Review of Literature
Bone is a highly specialized dynamic connective tissue, which provides mobility, muscle attachment, protection to internal organs and participates in metabolic homeostasis (Ducy et al., 2000; Teo et al., 2011; Teti, 2011; Weatherholt et al., 2012). Extracellular matrix constitutes the major component of bone which includes primarily collagen type-1 (~95%), proteoglycans and numerous non-collagenous proteins (5%) (van Apeldoorn et al., 2005). The organic matrix is mineralized with calcium and phosphate in the form of hydroxyapatite (Mahamid et al., 2011; Kliemt et al., 2013).

**Macroscopic structure of bone and types of bone**

Based on the structural organization, human bone is classified into axial skeleton (comprising the head and the trunk) and appendicular skeleton (the limbs). Based on their size and shape, they are further divided into long bones of the limbs (e.g. tibia, femur and humerus), short bones (phalanx), flat bones (skull, scapula and mandible) and irregular bones (the vertebrae) (Ferguson, 2004). All these skeletal sites show differing ratio of mechanical strength and based on which they are distinguished as trabecular (cancellous/spongy) bone and cortical (compact) bone (Kumar and Clark, 2002).

Trabecular bone is less dense and more elastic and undergoes active remodeling with a higher turnover rate (Hadjidakis and Androulakis, 2006). Trabecular bone is found at the end of long bones (epiphysis) and inside the cortex of flat bones, and consists of a network of interconnecting trabecular plates and rods within which lies hematopoietic or fatty marrow. The surface-to-volume ratio of cancellous bone is much greater than that of cortical bone, and the potential for metabolic activity is correspondingly higher (Compston, 2001). It is the site for the
action of bone cells [osteoblasts (OBs) and osteoclasts (OCs)] and a reservoir for minerals such as Ca, phosphorus (P) and magnesium (Mg) (Ferguson, 2004).

Cortical bone, which makes up the shafts (diaphysis) of the long bones and outer envelope of flat bones, constitutes approximately 80% of skeletal mass and plays an important role in the supportive, protective and mechanical functions of the skeleton (Henry, 2001). This compact bone is laid down concentrically around central canals or Haversian system, which contain blood vessels, lymphatic tissue, nerves and connective tissue. In cortical bone, densely packed collagen fibrils form concentric lamellae, and fibrils in adjacent lamellae run in perpendicular planes (Imai, 2014).

**Bone cells**

![Bone Cells Diagram](image)

(Adopted from Bonewald, 2011)

**Types of bone cells**

**Bone lining cells:** These cells that cover the bone are flat, elongated and inactive cells. They seem to regulate exchange of substrates between the bone fluid compartment and the extracellular fluid of bone marrow. They also serve as a
signaling link between the osteocytic network and the osteoclastic cell pool during bone resorption (Dierkes et al., 2009; Lupo et al., 2015).

**Osteoblasts:** They are cuboidal fibroblasts derived from mesenchymal cells. Transmembrane adhesion proteins (integrins, connexins, cadherins) and specific receptors (for cytokines, hormones, growth factors) maintain their function and responsiveness to metabolic and mechanical stimuli (Lecanda et al., 1998; Ferrari et al., 2000). The osteoid matrix secreted by osteoblasts consists primarily of collagen-I and a small percentage of other non-collagenous proteins (Sandberg, 1991). The osteoid is then mineralized leaving a thin layer of unmineralized matrix that covers all bone surfaces (Loveridge, 1999; Alcantara et al., 2011; Shibata et al., 2013). Differentiation of osteoblasts is a sequential process marked by changes in phenotypic expression of alkaline phosphatase (ALP), type I collagen, osteocalcin and osteopontin (Aubin, 1998). ALP is detected in preosteoblasts and increases to high level in mature osteoblasts then decreases on mineralization. Type I collagen is present from early proliferative stages, peaks at late matrix maturational stages. Osteoblasts lay down 0.5–1.5 \( \mu \text{m} \) osteoid per day and some osteoblasts trapped in their own calcified matrix develop into osteocytes. The lifespan of an osteoblast ranges up to 8 weeks in humans (Jowsey 1997; Mosley, 2000). Inadequate osteoblastic function is crucially involved in the pathogenesis of a number of common human metabolic bone diseases.

**Osteocytes:** They are mature osteoblasts that make up between 90% and 95% of the cellular component in mature adult bone tissue (Buckwalter et al., 1996; Noble, 2008; Carter et al., 2013). The constitutive molecular markers for osteocytes are limited to low collagen and ALP production, high casein kinase II and osteocalcin protein expression and high CD44 (Bonewald, 2007). It has long been proposed that
Osteocytes function as sensors for mechanical strain or increased load as part of the adaptive response of bone to maintain bone health (Burger and Klein-Nulend, 1999; Knothe-Tate et al., 2004; Kerschnitzki et al., 2013).

Osteoclasts: They are large, multinucleated and highly motile cells derived from the monocyte/macrophage lineage of hematopoietic stem cells. The earliest osteoclast precursor cells are the granulocyte-macrophage colony-forming units (CFU-GM) (Kurihara, 1990; Kurihara et al., 1991; Kerby et al., 1992; Menaa et al., 2000). Osteoclasts are polarized bone resorbing cells with ruffled border and enriched lysosomal enzymes (Edwards and Mundy, 2011).

