Discussion
Various types of fractures including femoral fractures are associated with frequent morbidity and mortality. Many herbal preparations are reported to facilitate fracture healing. Among them, *C. quadrangularis* has been demonstrated to be effective in bone fracture healing through experimental and clinical studies. In a clinical study, the external application of *C. quadrangularis* paste showed earlier formation of collagen fibers and callus at the fractured site (Udupa and Prasad, 1962). Further, *C. quadrangularis* is also found to counteract the anti-anabolic effects of cortisone and enhance the levels of collagen, mucopolysaccharides, calcium and phosphate at fracture site to facilitate the healing in experimental models like rats and dogs (Udupa and Prasad, 1964a, 1963, 1964b; Deka et al., 1994). Nevertheless, the molecular mechanism of action of *C. quadrangularis* on the bone fracture healing is not clearly understood.

BMPs have chondro- and osteoinductive functions and are the only molecules used in the clinical treatment to promote bone fracture healing. Since fracture healing is a well-orchestrated serial regenerative process involving many biomolecules including BMPs (Einhorn, 1998; Ducy et al., 2000; Dimitriou et al., 2005), the primary contention of the present study is to implicate whether BMPs play a role in *C. quadrangularis* induced femoral fracture healing. True to our contention, significant changes were observed in the expression of BMPs in the callus tissue at different time points (post fracture days 7, 14 and 21) studied on fracture healing processes of rats treated with *C. quadrangularis*.

Phytochemical analysis of *C. quadrangularis* revealed the presence of high levels of flavonoids, calcium, vitamin C and β-carotene, and these substances have established beneficial effects on bone (Adesanya et al., 1999; Mehta et al., 2001;
It is pertinent to suggest that the presence of phytochemicals in the *C. quadrangularis* could have influenced the expression of BMPs. In this regard, studies have shown that high levels of calcium increased the expression of BMP-2 in bone cells and mesenchymal stem cells (Nakade *et al.*, 2001; Barradas *et al.*, 2012; Danoux *et al.*, 2015). Such a possibility cannot be ruled out for the increased levels of various BMPs observed in the present study. However, further studies are warranted to confirm this using calcium-free *C. quadrangularis* treatment.

Earlier studies have demonstrated that flavonoids increase AP-1 binding to the promoter region of BMP-2 in rat osteoblasts (Wu *et al.*, 2008) and c-jun translocation and AP-1 DNA binding activity in MC3T3-E1 osteoblastic cells (Son *et al.*, 2008). These findings reveal that flavonoids can regulate BMP-2 at molecular level. In view of these, it is reasonable to suggest that flavonoids present in *C. quadrangularis* extract could have induced the expression of BMPs through increased intracellular AP-1 signaling. Thus, it appears that the flavonoids present in *C. quadrangularis* could have induced the expression of various BMPs possibly through AP-1 activation.

**β-sitosterol** is one of the major constituents of *C. quadrangularis* (Shirley and Sen, 1966; Enechi and Odonwodo, 2003; Singh *et al.*, 2007) which has been shown to exhibit estrogenic action in bone cells (Vivancos and Moreno, 2005). The presence of estrogen receptor (ER) in fracture callus suggests that estrogen may play an important role in the normal fracture healing process. A dynamic but transient 13-fold increase in ER mRNA by day 14 and its return to baseline by day 31 in the rat callus clearly demonstrates the direct action of estrogen *via* its receptor in fracture healing process (Boden *et al.*, 1989). Estradiol was found to selectively up-regulate the expression of BMP-6 in human osteoblastic cell lines (hFOB/ER3 and hFOB/ER9) (Rickard *et al.*, 1998). Later it was further delineated that estradiol activates BMP-2 mRNA
expression through ERα and ERβ acting via a variant estrogen-responsive element binding sites in the BMP-2 promoter (Song et al., 2006). Resveratrol, which has estrogenic effects also enhances BMP-2 mRNA mediated by ER through its binding sites in the BMP-2 promoter in mouse osteoblasts (Su et al., 2007). Probably, phytoestrogenic compounds present in C. quadrangularis could have stimulated ER/BMPs pathway at the femur fracture site.

