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Aspalathin from *Aspalathus linearis* (rooibos) reduces osteoclast activity and increases osteoblast activity *in vitro*

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ABSTRACT

Bone remodelling in a healthy body is in constant balance, maintaining an adaptive and robust skeletal system. In osteoporosis this balance is disrupted with the rates of osteoclastic bone resorption exceeding osteoblastic bone formation, resulting in lower bone mineral density. Rooibos tea, is a popular South African drink made from *Aspalathus linearis* leaves grown in the Western Cape. This tea is rich in phenolic compounds which have been widely investigated in recent years as a potential treatment for many ailments. In this study, aspalathin, a phenolic compound found exclusively in rooibos, increases osteoblast formation and function including increased osteoblast marker expression and mineralisation. In addition, aspalathin decreased differentiation and function of osteoclasts as well as reducing osteoclast formation in an osteoclast/osteoblast co-culture model. These results illustrate bone-protective effects of aspalathin *in vitro* through the reduction of osteoclast activity and promotion of osteoblast activity, with potential applications in the maintenance of bone density.

1. Introduction

Bone remodelling is an on-going process in adults that involves the resorption of bone by osteoclasts followed by the formation and mineralization of new bone by osteoblasts, with signal input from osteocytes in response to changes in mechanical loading or damage to bone (Lara-Castillo et al., 2015). Osteoblasts are small, mononucleated cells of mesenchymal cell lineage. Osteocytes produce differentiation factors such as osterix, runt-related transcription factor 2 (Runx2), and bone morphogenetic protein 2 (BMP-2) to promote osteoblast differentiation (Dirckx, Van Hul, & Maes, 2013; Manolagas & Jilka, 1995). Osteoblasts highly express alkaline phosphatase (ALP) which can be used as a biochemical marker for osteoblast activity (Garnero & Delmas, 1998). Sclerostin is an important inhibitor of osteoblasts during the resorption phase of bone remodelling (Winkler et al., 2003). Osteoclast precursors fuse and differentiate into large multinucleated bone resorbing osteoclasts in the presence of receptor activator of nuclear factor κB (RANKL) and macrophage colony stimulating factor (M-CSF) (Dooley, Tisbo, Lee, & Taylor, 2012; Vaananen & Laitala-Leinonen, 2008). RANKL signalling activates c-Fos and nuclear factor of activated T cells cytoplasmic 1 (NFATc1) the master regulator of osteoclasts (Wada, Nakashima, Hiroshi, & Penninger, 2006), leading to the formation of sturdy F-actin rings and the expression of key osteoclast genes such as carbonic anhydrase 2 (CAII), cathepsin K (CTSK), matrix metalloproteinase (MMP) −9 and tartrate-resistant acid phosphatase (TRAP) (Lorenzo et al., 2011; Vaananen, Zhao, Mulari, & Halleen, 2000).

Studies have shown that both black tea (*Camellia sinensis*) (Devine, Hodgson, Dick, & Prince, 2007; Hegarty, May, & Khaw, 2000) and green tea possess bone protective effects, with green tea showing more potent effects possibly due to its higher antioxidant content (Shen, Yeh, Cao, Chyu, & Wang, 2011). *Aspalathus linearis* (rooibos), a small bush indigenous to the Western Cape of South Africa, is farmed for its leaves which are used to make the popular rooibos tea (Dahlgren, 1960). In comparison to other teas, rooibos has been shown to exert higher antioxidant effects than black and oolong teas, and comparable effects to green tea (Von Gadow, Joubert, & Hansmann, 1997). *In vivo* studies have shown that rooibos potentially reduces prolonged stress (Schloms et al., 2014), and exerts potent anti-inflammatory (Baba et al., 2009) and chemoprotective effects (Marnewick et al., 2009). A recent study in rats showed that aspalathin-enriched green rooibos extract could