PU.1 transcription factor (TF) prevents the entry of these precursor cells towards the myeloid phenotype (Teitelbaum et al., 1997) but facilitates osteoclast formation and differentiation. PU.1−/− mice devoid of osteoclasts and macrophages is osteopetrotic (Tondravi et al., 1997). PU.1 also regulates the expression of receptor activator of nuclear factor κB (RANK) (Kwon et al., 2005), cathepsin K (Matsumoto et al., 2004) and tartrate-resistant acid phosphatase (TRAP) (Partington et al., 2004). Vesicles containing TRAP are fused into these vesicles, whose acidic pH allows cathepsin K to cleave the loop-peptide, generating TRAP with high specific activity (Vääräniemi et al., 2004; Ljusberg et al., 2005). TRAP is secreted from the ruffled border, dephosphorylates osteopontin and allows osteoclast migration; TRAP generates ROS that are targeted for degradation of the organic matrix components (Halleen et al., 1999).
Bone fracture

Bone fracture can be a complete or incomplete break in the anatomic continuity of bone that affects mechanical stability of the bone. A fracture is also associated with injury to the surrounding soft tissues, including blood supply, and in most cases with compromised function of the locomotor system. Fracture is often accompanied with an auto-activated healing involving many local and systemic growth factors, hormones and extracellular matrix components (Einhorn et al., 1995). Risk factors like low bone mineral density (BMD) and osteoporosis increase the incidence of fracture (Doblare et al., 2004).

Types of bone fracture

There are several types of bone fractures. Micro-fractures turn into macro-fractures due to continuous loading. Stress fractures occur in individuals who have increased repetitive-type physical activities. Traumatic fractures occur due to accidental excessive load on bone or intensive muscle contraction or deteriorated trabecular and microarchitecture. Pathologic fractures are due to the inability of the soft muscle tissues to absorb the external high energy forces (Doblare et al., 2004; Ulstrup, 2008; Bigham-Sadegh and Oryan, 2015).

![Bone fracture types](image)

(Adopted from Stone et al., 2003)
Modes of fracture

Based on the shape or pattern of the fractured fragments, fractures are divided into:

**Simple (closed)** - little or no bone displacement

**Compound** - fracture ruptures the skin and bone protrudes

**Transverse** - crack perpendicular to long axis of the bone - displacement may occur

**Oblique** - diagonal crack across the long axis of the bone

**Spiral** - diagonal crack involving a "twisting" of the bone about the longitudinal axis

**Comminuted (blowout)** - "crushing" fracture - more common in elderly - may require screws, rods, and wires - may cause permanent discrepancy in leg length

**Avulsion** - fragment of bone is pulled away by tendon (Hip flexors, adductors)

**Impacted** - one end of bone is driven up into the other - may result in length discrepancy

**Green stick** - occurs mostly in children whose bones have not calcified or hardened

**Depressed** - broken bone is pressed inward (skull fracture)

**Fracture healing and its classification**

Fracture healing is a complex regenerative process where bone regains its original condition and strength (Einhorn, 1998; Dimitriou et al., 2005; Brandi, 2010).
The healing process has been divided into two types 1) **Direct** (Primary) 2) **Indirect** (Secondary) fracture healing (Einhorn 1998; Marsell and Einhorn, 2011).

**Direct or primary fracture healing:** It is not a regular but more rapid healing process (Marsell and Einhorn, 2011; Aydin et al., 2012). The healing involves intramembranous ossification and direct cortical remodeling without callus formation (Isaksson et al., 2007).

**Indirect or secondary fracture healing:** Majority of fractures heal by this process through callus formation and endochondral ossification. During this process, some motions exist between the fracture ends which happen frequently after intramedullary nailing and external fixation procedures (LaStayo et al., 2003; Dimitriou et al., 2005; Marsell and Einhorn, 2011). The indirect healing process occurs in four main phases: (1) Inflammatory phase: The defect is initially filled with hematoma and there is intense inflammation (2) Repair phase: here the hematoma is quickly replaced by granulation tissue (3) Remodeling phase: a fibro cartilaginous callus is formed over the weeks (4) Mineralization phase: a hard callus is formed, becoming fusiform and slowly disappearing as Haversian remodeling progresses.

(Adopted from Wraighte and Scammell, 2006)
Mechanism of fracture healing

Fracture associated vascular damage leads to hemorrhage and inflammation that culminates in the development of a hematoma. The undifferentiated MSCs from the fracture gaps migrate proliferate and form the granulation tissue. This tissue forms the nucleus for subsequent healing process. The repair stage is characterized by the formation of callus, continued vascular in growth and the secretion of osteoid and collagenous fibers (Olsen et al., 2000). During intramembranous bone formation, mesenchymal cells differentiate directly into osteoblasts from the periosteum adjacent to the fracture site and synthesize bone matrix. Intramembranous bone formation mainly occurs in the flat bones of the skull, the mandible, and the part of the clavicle (Einhorn, 1998; Ducy, 2000).

Endochondral bone formation as a mode of fracture repair involves the indirect way of osteoblast formation. Initially, mesenchymal cells from the sub periosteal bone migrate, proliferate and differentiate into chondrocytes. These cells synthesize and secrete type II collagen and proteoglycans and form the bridging callus between the fracture ends to stabilize them (Reddi, 1998; Dimitriou et al., 2005). Then the chondrocytes become hypertrophic, mineralized and undergo apoptosis in a spatially organized manner (Barnes et al., 1999). The apoptotic chondrocytes are replaced by osteoblasts that secrete type I collagen matrix, ossified, and remodel to substitute the trabecular woven bone with compact bone. The repair process in many aspects recapitulates the stages of embryonic bone development (Bruder et al., 1994; Thompson et al., 2002) and expression of the same molecular markers of chondrogenesis and osteogenesis (Ferguson et al., 1999; Karsenty and Wagner, 2002; Thompson et al., 2002).
Role of hormones in bone fracture healing

The fracture associated healing is an auto-activated event involving many hormones (PTH, PTH-rP, GH etc.), local and systemic growth factors (BMPs, TGFβ, IGFs, PDGF, FGF, VEGF etc.) and extracellular matrix components (collagen, fibronectin etc.) (Simpson et al., 2006).