The impact of estrogen deficiency and the compensatory role of BMPs on bone loss and fracture healing are evident. Studies have shown that administration of rhBMP-2 rescues the bone loss in estrogen-deficient mice (Turgeman et al., 2002). BMP-7 treatment in ovariectomized rats registered a significant increase in bridging, higher callus volume, and increased bending stiffness and strength at the mid-shaft femoral fracture site (Blokhuis et al., 2012). Estrogen has recently been reported to stimulate osteoblastic differentiation through the activation of signaling mediated by BMP-4, which is structurally similar to BMP-2 (Matsumoto et al., 2013). It is quite possible that BMPs mediate the actions of estrogen in bone. If such is the case, estrogen may have a role in the expression of BMPs. Thus, the estrogenic compounds in C. quadrangularis extract could have contributed to the increased expression of BMPs observed in the present study.

The phytochemical analysis of C. quadrangularis has revealed that nearly 37% of dry weight is constituted by carbohydrates (Mishra et al., 2010). Fucoidan (a polysaccharide), which is abundant in brown algae has been reported to promote osteoblast differentiation through increased ALP activity, calcium accumulation and the expression of osteoblast-specific genes, such as ALP, runt-related transcription factor 2 (RUNX2), type I collagen-α 1 and osteocalcin in human alveolar bone marrow derived mesenchymal stem cells (Chizhov et al., 1999; Bilan et al., 2002;
Kim *et al.*, 2015). Further, fucoidan induced the expression of BMP-2 and stimulated the activation of extracellular signal-related kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase by increasing phosphorylation. These findings emphasize and reiterate that BMP-2 plays an essential role in bone formation (Park *et al.*, 2007; Hughes-Fulford and Li, 2011; Kuhn *et al.*, 2013; Lamplot *et al.*, 2013; Park, 2014).

In the present study, *C. quadrangularis* extract had positive influence on the expression of a list of BMPs studied. At present it is not known whether the regulatory mechanism employed by the principles present in the given *C. quadrangularis* extract is same or not. Of the BMPs studied, BMP-7 seems to be the least influenced. It is necessary to measure the quantity of different principles of *C. quadrangularis* present in the dose administered and their relevance to the observed increment in the expression and action of BMPs. However, it is clear that BMPs are certainly regulated by the constituents of the ethanolic extract of *C. quadrangularis*. It is interesting to note that plant principles which are widely used as dietary and therapeutic sources since our ancient times have bone fracture healing properties.

Vitamin C is a critical factor in the processes of cartilage and bone development (Sowers and Lachance, 1999). ATDC5 cell line exhibits the multistep chondrogenic differentiation observed during endochondral bone formation by promoting the formation of collagenous matrix (Altaf *et al.*, 2006). The addition of vitamin C to ATDC5 cultures shortened the pre-chondrogenic proliferation phase, facilitated earlier chondrogenic differentiation, heightened gene expression and robust hypertrophic differentiation (Temu *et al.*, 2010). Further, vitamin C has been reported to increase the expression of BMPs (Gunasekar *et al.*, 2014). Since *C. quadrangularis*...
has high levels of vitamin C, it may be the reason for increased expression of BMPs in fracture callus compared to control group. Thus *C. quadrangularis* appears to promote endochondral ossification by increasing the BMPs.

Previous study from our laboratory suggests that ethanolic extract of *C. quadrangularis* positively regulates the stimulatory IGF system components in human osteoblast like SaOS-2 cells *in vitro* (Muthusami *et al*., 2011b). In this study, we determined the effects of *C. quadrangularis* extract on IGF system components in rat femoral fracture model. IGFs are generally recognized to play an important role in fracture healing, which is achieved through the formation and maturation of callus and successive formation of cartilage and bone via endochondral ossification and intramembranous ossification. Thus, at the cellular level, successful fracture healing relies on the appropriate regulation of chondrocytes and osteoblasts (Trippel, 1998).