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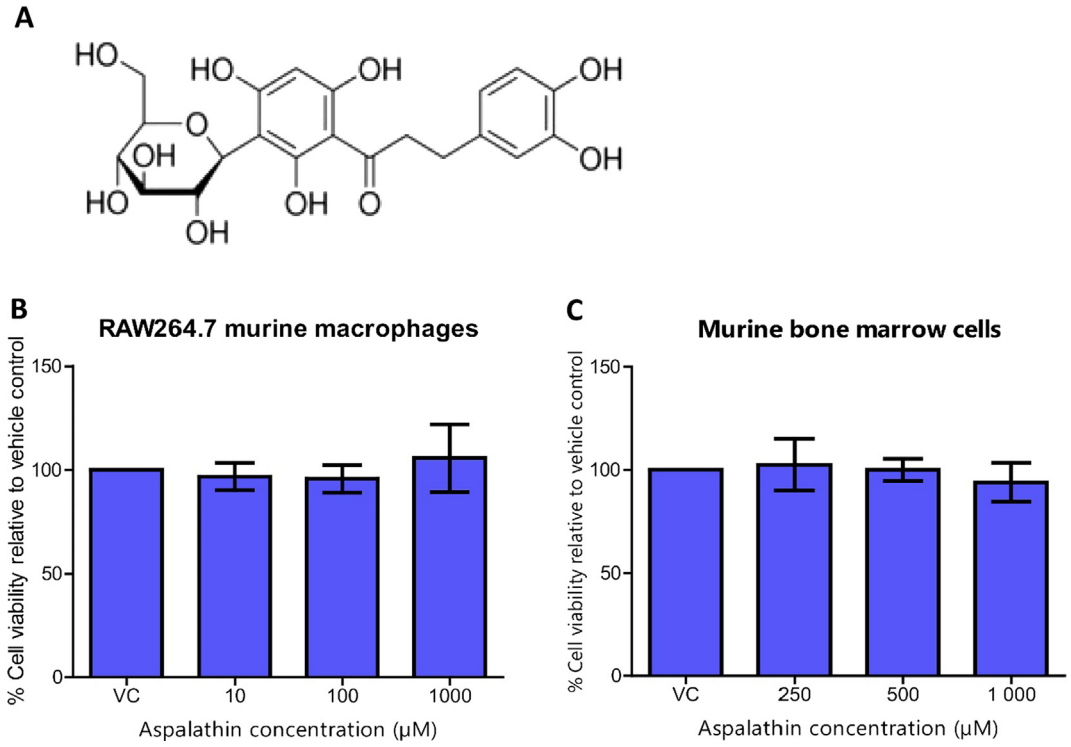

Fig. 1. Effects of aspalathin on cell viability. (A) Molecular structure of aspalathin. (B) Cell viability of RAW264.7 cells treated with aspalathin. Cells were treated with indicated concentrations of aspalathin for 48 h and cell viability was measured by Alamar blue assay. (C) Cell viability of primary murine bone marrow cells treated with the indicated concentrations of aspalathin for 48 h, measured by Alamar blue assay. Data are expressed as mean ± SD and are representative of three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

possess insulin sensitising effects (Mazibuko-Mbeje et al., 2019). Many of these effects may be attributed to the known effects of aqueous rooibos extract on the NFκB pathway (Shen et al., 2011). In our laboratories, aqueous rooibos tea extract has been shown to inhibit osteoclast differentiation and bone resorption in an *in vitro* study using RAW264.7 murine macrophage-derived osteoclasts (Moosa et al., 2018). In a study conducted by Nash et al., the effects of rooibos tea on osteoblast activity were investigated using Saos2 human osteoblast-like cells (Nash & Ward, 2016). The study showed that rooibos tea extract increased osteoblast differentiation and activity, as well as their capacity for mineralisation.