Parathyroid hormone (PTH)

The overall function of PTH is to reverse hypocalcemia and maintain normal extracellular calcium levels by enhancing gastrointestinal calcium absorption, renal calcium reabsorption, and osteoclastic bone resorption. Biologically active PTH (1-34) is reported to participate in femoral fracture repair in rats. PTH (1-34) stimulated the mRNA expression of collagen type-I, osteonectin, ALP and osteocalcin in the osteoblast of the femoral fracture callus (Nakijima et al., 2002).

PTH induced fracture healing mimicked endochondral bone formation as evident from the increased expression of chondrogenic and osteogenic markers in femoral fracture callus of male mice. Further, these authors have demonstrated the involvement of Wnts and β-catenin in PTH induced femoral fracture healing (Alkhiary et al., 2005; Kakar et al., 2007; Aspenberg et al., 2009; Peichl et al., 2011).

In in vitro condition, human recombinant PTH (teriparatide) treatment induced mesenchymal stem cell differentiation into osteoblast possibly through induction of Osterix (Osx) and Runx2 expression. In in vivo condition, teriparatide treatment enhanced cartilage formation and induced a significant increase in callus formation at 7, 10 and 14 days, post-fracture animal compared to control post-fracture animal in a mouse femoral fracture model. Thus, PTH enhances fracture healing through
enhanced differentiation of mesenchymal progenitor cells into mature osteoblast at the fracture site (Kaback et al., 2008).

Administration of exogenous PTHrP promoted fracture repair in Lepr \(^{-/-}\) mice by increasing callus formation and up regulated osteoblastic expression of ALP, Type-I collagen, Runx2 and IGF-I (Liu et al. 2015). PTHrP and its receptor are expressed in the outer and inner layers of periosteum, respectively. Conditional deletion of PTHrp affected the formation of callus, its ossification, impaired osteoblast formation and osteoclast activity in a tibial fracture mice model (Wang et al., 2015).

PTH is reported to facilitate healing in a mouse femoral allograft model by external callus formation and cartilage development (Takahata et al., 2012). In addition, PTH promoted remodeling and new bone formation so as to replace the graft (Jorgensen and Scwarz, 2011). These findings endorsed the positive effects of PTH (1-34) reported in an earlier study (Alkhiary et al., 2005). Thus, PTH not only increased cartilage formation but also increased coupled remodeling during fracture healing. PTH combination treatment with BMP-7 increased the tibial metaphysial volume and mechanical functions in a fracture healing rabbit model. However, neither treatment alone improved mechanical function (Morgan et al., 2008).

**Vitamin D3**

Administration of 25-OH-vitamin D after the experimental fracture significantly improved the mechanical strength of the fractured femur in aged female Wistar rats (Delgado-Martinez et al., 1998).
**Growth hormone (GH)**

GH has significant influence on the maintenance of bone mass by regulating bone remodeling. Though a few interventional studies with GH on accidental hip fracture patients and closed tibial fractures have accelerated fracture healing, open tibial fracture showed no significant healing (Weissberger *et al*., 2003; Yeo *et al*., 2003; Raschke *et al*., 2007). There is evidence from animal models and from *in vitro* studies that GH stimulates fracture healing (Bak *et al*., 1990; Raschke *et al*., 1999; Bail *et al*., 2002; Andreasen and Oxlund, 2003). In rat fracture model, GH increased stiffness and energy absorption at the highest dose level (Bak *et al*., 1990). They have further shown in an identical model that GH has an initial stimulatory effect on external callus formation but is poorly structured and remodeled (Mosekilde and Bak, 1993). In a rabbit model with unilateral tibial osteotomy, GH had no significant effect on fracture healing (Carpenter *et al*., 1992).

**Estrogen**

Decreased estrogen levels due to natural or surgically induced menopause lowered bone mineral density in humans (Eastell, 2006; Sambrook and Cooper, 2006). OBs contain both ERα and ERβ (Arts *et al*., 1997). The level of ERα increases during osteoblastic cell differentiation (Bodine *et al*., 1998). The main *in vivo* action of estrogen on the skeleton is to inhibit bone resorption with the regulation of cytokines and growth factors production in OBs. Estrogen is anti-apoptotic in OBs, and also has been shown to induce apoptosis in bone resorbing OCs (Kousteni *et al*., 2002). It exerts direct actions on OBs and OB progenitor cells that favor rather than suppress their phenotypic expression (Holzer *et al*., 2002). It has been shown that estrogen inhibits the synthesis of IL-1, IL-6, and TNF-α and IL-11, as well as IL-6.
synthesis in response to IL-1 (Jilka et al., 1992; Kimble et al., 1996; Jilka 1998; Manolagas, 2000). Estrogen has also been shown to induce the synthesis of BMP-6, OPG, TGF-β, NF-κB and c-Fos in hOB (Stein and Yang 1995; Rickard et al., 1998; Tau et al., 1998). Estrogen plus progestin increased BMD and reduced the risk of fracture in postmenopausal women (Cauley et al., 2003). Estrogen enhanced fracture healing in the long bones of mice (Beil et al., 2010).

Androgens

Testosterone has genomic and non-genomic anabolic actions on bone (Kang et al., 2003, 2004, 2008; Russell et al., 2012). The fracture healing property of testosterone has earlier been reported in traumatized bone of rats (Zafirau et al., 1996; Gordon et al., 1997). While it decreased the apoptosis of osteoblasts and osteocytes, it promoted the same in osteoclasts (Kousteni et al., 2001, 2002). Testosterone effect is confined to periosteum and acts through AR (Wiren et al., 2008). Recently, it has been reported that testosterone provided in a scaffold failed to induce callus formation in the fractured femur of AR knock out (ARKO) mice (Cheng et al., 2013). However, in wild mice, testosterone is able to bridge the femur fracture. Further, the healing effects of testosterone is comparable to that of BMP-2 and their combination was more effective (Cheng et al., 2013).

