Rooibos tea extracts inhibit osteoclast formation and activity through attenuation of NF-κB activity in RAW264.7 murine macrophages

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Abstract

Rooibos tea is a naturally sweet and aromatic tea that is native to the Western Cape province of South Africa. Rooibos is usually fermented to produce the traditional reddish brown colour and has been found to have numerous health benefits. These include beneficial effects on osteoblasts, however, its effects on osteoclast formation and activity is unknown. Osteoclasts are large, multinucleated cells responsible for bone resorption. Binding of RANKL to its receptor on osteoclast precursors triggers the NF-κB signalling pathway leading to the formation of osteoclasts. Certain bone destructive diseases, such as osteoporosis, are characterised by overactive osteoclasts. Inhibition of osteoclasts may offer a potential mode to prevent these diseases. The polyphenol content of both fermented and unfermented tea extracts were similar although the radical scavenging activity of fermented rooibos tea was lower. Both tea extracts were not cytotoxic and inhibited osteoclast formation. Fermented rooibos tea extract caused a greater reduction in the osteoclast resorption and associated gene expression when compared with unfermented rooibos tea. Both tea extracts were shown to attenuate NF-κB activity. Fermented rooibos was found to have a more potent inhibitory effect on osteoclasts than unfermented rooibos extract and therefore may have a beneficial effect on bone health.

Introduction

Due to the crucial task of the skeleton in maintaining structure, movement and stability, it is imperative that it constantly remains in good condition.[1, 2] Osteoporotic fractures place a heavy burden on the individual and the health care systems. Dietary intervention and development of nutraceutical products can prevent the development of osteoporosis reducing the risk of fractures. Several dietary factors such as following a Mediterranean diet and a diet rich in protein, fruits and vegetable have been shown to have beneficial effects.[3] Fruits and vegetables are rich in flavonoids and several of these such as luteolin and quercitin have been shown to have a beneficial effect on bone health. [4, 5]

Bone is continuously being remodelled and a balance between bone synthesis by osteoblasts and resorption by osteoclasts is essential. Excessive bone resorption by osteoclasts leads to the development of osteoporosis.[6] Osteoclasts are large multinucleated cells derived from haematopoetic stem cells of the monocyte lineage.[7] Osteoblasts secrete two important cytokines necessary for osteoclast formation, namely, macrophage colony stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL). M-CSF binds to c-fms receptor on osteoclast precursors and stimulates proliferation and survival whereas RANKL binding to its accompanying receptor, RANK (receptor activator of NF-κB), triggers osteoclast precursors to differentiate and fuse into mature osteoclasts.[8] Coupling of RANK/RANKL triggers a cascade of downstream events, specifically the activation of NF-κB which further induces key regulatory factors important for formation and survival of multinucleated osteoclasts.[8] Such factors include dendritic cell-specific transmembrane protein (DC-STAMP), which plays a role in cell-to-cell fusion,[9] and tartrate-resistant acid phosphatase (TRAP), cathepsin K (CTSK), and matrix metalloproteinase 9 (MMP-9), which play important roles in resorption. [10]

Aspalathus linearis (rooibos) is a short bush-like plant that is local to the Western Cape region of South Africa. Leaves can be harvested and dried producing green or unfermented rooibos.[11] To manufacture the more traditional red brown coloured rooibos tea, the leaves undergoes a fermentation process during which the polyphenols in rooibos, become oxidized.[11] Rooibos tea is rich in flavonoids which are known to possess various beneficial characteristics including antioxidant, antitumor, anti-atherosclerotic, anti-inflammatory, antiviral and estrogenic activities.[12-14] The flavonoids in rooibos are aspalathin, nothofagin, quercitin, luteolin, orientin, iso-orientin and chrysoeriol.[15] Some of these compounds have been shown to have beneficial effects on bone health. Luteolin has been shown to reduce osteoclastic differentiation and resorption in murine bone marrow macrophages as well as reduce ovariectomy induced bone loss in mice.[4] In New Zealand white rabbits with bone defects,

quercitin was shown to increase local bone formation showing potential as a bone grafting material.[5] Furthermore, it has been reported that chrysoeriol can promote growth and mineralization in MC3T3-E1 murine pre-osteoblast cells.[16] These studies may suggest that rooibos could possibly improve bone health by inhibiting osteoclast function and promoting osteoblast mineralization.

Rooibos tea is rich in flavonoids and the effect of rooibos as consumed on osteoclastogenesis is unknown. The aim of this study is to investigate the effects of fermented and unfermented rooibos tea extracts on RANKL-induced osteoclast formation and bone resorption in RAW264.7 murine macrophages.

2. Materials and Methods

2.1 Reagents and materials

Trypan blue, crystal violet, antibiotic-solution containing: streptomycin, fungizone and penicillin, and primary antibodies for western blotting were purchased from Sigma-Aldrich (St Louis, MO, USA). Dulbecco's minimum essential medium (DMEM) was provided by Sterilab Services (Harrogate, UK) and foetal bovine serum (FBS) was supplied by Biochrom (Berlin, Germany). RANKL was acquired from Research and Diagnostic Systems (R&D Systems, Minneapolis, MN, USA). Cell cluster plates and glass cover slips were purchased from LASEC (Cape Town, South Africa (SA)). Osteoassay plates were purchased from Corning inc. (Corning, NY, USA). Fermented rooibos tea (red rooibos) was obtained from National Brands Limited (Rivonia, Durban, SA). Unfermented rooibos tea (green rooibos) was obtained from Biedouw Valley (Clanwilliam, Western Cape, SA). Phalloidin-conjugate and Hoechst 3342 were purchased from Life Technologies (Carlsbad, CA, USA). IκB and GAPDH rabbit polyclonal antibodies were purchased from Abcam (Cambridge, MA, USA). Secondary antibodies for western blotting were purchased from Bio-Rad (Hercules, CA, USA). Reverse transcriptase was purchased from New England Biolabs (Ipswich, MA, USA). Oligo (dT) primers were purchased through Thermo Scientific (Waltham, MA, USA). Other PCR reagents (dNTPs, DNA polymerase, DNA ladder, loading dye, etc.) were supplied by KAPA Biosystems (Cape Town, SA).

2.1 Tea extract preparation

Concentrations were based on previous studies on tea extracts that showed anti-osteoclastogenic activity.[17] Tea extracts were prepared as previously described.[17] In short, tea leaves at a concentration of 125 mg ml^{-1} were steeped in deionized boiling water for 30 min to maximize polyphenol content.

Traditionally rooibos tea is boiled in water and allowed to steep for long periods.[18] Thereafter, the extracts were filtered using Whatman filter paper (grade 1) and 0.22 µm syringe filter and aliquots were stored at -20°C in the dark. A vehicle control of sterile distilled water was processed as described above and was used in all experiments. Tea extracts used in all experiments were from the same batch.

