

Comparative Effects Between Green Tea and Black Tea Polyphenols in Suppressing Adverse Effects of TNF- α Induced Inflammation in Osteoblasts

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Abstract: The aim of the study was to compare the osteoprotective Effects of Green Tea (GTE) and Black Tea (BTE) Extracts on Normal Human Osteoblast (NH_{Ost}) cells in non-inflammatory and inflammatory conditions. NH_{Ost} cells were treated with GTE and BTE 5, 10, 50 and 100 $\mu\text{g}/\text{mL}$ for 2, 5 and 10 days. The experiments were performed in the absence and presence of Tumour Necrosis Factor- α (TNF- α) to emulate non-inflammatory and inflammatory conditions, respectively. All concentrations of GTE and BTE exhibited = 80% cell proliferation at all-time points. In the absence of TNF- α , 5 $\mu\text{g}/\text{mL}$ of GTE and BTE significantly up-regulated Osteo Prote Gerin (OPG) level compared to control and 100 $\mu\text{g}/\text{mL}$ of the extracts reduced Receptor Activator of Nuclear factor Kappa B Ligand (RANKL) level on day 5 and 10. In inflammation, 5 and 50 $\mu\text{g}/\text{mL}$ BTE significantly elevated OPG level while with GTE only 5 $\mu\text{g}/\text{mL}$ gave a similar effect. Higher concentrations 50 and 100 $\mu\text{g}/\text{mL}$ of both extracts significantly suppressed RANKL expression. The 100 $\mu\text{g}/\text{mL}$ GTE and all BTE concentrations tested except 100 $\mu\text{g}/\text{mL}$ significantly increased Alkaline Phosphatase (ALP) activity by day 5 in non-inflammatory condition. About 5 $\mu\text{g}/\text{mL}$ GTE increased the ALP activity in inflammatory condition. Likewise, BTE was also found to reverse the TNF- α effect by elevating the ALP activity. GTE and BTE increased formation of mineralized nodules in both conditions at each time points. BTE and GTE exert protective effects on osteoblast activities including reverting the TNF- α -induced adverse effects and these effects are more pronounced in BTE treatment.

Key words: Black tea, polyphenols, chronic inflammation, osteoblasts, OPG, RANKL

INTRODUCTION

Chronic inflammatory diseases such as Rheumatoid Arthritis (RA), psoriasis, ankylosing spondylitis, systemic lupus erythematosus, multiple sclerosis inflammatory bowel diseases, pemphigus vulgaris, chronic periodontitis and others are frequently associated with bone loss and increased skeletal fragility (Dimitroulas *et al.*, 2013; Straub *et al.*, 2015). Inflammation-induced pathologic bone loss occurs as a result of disturbances in the normal bone remodelling process. Normal bone remodelling is a balance between bone-forming osteoblast and bone-resorbing osteoclast activities. Osteoblasts are responsible for mineralization of bone and modulation of osteoclast differentiation (Baum and Gravallesse, 2014). Osteoblasts regulate osteoclast differentiation through its

production of several factors including RANKL and OPG (Baum and Gravallesse, 2014). RANKL interacts with the RANK receptor on the osteoclast precursors to stimulate osteoclastogenesis. OPG functions as a decoy receptor for RANKL to inhibit the binding of RANKL to RANK receptor, thus, limiting osteoclastogenesis and protecting against excessive bone resorption (Baum and Gravallesse, 2014; Weitzmann, 2013). Chronic inflammation is associated with excessive production of pro-inflammatory cytokines including TNF- α and Inter Leukin-6 (IL-6) and their occurrence in the bone microenvironment inhibits the actions of osteoblasts, resulting in uncoupling of resorption and formation in favour of excess resorption.

Polyphenols, abundantly exist as constituents of fruits, vegetables, cereals, dry legumes, chocolate and

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beverages such as tea, coffee or wine, have been reported to exert anti-inflammatory and anti-oxidative activities (Perez-Jimenez *et al.*, 2010; Zhang and Tsao, 2016). Green Tea Polyphenols (GTP) and Black Tea Polyphenols (BTP) originate from the leaves of *Camellia sinensis* (L.) Kuntze and they are believed to contribute to the health benefits associated with drinking tea which is the most commonly consumed beverage globally next to water (Shen *et al.*, 2009). Both types of tea differ in their polyphenolic constituents as a result of their manufacturing process (Shen *et al.*, 2011). Catechins account for the major constituents of GTP and they include (-)-Epi Gallo Catechin Gallate, (EGCG), (-)-Epicatechin Gallate (ECG), (-)-Epicatechin, (EC) and (-)-Epi Gallo Catechin (EGC) with EGCG being the most abundant polyphenols (Chacko *et al.*, 2010; Singh *et al.*, 2010). During the manufacturing of black tea which involves the fermentation of tea leaves, a large portion of catechins are oxidized into theaflavins and thearubigins (Obuchowicz *et al.*, 2011; Chaturvedula and Prakash, 2011).