Glucocorticoids (GC)

The major complication of long-term systemic GC excess is the detrimental effect on bone as it is associated with increased fracture risks particularly in hip and spine (Lukert and Raisz, 1990; Van Staa et al., 2000; Alesci et al., 2005). Supra-therapeutic or prolonged treatment with glucocorticoids interfered with healing
process in the tibial and ulna osteomic models in rabbits (Bostrom et al., 2000; Waters et al., 2000; Luppen et al., 2002).

**Role of growth factors in bone fracture healing**

Growth factors (GFs) regulate the cellular stages of tissue regeneration (Chen et al., 2010; Javed et al., 2011). Several growth factors such as BMPs, PDGF, IGFs, TGFβ, FGF and VEGF are involved in bone fracture healing process. These factors act in an autocrine, paracrine and endocrine fashion to regulate the various stages of bone fracture healing. Some growth factors act as mitogenic and angiogenic signals during the initial steps of bone regeneration (Ferriera et al., 2013).

**BMPs**

Of the 20 different isoforms of BMP described, BMP-2, -4 and 7 are the most extensively investigated for their roles in the enhancement of skeletal repair (Friedlaender et al., 2001; Kloen et al., 2003; Yu et al., 2010). Recombinant human BMP-7 selected to treat tibial non-unions patients although proved to be safe and effective, the data showed no improvement compared with autologous bone grafting. Patients with open tibial-shaft fractures received a statically locked intramedullary nail (standard of care) or standard of care plus either 0.75 mg/kg or 1.50 mg/kg BMP-2 embedded in a type I collagen sponge (Govender et al., 2002). The group received high dose of BMP-2 reduced the need for secondary intervention and also fewer failures of the nail, fewer infections and faster wound-healing. In view of these, rhBMP-2 got the FDA premarket approval and is made available for the treatment of fresh, open tibial fractures. Nevertheless, further clinical studies have shown that rhBMP-2 was not augmenting the impact of standard of care alone in patients with
closed tibial fractures (Aro et al., 2011; Lyon et al., 2013). An alternative biomaterial of hybrid nanofibre mesh/alginate used to deliver rhBMP-2 yielded greater bone connectivity compared with the collagen sponge (Boerckel et al., 2011). Mesenchymal stem cells transfected with BMPs for the treatment of non-union fracture were also explored (Liebergall et al., 2013). Collectively, the clinical findings on the use of rhBMPs have been disappointing and prohibitive due to high dose and cost involvement.

In contrast to these clinical findings, in a mouse model BMPs participate in all three phases of fracture healing (Tsuji et al., 2006; Yang et al., 2014; Yang et al., 2015). In consonance with these findings, the mRNA expression of BMP-2, 4, 5 and -7 in the chondrocytes and in the mesenchyme surrounding cartilaginous anlage revealed their relevance in endochondral bone formation (Kingsley, 1994; Lyons et al., 1995; Rosenweig et al., 1995; Rosen et al., 1996).

**IGFs**

Insulin-like growth factors (IGF-I and II) are the most abundant growth factors stored in bone, and are likely to be important local regulators of fracture healing (Trippel, 1998). IGFs promote cell proliferation and matrix synthesis by chondrocytes and osteoblasts (Guenther et al., 1982; McCarthy et al., 1989), the two cell types largely responsible for the formation of fracture callus. During fracture healing, IGF-I is involved in cell proliferation and differentiation of mesenchymal cells, periosteal cells, osteoblasts, osteoclasts and chondrocytes in an autocrine and/or paracrine fashion. IGF-I regulates osteoblastic proliferation, differentiation, survival and the synthesis of bone matrix in vivo (Pfeilschifter et al., 1990; Bikle et al., 2002; Nakae et al., 2001). IGF-I stimulates VEGF secretion from both osteoblasts and
chondrocytes to promote angiogenesis (Deckers et al., 2000). Moreover, osteoblasts and chondrocytes promote each other’s differentiation process (Nakaoka et al., 2006).

Osteoblasts orchestrate interactions between different cell populations, playing a central role in fracture healing by coordinating the actions of different cell types involved, and that most of those cell-cell communication functions are dependent on IGF-I signaling. IGFBP-5 may play an important role in endochondral ossification and bone remodeling interacting with IGF-I (Koh et al., 2011). IGF-I and its receptors are widely expressed in periosteal cells, mesenchymal cells, proliferating chondrocytes and osteoblasts in calluses during fracture healing (Eingartner et al., 1999; Okazaki et al., 2003) emphasizing the role of IGF-I in fracture healing. Stimulation of fracture healing by local application of IGF-I has been demonstrated in rats, pigs and mouse models (Schmidmaier et al., 2001; Raschke et al., 2002; Fowlkes et al., 2006). IGF-I secreted from osteoblasts induces discrete migration of osteoblasts, promotes cell spreading and regulates cell polarization in a monolayer model of wound healing (Nakasaki et al., 2008).