2.2 Tea analysis

2.2.1 Total polyphenolic content (TPC)

Total polyphenolic content (TPC) was analysed using a Folin-Ciocalteu (F-C) assay as described by Amin *et al.*[19] The F-C assay is an *in vitro* colorimetric assay that measures the reducing capability of a sample. Polyphenols readily react with the reagent and colour change can be used as a measure of TPC. Gallic acid (GA) was used to prepare a calibration/standard curve using a final concentration range of 0-0.04 mg/ml. The generated equation was then used to determine the tea's phenolic content in mg gallic acid equivalents (GAE)/g. Tea samples (125 mg ml^{-1}) were diluted to 10% in double distilled deionized water. Samples and standards (10 µl each) were added to a 96-well plate followed by 50µl of F-C (diluted 1:15 F-C reagent in distilled water) and 50µl of a 7.5% sodium carbonate solution. After 1 hr, absorbance was read at 630nm and the data were expressed as mg GAE/g.[19, 20]

2.2.2 Trolox equivalent antioxidant capacity (TEAC) assay

Trolox equivalent antioxidant capacity (TEAC) assay was performed as previously described.[21] This assay measures the ability of antioxidants to scavenge 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) radical cation (ABTS$^+$) a chromophore which has a decrease in intensity in the presence of antioxidants. In short, ABTS$^+$ stock solution was produced by the reaction of 3 mM $K_2S_2O_8$ with 8 mM of ABTS in 10 ml PBS. This mixture was then left to stand in the dark for 12 hr. ABTS$^+$ stock solution was diluted 1:30 (v/v) in PBS to create a working solution. A solution of 1 mM Trolox equivalents (TE) was used to create a standard curve with a final concentration range of 0-0.0027 mM TE. The generated equation was then used to determine the tea's antioxidant capacity. A 10 µl volume of 1% tea samples or standards were added to a 96-well plate and then 290 µl of ABTS$^+$ working solution was added. After 30 min, absorbance was read at 734 nm and the results were expressed as mM TE/g.

2.3 Cell culture maintenance

The RAW264.7 murine macrophage cell line (TIB-71) was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). RAW264.7 macrophages express c-fms as well as M-CSF and

therefore only require RANKL to differentiate.[22] Cells were propagated as a monolayer in DMEM containing 10% heat-inactivated FBS and antibiotics (100 µg ml^{-1} streptomycin, 0.25 µg ml^{-1} fungizone and 100 U ml^{-1} penicillin), (complete medium) at 37°C. The atmosphere was humidified at 95% air and 5% CO_2.

2.4 Alamar blue assay

The effect of fermented and unfermented rooibos tea extracts on the viability of proliferating RAW264.7 macrophages was determined, using an Alamar blue assay (Life Technologies). Viable cells determined with the trypan blue exclusion assay were seeded in 96-well culture plates at 5 000 cells/well in complete media and was then incubated at 37°C for 24 hr to allow for attachment. Thereafter, the medium was changed and the cells were exposed to 0-500 µg ml^{-1} of fermented or unfermented rooibos extracts at increasing concentrations along with the vehicle control of sterilized distilled water. After 48 hr, the cells were incubated with 10% Alamar blue for 4 hr at 37°C in the dark. The absorbance was measured on an ELX 800 Micro plate reader (BioTek, Winooski, VT, USA) at a wavelength of 570/600 nm, and expressed as percentage compared to control. Three independent experiments were conducted (n=6).

2.5 Osteoclast formation

Viable RAW264.7 cells were seeded in 48-well culture plates at 15 000 cells/well and were cultured in complete media in the presence of 15 ng ml^{-1} RANKL. These cells were exposed to 0-500 µg ml^{-1} aqueous rooibos extracts. Media and all factors were replaced on day 3 and experiments were terminated on day 3, 4 and 5.

2.5.1 TRAP staining for multinucleated osteoclasts

To identify multinucleated osteoclast formation the media were removed and cells were fixed with 3.7% formaldehyde in PBS and stained using a TRAP staining kit (#387A, Sigma-Aldrich) according to the manufacturer's instructions. Thereafter, the cells were counterstained with haematoxylin for 2 min to stain the nucleus. Cells containing three or more nuclei were identified and counted as mature osteoclasts. Photomicrographs were taken with an Olympus SC30 camera attached to an Olympus BH2 microscope (Olympus, Tokyo, Japan).

2.5.2 Fluorescent microscopy for visualization of F-actin rings in osteoclasts

RAW264.7 cells were seeded at 10 000 cells/well on sterilized glass cover slips placed in the wells of a 24-well plate, in the presence of 30 ng ml^{-1} RANKL and aqueous tea extracts at 125, 250 and 500 µg ml^{-1}, along with a vehicle control of sterile distilled water. After 3 days the culture media and all factors were replaced. Termination of this experiment took place 5-7 days after the onset of the experiment. Cells were fixed with 3.7% formaldehyde in PBS for 5 min. Thereafter, 0.1% Triton-X in PBS was added in order to permeabilize the membrane and then a fluorescent phalloidin conjugate solution was added and left to stand for 40 min at room temperature in the dark. After staining, cells were washed with PBS and then counterstained with Hoescht 3342. Actin rings and nuclei were visualized and photographed using a Zeiss inverted Axiovert CFL40 microscope attached to a Zeiss Axiovert mRM monochrome camera using the appropriate filter sets (Hoechst (Excitation: 352 nm, Emission: 455 nm); Phalloidin (Excitation: 502 nm, Emission: 525 nm) (Zeiss, Germany)).

2.5.3 Bone resorption assay on bone mimetic plates

Cells were seeded at 10 000 cells/well on 24-well Corning Osteoassay plates coated with calcium phosphate apatite (Corning Inc.) in the presence of 30 ng ml^{-1} RANKL, as described before,[23] and tea extracts or vehicle. After 3 days the culture media and all factors were replaced. The experiment was terminated after 5-7 days. The osteoassay plates were rinsed with 5% sodium hypochlorite for 5 min to remove the cells. The plate was then rinsed with distilled water and left to dry. A modified von Kossa staining method was used to stain the plates.[17] Briefly, 5% silver nitrate was added to each well and incubated at room temperature for 30 min in the dark. After washing, 5% sodium bicarbonate was added for 4 min. A Zeiss Axiocam ERc5 microscope attached to a Zeiss Axiocam MRc5 camera (Zeiss) was used to capture the resorbed pits. Three images were captured per well and the resorption area was quantified with Image J software.[24]

2.5.4 Quantification of mRNA expression using RT- PCR

Cells were seeded at 30 000 cells/well on a 24-well plate and treated with vehicle control or tea extracts (250 – 500 µg ml^{-1}) in the presence of RANKL (15 ng ml^{-1}) for 5 days. At the end of culture, cells were lysed using a TRI Reagent® (Sigma-Aldrich) and the total RNA was extracted. RNA was converted to cDNA using M-MuLV reverse transcriptase (New England Biolabs) as per manufacturer's instructions. FastStart Essential DNA Green Master Mix (Roche) was used as the fluorescent probe for the quantitative real time PCR (qRT-PCR) assay using the cycling protocol shown in Table 1. Analysis of gene expression was done using the 2$^{-\Delta\Delta}$ CT method using a LightCycler® Nano System (Roche Diagnostics) and

normalized to the house keeping gene, GAPDH.[25] Primers (Table 2) were synthesized by Inqaba Biotec (Pretoria, SA).