GTP have been reported to exhibit a number of anti-inflammatory effects in *in vivo* and *in vitro* models (Cyboran *et al.*, 2015; Okuda *et al.*, 2014) and to inhibit NF- κ B *in vitro* in intestinal epithelial cells (Yang *et al.*, 2001; Gossiau *et al.*, 2011). In addition, GTP have been shown to exert osteoprotective effects in chronic inflammation-induced bone loss in animal models (Shen *et al.*, 2010).

Black Tea Polyphenols (BTP) have also been described for their anti-inflammatory effects (Chatterjee *et al.*, 2012; Aneja *et al.*, 2004; Gossiau *et al.*, 2011). Furthermore, BTP have been reported to prevent ovariectomy-induced bone loss in rats and was associated with reduced number of active osteoclasts in addition to diminished serum levels of bone resorbing-cytokines (IL-6, TNF- α), RANKL and resorption markers (tartrate-resistant acid phosphatase and hydroxyproline) (Das *et al.*, 2009).

Despite, the extensive therapeutic studies on GTP, their effect on osteoblasts in an inflammatory model, need to be investigated. In addition, there is scarce information on the effects of BTP on inflamed osteoblasts. The present study, thus, aimed to compare the osteoprotective effects of GTE and BTE on osteoblasts in non-inflammatory and inflammatory conditions.

MATERIALS AND METHODS

Materials: Dry and ground black tea powder and green tea leaves were obtained from BOH Plantations (Malaysia) and Cameron Bharat Plantations (Malaysia), respectively.

Folin-Ciocalteu's (FC) phenol reagent, gallic acid and sodium carbonate were purchased from Merck (Germany). Normal Human Osteoblast (NHOst) cells were obtained from LONZA (USA). Cells were cultured in T-25 and T-75 flasks (Orange Scientific, Belgium) in Osteoblast Basal Medium (OBM) supplemented with 10% Foetal Bovine Serum (FBS), 0.1% ascorbic acid, 0.1% gentamicin and 0.1% amphotericin-B (OBM™, LONZA, USA). Reagent for the cell proliferation assay, an MTS-based assay, Cell Titer 96® Aqueous One solution was obtained from Promega (USA). Recombinant Human TNF- α was purchased from Gibco® (USA). Osteogenesis Assay kit (ECM815) was purchased from Milipore (USA). Alkaline phosphatase substrate kit was purchased from BioVision Research Products (USA). Human Osteoprotegrin Instant ELISA kit and Human sRANKL ELISA kit were purchased from Bender Medsystems GmbH (Austria) and Koma Biotech Inc. (Korea), respectively.

Extraction of black and green tea bioactive compounds:

Black tea powder and green tea leaves were separately soaked in 200 mL boiling deionized water for 10 min (Danrong *et al.*, 2009). The infusion was filtered, diluted until 1000 mL of deionized water and cooled to room temperature. The Black Tea (BTE) and Green Tea Extracts (GTE) were freeze-dried and kept at -20°C in plastic containers.

Determination of total phenolic contents of black and green tea:

The total phenolic content was determined according to the Folin-Ciocalteu reagent using gallic acid as standard (Parthasarathy *et al.*, 2009). BTE and GTE were dissolved to the concentration of 1 mg/mL (5 mg of extracts dissolved in 5 mL of ethanol) and 1 mL aliquots were mixed with 5 mL Folin-Ciocalteu reagent. After 5 min of incubation, 4 mL of Na₂CO₃ was added, mixed and incubated at room temperature for 60 min. The absorbance was measured against blank sample at 765 nm in a spectrophotometer. Total phenol content was expressed as milligrams of Gallic Acid Equivalent per gram of dry weight (mg GAE/g) of extracts.