Many herbal compounds such as soy protein, dried plum, and seed extracts of safflower, Rynchosia volubilis and calcium have been shown to increase osteoblastic function and have positive influence on bone metabolism by regulating IGF synthesis (Honda et al., 1995; Arjmandi et al., 2002; Gann et al., 2005; Kim et al., 2005; Lee et al., 2009). IGF-IR is not only involved in osteoblast differentiation during fracture repair, but also integrates the responses of chondrocyte, osteoclast and endothelial cells towards endochondral bone formation required for normal fracture repair (Wang et al., 2015).
Vascular Endothelial Growth Factor

The role of vasculature in osteogenesis was appreciated, when Haller, (1763) suggested that blood vessels are responsible for bone formation. Endogenous vascular endothelial growth factor (VEGF) is important for bone formation. VEGF is produced by endothelial cells, macrophages, fibroblasts, smooth muscle cells, osteoblasts, and hypertrophic chondrocytes (Josko et al., 2000; Deckers et al., 2002; Bluteau et al., 2007). Like most peptide growth factors, VEGF binds to receptors (VEGFR-1 and 2) on the cell surface of its targets (Ferrara et al., 2003).

Primary human osteoblasts express high levels of VEGFR-1 and signaling through VEGFR-1 on osteoblasts induces a strong chemotactic response (Mayr-Wohlfart et al., 2002). VEGF also indirectly induces proliferation and differentiation of osteoblast precursor cells through increased secretion of endothelin-I and IGF-I (Wang et al., 1997). VEGF has also been shown to promote mesenchymal stem cell (MSC) chemotaxis (Mishima and Lotz, 2008). VEGF plays an important role in every step of the fracture repair cascade from being concentrated in fracture hematoma, to the final remodeling stages of fracture repair (Beamer et al., 2010).

Fibroblast Growth Factor

Fibroblast growth factor (FGF) and its receptor (Fgfr) gene families encode essential signaling molecules that function through all stages of endochondral bone development. Recombinant human FGF-2 was used to enhance tibial-shaft fracture healing in 70 patients (Kawaguchi et al., 2010). Patients were injected into their fracture with low and high doses of FGF-2 hydrogel. The treatment induced the
fracture union without any differences between the high-dose and low-dose FGF-2 and any type of adverse effects.

Kawaguchi et al. (1994) revealed that a single dose of recombinant basic FGF increased the structural integrity of fibula fracture in rats. FGF9 and FGF18 signaling regulate hypertrophic chondrocyte differentiation, skeletal vascularization, and osteoblast/osteoclast recruitment to the growth plate (Liu et al., 2002; Hung et al., 2007; Liu et al., 2007).

**PDGF**

Recombinant human homodimeric PDGF subunit B (PDGF-BB) has been extensively studied for the enhancement of skeletal repair. In clinical trial studies, PDGF-BB combined with a β-tricalcium phosphate matrix was used to treat the patients requiring hind foot or ankle arthrodesis, as an alternative to the autologous bone graft from the iliac crest (DiGiovanni et al., 2013). Eventhough, PDGF as well as autologous graft showed better fusion, patients given PDGF had less pain and an improved safety profile.

**Wnt signaling**

Wnt signaling is activated during bone fracture repair (Hadjiargyrou et al., 2002). Study has revealed the up-regulation of Wnt signaling pathway genes, including Wnt-4, Wnt-5b, Dv11-3, CKIA1, TCF1, LRP-5 and Wnt target genes such as En-1, PPARD and CD44 in a rat closed fracture model. LEF-1 is a known repressor of Runx2, therefore the down-regulation of LEF-1 was deemed necessary during the early phase bone repair to occur (Zhong et al., 2006). In addition,
β-catenin and Dishevelled (Dvl) proteins were localized in proliferating and differentiating chondrocytes and osteoblasts within the fracture callus.

Further, in the early phases of fracture healing, β-catenin tightly regulates the differentiation of mesenchymal cells into osteoblasts and chondrocytes lineages. Several Wnt ligands and receptors were also up-regulated during fracture repair including Wnt-4, 5b, 5a, 10b, 11 and 13, and the receptors Fz-1, 2, 4 and 5, and LRP-6, indicating that both canonical and non-canonical signaling pathways are activated (Chen et al., 2007a). Wnt-1-induced secreted protein 1 (WISP-1) may play a role during bone fracture repair as demonstrated in a mouse femur fracture model (French et al., 2004).

**Oxidative stress in fracture healing**

Large amount of oxygen free radicals are generated in the process of fracture healing. Consequential oxidative stress prolong the inflammatory and repair periods of fracture healing (Turgut et al., 1999; Kelpke et al., 2001; Prasad et al., 2003; Yeler et al., 2005). Due to the presence of oxidative stress in fracture site, there is a defense system to alleviate the harmful effects of these oxidants including enzymatic [e.g., superoxide dismutase (SOD) and glutathione reductase (GR)] and non-enzymatic antioxidants [such as glutathione (GSH) and vitamins C and E] (Phillips and Yeowell, 1997; Day et al., 2000). These antioxidants are regulated by the transcription factor Nuclear factor erythroid-derived 2 like (Nrf2), which acts as a master regulator of cellular protection (Wruck et al., 2007, 2008). In particular, Nrf2 has been upregulated in mesenchymal cells that participate in callus formation being the critical step in fracture repair (Takahata et al., 2009). Thus Nrf2 protects the chondrogenic and osteogenic precursor cells from the onslaught of ROS generated at
the fracture site. Recent studies have underscored the essentiality of Nrf2 in bone anabolic activities (Park et al., 2014) and the impaired femur fracture healing in Nrf2 deficient mice (Lippross et al., 2014). These observations emphasize that oxidative stress can also be a therapeutic target aimed to improve fracture healing.

Herbs and bone

Search for therapy is a continuous process as we are yet to identify the drug for the disease. Though, phytotherapy is an ancient system of medicine in practice, it is incompletely understood and remains a potential area for research, especially as an alternative system of medicine. There are certain natural herbs with therapeutic values for bone fracture healing (Huang and You, 1997). A meta-analysis with 14 randomized controlled trials involving 780 patients has concluded that phytotherapy was as effective as hormonal therapy in the prevention of postmenopausal bone loss at lumbar, femoral and forearm sites (Xu et al., 2009). Interestingly, phytotherapy had much lower incidence of uterine bleeding and breast pain in postmenopausal women than those treated with hormonal therapy.

