Discussion
Osteoporosis is characterized by low bone mass and microarchitectural deterioration of bone tissue, increasing susceptibility to fracture (Heinemann DF, 2000). Although osteoporosis is often described as a silent disease because it is typically asymptomatic until a fracture occurs, the disease negatively and significantly impacts morbidity and mortality as it can lead to severe pain, deformity, disability, and death (Heinemann DF, 2000; Salkeld et al., 2000). While traditionally considered a condition afflicting predominantly postmenopausal women, osteoporosis is also an under recognized disorder in men (Ebeling PR, 2008; Adler RA., 2011) as analysis of hip fracture incidence indicates that one-third occur in men (Burge et al., 2007). As compared to females, males often develop fractures 10 years later in life (Adler RA, 2011) and are less likely to survive with a mortality rate as high as 37.5% after hip fracture (Bass et al., 2007; Jiang et al., 2005). Apart from bone fractures due to osteoporosis, bone parameters across different types of hip osteoarthritis and their relationship to osteoporotic fracture risk have also been widely studied (Castaño-Betancourt et al, 2013).

Inflammatory cytokines, especially tumor necrosis factor α (TNF-α), have been shown to inhibit osteogenic differentiation of mesenchymal stem cells (MSCs) and bone formation in estrogen deficiency-induced osteoporosis, but the mechanism responsible remains poorly understood. MicroRNAs (miRNAs) have been shown to regulate MSC differentiation (Yang et al., 2013). Recent study reported by Yang et al., 2013 has identified a novel mechanism whereby TNF-α, suppressing the functional axis of a key miRNA (miR-21) contributes to estrogen deficiency-induced osteoporosis. In the same study, they screened differentially expressed miRNAs in MSCs derived from estrogen deficiency-induced osteoporosis and found miR-21 was significantly downregulated. miR-21 was suppressed by TNF-α during the osteogenesis of MSCs. Furthermore, miR-21 was confirmed to promote the osteoblast differentiation of MSCs by repressing Spry1, which can negatively regulate the osteogenic differentiation of MSCs. Up regulating miR-21 partially rescued TNF-α-impaired osteogenesis of MSCs. Blocking TNF-α ameliorated the inflammatory environment and significantly enhanced bone formation with increased miR-21 expression and suppressed Spry1 expression in ovariectomised (OVX) mice. Our results revealed a novel function for miR-21 and suggested that suppressed
miR-21 may contribute to impaired bone formation by elevated TNF-α in estrogen deficiency-induced osteoporosis (Yang et al., 2013).

A number of studies have reported increased production of TNF by cultures of mononuclear cells derived from osteoporosis patients, an effect reversed by estrogen replacement (Shevde et al., 2000) and that ROS may play a role in bone loss in osteoporosis patients by generating a more oxidized bone microenvironment (Almeida and O'Brien, 2013). Based on these finding, we probed here the management and/or regulation of ROS and TNF-α activation in osteoporosis patient monocytes. The mechanisms of cellular activation as well as TNF-α and ROS enhancement would definitely help in better understanding the pathogenesis of osteoporosis.

TNF-α is implicated in the pathophysiology of bone metabolism. The presence of elevated levels of TNF in the bone marrow of ovx animals and in the conditioned media of peripheral blood cells of postmenopausal women as well as osteoporosis patient is well documented (Pacifici R, 2010). Moreover, ROS are also involved in the etiopathology and progression of osteoporosis as reported earlier, and that, radicals generated in cells of osteoporosis patient stimulate TNF-α, causing accelerated bone loss.

We used allicin from garlic and EGCG from green tea in the present study whose exact mechanism underlying their antioxidant activity still remains poorly understood. Allicin-induced enhancement of GPx activity has been reported (Perchellet, 1986; Bryk et al, 2002). To the best of our knowledge, we show for the first time that allicin exerts potent anti-inflammatory effects on osteoporosis patient mononuclear cells.

Since higher doses of allicin (>100 μM) and EGCG (23 μg/ml) have proven toxic (Ankri et al, 1997; Hasan et al, 2006; Kawai et al 2005), thus prior to all investigations, cell viability and potential cytotoxicity were determined for the doses employed in this study using trypan blue and MTT assay where viability of ~98–99% was observed with the lower doses selected in the present study (data not shown). Moreover, our laboratory has previously demonstrated that the doses employed in the present study for allicin or EGCG had no effect on human housekeeping genes.
Discussion

namely R18, thereby demonstrating that the effect of allicin or EGCG, was not mediated by cellular death, but rather by specific inhibition of expression and secretion of pro-inflammatory molecules (Hasan et al., 2006; Singh et al, 2002; Fatima et al, 2012). Hence, data has not been shown here.