Table 1: PCR cycling protocol for quantitative PCR.

Step	Temperature	Duration	Cycles
Hold	95°C	10 min	1
3-Step amplification	95°C	20 sec	35
	60°C	20 sec	
	72°C	20 sec	
Melting	65°C	1 min	1
	95°C	1 sec	

Table 2: Primer sequences

GAPDH: Forward: 5'-GATGACATCAAGAAGGTGGTGAAGC-3', Reverse: 5'-ATACCAGGAAATGAGCTTGACAAA-3'
TRAP: Forward: 5'-CCACCCTGAGATTTGTGGCT-3', Reverse: 5'-ACATACCAGGGGATGTTGCG-3'
Cathepsin K: Forward: 5'-CTGGAGGGCCAACTCAAGA-3', Reverse: 5'-CCTCTGCATTTAGCTGCCTT-3'
MMP-9: Forward: 5'-GTCATCCAGTTTGGTGTCGCG-3', Reverse: 5'-AGGGGAAGACGCACAGCTC-3'
NFATc1: Forward: 5'-GTGGAGAAGCAGAGCAC-3', Reverse:5'- ACGCTGGTACTGGCTTC-3'
DC-STAMP: Forward: 5'-ATGACTTGCAACCTAAGGGAAAG-3', Reverse: 5'-GTCTGGTTCCAAGAAACAAGGTCAT-3'
CA II: Forward: 5'-GAGTTTGATGACTCTCAGGACAA-3', Reverse: 5'-CATATTTGGTGTTCCAGTGAACCA-3'

2.6 NF-κB Alkaline phosphatase reporter gene assay

RAW264.7 cells were stably transfected with pNiFty2-SEAP plasmid (InvivoGen, San Diego, USA) using Genecellin transfection reagent (BioCellChallenge, Paris, France) and selected using Zeocin (InvivoGen). Transfected RAW264.7 cells were seeded in 96-well plates at 5 000 cells/well and cells were cultured in complete media. In addition, 50 µg ml^{-1} Zeocin was added to media. Transfected cells were stimulated with 35 ng ml^{-1} RANKL[26] and tea extracts (62.5-500 µg ml^{-1}) or vehicle control. After 48 hr media were collected and analysed with a SEAP assay according to the manufacturer's instructions.[26]

2.7 Western blot

RAW264.7 cells were seeded in complete media at $1x10^6$ cells/well in 6-well plates and left overnight to attach. The next day, cells were pre-exposed to the teas (500 µg ml^{-1}) and vehicle control and left to

incubate at 37°C for 4 hr, to ensure sufficient exposure to the teas before RANKL (15 ng ml^{-1}) was added. The cells were further incubated for 30 min. Thereafter, cell lysates were prepared using RIPA buffer supplemented with PMSF and protease and phosphatase inhibitors. Protein content was quantified with the BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). The purified proteins were resolved on 12% SDS-PAGE gels before being electrotransferred onto a nitrocellulose membrane. Protein binding sites were blocked with Tris buffered saline (TBS) containing 5% BSA and 0.2% Tween 20. Membranes were incubated overnight with a rabbit polyclonal primary antibody (1:1 000) at 4 °C and detection was achieved after 1 hr incubation with goat anti-rabbit HRP conjugated secondary antibody (1:20 000) using an ECL plus detection system (Bio-Rad). Digital images were captured with a Chemidoc MP (Bio-Rad).

2.8 Statistical analysis

The data was analyzed by two-way analysis of variance (ANOVA) with Bonferonni post hoc testing using GraphPad Prism software (GraphPad software Inc, California, USA). Testing was done at the 0.05 level of significance. Three independent experiments were conducted with different cell passages. All experiments were done in triplicate and were expressed as mean ± standard deviation (SD), unless otherwise stated.

3. Results

3.1 Total polyphenol content (TPC) and antioxidant activity

Analysis of both fermented and unfermented teas revealed no statistical differences in TPC (Figure 1A) between the teas. The antioxidant activity of both teas was then determined by TEAC assay. Fermented rooibos tea extract exhibited significantly lower anti-radical capacity compared to unfermented rooibos tea extract (Figure 1B).

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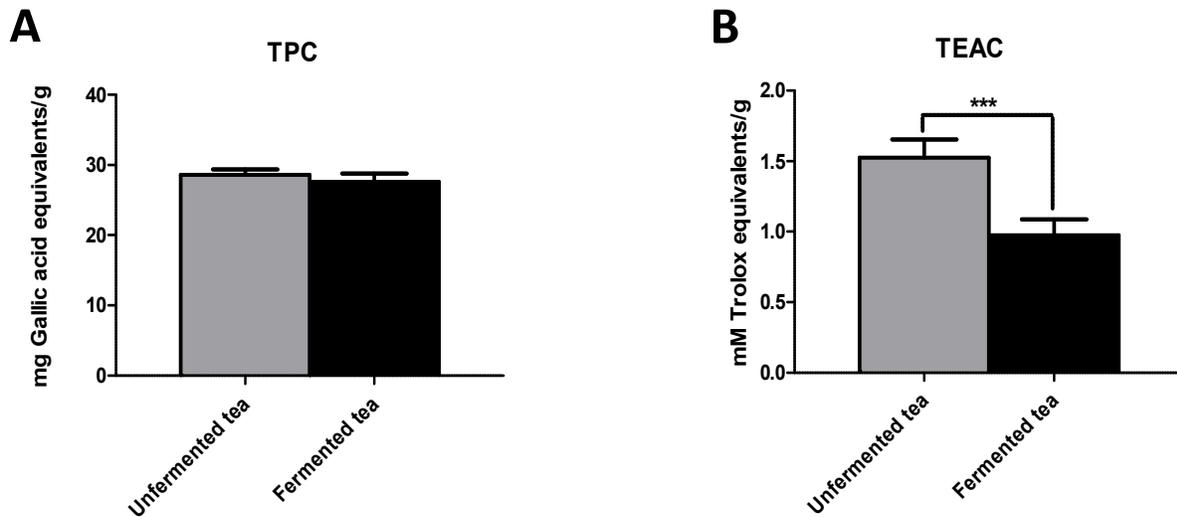
Figure 1. Analysis of fermented and unfermented rooibos extract. (A) Total polyphenol content (TPC) determined with the Folin-Ciocalteu assay. **(B)** Antioxidant activity of fermented and unfermented rooibos tea extracts was determined with the TEAC assay. Data expressed as mean ± SD and are representative of three independent experiments. ***$p < 0.001$ *vs* other tea.