Traditional methods of fracture healing

The first research study demonstrating the ability of Cissus quadrangularis to accelerate bone fracture healing was made in 1960 followed by a series of studies thereafter mostly in experimental animals (Udupa and Prasad, 1962, 1964, 1964; Prasad and Udupa, 1963; Singh and Udupa, 1962). Even before these research observations, there were references regarding the folkloric use of the C. quadrangularis for bone fracture healing, collected through interviews of native healers (Gupta and Sharma, 2008). The plant material was applied as paste at fracture
site (human) or extracts prepared in organic solvents through injection (experimental animals). Herbal drugs are preferred over synthetic drugs because of their low cost and lack of obvious side effects. Moreover, researchers around the world are interested in providing scientific validation to strengthen the therapeutic values of herbal medicines (Vengatesh et al., 2003; Verma and Singh, 2008; Chavi et al., 2011).

The following is the list of medicinal plants investigated for their effects on bone formation and bone fracture healing

<table>
<thead>
<tr>
<th>S.No</th>
<th>Plants</th>
<th>Part used</th>
<th>System of medicine</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Eucommia ulmoides</em></td>
<td>Leaves</td>
<td>Chinese</td>
<td>Lin <em>et al.</em>, 2011</td>
</tr>
<tr>
<td>2</td>
<td><em>Rehmannia glutinosa</em></td>
<td>Roots and leaves</td>
<td>Chinese</td>
<td>Lim and Kim, 2013</td>
</tr>
<tr>
<td>3</td>
<td><em>Morinda officinalis</em></td>
<td>Root</td>
<td>Chinese</td>
<td>Li <em>et al.</em>, 2014</td>
</tr>
<tr>
<td>4</td>
<td><em>Puerariae lobata</em></td>
<td>Roots and leaves</td>
<td>Chinese</td>
<td>Huh <em>et al.</em>, 2006</td>
</tr>
<tr>
<td>5</td>
<td><em>Camellia sinensis</em></td>
<td>Leaves</td>
<td>Ayurveda</td>
<td>Das <em>et al.</em>, 2005</td>
</tr>
<tr>
<td>6</td>
<td><em>Cimicifuga racemosa</em></td>
<td>Roots and rhizomes</td>
<td>Native Americans</td>
<td>Chen <em>et al.</em>, 2002</td>
</tr>
<tr>
<td>7</td>
<td><em>Angelica sinesis</em></td>
<td>Leaves and root</td>
<td>Chinese</td>
<td>Yang <em>et al.</em>, 2002</td>
</tr>
<tr>
<td>8</td>
<td><em>Curculigo orchioides</em></td>
<td>Rhizome</td>
<td>Chinese</td>
<td>Wang <em>et al.</em>, 2010</td>
</tr>
<tr>
<td>9</td>
<td><em>Psoralea corylifolia</em></td>
<td>Fruit</td>
<td>Chinese</td>
<td>Li <em>et al.</em>, 2014</td>
</tr>
<tr>
<td>10</td>
<td><em>Fructus ligustri lucidi</em></td>
<td>Fruit</td>
<td>Chinese</td>
<td>Zhang <em>et al.</em>, 2008</td>
</tr>
</tbody>
</table>
Eucommia ulmoides

The plant extract as well as active principles are reported to have anabolic effects on bone both in vivo (Zhang et al., 2009, 2014) and in vitro (Ha et al., 2003; Lin et al., 2011). However, the active principles of this plant inhibited osteoclast activity in vitro (Ha et al., 2003; Lin et al., 2011). Nevertheless, there is no information on its fracture healing abilities.

Rehmannia glutinosa

Rehmannia glutinosa Libosch which is widely used to maintain haemostatic and diuretic agent in Eastern Asia, also registered anabolic activities on osteoblasts (Oh et al., 2003) and increased the BMD (Lim and Kim, 2013). This plant has also not been tested for its fracture healing properties.
**Morinda officinalis**

The roots of this plant (named, Bajitian) have been recorded in pharmacopeia of the People’s Republic of China and helps to strengthen the bones and kidneys and enhance the immune system function. An ethanolic root extract of *M. officinalis* rich in anthrax quinones prevented bone loss in ovariectomized rats (Li et al., 2009; Li et al., 2014). This can be attributed to its antiosteoclastic activity and thus bone resorption (Bao et al., 2011). However, its effect on fracture healing is not known.

**Puerariae lobata**

The root of *Puerariae lobata*, a wild creeper leguminous plant is one of the earliest and most important crude herbs used in oriental medicine. Puerarin, an isoflavone found in this herb acts through estrogen receptors (Wang et al., 2013, 2014). This isoflavone while increased osteoblast activity, decreased ostoclastic activity in vitro (Li and Yu, 2003; Zhang et al., 2007). Further, this principle prevented OVX induced bone loss in rats (Urasopon et al., 2008; Lim et al., 2013). Nevertheless, its impact on fracture healing is not known.

**Camellia sinensis**

It is black tea plant. The phytoestrogenic property of aqueous extract not only prevented bone loss but also restored the same in OVX rats (Das et al., 2004, 2005, 2009). Epigallocatechin-3- gallate (EGCG), a polyphenol present in green tea, induced osteoblast activity (Choi and Hwang, 2003). However, it decreased osteoclast activity (Morinobu et al., 2008; Lin et al., 2009) and suppressed MMP-2 and MMP-9 activity in osteoclast precursor cells (Oka et al., 2012).
**Cimicifuga racemosa**


**Angelica sinesis**

*Angelica sinesis* is a Chinese herb used for cleansing blood and increasing circulation and as a valuable remedy for anemia, menstrual irregularities and constipation. Z-ligustilide isolated from *A. sinesis* prevented OVX induced bone loss (Ma and Bai, 2012; Lim and Kim, 2014) and its extract inhibited the osteoclast differentiation (Kong *et al.*, 2014). However, there is no report on its effects on fracture healing.