Preparation and maintenance of Normal Human Osteoblast (NHOst) cells:

NHOst cells were grown in Osteoblast Basal Medium supplemented with 10% Foetal Bovine Serum (FBS), 0.1% ascorbic acid, 0.1% gentamicin and 0.1% amphotericin-B at in a CO₂ incubator with 90% humidity, 5% CO₂ and 37°C. As soon as the cells reached 70% confluency, they were detached with Accutase (Gibco, USA) and sub cultured every 7-8 days. The media was changed every 3 days. Cells at passage 7 were used for every experiment.

BTE and GTE treatments of NHOst cells: BTE and GTE powder were dissolved in absolute ethanol and subsequently dissolved with media to the following concentrations: 5, 10, 50 and 100 µg/mL. NHOst cells were treated with all concentrations for various time points in independent experiments.

Cell viability (MTS) assay: Prior to GTE and BTE treatments, NHOst cells were seeded in a 96-well flat bottom plate at a density of 1.5×10^4 cells per well with 100 µL of media. NHOst cell proliferation was measured using the MTS assay, CellTiter96[®] Aqueous One Solution. Briefly, cells were treated with 5, 50 and 100 µg/mL of BTE or GTE for 2, 5 and 10 days in the presence and absence of 1 ng/mL TNF-α and measured for cell viability.

RANKL and OPG protein determination: Protein concentrations of both RANKL and OPG markers were assayed using specific ELISA assays. NHOst cells were cultured in 25 cm² flasks at a density of 5×10^3 cells with 5 mL of media prior to BTE and GTE treatments. The protein concentration was determined in cell supernatants collected from BTE and GTE-treated cells in absence and presence of TNF-α-induced inflammation as well as the corresponding controls. The assays were conducted according to the manufacturer's instructions. All samples were assayed in triplicates.

ALP activity: Prior to GTE and BTE treatments, NHOst cells were cultured in 25 cm² flasks at a density of 5×10^3 cells with 5 mL of media. ALP activity was measured in cells treated with BTE and GTE with and without 1 ng/mL TNF-α for 2, 5 and 10 days. Cell culture media was collected and ALP activity was photometrically measured using p-Nitro Phenyl Phosphate (pNPP) as the substrate. The assay was conducted according to the manufacturer's instructions.

Alizarin red-S staining: NHOst cells were cultured in 24-well plates at a density of 5×10^4 cells per well with 1.5 mL media prior to BTE and GTE treatments. NHOst cells were supplemented with hydrocortisone and β-glycerophosphate to initiate the differentiation process. The mineralisation of BTE- and GTE-treated NHOst cells in absence and presence of TNF-α-induced inflammation was determined. The assay was conducted according to the manufacturer's instructions.

RESULTS AND DISCUSSION

Total phenolic contents of BTE and GTE: The total phenolic content of 5 g of each BTE and GTE were

Table 1: Total phenolic contents in GTE and BTE

Tea extracts	Total Phenolic Content (TPC) (mg GAE/g)
GTE	78.5±3.8
BTE	84.6±4.9

Data are expressed as mean value±SD for three replicates

equivalent to 84.57 ± 4.9 and 78.48 ± 3.8 mg of gallic acid, respectively (Table 1). The higher phenolic content in BTE, found in this study, could be explained by the differential rate of its release into the hot water infusion. According to Liebert *et al.* (1999) the liberation of the phenolic content from green tea into water infusion is more gradual in comparison to that from black tea.

Effects of BTE and GTE on NHOst cell proliferation: NHOst cells incubated with BTE or GTE in inflammatory and non-inflammatory conditions showed cell viability above 80% at all-time points and concentrations used (data not shown). No significant differences were observed between treatment and control groups indicating that all concentrations of BTE and GTE used for the experiments were non-toxic to the cells.