3.2 Rooibos tea extracts does not affect cell viability in RAW264.7 macrophages

RAW264.7 murine macrophages were exposed to 62.5-500 µg ml^{-1} fermented or unfermented rooibos extract and cell viability was analysed by Alamar blue assay. Both teas were not cytotoxic at the concentrations evaluated and these concentrations were used throughout the study.
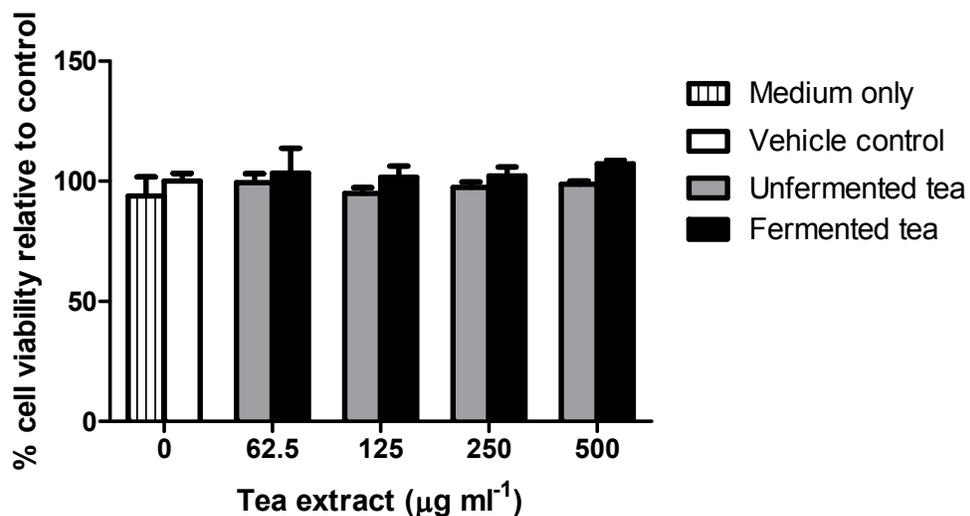
Figure 2. Effects of unfermented and fermented rooibos tea extracts on cell viability in undifferentiated RAW264.7 macrophages. Cells were seeded at 5 000 cells/well in 96-well plates and treated for 48 hr with vehicle control or tea extracts (62.5-500 µg ml^{-1}). Cell viability expressed as percentage relative to control determined with the Alamar blue assay. Data are expressed as the mean ±SD.

3.3 Rooibos tea extracts inhibit RANKL-induced osteoclastogenesis

To determine whether the rooibos tea extracts affected the osteoclast cell formation and size, TRAP stained images were evaluated (Figure 3A). TRAP is expressed during osteoclast precursor fusion and activation, and in this study is used as a marker for osteoclastogenesis *in vitro*.[27] TRAP positive, multinucleated with 3 or more nuclei were identified as osteoclasts and counted. Small osteoclasts appear in the RANKL positive control at day 3. Few osteoclasts are also present in the cells exposed to unfermented tea extract at 62.5 µg ml^{-1}. Osteoclasts were not seen when exposed to any of the other concentrations of tea at day 3. At day 4 and 5 fermented rooibos extract showed a dose dependent reduction in osteoclast number. Unfermented rooibos extract only showed a significant decrease in osteoclastogenesis at 125-500 µg ml^{-1} on day 4 but only at the highest concentrations tested [250 µg ml^{-1} ($p<0.05$) and 500 µg ml^{-1} ($p<0.001$)] on day 5. Differences between fermented and the unfermented rooibos tea were significantly different at all concentrations evaluated on day 4 and 5 (Figure 3E).
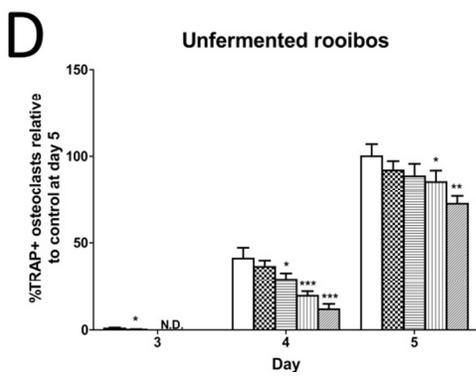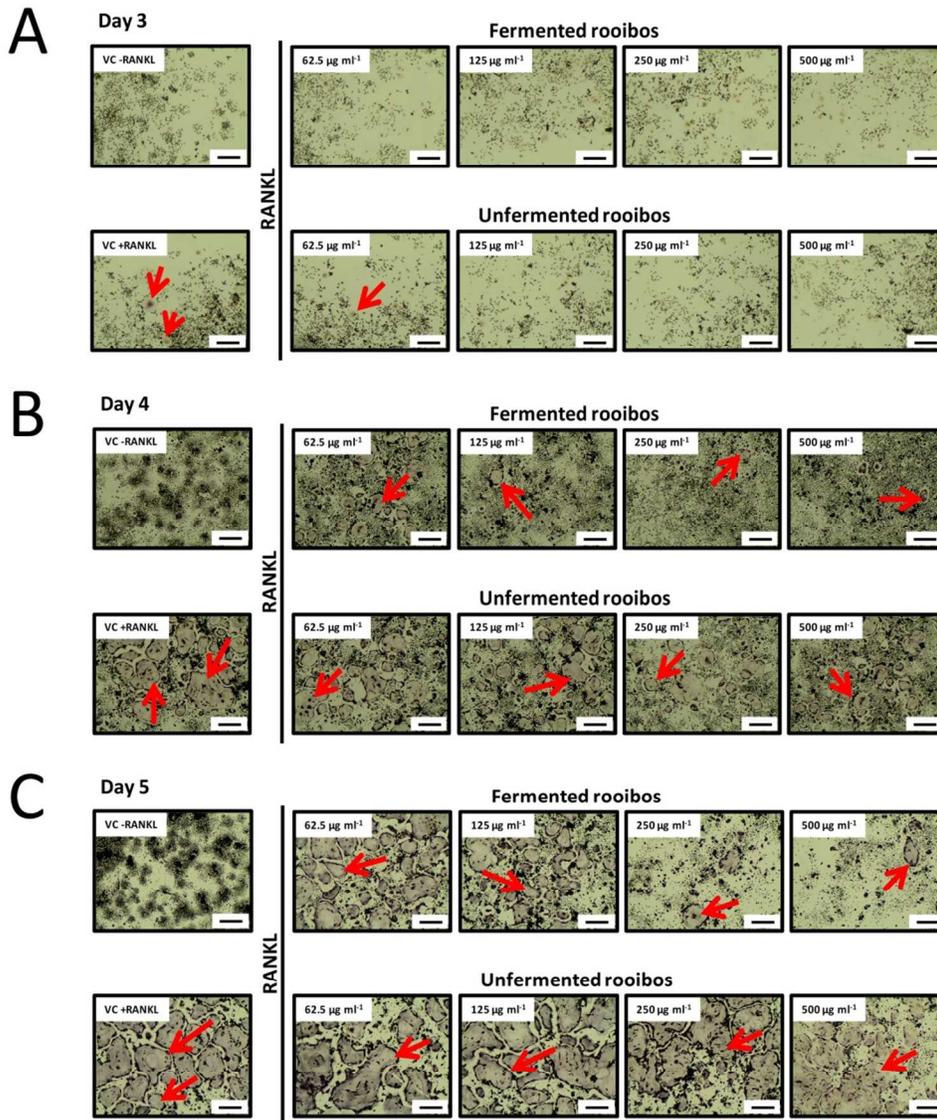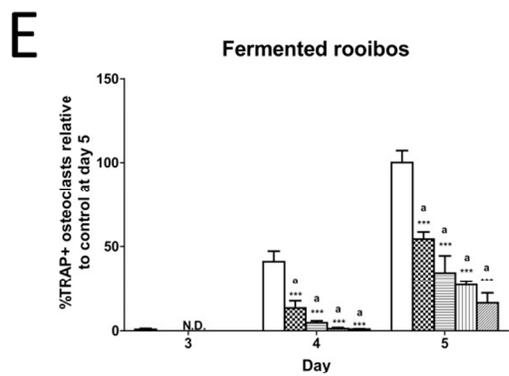
Figure 3. Effect of fermented and unfermented rooibos extract on osteoclastogenesis. Cells were seeded at 15 000 cells/well in 48 well plates and were treated with vehicle control or tea extracts in the presence of RANKL. After 3 **(A)**, 4 **(B)**, and 5 **(C)** days, the cells were stained for the presence of TRAP and multinucleated osteoclasts stain purple (red arrows). Scale bar = 500 μm. **(D and E)** TRAP+ cells with 3 or more nuclei were counted under a microscope. VC+RANKL: vehicle control with RANKL. VC-RANKL: vehicle control without RANKL. *p<0.05, ***p<0.001 *vs* vehicle control. a = fermented *vs* unfermented tea extract with p<0.001.

