acid is found in green plants and is the representative of hydroxyl cinnamic acids. Caffeic acid is reported to inhibit osteoclastogenesis in mouse bone marrow derived macrophage precursor cells (BMMs) (Sandra et al., 2011)

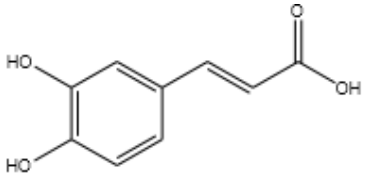

Figure 2.22 Chemical structure of caffeic acid

III. MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and reagents

The solvents used in extraction and fractionation, like petroleum ether, chloroform, ethyl acetate, n-butanol and ethanol was purchased from Loba Chemie Pvt. Ltd., Mumbai India. The chemicals used in phytochemical evaluation, 1, 1-diphenyl-2picrylhydrazyl (DPPH), vanillin, aluminium trichloride (AlCl3), naphthylethylenediamine dichloride, and tannic acid from Himedia Laboratories, Pvt. Ltd. Mumbai, India. Hydrogen peroxide were purchased from Finar chemicals Pvt. Ltd. Ahmedabad India. Lead acetate was purchased from Central drug house Pvt. Ltd. Delhi. Standard markers used in High performance liquid chromatography (HPLC) studies like gallic acid was purchased from Oxford Laboratory Pvt. Ltd. Mumbai, quercetin were purchased from Sigma Aldrich Chemicals, Bangalore, India. Ferric chloride, Trichloroacetic acid (TCA), Folin-Ciocalteu's reagent, Ehrlich's reagent were purchased from Loba chem. Pvt. Ltd., Mumbai India. For immunomodulatory assay Dulbecco's modified Eagle's medium (DMEM), RPMI- 1640, penicillin and streptomycin were purchased from Himedia Laboratories, Mumbai, India. Fetal bovine serum (FBS) from Gibco, US, DMSO, Con A and MTT from Himedia Laboratories, Mumbai, India. Formaldehyde solution was purchased from CDH Pvt. Ltd. New Delhi, India. All other chemicals and reagents used in the study were of analytical grade. The biochemical kits were procured from Erba diagnostics Mannheim, Nalagarh road, Baddi, Distt Solan, H.P.

3.1.2 Equipment and Instruments

Major instruments like ELISA plate reader from Biotech instruments, USA; Trinocular microscope attached with the camera (Model RXL-5HT) from Radical microscope Ambala, India; UV (Ultra violet) double beam spectrophotometer from Thermoscientific, India; Rota evaporator from Ika USA Pvt Ltd; Real-time PCR analyzer CFX96 from Bio-Rad, India were used. Water bath and Vortex shaker from REMI Mumbai, India, refrigerated centrifuge (Remi Electronik Ltd., Model no. CPR24 plus); deep freezer from Cell frost Innovation Pvt. Ltd. Model-CF 300, were used. Sonicator (Ultrasonic cleaner; Model Cleaner 30A). HPTLC having CAMAG Linomat 4 sample applicator (Camag Muttenz, Switzerland) equipped with syringe (Hamilton), twin trough chamber (CAMAG), TLC scanner 2 conjugated with winCATS software.

3.2 Methods

3.2.1 Collection of plant material

The stem bark of three Pinus species were collected from their native locations as P. roxburghii from local areas of Solan, HP (latitude 30.904486; longitude 77.096733 altitude: 1452 m); P. wallichiana from Shimla, HP (latitude 31.104605, longitude 77.173424. altitude: 2195 m) and P. gerardiana from Rekongpeo, Kinnaur, HP (latitude 31.583331 longitude 78.416665 altitude: 2759 m). The plant drug samples of P. roxburghii, P. wallichiana and P. gerardiana were duly authenticated from Department of Forestry, YS Parmar University of Horticulture and Agriculture Sciences, Nauni, HP, India and the voucher specimen with field book number, 13488, 13489 and 13506 were kept in

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institutional herbarium respectively. The plant part was dried in shade (up to 40ºC), coarsely powdered (to mesh size 10-20) using a mechanical grinder and stored in air tight container till further use.

3.2.2 Plant drug extraction

The coarsely powdered drug was defatted using petroleum ether as a nonpolar solvent for 18- 24 h at 50-55ºC and then extracted using 90% v/v ethanol in water (hydroalcoholic solvent) using Soxhlet apparatus at a temperature of 65-72ºC for 72 h. The solvent was recovered by evaporation under reduced pressure using rota evaporator. The semisolid mass was further freeze-dried using Lyophilizer at -80oC for 24 h to obtain freeze-dried powder and stored at 4- 8ºC till further use.