Mineralization of bone matrix: In the present study, we could show that day 5 of mineralization gave the optimal number of crystal nodules. BTE and GTE increased significantly the deposition of hydroxyapatite crystals by the non-inflamed osteoblasts in comparison to the untreated controls. The most effective concentrations were 5 µg/mL BTE and 100 µg/mL GTE. In inflammatory condition, TNF-α reduced the ability of osteoblasts to deposit hydroxyapatite crystals after 5 days of mineralisation. Both BTE and GTE were able to overcome the TNF-α-induced reduction in mineralisation. The most effective concentrations were 5 µg/mL BTE and 50 µg/mL GTE. Overall, BTE was more effective in increasing mineralisation at a lower concentration than GTE. However, in inflammatory condition, GTE was able to overcome the TNF-α-induced reduction more effectively, although, GTE did so at a higher concentration than BTE. Our results were similar with previous findings which indicated that EGCG, a rich compound in green tea, enhanced the differentiation of osteoblasts in which mineralization of bone matrix was increased and more bone nodules were formed (Vali *et al.*, 2007). In addition, regardless of the types of tea, it has been proven that tea drinkers have ~5% greater Bone Mineral Density (BMD) and content at various sites compared to non-tea drinkers, underscoring the advantage of habitual tea drinking (Kara *et al.*, 2007). In contrast to the absence of nodules observed in control cells, bone nodules were detected as early as 2 days in inflammatory condition (Fig. 1). TNF-α is a pro-inflammatory cytokine and it induces RANKL, a

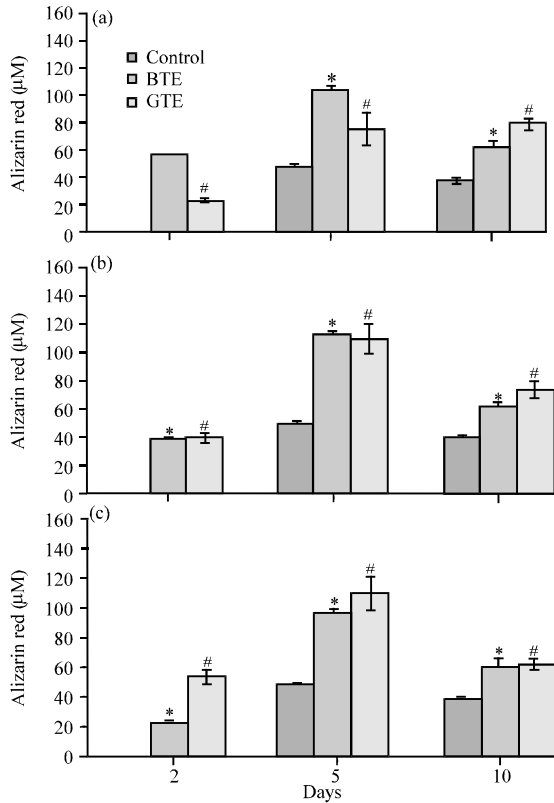


Fig. 1: a) 5 µg/mL; b) 50 µg/mL and c) 100 µg/mL; Quantitative analysis of bone mineralization. Effects of 5, 50 and 100 µg/mL GTE and BTE to NHOst cells in non-inflammatory condition for 2, 5 and 10 days compared to control group. GTE and BTE increased mineralization at all concentrations and time points. Data are expressed as mean value±SD; #p<0.05 (GTE); *p<0.05 (BTE)

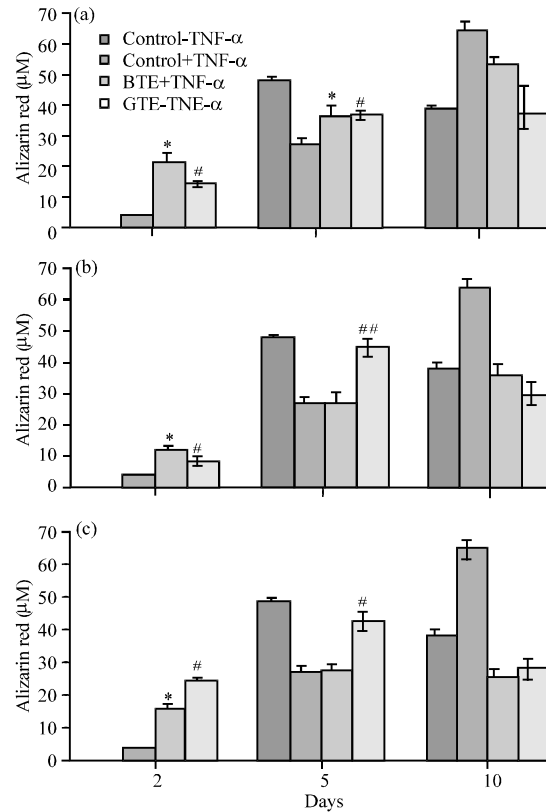


Fig. 2: a) 5 µg/mL; b) 50 µg/mL and c) 100 µg/mL; Quantitative analysis of bone mineralization. Effects of 5, 50 and 100 µg/mL GTE and BTE to NHOst cells in inflammatory condition for 2, 5 and 10 days compared to control group (control+TNF-α). Data are expressed as mean value±SD; #p<0.05 and ##p<0.01 (GTE); *p<0.05 (BTE)