Aspalathin (Fig. 1A) is a dihydrochalcone C-glucoside polyphenol uniquely found in the *Aspalathus linearis* plant (Dahlgren, 1960). Several studies have shown aspalathin to be the major phenolic compound detected in aqueous extracts of rooibos (Bramati et al., 2002; Bramati, Aquilano, & Pietta, 2003; Joubert, Winterton, Britz, & Gelderblom, 2005). Snijman et al. compared the antioxidant capabilities of aspalathin to other phenolics found in rooibos (Snijman et al., 2009). Results showed that among other phenolics found in rooibos, such as quercetin, nothofagin, and catechins, aspalathin showed the most potent free radical scavenging ability with an IC_{50} at 3.33 μM. These results show that aspalathin displays very high antioxidant capabilities, rivalling the well-studied quercetin (Snijman et al., 2009). The objective of this study was to identify the potential bone protective effects of aspalathin using murine RAW264.7 macrophages and primary extracted mouse bone marrow cells to study the effects on osteoclasts and osteoblasts specifically.

2. Methods

Ethics approval for this study was obtained from the Research Ethics Committee of the Faculty of Health Sciences, University of Pretoria (Ethics Reference Number: 328/2017).

2.1. Reagents

Dulbecco's Modified Eagle Medium (DMEM), alpha-Minimum Essential Medium (αMEM), and fetal bovine serum (FBS) were obtained from GIBCO (Grand Island, USA). Murine RANKL (562-TR) was purchased from Research and Diagnostic Systems (Minneapolis, USA). Penicillin/streptomycin/fungizone mix (#226), Corning OsteoAssay plates, TRI - reagent®, Acid Phosphatase, Leukocyte (TRAP) Kit (387A), aspalathin (03520585) and all other research-grade materials were obtained from Sigma-Aldrich Inc. (St Louis, USA). Alamar Blue reagent and Alexa Fluor 568 were obtained from Thermo Fisher Scientific (Waltham, USA). M-MuLV reverse transcriptase was purchased from New England Biolabs (Hitchen, UK). Osteoclast PCR Primers were obtained from Inqaba Biotec (Pretoria, South Africa) and osteoblast PCR primers from Thermo Fisher Scientific (Waltham, USA). Kapa Robust Hotstart Readymix PCR kit was purchased from Kapa Biosystems (Cape Town, South Africa). FastStart Essential DNA Green Master was obtained from Roche Diagnostics (Randburg, South Africa). Plastic consumables were purchased from Lasec (Cape Town, South Africa) or Labotec (Midrand, South Africa) depending on availability.

2.2. Preparation of aspalathin solution

A 1 M stock solution of aspalathin was prepared in DMSO (vehicle) and frozen as aliquots in −80 °C until further use. Stock solutions were freshly diluted to working concentrations in culture medium before experiments. The final DMSO concentration in the culture medium did not exceed 0.1% (v/v).

2.3. Isolation of murine bone marrow cells and cell culture

For experiments involving primary mouse cells, 10–12 week old male and female C57BL/6 mice were used. The local Institutional Animal Care Committee and the local authorities approved all

Table 1
List of primer sequences used.

Gene	Forward primer	Reverse primer
GAPDH	5′ GATGACATCAAGAAGGTGGTGAAGC 3′	5′ ATACCAGGAAATGAGCTTGACAAA 3′
c-Fos	5′ CCCATCGCAGACCAGAGC 3′	5′ ATCTTGCGAGGCAGGTCGGT 3′
NFATc1	5′ GTGGAGAAGCAGAGCAC 3′	5′ ACGCTGGTACTCCCTTC 3′
Cathepsin K	5′ CTGGAGGGCCAACTCAAGA 3′	5′ CCTCTGCATTTAGCTGCCTT 3′
CA II	5′ GAGTTTGATGACTCTCAGGACAA 3′	5′ CATATTTGGTGTTCCAGTGAACCA 3′
Runx2	5′ CTGACTGGAAGAGCGGAGAG 3′	5′ GGGAATCCATCGGTCATGCT 3′
Osterix	5′ AGTGGTAGGAAGGAGTTGGTG 3′	5′ AACATCAACCAACACACAGT 3′
BMP-2	5′ ACATCCACTCCACAAACGAG 3′	5′ GTCATTCCACCCCACATCAC 3′
Sclerostin	5′ CGTGCCTCATCTGCCTACTTGTGCA 3′	5′ GAAGTCCTTGAGCTCCGACTGGTTGTG 3′

