4.1 Summary

Using a combination of *in vivo, in vitro* and molecular approaches, we investigated the osteogenic effect of total extract, standardized fraction and a novel compound obtained from an Indian medicinal plant *U. wallichiana* that is traditionally known for its enhanced fracture healing property. The study was divided into three sections. Major findings from each section are given bellow.

Section A

This section deals with the *in vivo* evaluation of total ethanolic extract (TEE) and a standardized fraction (BF) prepared from the stem-bark of *U. wallichiana* in rat skeleton. The major findings of the study are as follows:

1. Both TEE and BF treatment promoted peak bone mass achievement in growing female rats evident from increased BMD, bone strength, MAR and BFR. The effect was possibly mediated by increased osteoprogenitor cells in the bone marrow of the treated rats. Interestingly, BF rich in *C*-glucosylated flavonoids had a similar effect as TEE at a dose that was 15 times lower than TEE.
2. Both TEE and BF treatment prevented OVx induced decreases in BMD in femur, tibia and LV4. The effect was better than estrogen treated OVx group. In addition, both TEE and BF improved femoral bone strength and its trabecular microarchitecture. In this case also, BF had a comparable effect to TEE at a dose that was 15 times lower than the later. The preventive effect of the extracts was possibly mediated by a decrease in the bone turnover rate.

3. Both TEE and BF was devoid of uterine estrogenicity in rats, suggesting their safety in terms of undesirable uterine effects and one of the prerequisites for human use under the postmenopausal condition.

Our study findings show the butanolic fraction (BF) of *U. wallichiana* stem-bark is more effective at preventing postmenopausal bone loss in rats than the total ethanolic extract (TEE). Furthermore, substantial enrichment of *C*-glycosylated flavonoids in BF over TEE might have contributed in the increased biopotency of the former over the later. Out of the four flavonoids, GTDF was further studied because of its novel structure and abundance in the stem bark extracts.

Section B

This section deals with the *in vitro* evaluation of GTDF in osteoblasts and understanding its mode of action. The major findings of the study are as follows:

1. GTDF stimulated proliferation, survival, differentiation and mineralization of osteoblasts.
2. Although GTDF had no direct effect on osteoclatogenesis, it may have antiosteoclastogenic effect *in vivo* because it increased OPG-to-RANKL ratio in osteoblasts, which is an important determinant of osteoclastogenesis.
3. GTDF transactivated AhR but not ERs (activated by most of the phytochemicals). In addition, pharmacological inhibition of AhR or AhR silencing methodologies demonstrated that AhR was vital for mediating the osteogenic effects of GTDF. Moreover, activation of AhR by GTDF in mediating osteoblast differentiation was indirect and involved stimulation of cAMP production, the second messenger which is responsible for AhR's nuclear import and the resultant transcriptional regulation.
4. The AhR activation by GTDF was different from TCDD (an environmental toxicant known for AhR activation) in maintaining an AhR protein in osteoblasts, which was irreversibly lost by TCDD treatment.

Results of this study demonstrate strong osteoblast stimulating effect of GTDF and this effect is mediated by AhR. Furthermore, these studies for the first time demonstrate AhR as a possible target for developing novel osteogenic agents.

Section C

This section deals with the *in vivo* evaluation of GTDF in different animal models. The major findings of the study are as follows:

1. GTDF treatment to OVx rats in preventive mode had positive impact on BMD, bone strength and microarchitectural parameters over OVx control and OVx+17β-E2 groups. The effect appears to be mediated by decreased bone turnover rates.
2. GTDF treatment in OVx rats had no uterine hyperplastic effect.
3. GTDF treatment to growing rats promoted attainment of peak bone mass by positive modifications of BMD, bone strength, MAR and BFR. In addition, the GTDF treated rats exhibited better static histomorphometric parameters at the femur and tibia shaft (cortical bones). Data indicate that GTDF promoted modelling-directed accretion of bone mass.
4. GTDF treatment promoted bone regeneration in the drill-hole injury in the femur shaft of normal adult as well as osteopenic rats assessed by mineral deposition and microarchitectural parameters at the site of injury.
5. Finally, GTDF treatment restored the OVx-induced osteopenia in adult rats by stimulating new bone formation, number of osteoprogenitor cells in the bone marrow and improving microarchitectural parameters at femur and tibia trabeculae over the vehicle treated OVx rats. Restorative effect of GTDF treatment was more or less comparable to the rats receiving iPTH treatments and significantly better than Aln, which suggested a substantial anabolic effect of GTDF.

Our study demonstrates that daily oral administration of GTDF at a favorable dose of $5.0mg.kg^{-1}$ daily mitigates OVx-induced deterioration of bone strength and maintains trabecular microarchitecture without a uterine hyperplastic effect. In addition, GTDF accelerates fracture repair and exerts anabolic effect on osteoporotic bone that is comparable to iPTH by stimulatory effect on osteoblast differentiation of osteoprogenitors. Together, these data make GTDF an attractive alternative anabolic strategy for the development of new treatment against postmenopausal osteoporosis.

4.2 Future Directions

The following unanswered questions need to be addressed in future.

1. Our *in vitro* results suggested that GTDF had no direct on osteoclastogenesis yet it effectively mitigated OVx-induced bone loss when administered in preventive mode, suggesting anti-resorptive mode of action of GTDF. We surmised that the anti-resorptive effect of GTDF could be mediated indirectly by increasing OPG-to-RANKL ratio in osteoblasts. However, this hypothesis requires validation in osteoblast-osteoclast co-culture system in the presence of GTDF. Additionally, it needs to be demonstrated that OPG-to-RANKL ratio in bones of GTDF treated OVx rats is higher than the vehicle treated OVx rats with corresponding decrease in bone marrow osteoclastogenesis in *ex vivo* cultures.
2. One of the striking findings of our study is the identification of AhR as a putative bone anabolic target. However, it appears from our data that GTDF first stimulates osteoblast cAMP production before transactivating AhR. As cAMP is mainly increased when some G-protein coupled receptors (GPCR) get activated, so there is a need to identify the most proximal target (most probably a GPCR) of GTDF in osteoblasts.
3. As GTDF only activated AhR but not ERs *in vitro*, studies in AhR- and ER *null* mice rendered estrogen deficient are needed to further establish the specificity of the receptor types (AhR vs ER) in mediating bone anabolic effect of GTDF.
4. Pharmacokinetic studies (data not shown in the thesis but presented in [139]) show a low oral bioavailability of GTDF. Increasing the bioavailability enhances the biological effect of any agent. Furthermore, bone formation by osteoblasts is a far more protracted event than resorption in the bone remodeling cycle. It is therefore surmised that augmenting the bioavailability at the tissue level by either targeted material conjugates or sustained release nanoparticle conjugates would enhance the osteogenic effect of GTDF.
5. GTDF increased linear bone growth in appendicular skeleton and accelerated fracture repair. Both the processes involve increased chondrocytes proliferation and deposition of cartilaginous template before proper bone formation. Therefore, effect of GTDF on chondrocyte function should be studied.