family member of the TNF-α superfamily, osteoclast formation and bone resorption. Its effect on osteoblast cells has been little explored. Although, mainly produced by immune cells, other cells have receptors for TNF-α. Pre-osteoblasts and osteoblasts have an array of TNF-α receptors: TNFR1, DR3, DR5, Fas etc. Activation of the DR3 receptors leads to osteoblast differentiation which would explain the capability of the cells to form hydroxyapatite nodules (Robinson *et al.*, 2007). However, prolonged exposure (5 days) to TNF-α as shown in Fig. 2 leads to a reduction of bone mineralisation capability. Lu *et al.* (2006) proposed that the duration of exposure is very important for TNF-α to exert apparent effect on bone remodelling. According to Lu *et al.* (2006), short-term exposure of TNF-α significantly induced greater osteogenic differentiation by promoting production of endogenous Bone Morphogenetic Protein-2 (BMP-2) in

human primary osteoblasts. However, there was also a marked increase in nodule formation in inflammatory condition on day 10, thus, contradicting the above statement. The effect of BTE and GTE on mineralisation in TNF-α treated NHOst cells were not uniform. The explanation for the underlying mechanisms requires more in-depth studies. It has been reported that TNF-α strongly triggers ALP activity in human mesenchymal and osteoblast cells (Lencel *et al.*, 2001; Ding *et al.*, 2009). This is a plausible mechanism; however, we could not observe an increase of ALP activity in our cell culture model.

Long-term treatment with black tea extract increases alkaline phosphate: Alka Line Phosphatase (ALP) (phosphate-monoester phosphohydrolase; EC 3.1.3.1) is a metalloenzyme and exists in multiple isoforms. It is present in bone as tissue non-specific ALP. Although, the

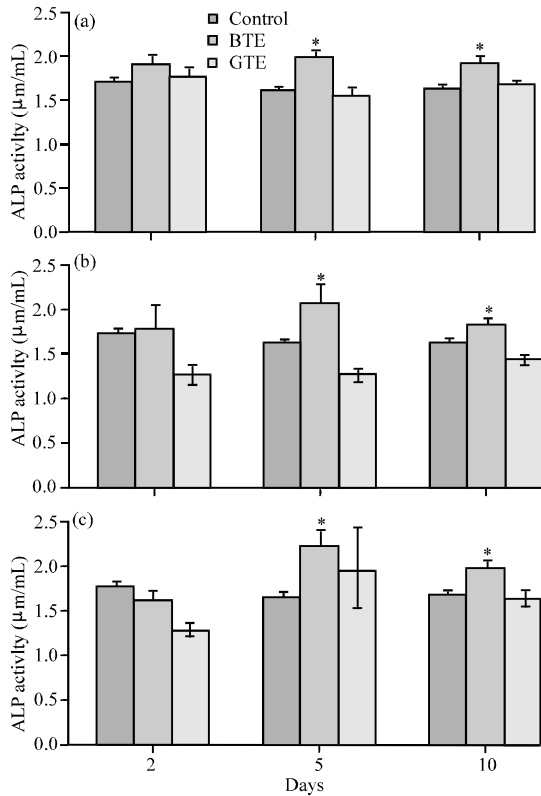


Fig. 3: a) 5 µg/mL; b) 50 µg/mL and c) 100 µg/mL; ALP activity. Effects of 5, 50 and 100 µg/mL GTE and BTE to NHOst cells in non-inflammatory condition for 2, 5 and 10 days compared to control group. BTE significantly increased ALP activity at all concentrations on day 5 and 10. Data are expressed as mean value±SD; *p<0.05 (BTE)