In the present study, *C. quadrangularis* increased the mRNA expression of IGF-I and II at different time points (7, 14 and 21 days) studied in the femoral fracture callus. An earlier study by Udupa and Prasad (1964) reported the hastening of fracture healing by *C. quadrangularis*. Taken together, it is pertinent to suggest that the fracture healing effect of *C. quadrangularis* might be mediated through increased production of IGFs by osteoblasts as IGFs have been shown to hasten fracture healing (Nakasaki *et al*., 2008).

IGF mRNA and protein levels are induced by hormones that induce the production of cAMP. cAMP activates PK-A, which in turn activates the transcription factor CCAAT/enhancer binding protein δ (C/EBP δ), which binds to a hormone responsive element in the IGF gene promoter termed HS3D. Flavonoids have been shown to inhibit cAMP phosphodiesterase from different species and therefore can mimic cAMP effects (Beretz *et al*., 1978; Ferrell *et al*., 1979; Umayahara *et al*.,
1997). *C. quadrangularis* being rich in flavonoids could have increased the levels of cAMP, which in turn could have induced the mRNA expression of IGFs.

The increase in mRNA expression of IGF-I and IGF-II after treatment with *C. quadrangularis* might also be mediated through the phytoestrogens present in the plant, which could have mimicked estradiol in inducing the expression of IGFs. Ethanolic extract of *C. quadrangularis* has been found to contain β-sitosterol, which is considered as a weak agonist for estrogen receptors ERα and β and preferentially binds to ERβ (Gutendorf and Westendorf, 2001).

Estrogen has been shown to induce the IGF-I mRNA expression in human osteoblasts. β-sitosterol has estrogen like actions in osteoclastic precursor RAW 264.7 cells and their effects were reversed by estrogen receptor blockers. Although no consensus ERE has been identified in the characterized portion of the IGF-I gene promoter (Kim *et al.*, 1991; Hall *et al.*, 1992; Kim *et al.*, 2005; Mochizuki *et al.*, 2005), the activator protein (AP)-1 site has been identified in the 5′flanking sequence of the IGF-I and IGF-II genes (Umayahara *et al.*, 1994). ERα and ERβ can both interact with the AP-1 site to stimulate gene expression. Flavonoids have been shown to regulate AP-1 signal transduction pathways in (HEK) 293 cells (Frigo *et al.*, 2002). Naringin, a flavonoid, has been shown to increase AP-1 binding to the promoter region of BMP-2 in primary rat osteoblasts (Wu *et al.*, 2008). Quercetin has been shown to increase c-jun translocation and AP-1 DNA binding activity in MC3T3-E1 osteoblastic cells (Son *et al.*, 2008). Therefore, flavonoids present in *C. quadrangularis* could have induced the mRNA expression of IGF-I and IGF-II, through increased intracellular cAMP signaling and/or through AP-1 signaling.
In this study, IGF-IR mRNA expression was significantly increased by *C. quadrangularis* treatment compared to control. Okazaki *et al.* (2003) revealed that the distribution of IGF-IR was similar to that of IGF-I throughout the rat fracture healing as determined by immunohistochemistry and *in situ* hybridization. In a rat model, the local administration of IGF-I had a greater stimulating effect on fracture healing than TGF-β, and the application of both growth factors resulted in a significantly higher maximum load and torsional stiffness (Schmidmaier *et al*., 2003, 2006). The biological actions of both IGF-I and IGF-II are mediated by IGF-IR. The IGF-IR promoter is a TATA-less, CAAT-less, G-C rich and initiator type of promoter. The IGF-IR gene contains many Sp1 binding sites in both the 5’-flanking and 5’-untranslated regions. These Sp1 binding sites are active and are involved in IGF-IR gene regulation (Werner *et al*., 1992). Studies have shown that retinoic acid and quercetin activate Sp1 promoter activity in human bronchioalveolar carcinoma cells (Maeno *et al*., 2002) and human hepatoma cells (Kim *et al*., 2008), respectively. The ethanolic extract of *C. quadrangularis* contains quercetin and the retinoic acid precursor β-carotene (Singh *et al*., 2007). Therefore it is possible that quercetin and/or β-carotene present in the *C. quadrangularis* extract could have increased the Sp1 which in turn could have upregulated the expression of IGF-IR.