3.4 Rooibos tea extracts attenuate actin ring formation

Presence of actin rings were identified with phalloidin conjugate staining and the nuclei were localised with Hoescht 3324 staining. At all concentrations evaluated fermented rooibos extract caused a decrease in the number of actin rings and at the highest concentration, 500 μg ml^{-1}, evaluated actin rings were absent (Figure 4). Unfermented rooibos extract caused less inhibition than the fermented tea, with fewer actin rings seen only at the highest concentrations tested.
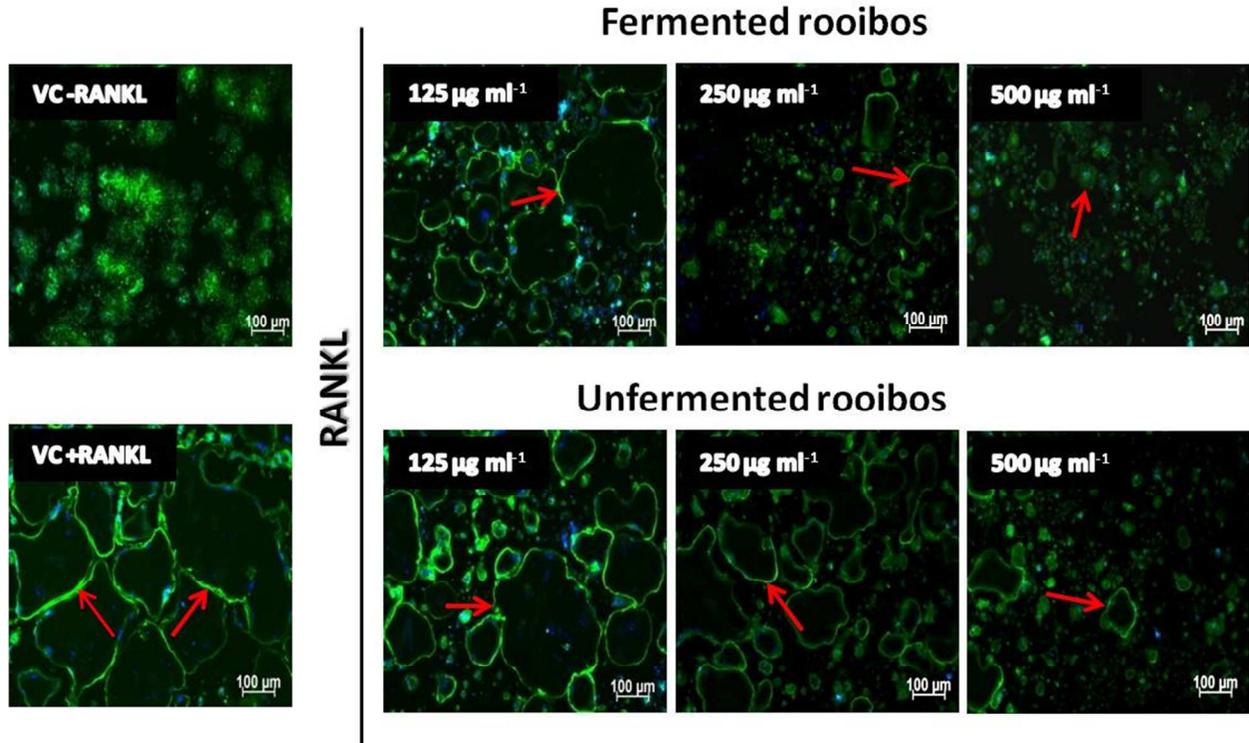
Figure 4. Effect of fermented and unfermented rooibos tea extracts on actin ring formation. Cells were seeded on glass coverslips at 10 000 cells/well in 24-well plates and treated with vehicle control or tea extracts (125, 250 and 500 µg ml^{-1}) in the presence of RANKL. After 5 days, cells were fixed and stained for actin with phalloidin (green) and nuclei with Hoechst 3324 (blue). Actin rings are indicated with red arrows. VC+RANKL: vehicle control with RANKL. VC-RANKL: vehicle control without RANKL. Scale bar = 100 µm.

3.5 Rooibos tea extracts decrease bone resorption

The effect of the tea extracts on osteoclast function was determined using bone resorption assay (Figure 5A and B). A dosage dependent decrease in bone resorption was observed for fermented tea rooibos extracts. A significant decrease in bone resorption was only observed at 250, 375 and 500 µg ml^{-1} for unfermented rooibos tea. Differences between unfermented and fermented rooibos tea was significant at 125 – 500 µg ml^{-1}.