The real time RT-PCR results indicate an appreciable / high degree of down-regulation in endogenous TNF-α mRNA expression by allicin and EGCG in osteoporosis patient monocytes. Similarly, both allicin and EGCG down-regulated the augmented OPG mRNA expressions in osteoporosis patient monocytes. Our results are in accordance to similar pattern observed by other workers with different antioxidants than ours (Nazrun et al., 2012). TNF-alpha production in monocytes is regulated at multiple intracellular levels, beginning with transcription (Raabe et al., 1998). Augmented expression of TNF-α mRNA and activation of a relevant transcription factor, NF-κB, have been reported in monocytic cells derived from osteoporosis patient. Consistent with earlier finding, we also report here the upregulation of TNF-α mRNA expression as well as activation of NF-κB in human monocytes derived from osteoporosis patients.

The induction of TNF-α expression was observed to be mediated through activation of NF-κB, as evidenced by the suppression of TNF-α mRNA in the presence of SN50, an inhibitor of NF-κB. On the contrary, the control SN50/M, an inactive analogue of SN50, failed to show any such effect. It has been well established that TNF-α induced nuclear translocation of NF-κB was inhibited by SN50 peptide as demonstrated in EMSA (Lin et al., 1995).

In view of it, our data demonstrated that this effect involved inhibition of the NF-κB pathway induced by allicin as well as EGCG, probably by inhibiting the degradation of IκBa. The NFκB heterodimer is retained in the cytoplasm in an inactive form through association with one of the IκBs inhibitory proteins. As a consequence of stimulation by TNF-α, the IκBa gets phosphorylated by a specific kinase complex (IKK) leading to its ubiquitination, and subsequent proteolysis by the 28S proteosome (Lang et al., 2004; Li and Verma, 2002). The degradation of IκB releases active NF-κB, which translocates to the nucleus and regulates gene expression by binding to κB binding sites or by interacting with other transcriptional
factors (Brown et al., 1995). Since a number of genes involved in inflammatory responses are regulated by NF-κB pathway, thus a high magnitude downregulation of the NF-κB pathway by allicin and or EGCG would predictably reduce the elaboration of NF-κB-mediated TNF-α mRNA expression. In addition, both allicin and EGCG exerted a higher degree of neutralizing effects than NAC on TNF-α induced actions in osteoporosis patient monocytes.

It’s well established that glutathione directly reacts with ROS, and GPx catalyzes the removal of hydrogen peroxide (Mesiter and Anderson, 1983). Down-regulation or decrease in GPx activity indicates impairment of hydrogen peroxide-neutralizing mechanisms (Rukmini et al., 2004). Here, we observed an appreciably suppressed GPx activity in osteoporosis patient monocytes that were untreated with allicin or EGCG, thereby correlating with earlier reports that substantial amounts of ROS are being generated in bone cells of osteoporosis patient due to lowering of antioxidant defence in such cells. Enhancement of GPx activity in osteoporosis patient monocyte cultures after addition of NAC, a precursor of in vivo antioxidant glutathione, indicates reversal of impaired neutralizing mechanisms. Surprisingly, here slightly augmented GPx activity was observed when allicin or EGCG was co-cultured instead of NAC, indicating allicin and EGCG to be an effective natural antioxidant combating ROS, generated as a consequence of cellular activation in osteoporosis patient monocytes. Thus, our study shows amelioration in GPx activity by allicin and EGCG, which in turn, correlated inversely with the downregulation of TNF-α mRNA expression and ROS in monocytes of osteoporosis patients.

In our study, peripheral blood mononuclear cells (PBMCs) were used directly for the generation of osteoclast precursors. The multinucleated osteoclast precursors were observed to appear on day 3 and the number increased after 5 days of culture, as revealed by Tartrate Resistant Acid Phosphatase (TRAP) staining. However, there was no appearance of osteoclast precursors after 24 h (1 day) of culture (data not shown). The number of multinucleated preosteoclasts, arising from PBMCs isolated from the blood of normal healthy individual (data not shown) and osteoporotic patients were counted by TRAP staining. Interestingly, we observed an individual variation in osteoclast generation from different donors.
Our data exhibits that the multinucleated cells in monocyte cultures have numerous of the characteristics of osteoclasts. The data shows that cells in our cultures were large and multinucleated with ultrastructural features of osteoclasts, including a peripheral cytoplasmic clear zone devoid of subcellular organelles, and extensive cell surface folds and branched pleomorphic mitochondria. Such a finding is in agreement with earlier reporting (Ibbotson et al., 1984). These particular ultrastructural features are found in freshly isolated osteoclasts and in osteoclasts not lying adjacent to an endosteal bone surface (Ibbotson et al., 1984; Osdoby et al., 1982). Although numerous plasmalemmal folds and indentations were also regularly observed in our cultured cells, we did not identify the classical ruffled borders as reported for osteoclasts found adjacent to bone in Howship's lacunae in vivo (Ibbotson et al., 1984; Holtrop and king, 1997).