3.2.3 Plant drug fractions

The freeze-dried powder of hydroalcoholic extract was suspended in distilled water in a separating funnel and fractioned using solvents of graded polarities. The dichloromethane, ethyl acetate, n-butanol, and aqueous fractions thus obtained were subjected to evaporation under pressure using rota evaporator.

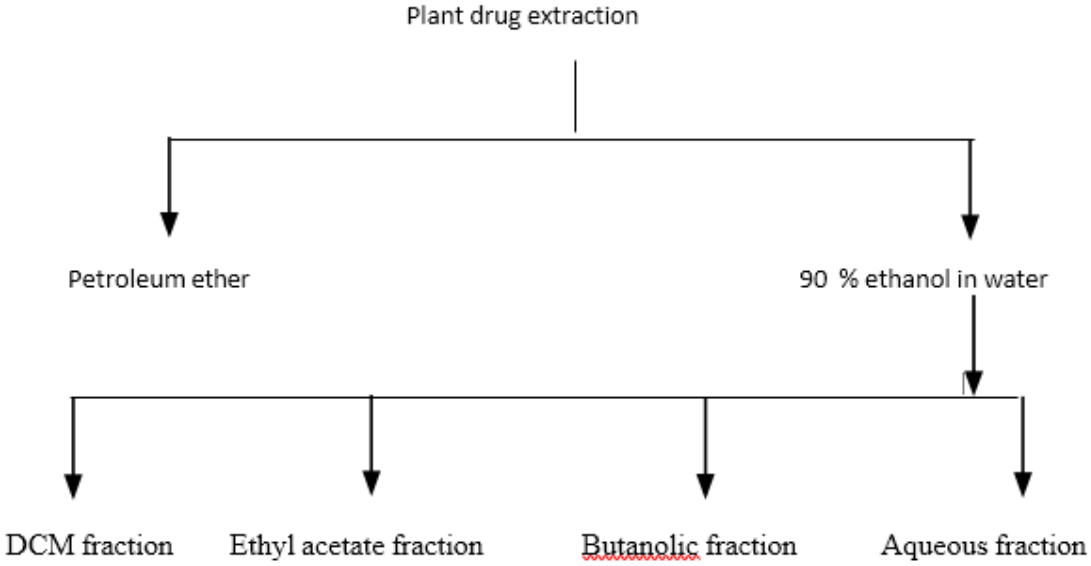

Figure 3.1 Flow chart for plant drug extractions

IV. RESULTS

Plant based products are recognized as major source of therapeutics, throughout human history to maintain health due to potentially wider safety margin. The use of natural products and based medicine has received more attention because of its compatibility and acceptability when compared to the synthetic drugs. The existing approaches for treating the devastating condition of bone porosity has some serious health risks like breast cancer, hot flashes, breathing trouble, chest pain, stomach upset, and inflammation in esophagus (Lewiecki, 2011). Thus, an emergence of treating the fatal state of osteoporosis is sought and may be resolved with the tool of modern drug discovery.

V. SUMMARY AND CONCLUSION

5.1 Summary

Some Pinus species are native to Himalayan states and stem bark of P. roxburghii and P. wallichiana is used in the form of paste to cure bone fractures. It has been reported to contain bioactive compounds from the class flavonoids, terpenes,

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tannins, steroid, and glycosides. Osteoporosis is a metabolic and hormonal disease, highly prevalent in postmenopausal women and the recommended medication is associated with severe side effects. The aim of the present study was to evaluate the pharmacological activity of stem bark of Pinus plants in surgically induced osteoporosis model in female rats.