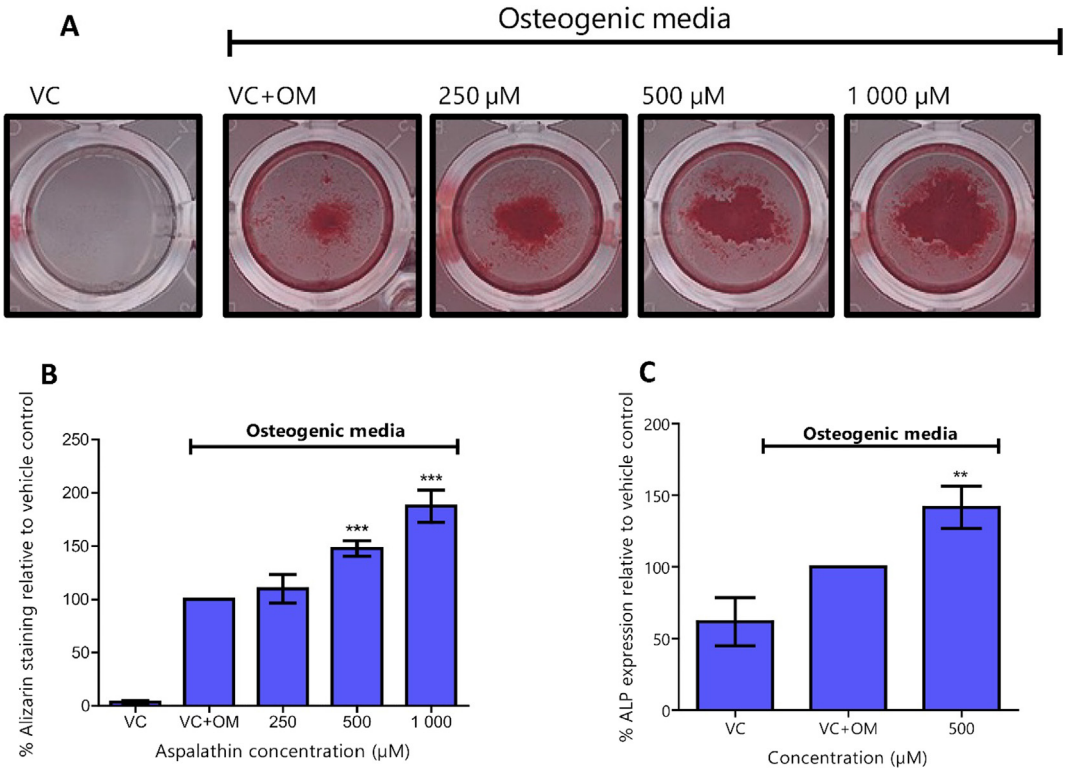

Fig. 2. Effects of aspalathin on osteoblast mineralization and ALP activity. (A) Primary murine bone marrow cells were treated with osteogenic media in the presence or absence of aspalathin as mentioned in methods for 21 days, then stained for mineralization using Alizarin Red S staining. B) Mineralization staining was dissolved and optical density was measured at 540 nm. (C) Primary murine bone marrow cells were treated with osteogenic media in the presence or absence of aspalathin as mentioned in the methods for 10 days. ALP expression was calculated relative to total protein expression. Data are expressed as mean ± SD and are representative of three independent experiments. (VC = Vehicle control. OM = Osteogenic media. $^{**}P < 0.01$ vs. VC + OM; $^{***}P < 0.001$ vs. VC + OM). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