The multinucleated cells in our cultures had other characteristics of osteoclasts. This was evident from the observation that they were responsive to treatment with the autocrine cytokine namely TNF-alpha that influence osteoclastic activity. TNF-alpha (2 ng/ml) increased the numbers of multinucleated cells in the cultures. This effect was inhibited by natural antioxidants like allicin and EGCG. Osteoclasts have been shown before to form by fusion (Jee and Nolan, 1963; Ibbotson et al., 1984), and the results of the current investigation suggest that this process is probably stimulated by TNF-alpha and inhibited by natural antioxidants like allicin and EGCG. Our data suggests that the mononuclear precursor cell of these multinucleated cells is immature marrow monocyte macrophage. The progenitor cells stained heavily with nonspecific esterase were phagocytic and appear similar to other monocytes-macrophages with Wright's-Giemsa staining (data not shown).

Interestingly, we observed that co-culturing of PBMCs with EGCG (20 µg/ml) or Allicin (500 ng/ml) in osteoclastogenic medium for 3 and 5 days resulted in an appreciable amount of reduction in appearance of multinucleated osteoclast precursors, thereby reflecting the potential of EGCG and allicin to exert regulatory effect in osteoclast generation and differentiation. Nearly 20-25% suppression in appearance of multinucleated cells was observed in cultures receiving 15 and 20 µg/ml of EGCG relative to control cultures devoid of any EGCG. Interestingly, around 30-35% suppression in appearance of multinucleated cells was observed in
cultures receiving 250 and 500 ng/ml of allicin relative to control cultures devoid of any allicin.

In addition to the above, our data revealed that allicin down regulated / suppressed the secretion of sRANKL by around 17.34%, 40.93%, 68.83% and 86.92% with 50, 100, 250 and 500 ng/ml allicin respectively. The IC$_{50}$ was computed out to be in between 100 - 125 ng/ml. Similarly, analysis of the data revealed that EGCG down regulated / suppressed the secretion of sRANKL by around 14.82%, 31.24%, 47.04%, 59.96% and 71.08% with 2, 5, 10, 15 and 20 µg EGCG respectively. The IC$_{50}$ was computed out to be in between 7.5 – 10 µg/ml of EGCG. Furthermore, our data shows that TNF-alpha up-regulated the levels of sRANKL in culture monocytes of osteoporosis patients. On the contrary, when TNF-α was co-cultured along with allicin or EGCG, then allicin inhibited the secretion of sRANKL by around 60% whereas EGCG inhibited the same by around 44%. Therefore, the present study provides for the first time evidences that Allicin from garlic as well as EGCG from green tea inhibits RANKL mediated signalling events that lead to osteoclast differentiation and function in monocyte cultures. Incubation of osteoclast progenitor cells with allicin or EGCG inhibited TRAP activity and OPG expression in a dose dependent manner.

These findings are novel and contribute to define a mechanism for the altered bone turnover in osteoporosis patients that, in turn, may protect patients from early bone loss or lead to increased bone mass. Bone mass is tightly regulated by osteoclastic and osteoblastic bone remodelling. The contribution of natural antioxidants like allicin from garlic as well as green tea polyphenol namely EGCG to bone remodelling in vivo is poorly understood. Our findings are suggestive that allicin and EGCG to inhibit osteoclastogenesis, likely by preventing the formation of pre-osteoclast cells capable of fusing into multinucleated osteoclasts.

ROS including superoxide anion and hydrogen peroxide (H$_2$O$_2$) have been recognized as major intermediaries in the formation and activation of osteoclasts in vitro and in vivo (Wittrant et al., 2008; Suda et al., 1993; Garrett et al., 1990; Lee et al., 2005). Recent studies indicate that RANKL-mediated ROS production serves to regulate RANKL signalling pathways required for osteoclast differentiation (Koh et al., 2006; Ha et al., 2004; Wittrant et al., 2008). In RAW264.7 and BMM precursor cells,
RANKL increases ROS, whereas expression of catalase in BMM cells blocks RANKL-induced ROS production and inhibits the formation of TRACP (+) osteoclasts (Wittrant et al., 2008; Lee et al., 2005). These effects were reported to be correlated with the decreased formation of multinucleated osteoclast-like cells in the TRACP assays and likely contributed to the inability of these cells to differentiate in response to RANKL.

Therefore, in summary, our data regarding regulation of TNF-alpha, sRANKL, OPG, GPx activities, osteoclast formation and differentiation, etc by allicin from garlic as well as EGCG from green tea could provide a valuable tool in probing the control molecular mechanism of osteoporosis, and in turn, such natural antioxidants may serve as adjuncets in the management of osteoporosis.