The stem bark of three Pinus species P. roxburghii, P. wallichiana and P. gerardiana was defatted using petroleum ether and extracted with soxhlet apparatus using 90 % v/v ethanol in water (hydro-alcoholic extraction). The freeze dried extract powder fractioned using solvents of graded polarities; dichloromethane, ethyl acetate, n-butanol and aqueous fractions were prepared. The phytochemical screening of the plant extracts and fractions was performed for the presence of bioactive constituents like alkaloids, glycosides, flavonoids, phenolics, carbohydrates, tannins, terpenoids and steroids etc. The presence of gallic acid, quercetin, ascorbic acid and tannic acid was quantified in plant extracts and in bioactive fraction using HPLC study. Moreover, the presence of quercetin, gallic acid, tannic acid, catechin and caffeic acid were also quantified using HPTLC analysis. Hydroalcoholic extracts and fractions were standardized using quercetin, gallic acid, catechin, caffeic acid and corresponding Rf values obtained are 0.65, 0.40, 0.22 and 0.60. The antioxidant activity was evaluated using free radical scavenging methods for DPPH, nitric oxide and hydrogen peroxide radicals' assays. Anti-inflammatory activity was carried out using albumin denaturation and HRBC membrane stabilization assays. In-vitro osteoblastic proliferation by a hydroalcoholic extract of Pinus extracts was assessed on UMR-106 cell lines. Acute toxicity study of prepared extracts was conducted as per OECD423 guidelines. In in vivo antiosteoporotic activity female rats were subjected to surgical ovariectomy. Surgical removal of ovaries (ovariectomy) in the female rat of age 12-14 weeks is a scientifically validated method for inducing osteoporosis which resembles the postmenopausal state in women. The in-vivo anti-osteoporotic activity of Pinus extracts 100, 200 mg/kg at and active fraction 100 mg/kg was carried out at on female wistar rats using surgical ovariectomy model. Biochemical studies were carried out include serum calcium, phosphorus, alkaline phosphatase, estrogen and urine hydroxyproline. The biomechanical studies include three point bending of tibia, femoral neck loading test and compression of fourth lumbar vertebrae were done including assessment for BMD. RNA expression of RANKL, cathepsin K and OPG was estimated in tibia bone tissue. Bone tissue histology studies was done on femur bone.

In the present study the hydro alcoholic extract of each Pinus species showed the presence of alkaloids, glycosides, flavonoids, phenolics, carbohydrates, tannins, terpenoids and steroids. The presence of tannins, flavonoids, steroids, alkaloids are found absent in dichloromethane fraction of three pine species and found in other three fractions ; ethyl acetate, n-butanolic and aqueous fractions. In present study, the HPLC analysis quantitatively confirmed the presence of quercetin, gallic acid ascorbic acid and tannic in each Pinus plant extract and also in bioactive n-butanolic fraction. In free radical scavenging assays of different Pinus extracts and fractions, extracts of P. gerardiana and P. roxburghii with their n-butanolic fraction showed maximum antioxidant efficacy. The restoration of the trabecular network with less inter-trabecular spaces was observed in groups treated with Pinus plant extract and n-butanolic P.gerardiana fraction. The results indicated that n-butanolic fraction of P.gerardiana exhibited potent antiosteoporotic activity correlated with the histopathological studies, over other extracts and fractions and was comparable to standard raloxifene thus formulated into tablet. The presence of phytoconstituents: quercetin, gallic acid, tannic acid is reported in the present study. These phytoconstituents are reported in literature to inhibit bone loss, oxidative stress and suppress the release of inflammatory mediators. These may be responsible for the protective effect of extracts and fractions against the experimental model of osteoporosis. The most active n-butanolic fraction of P. gerardiana was formulated into tablet and evaluation was done by testing the hardness, friability, and disintegration time.

5.2 Conclusion

The findings from present investigation may conclude that the Pinus plant species has efficacy to ameliorate the pathological state of osteoporosis via modulation of estrogen, RANKL, OPG and cathepsin signaling. This effect may be due to the presence of quercetin, gallic acid, ascorbic acid, catechin, caffeic acid and tannic acid phytoconstituents.

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VI. RECOMMENDATIONS AND FUTURE DIRECTIONS

Pinus species stem bark is used traditionally as a paste to prevent or treat bone related disorders and fractures. Osteoporosis is a metabolic and hormonal disease, highly prevalent in postmenopausal women. The synthetic treatments that are available are associated with side effects Thus, an emergence of treating the fatal state of osteoporosis may be resolved with the tool of modern herbal drug discovery. The present research focuses on the investigation anti- osteoporotic activity of a hydroalcoholic extract of P. roxburghii, P. wallichiana and P. gerardiana and their potent bioactive fraction with mechanistic elucidation and phytochemical evaluation. The presence of phytoconstituents: quercetin, gallic acid, ascorbic acid tannic acid, caffeic acid, catechin is reported in the present study. In our study, n-butanolic fraction of P. gerardiana displayed antiosteoporotic activity. An oral herbal formulation of bioactive fraction of Pinus in recommended dosage has been prepared. In future,

- Phytoconstituents of Pinus plants can be further studied in view of designing new pharmacological strategies for therapeutics of bone disorder like osteoporosis
- Moderately active fractions could also be mixed to form a formulation and tested for enhanced activity.
- Synergistic study of Pinus plants on post-menopausal osteoporosis can also be carried out.
- Plant material extracts from different geographical area may be analyzed for phytochemical and biomedicinal properties.

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