**Curculigo orchioides**

*Curculigo orchioides* is used as a traditional herbal medicine in India and China. Curculigoside (CCG) is the main bioactive compound present in the rhizome of *C. orchioides*. The antioxidant properties of CCG have been exploited to reveal its pro-anabolic and anti-catabolic activities on bone cells (Jiao *et al.*, 2009; Wang *et al.*, 2010, 2012). CCG also antagonized the pro-osteoclastic activities induced by dexamethasone in rat calvarial cells (Zhu *et al.*, 2015).
**Psoralea corylifolia**

Scurfpea fruit (or) the fruit of *Psoralea corylifolia* L. (Fabaceae) is a commonly used herb in China and other Asian countries for thousands of years. The major active constituents of *P. corylifolia* are psoralen, isopsoralen, bavichin (BA), isobavichin and bakuchiol (BK). These principles protected OVX rats bone loss and also stimulated many osteoblastic activities (Li *et al*., 2014) through up regulation of Wnt signaling components *in vitro* (Weng *et al*., 2015).

**Fructus ligustri lucidi**

*Fructus ligustri lucidi* (FLL) is the fruit of *Ligustrum lucidum* Ait. FLL has been used in traditional Chinese medicine for over thousands years. Ethanolic as well as aqueous extracts of FLL increased BMD in selected skeletal sites of OVX rats (KO *et al*., 2010; Liu *et al*., 2015). Treatment of FLL ethanolic extract accelerated the formation of calcified matrix and increased extracellular calcium and phosphorous deposition in UMR-106 cells *in vitro* (Zhang *et al*., 2008).

**Eurycoma longifolia**

*Eurycoma longifolia* (EL) is a traditional medicinal plant known locally as “Tongkat Ali” and is a native herb in the Southeast Asia region, especially in Malaysia, Indonesia, Cambodia, Laos and Vietnam (Kuo *et al*., 2004). EL increased trabecular bone connectivity and increased the OPG gene expression in orchidectomized rats (Ramli *et al*., 2012; Shuid *et al*., 2012).
**Labisia pumila**

*Labisia pumila*, a herbal plant from the family of Myrsinaceae present in three types (*Labisia pumila*, *i.e.* *Labisia pumila var. alata* (LPva), *Labisia pumila var. pumila* (LPvp) and *Labisia pumila var. lanceolata* (LPvl)) with potential contents having estrogeneric activities (Fazliana *et al.*, 2009). Aqueous extracts of LPva served as stimulator of antioxidants and enhanced the mechanical properties of bone in OVX rats (Fathilah *et al.*, 2013; Effendy and Shuid, 2014; Effendy *et al.*, 2015).

**Piper sarmentosum**

It is used in Malaysia as a traditional medicine for diabetes, hypertension, inflammation, dermatitis and joint pain. It has antioxidant properties. Its extract hastened the fracture healing process in OVX animals (Estai *et al.*, 2011).

**Herba epimedi**

It is one of the most frequently prescribed herb for the treatment of osteoporosis in China. A short-term clinical study involving postmenopausal women demonstrated that aqueous extract of Herba epimedii could prevent bone loss and increase osteocalcin and E₂ levels (An *et al.*, 2000). A similar observation was made in OVX rats (Jiang *et al.*, 2002). Further, a combination with Ligustri lucidi extract increased the BMD in OVX rats (Liu *et al.*, 2015).

The flavonoid fraction of Herba epimedii promotes proliferation and differentiation of primary rat calvarial osteoblasts *in vitro* (Han *et al.*, 2003). The flavonoids also have the ability to induce the commitment of mesenchymal stem cells.
into osteoblasts and suppress their differentiation into adipocytes (Zhang et al., 2009; Zhang et al., 2016).

*Sambucus williamsii Hance* (SWH)

It is widely distributed in China. The major active chemical constituents of the plant are lignans, steroids, triterpenoids, phenolic acid, tianshic acid and methyl ester of tianshic acid. The latter two compounds possessed while stimulating osteoblastic activity of UMR-106 cell with increased osteogenic markers and anti-osteoclastic markers (Yang et al., 2006; Zhang et al., 2011; Xiao et al., 2014). Recently, it has been demonstrated that the root bark of SWH promoted cell proliferation and differentiation of MC3T3-E1 cells into osteoblastic lineage (Yang et al., 2015). Oral administration of 60% alcoholic extracts of SWH to OVX rats significantly increased serum Ca levels as well as decreased urinary Ca excretion (Yang et al., 2005).

*Drynaria fortunei*

It is one variety of the traditional Chinese medical herb Gusuibu. *Drynaria fortunei* total flavonoids (DFTF) improved bone quality in OVX mice. DFTF significantly increased total BMD, trabecular BMD, distal femur and proximal tibia BMD in OVX mice (Hung et al., 2010). In *in vitro* condition, DFTF while significantly increased the osteoblastic activity, it decreased the osteoclastic activity (Wong et al., 2013).
**Terminalia arjuna (Ta)**

It is a large tree distributed throughout India. Its bark is used to isolate principles with therapeutic values in the treatment of cardiac diseases. Indian Vedic Ayurveda, the age-old traditional healthcare system has identified Arjun (Ta) as the most vital herb in treatment of bone fractures and bone loss. It is known to slow down bone loss and increase BMD. The bark paste of Ta plant is being successfully used for the treatment of fractured bone of animals as well as human being. The decoction of the bark is used therapeutically to relieve the pain and inflammation (Patnaik *et al.*, 2007).