procedures. In short, mice were kept in standard conditions and fed standard diet with water *ad libitum,* as previously described (Baschant et al., 2016). Mice were humanely sacrificed under general anaesthesia. After cervical dislocation, the long bones from the upper limbs of adult mice were removed. Skin, muscle, and connective tissue were removed, and the bones were placed in PBS on ice. The bone marrow, including mesenchymal osteoblast progenitors, was then flushed from the bones into sterile tubes containing 10 ml DMEM supplemented with 20% FBS. For osteoblast differentiation, bone marrow cells were counted using trypan blue exclusion, and seeded in DMEM with 10% FBS. Osteogenic media consisted of DMEM supplemented with 10% FBS, 100 μg/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml fungizone, 10 mM beta-glycerophosphate and 100 μM ascorbic acid 2-phosphate (Wutzl et al., 2010).

2.4. RAW264.7 macrophage culture

RAW264.7 murine macrophages (#TIB-71) were purchased from American Type Culture Collection (ATCC, Rockville, MD) and maintained in DMEM with 10% FBS, 100 μg/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml fungizone. These cells have been shown to differentiate into multinucleated, bone resorbing cells in the presence of RANKL (Collin-Osdoby & Osdoby, 2012). Cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2. All cell culture media and factors were replaced every three days.

2.5. Alamar blue assay for cell viability

Cells were seeded in 96-well plates and allowed to adhere for 24 h followed by exposure to increasing concentrations of aspalathin (10–1000 μM) for 48 h. Murine bone marrow cells were seeded at a density of 3×10^5 cells/well while RAW264.7 cells were seeded at a density of 5×10^3 cells/well. Alamar blue assay was conducted as per manufacturer's instructions (Life Technologies). Absorbance was measured at 570 nm with 600 nm as reference wavelength on a microplate reader (BioTek Instruments Inc., Winooski, VT, for RAW264.7 cells;