Bioavailability and actions of IGFs are regulated by IGFBPs (Conover, 2008). IGFBPs in bone can either inhibit or stimulate the actions of IGFs on osteoblasts. While IGFBP-1, -2, -4, and -6 inhibit IGF action, IGFBP-3 and -5 stimulate IGF action in bone (Govoni *et al*., 2005). IGFBP-5 is the most abundant IGFBP stored in bone and has been reported to enhance IGF action in bone *in vitro* and *in vivo* (Richman *et al*., 1999). IGFBP-5 also play an important role in endochondral ossification and bone remodeling by interacting with IGF-I. In the present study,
C. quadrangularis increased both IGFBP-3 and IGFBP-5 gene expression which could have favoured bone fracture healing. Decreased serum IGF-I is associated with an increased risk of osteoporotic fractures (Garnero et al., 2000) and IGFs hasten the process of fracture healing (Nakasaki et al., 2008). Thus the increase in IGFs, IGF-IR, IGFBP-3, IGFBP-5 in femoral fracture callus by C. quadrangularis treatment could have facilitated the fracture healing process.

Exogenous VEGF enhanced not only blood vessel formation but also ossification and callus maturation in murine femur fractures (Street et al., 2002). BMPs are expressed in fracture callus and are bound to the underlying extracellular matrix along with VEGF (Kloen et al., 2002). Both VEGF and BMPs increase the differentiation and metabolism of preosteoblasts, and BMP-4 is known to work synergistically with VEGF in promoting bone formation (Carano and Filvaroff, 2003). Therefore the increase in VEGF by C. quadrangularis could have hastened the fracture healing.

In order to determine the effects of C. quadrangularis on bone remodeling in the femoral fracture callus, the activities of TRAP (the marker enzyme for osteoclast) and ALP (the marker enzyme for osteoblast) were estimated. Bone fracture reduced the activities of ALP in the femoral fracture callus of rats. This could be possibly due to induction of oxidative stress as osteoblasts have appreciable amounts of polyunsaturated fatty acid (PUFA) and hence are highly susceptible to oxidative stress (Raisz, 1993). An in vitro study also reported that oxidative stress affects the mineralization and downregulates the osteogenic markers Runx2 and ALP in MC3T3-E1 cells treated with hydrogen peroxide (Arai et al., 2007). Interestingly, the administration of C. quadrangularis increased the activities of ALP. C. quadrangularis contains quercetin and β-carotene, both of which have been shown
to increase ALP mRNA expression and activity in various osteoblastic cells such as MG-63, U2OS and SaOS-2 cells (Prouillet et al., 2004; Orimo and Shimada, 2005). Hexane extract of *C. quadrangularis* contains several phytochemicals including triterpenes, fatty acid methyl esters, glycerolipids, steroids, phytols and cerebrosides. The glycerolipids and squalene stimulated ALP activity during the differentiation of MC3T3-E1 osteoblastic cells (Pathomwichaiwat et al., 2015). Therefore, the observed increase in the ALP activity in the present study might be mediated through β-carotene, quercetin and other phytochemicals present in the extract.

There are clear evidences that oxygen derived free radicals are produced at the resorptive interface of bone and are required for active resorption (Garrett et al., 1990; Key et al., 1990; Hall et al., 1995; Arai et al., 2007). The bone resorbing osteoclasts generate high levels of superoxide anion and hydrogen peroxides (Lean et al., 2005). These free radicals modulate inter- and intracellular signaling responsible for bone loss (Lean et al., 2003). In the present study, both the levels of TRAP and H$_2$O$_2$ were increased in femoral fracture callus, suggesting increased bone resorption.

Fracture healing process shows the involvement of oxidative stress (Ikeda et al., 1989; Oda et al., 1992; Rangan and Bulkley, 1993). While the first three days of fracture healing shows no sign of oxidative stress, the various cells that participate in callus formation increase the production of oxygen free radicals. These radicals may cause oxidative injury to the fractured bone (Cornell and Lane, 1992; Symons, 1996). Oxidative stress clearly prolongs the inflammation and delay the repair periods of fracture healing (Turgut et al., 1999; Prasad et al., 2003). Production of oxygen free radicals during fracture healing is highest particularly on the 7$^{th}$ and 21$^{st}$ post-fracture days. Oxidative stress occurs after sustaining a fracture (Yeler et al., 2005;
Paskalev, 2011). Göktürk et al. (1995) evaluated oxidant status during bone fracture healing in rats, measuring MDA levels in bone specimens as an indicator of oxidative stress. A significant increase in MDA levels was observed on days 7 and 14 in fracture induced rats.