**Cissus quadrangularis**

*Cissus quadrangularis (C. quadrangularis)* is an indigenous medicinal plant of India. The use of this plant by the common folk for promoting fracture healing process is an old practice. Commonly known as “bone setter”, the plant is referred to as “Asthesamdhani” in Sanskrit and Hadjod in Hindi because of its ability to join bones. It has been prescribed as a general tonic especially for the fractured patient in ancient Ayurvedic texts by *Bhava Prakash* and *Chakra Dutta*. *C. quadrangularis* is widely used for the treatment of bone fractures, scurvy tumours and hemorrhoids (Nadkarni, 1954; Oliver-Bever, 1983). It is shown to neutralize the anti-anabolic effect of steroids like cortisone in healing of fractures.
Cissus quadrangularis

(Adopted from Chidambara Murthy et al., 2003)

*C. quadrangularis* caused increase in ALP levels during fracture healing in adult dogs (Udupa and Prasad, 1963; Chopra *et al.*, 1975). A trial undertaken to evaluate the effect of *C. quadrangularis* extract revealed faster healing of experimentally fractured radius-ulna of dog (Deka *et al.*, 1994).

‘Lakshadi Guggulu’ is an ayurvedic polyherbal preparation that had *Cissus quadrangularis* as one of the constituents along with other herbal constituents like *Commiphora mukul*, resin of *Ficus religiosa* tree, *Withania somnifera* and *Grewia hirsuta*. This preparation was extremely effective in relieving pain, reduction of swelling and promoting the process of healing of the simple fractures as well as in curing the allied disorders associated with fractures (Oliver-Bever, 1983). *C. quadrangularis* treatment to patients with mandible fracture hastened the healing process which is also associated with an increased osteopontin expression (Singh *et al.*, 2013).

The stem part of *C. quadrangularis* has been reported to contain triterpenes including α and β-amyrins, β-sitosterol, glycerolipids ketosteroid, β-carotene
cerebrosides, quercetin & vitamin C (Attawish et al., 2002). These phytoconstituents are known to induce osteoblastic differentiation and mineralization of matrix through MAPK activity (Parisuthiman et al., 2009). The positive effect of ethanolic extract of *C. quadrangularis* has further been supported by the increased expression of IGFs and IGF-IR by human SaOS-2 cells (Muthusami et al. 2011a, b). *C. quadrangularis* extract significantly increased the expression of type II collagen and aggrecan in primary chondrocytes cells (Kanwar et al., 2015). Some of the active principles *viz.*, glycerolipids and squalene derived from *C. quadrangularis* stimulated the differentiation of MC3T3-E1 osteoblastic cells (Pathomwichaiwat et al., 2015). While stimulating osteoblast functions, *C. quadrangularis* down regulated the gene expression of cytokines (IL-1β, IL-18, TNF-α) and matrix metalloproteinases (MMP-3,-9,-12,-13) that participate in the activation of osteoclasts.

The positive anabolic effects of *C. quadrangularis* are also evident from *in vivo* studies. The whole extract as well as phytoestrogen-rich fraction of the aerial parts of *C. quadrangularis* treatment prevented the loss of BMD in OVX rats (Shriwalkar et al., 2003; Aswar et al., 2012). Its effects were more on the cancellous bone of femur followed by tibia. The positive effects of *C. quadrangularis* even penetrated to the progeny which presented with increased bone formation after maternal treatment (Potu et al., 2008).

Quercetin in a collagen matrix used as graft material increased new bone formation (Wong and Rabie, 2008). Quercetin stimulated the osteogenic differentiation of rBMSCs by promoting the gene expression and secretion of osteogenic markers without obvious influence on the cellular morphology (Zhou et al., 2015). Quercetin metabolites such as rutin and hyperoside increased ALP activity in SaOS-2 cells (Nash et al., 2016).
Daidzein, an isoflavonoid in collagen matrix increased new bone formation locally in white rabbits and suggested to be used for bone grafting (Wong and Rabie, 2009). Daidzein analogs increased expression of osterix, ALP, OPN, RUNX2 and COL1A1 and IGF-I in human mesenchymal stem cells and adipose derived stem cells (Strong et al., 2013, 2014). Equol, a metabolite of daidzein increased the BMD of growing female rats without showing a significant effect on the weight of their reproductive organs (Tousen et al., 2015).
**Scope of the present investigation**

The perusal of literature revealed the importance of BMPs, IGF system components and VEGF to promote the bone fracture healing. Various hormones and herbal compounds have been shown to promote bone fracture healing. Even though, *C. quadrangularis* has been reported to hasten bone fracture healing, the molecular mechanism behind its action has not been understood. In this regard, femur fractures were developed in female Sprague-Dawley rats and treated with ethanolic extract of *C. quadrangularis*. The callus was harvested and analyzed for the mRNA and protein expression of BMPs, IGF system components and VEGF at different time points of fracture healing.
AIM

The aim of the present study was to determine the effects of ethanolic extract of *C. quadrangularis* on the expression of BMPs, IGF system components and antioxidant system in the femur fracture callus of female Sprague-Dawley rats.

HYPOTHESIS

It is hypothesized that *C. quadrangularis* extract may induce bone fracture healing by modulating the expression of specific growth factors and enhancing the antioxidant system in the callus of rats.

OBJECTIVES

1. To investigate the effects of *C. quadrangularis* on the mRNA and protein expression of BMPs in the fracture callus of female rats.

2. To determine the effects of *C. quadrangularis* on the mRNA and protein expression of IGF system components and VEGF in the fracture callus of female rats.

3. To evaluate the effects of *C. quadrangularis* on the antioxidant system and bone markers in the fracture callus of female rats.