B
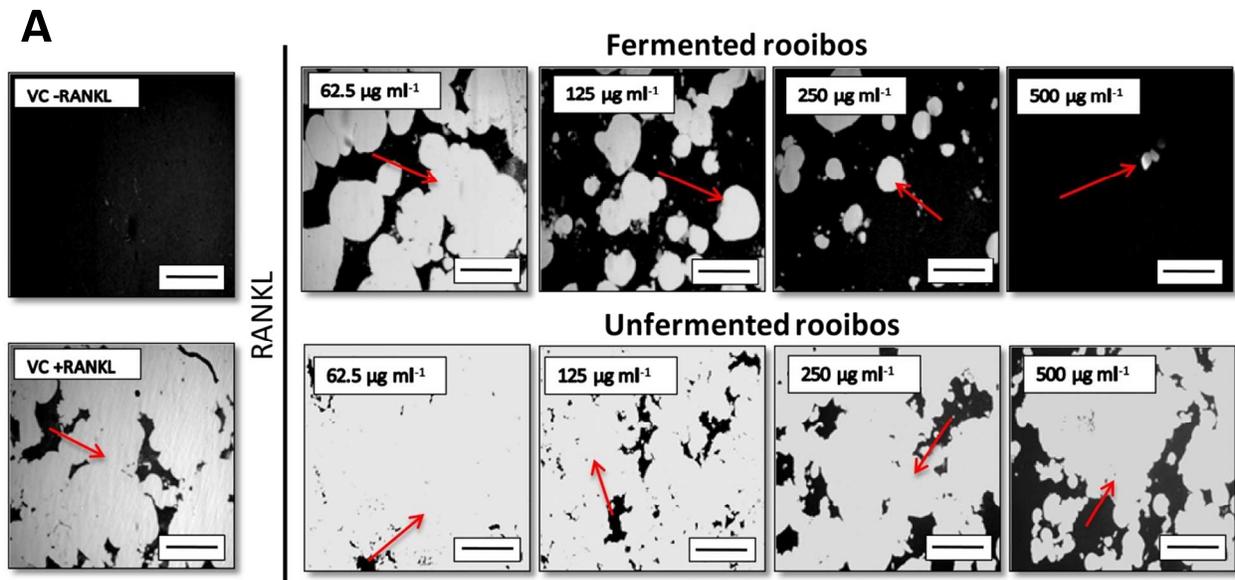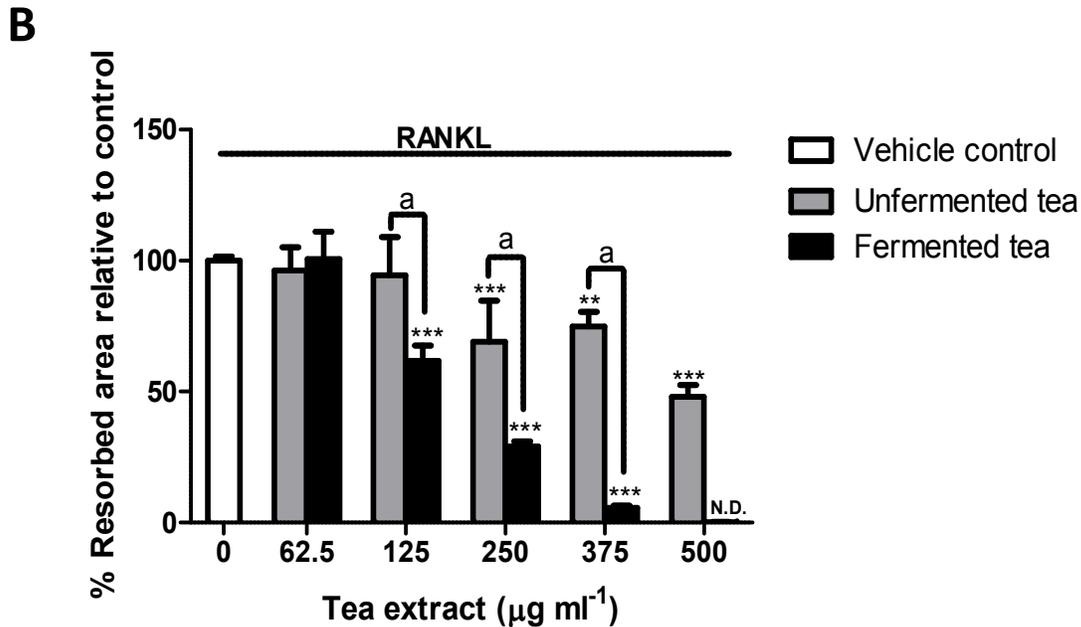
Figure 5. Effects of fermented and unfermented rooibos tea extracts on bone resorption. Cells were seeded into osteoassay plates and exposed to vehicle control or tea extracts (62.5-500 µg ml^{-1}) for 5 days. **(A)** Photomicrographs of resorption pit formation in 24-well osteoassay plates. Light areas are resorbed surfaces (red arrows). Scale bar = 200 µm. **(B)** Resorption pits was quantified using Image J software for cells exposed to fermented and unfermented tea extracts. Results are expressed relative to the vehicle control. **$p<0.01$; ***$p<0.001$ *vs* vehicle control. a= fermented *vs* unfermented tea extract with $p<0.001$. N.D.: Not detected. VC+RANKL: vehicle control with RANKL. VC-RANKL: vehicle control without RANKL.

3.6 Rooibos tea extracts inhibit osteoclast specific gene expression

The effect of the tea extracts on downstream genes that are important for osteoclast fusion and resorption was quantified with qRT-PCR. Compared to the vehicle control, for the unfermented rooibos tea a significant decrease in the expression of NFATc-1, DC-STAMP and to a lesser degree CA II, TRAP, Cathepsin K and not MMP-9 was observed. In contrast, fermented rooibos extract caused a decrease in the expression of all the genes evaluated (Figure 6). Differences between unfermented and fermented rooibos tea extract was significant for CA II at 250 µg ml^{-1}.

NFATc1

DC-STAMP

CA II

TRAP

Cathepsin K

MMP-9
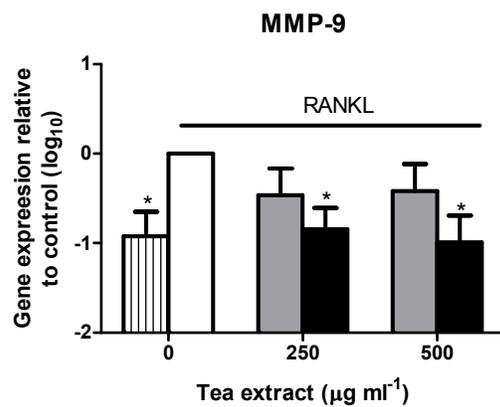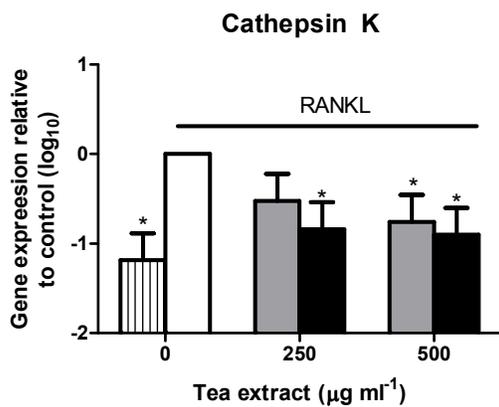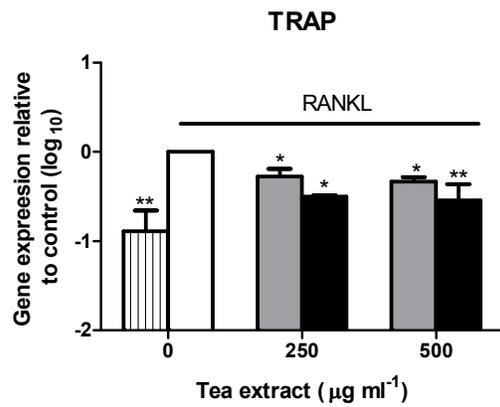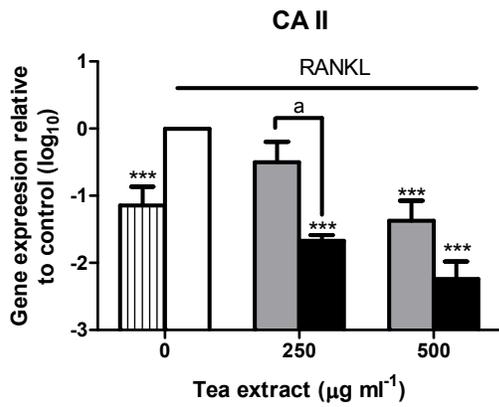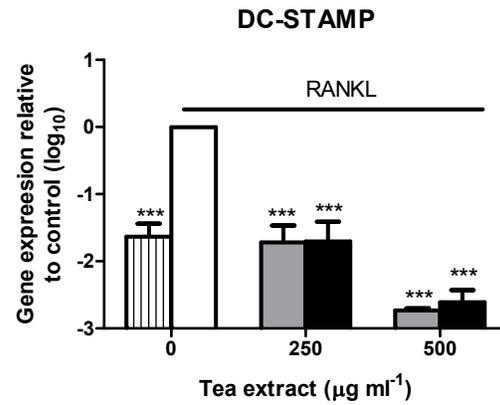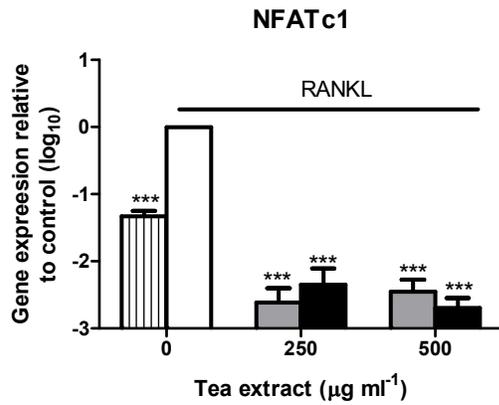
Legend: No RANKL | Vehicle control | Unfermented tea | Fermented tea