exact regulatory pathway is unclear is many experiments have shown that ALP is important for bone mineralization. It is considered as a marker for osteogenic activities (Golub and Boesze-Battaglia, 2007). The osteoblast cells used for our experiments were a mixture of early and late mature osteoblasts (Aisha *et al.*, 2014) in which ALP is fully expressed. The incubation of NHOst cells for 10 days did not lead to a change in ALP activity. However, elevated ALP activity was observed when the cells were incubated with BTE and GTE. The highest increase in ALP activity was observed with 100 µg/mL BTE after 5 days of incubation. Overall, the results for ALP in the NHOst cells incubated with BTE showed the similar time-dependent pattern like the corresponding experiments for mineralization. GTE treatment of NHOst cells did not significantly increase the activity of ALP except for 5 µg/mL GTE at 2 days and 100 µg/mL GTE at 5 days. In contrast to BTE treatment, we also observed some inhibitory effects in cells incubated with GTE.

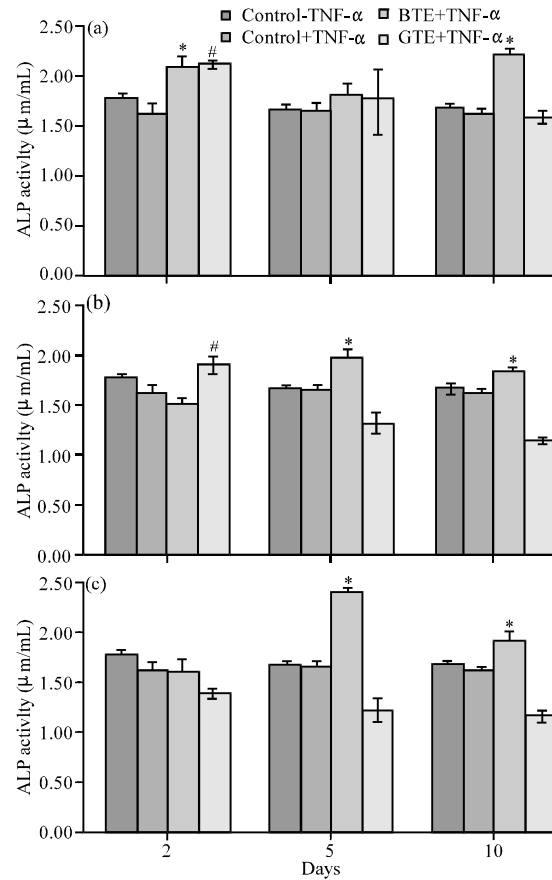


Fig. 4: a) 5 µg/mL; b) 50 µg/mL and c) 100 µg/mL; ALP activity. Effects of 5, 50 and 100 µg/mL GTE and BTE to NHOst cells in inflammatory condition for 2, 5 and 10 days compared to control group (control+TNF-α). BTE showed more significant increased ALP activity. Data are expressed as mean value±SD; #p<0.05 (GTE); *p<0.05 (BTE)

Interestingly, there was no significant change in ALP activity between control and TNF-α stimulated cells and over time with TNF-α stimulated cells. Both BTE and GTE increased ALP activity in TNF-α stimulated cells. The presented results were in parallel with studies performed by Datta *et al.* (2014). They reported that theaflavin, the major flavonoid in black tea was able to alter the alkaline phosphatase level in rats and mice thus preventing bone loss due to arthritis and ovarian hormone deficiency, respectively (Fig. 3 and 4).

Effects of BTE and GTE on NHOst cells functions-OPG and RANKL protein levels: In this present study, the findings suggest that in non-inflammatory condition, both GTE and BTE are capable to reduce the production of RANKL. It was observed that BTE acted more rapidly