Many findings show that oxidative stress clearly increases after a fracture has been sustained. Endogenous antioxidant defense systems may be insufficient to overcome the increased oxidative stress during fracture healing. Thus, the antioxidant treatment would be beneficial during fracture healing in order to eliminate the deleterious effects caused by oxygen free radicals. *C. quadrangularis* extract possesses antioxidant property. This property of *C. quadrangularis* has beneficial effects on the fracture healing process (Chidambara Murthy et al., 2003; Agbor et al., 2005; Muthusami et al., 2005).

The onset of oxidative stress can be evaluated by assessing the production of \( \text{H}_2\text{O}_2 \), lipid peroxidation and the activities of SOD, GST and GPx. In the present study, there was a significant decrease in the levels of antioxidant enzymes viz. SOD, GPx and GST in the femoral fracture callus of rats. The decrease in SOD activity in femoral fracture callus could have resulted in more accumulation of \( \text{O}_2^- \), which has been shown to inhibit other antioxidant enzymes (Kono and Fridovich, 1982). Similarly, when GPx fails to eliminate \( \text{H}_2\text{O}_2 \) from the cell, the accumulated \( \text{H}_2\text{O}_2 \) has been shown to inactivate SOD (Sinet and Garber, 1981). SOD is responsible for \( \text{O}_2^- \) dismutation to \( \text{H}_2\text{O}_2 \), a species that is more reactive than \( \text{O}_2^- \). Simultaneous administration of *C. quadrangularis* prevented the decline in the activities of SOD in femoral fracture callus and indeed raised SOD activities higher than the control level. The higher activity of SOD in the tissue quenches the increased levels of \( \text{H}_2\text{O}_2 \) and \( \text{O}_2^- \) concentrations.
Simultaneous administration of *C. quadrangularis* enhanced the activities of GPx and GST in femoral fracture callus. Bone fracture registered a decrease in the activities of antioxidant enzymes in the femoral callus. The increase in the activities of the antioxidant enzymes after *C. quadrangularis* treatment could prevent the oxidative stress caused by fracture.

The increase in the levels of H$_2$O$_2$ in the femoral callus after fracture induction might have induced the peroxidation of polyunsaturated fatty acid and led to the formation of MDA, one of the byproducts of lipid peroxidation. Since MDA has got high reactivity towards amino groups, it inhibits the synthesis of nucleic acids and proteins and also deactivates the enzymes (Bird and Draper, 1980). Thus, the decrease in femoral callus antioxidant enzymes observed could have increased the lipid peroxidation.

*C. quadrangularis* effectively prevented the fracture-induced increase in the levels of H$_2$O$_2$. This may be due to augmented activities of GPx and GST. The extract also possesses β-sitosterol, which has binding affinity for ERs (Gutendorf and Westendorf, 2001). β-sitosterol has been reported to increase the SOD and GPx activities over untreated cells by 26% and 34% in RAW 264.7 cells. The effect of β-sitosterol in these cells has been shown to be mediated through ERs as the effects were blocked by the ER antagonists tamoxifen and ICI 182, 780 (Vivancos and Moreno, 2005). β-sitosterol treatment also recovered GSH/total glutathione ratio, suggesting the ROS scavenging activity. The augmented activities of SOD, GPx and GST observed in the femoral fracture callus treated with *C. quadrangularis* extract might be mediated through β-sitosterol present in the extract and this could be responsible for the increased resistance to oxidative stress observed in femoral fracture callus. Taken together, the present study demonstrates that *C. quadrangularis*
treatment decreased the free radical toxicity induced by inflammatory reactions at the fracture site and increased the antioxidant enzymes which could have hastened the bone fracture healing.