X-axis (all panels): Tea extract ($\mu g\ ml^{-1}$)

Y-axis (all panels): Gene expreesion relative to control ($\log_{10}$)

Figure 6. Effect of fermented and unfermented rooibos tea extracts on osteoclast specific gene expression. Cells were seeded at 30 000 cells/well into a 24-well plate and treated with vehicle control or tea extracts (250 – 500 µg ml^{-1}) in the presence of RANKL for 5 days. Gene expression of osteoclastic markers NFAT-c1, DC-STAMP, CAII, TRAP, cathepsin K and MMP-9 were determined by qRT-PCR. GAPDH was used as a house-keeping gene. Results are expressed relative to vehicle control. *p<0.05 **p<0.01; ***p<0.001 *vs* vehicle control. a= fermented *vs* unfermented tea extract with p<0.001.

3.7 Rooibos tea extracts suppress the NF- κB pathway

The activation of NF-κB, expressed downstream of RANK/RANKL binding, is vital towards osteoclast formation. NF-κB activation requires the ubiquitination and degradation of IκB.[28] At 500 µg ml^{-1} both fermented and unfermented rooibos extract prevented the degradation of IκB (Figure 7A). Using the NF-κB-SEAP reporter assay, both unfermented and fermented rooibos tea extracts significantly decreased NF-κB expression at increasing concentrations (Figure 7B). Differences between unfermented and fermented rooibos tea was only significant at 62.5 µg ml^{-1} and 500 µg ml^{-1}.
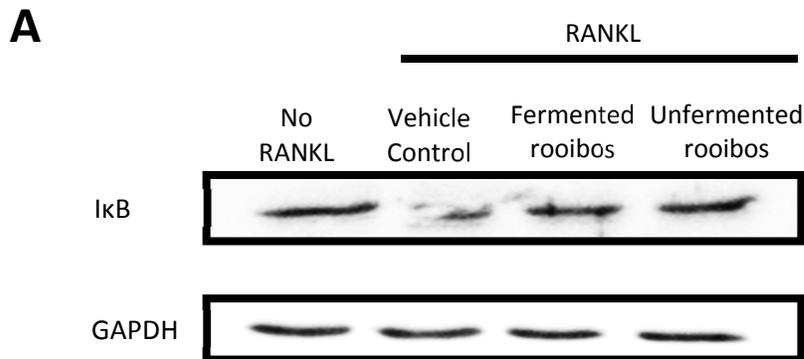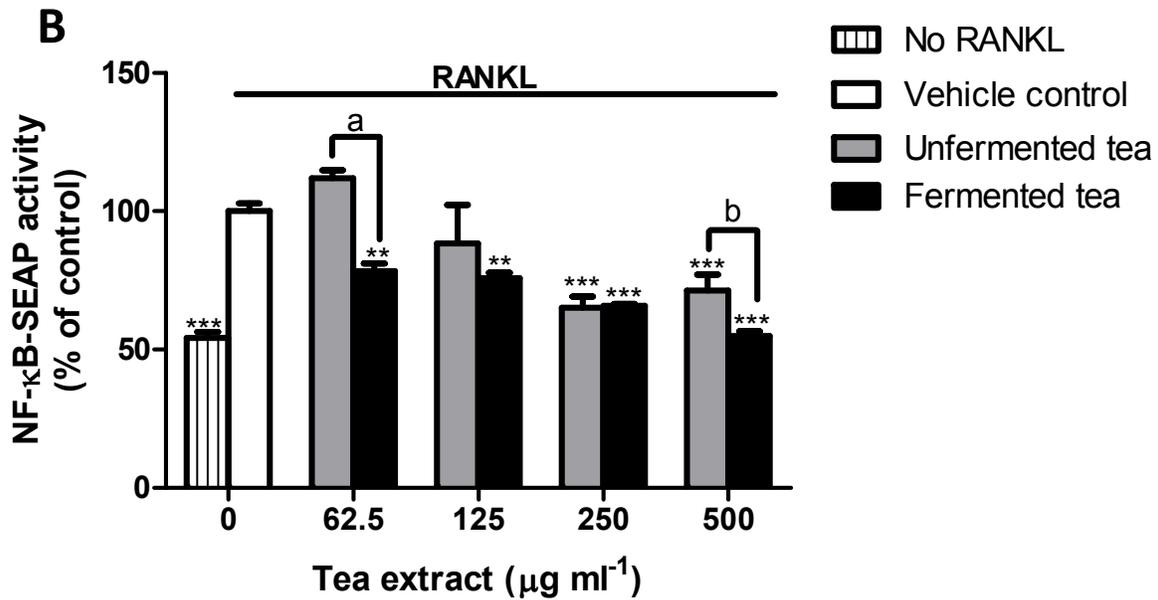
Figure 7. Effects of fermented and unfermented rooibos tea extracts on NF-κB pathways. (A) RAW264.7 macrophages were pre-exposed to 500 μg ml^{-1} fermented or unfermented rooibos extract or vehicle for 4 hrs and then exposed to RANKL for 30 min. Cell lysates were prepared and IκB protein expression was determined by western blot with GAPDH as loading control. **(B)** Transfected RAW264.7 macrophages were seeded at 5 000 cells/well in 96-well plates and treated with vehicle control or tea extracts (62.5 – 500 μg ml^{-1}) in the presence of RANKL. After 2 days, conditioned media were collected and analyzed for NF-κB expression, using a SEAP assay. Results are expressed as a percentage relative to control. ** $p < 0.01$; *** $p < 0.001$ *vs* vehicle control. a= fermented *vs* unfermented tea extract with $p < 0.001$, b=fermented *vs* unfermented tea extract with $p < 0.01$.