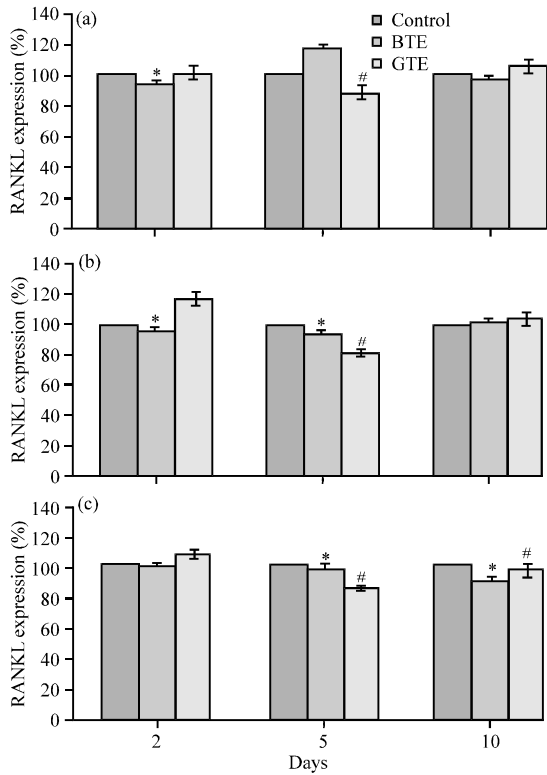


Fig. 5: a) 5 µg/mL; b) 50 µg/mL and c) 100 µg/mL; RANKL protein expression. Effects of 5, 50 and 100 µg/mL GTE and BTE to NHOst cells in non-inflammatory condition for 2, 5 and 10 days compared to control group. Data are expressed as mean value±SD; #p<0.05 (GTE); *p<0.05 (BTE)

than GTE when it started to reduce RANKL protein as early as day 2 of treatment (Fig. 4). Simultaneously, OPG level was shown to increase with only 5 µg/mL of GTE and BTE on day 2, 5 and 10 of experiment to a level of statistical significant (Fig. 5). The results exhibited that both treatments affected RANKL and OPG production as expected, therefore suggesting an estrogenic effects of GTE and BTE on bone (Bhuyan *et al.*, 2013). However, the effects of GTE and BTE treatments in non-inflammatory condition did not show a similar impact as that seen in inflammatory condition. This is due to the fact that RANKL and OPG have been at the basal levels where the bone remodelling could accordingly occur in basal physiological condition. Additionally in the present study, treatments with 100 µg/mL GTE and BTE significantly reduced the RANKL production on day 5 and 10. Previous studies have shown that TNF-α stimulated the expression and production of RANKL, consequently resulting in an increase in osteoclast formation and activity *in vitro* and *in vivo* (Bu *et al.*,

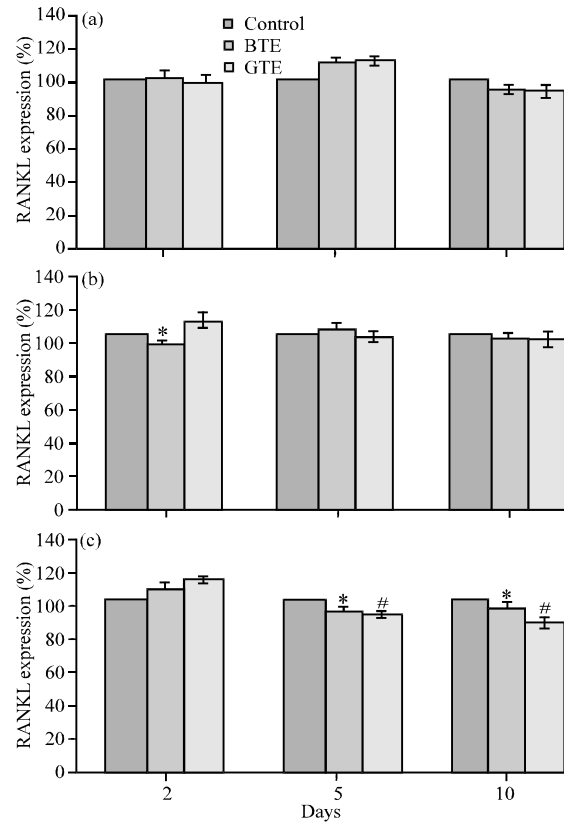


Fig. 6: a) 5 µg/mL; b) 50 µg/mL and c) 100 µg/mL; RANKL protein expression. Effects of 5, 50 and 100 µg/ml GTE and BTE to NHOst cells in inflammatory condition for 2, 5 and 10 days compared to control group (control+TNF-α). Data are expressed as mean value±SD; #p<0.05 (GTE), *p<0.05 (BTE)