4. Discussion

Rooibos tea, is a caffeine free herbal tea which provides many health benefits including antioxidant and anti-inflammatory activity.[29] Nash *et al.* have reported an increase in mineralization, alkaline phosphatase activity and osteoblast differentiation markers in rooibos-tea treated osteoblast-like cells, *in vitro*, indicating possible beneficial effects of fermented rooibos tea at 1 and 10 μg ml^{-1} GAE of tea on bone health.[31] However little is known on the effects of rooibos on osteoclast formation and function. In this present study we made use of concentrations of 62.5-500 μg ml^{-1} (about 1-14 μg ml^{-1} GAE) of rooibos tea extracts, similar to the concentrations reported by Nash *et al.* to promote osteoblast differentiation. However, as the absorption of some rooibos compounds is low,[32] the concentrations

used in this present study may not be achievable in the plasma through diet alone and supplementation may be necessary. Nevertheless, this study identifies the potential anti-osteoclastogenic effects of rooibos tea extracts.

The most commonly consumed form of rooibos tea is the fermented type of tea as it has a richer aroma and flavour. The fermentation process has been shown to decrease the total polyphenol content and the flavonoid content, especially of aspalathin, as well as associated total antioxidant activity.[33] Similar to Joubert et al.[34] we have shown no difference in total polyphenolic content between aqueous extracts of unfermented and fermented rooibos, and further shown that associated antioxidant activity was statistically lower in fermented rooibos compared to unfermented rooibos. These findings are further confirmed by Bramati et al., who have reported lower levels of antioxidant activity in fermented compared to unfermented aqueous rooibos extract; however they further reported that unfermented rooibos had greater levels of polyphenols.[33] The source of the rooibos tea and differences in steeping times may explain differences between the Bramati et al. and this present study (Bramati et al. steeped for 10 min while we steeped the tea for 30 min). A longer steeping time was used in this study to maximize the polyphenol content in the tea extracts. Increasing steeping time has been shown to increase the polyphenolic content and antioxidant activity of fermented and unfermented rooibos.[18, 35] Traditionally rooibos tea is boiled in water and allowed to steep for long periods of time.[18]

At the concentrations evaluated, both extracts were not cytotoxic to the cells. Fermented rooibos extract reduced the number of differentiated osteoclasts and further showed a greater inhibitory effect on actin ring formation and the capacity for resorption than unfermented rooibos extract. The decrease in actin rings and inhibition of resorption is possibly due to the presence of fewer differentiated osteoclasts. These results suggest that, even though the fermentation process does lower the antioxidant activity of rooibos, it may enhance the anti-osteoclastogenic effect of the tea.

In addition to the effects on osteoclastogenesis and bone resorption, we further evaluated the effects of both fermented and unfermented rooibos extracts on genes involved in the formation of osteoclasts (NFAT-c1, DC-STAMP) and in the resorption process (TRAP, CA II, Cathepsin K and MMP-9). DC-STAMP plays an important role in cell-to-cell fusion which is necessary for the formation of osteoclasts.[9] Cathepsin K and MMP-9 are both lysosomal enzymes that are important for the degradation of the organic component of bone.[36, 37] TRAP is believed to play a role in the formation of reactive oxygen species (ROS) required in the resorption process.[38] Carbonic anhydrase (CA) II is the enzyme responsible

for the catalyzation and hydration of CO_2 into H^+ and HCO_3^- during bone resorption.[8] NFAT-c1 promotes the expression of genes such as DC-STAMP, TRAP, MMP-9 and cathepsin K, resulting in the formation of bone resorbing osteoclasts.[39] Down regulation of NFATc1 and DC-STAMP was statistically significant for both unfermented and fermented rooibos tea extracts which indicates that both teas inhibit the formation of osteoclasts. Both teas further inhibited significantly CAII, TRAP and cathepsin K expression. However, only fermented tea significantly reduced MMP-9 expression.

To elucidate the mechanism of action of the tea extract, the effects of the rooibos extracts on the NF-κB signalling pathway was investigated. Studies have shown that inhibition of this pathway can prevent osteoclast formation and bone resorption.[40] Binding of RANKL to RANK leads to the degradation of IκB allowing for the nuclear translocation of NF-κB.[41] Activation of NF-κB binding sites in the nucleus stimulates NFAT-c1, the master regulator of osteoclast formation and function.[39] Cells exposed to both fermented and unfermented rooibos extracts had decreased NF-κB activity with fermented rooibos extract having a stronger inhibitory effect. Both extracts further inhibited the degradation of IκB offering a potential mode for the inhibitory effect of rooibos extracts on osteoclasts.

NF-κB plays a crucial role to the cellular response to oxidative stress and pro-inflammatory signals.[42] Indeed, oxidative stress has been associated with increases in resorption of bone and decreased bone mass in healthy women.[43] Inhibition of pro-oxidative pathways has been shown to decrease osteoclast activity.[44] Furthermore, pro-oxidants have been shown to increase the number of TRAP+ multinucleated giant cells from the human marrow.[43] Joubert *et al.* reported that pure aspalathin in the presence of Fe(III) has pro-oxidant activity.[45] It was further shown that the fermentation process decreased the pro-oxidant effect of aqueous rooibos extracts, possibly due to the decrease in aspalathin during fermentation.[45] The decrease in pro-oxidant activity in fermented rooibos tea may contribute to the stronger anti-osteoclastogenic effects of this tea compared to the unfermented tea extract that we observed. Differences in aspalathin levels between unfermented rooibos and fermented rooibos could further explain the differences seen in NF-κB activity in our present study.

Consumption of both unfermented and fermented rooibos tea by healthy humans increases the total antioxidant capacity (TAC) of plasma with a TAC of 2.62 and 2.04 mmol Trolox/l for unfermented and fermented rooibos tea respectively.[46] This suggests that the polyphenols present in rooibos tea may be readily absorbed and could possibly have a beneficial effect on bone health when consumed regularly. However, further studies on the effects of flavonoids present in rooibos tea on osteoclast activity are needed.

5. Conclusion

In conclusion, this study demonstrates that both fermented and unfermented rooibos extracts can inhibit osteoclast formation and activity with the fermented rooibos being a more potent inhibitor than unfermented rooibos extract. This was despite similar total polyphenol content and lower antioxidant activity reported in fermented rooibos extract compared to unfermented rooibos extract. Inhibition of formation and resorption were coupled with decreases in the expression of osteoclastogenic (NFAT-c1, DC-STAMP) and resorption (TRAP, MMP-9 Cathepsin K and CA II) genes especially by fermented rooibos tea extract. Moreover, NF-κB signalling was abrogated by both rooibos extracts offering a possible mechanism of action for effects of rooibos extract in osteoclasts. Coupled with its stimulatory effects on osteoblasts,[31] this study suggests that rooibos could potentially have beneficial effects on bone health.

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