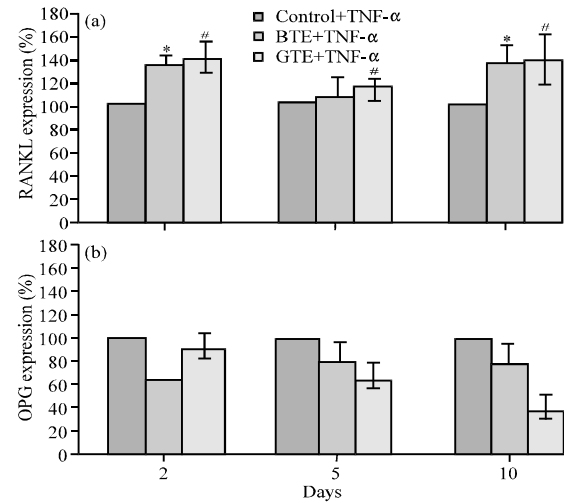


Fig. 7: Continue

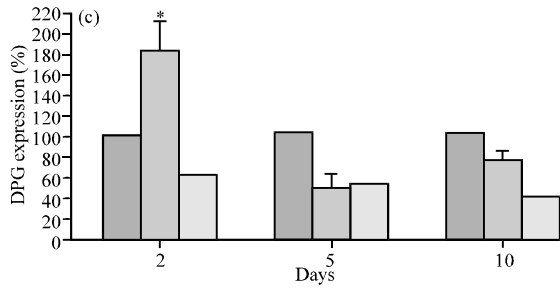


Fig. 7: a) 5 µg/mL; b) 50 µg/mL and c) 100 µg/mL; OPG protein expression. Effects of 5, 50 and 100 µg/mL GTE and BTE to NHOst cells in non-inflammatory condition for 2, 5 and 10 days compared to control group. Data are expressed as mean value±SD; #p<0.05 (GTE); *p<0.05 (BTE)

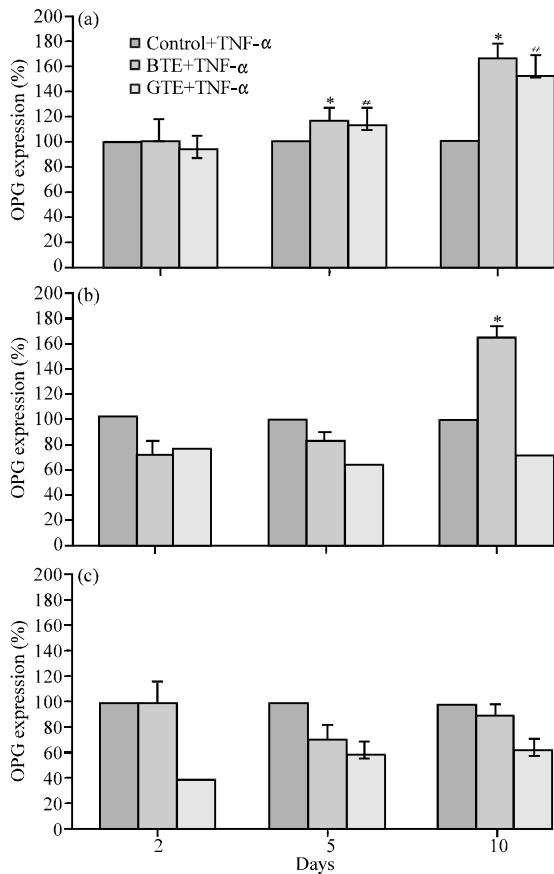


Fig. 8: a) 5 µg/mL; b) 50 µg/mL and c) 100 µg/mL; OPG protein expression. Effects of 5, 50 and 100 µg/mL GTE and BTE to NHOst cells in inflammatory condition for 2, 5 and 10 days compared to control group (control+TNF-α). Data are expressed as mean value±SD; #p<0.05 (GTE); *p<0.05 (BTE)

2009; Goto *et al.*, 2011; Qian *et al.*, 2014). It is suggested that GTE and BTE appear to neutralize the TNF-α effect on RANKL production, thus reducing the osteoclast formation, even though other concentrations at different time points did not show favourable results. It can be observed that the fluctuations in the results of the treatment groups were not apparent to the control group (Fig. 6). In comparison to RANKL, OPG production was significantly inclined with only 5 µg/mL GTE and BTE treatments at the same time points as RANKL reduction (Fig. 7 and 8).

CONCLUSION

BTE treatment to NHOst cells resulted in a significant increase in nodule deposition as well as ALP activity in both non-inflammatory and inflammatory conditions indicating promotion of matrix formation in bone. Simultaneously, results of the principal markers involved in bone remodelling which include RANKL and OPG, proved that BTE is commensurate to GTE at feasible doses to restore the equilibrium in bone metabolism and restrict the bone loss.

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