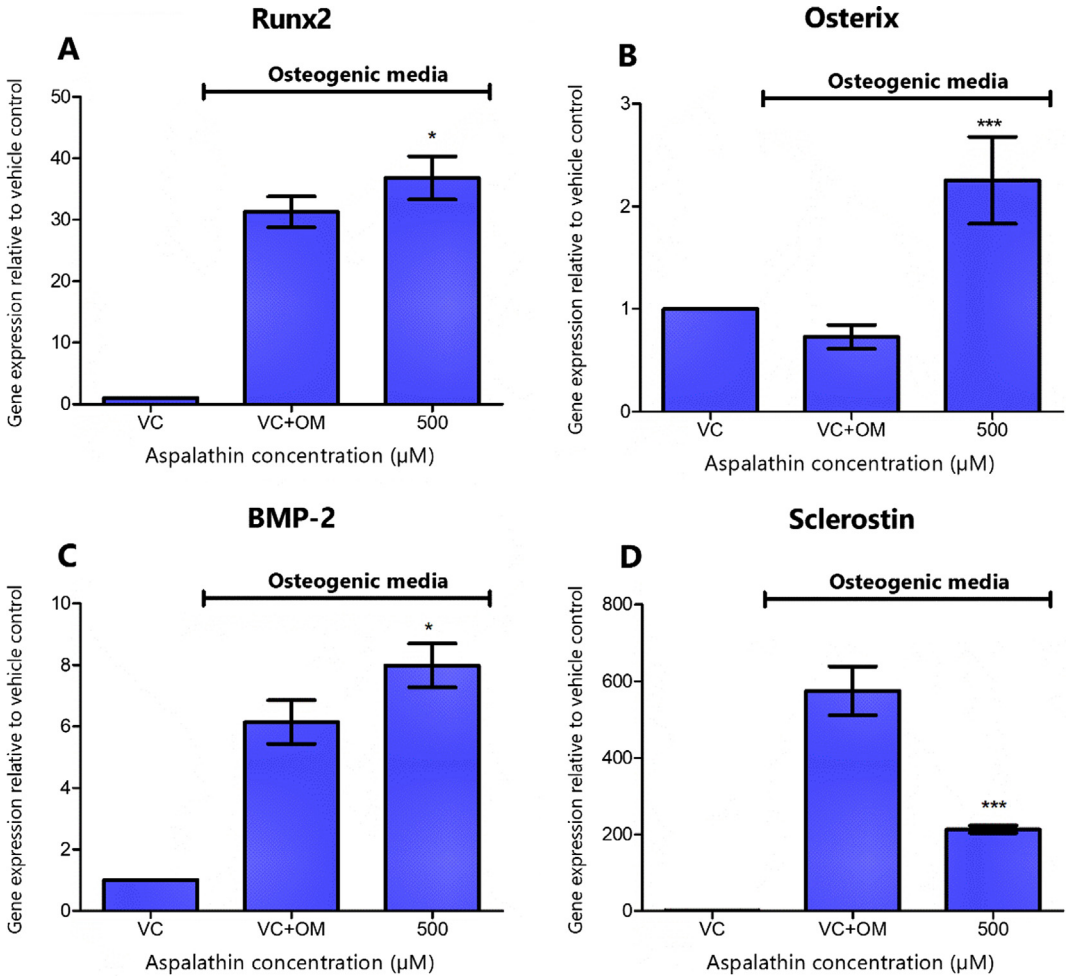

Fig. 3. Effects of aspalathin on osteoblast marker gene expression. Murine bone marrow cells were exposed to osteogenic media and aspalathin for either 10 days (Runx2; Osterix) or 21 days (BMP-2; Sclerostin) after which qRT-PCR analysis was conducted. (A) Gene expression analysis of Runx2. (B) Gene expression analysis of osterix. (C) Gene expression analysis of BMP-2. (D) Gene expression analysis of sclerostin. The GAPDH gene primer was used as a loading control. Data were analyzed using $2^{-\Delta\Delta CT}$ method. Gene primer sequences are shown in Table 1. Data are expressed as mean ± SD and are representative of two independent experiments. (VC = vehicle control. OM = Osteogenic media. $^{*}P < 0.05$ vs. VC + OM; $^{***}P < 0.001$ vs. VC + OM).

BMG Labtech, Ortenburg, Germany, for murine bone marrow cells). Results were expressed relative to the vehicle control. Three independent experiments were conducted with four replicates per treatment.

2.6. Alizarin Red S assay for osteoblast mineralisation

The mineralisation of primary mouse osteoblasts was measured using an Alizarin Red S assay, which stains mineral deposits red (Gregory, Gunn, Peister, & Prockop, 2004). Freshly extracted primary bone marrow cells were seeded in 48-well cell culture plates at a density of 7.5×10^5 cells/well and incubated at 37 °C for 24 h to allow attachment to occur. Cells were exposed to aspalathin (250–1000 μM) in the presence of osteogenic media and incubated at 37 °C for 21 days with full media changes every other day. At the end of culture, cells were fixed using 10% paraformaldehyde. Alizarin red S analysis of osteoblast mineralisation was conducted as previously described (Gregory et al., 2004). Plates were scanned using a Canon CanoScan FB620U Flatbed Scanner before adding 100 mM cetylpyridinium chloride to each well in order to dissolve the stain. Absorbance was read using a FLUOstar Omega microplate reader (BMG Labtech, Ortenburg, Germany) at 540 nm. Results were quantified by calculating the change in absorbance relative to the vehicle control. Three independent experiments were conducted with three replicates per treatment.

2.7. ALP expression in osteoblasts

Primary murine bone marrow cells were seeded in 12-well cell culture plates at a density of 4×10^6 cells/well and incubated for 24 h to allow attachment to occur. Cells were then exposed to osteogenic media alone or in combination with aspalathin at 500 μM, due to the effects of this concentration in the previous experiment. Cells were then incubated at 37 °C for 10 days with complete media changes every other day. ALP expression was analysed as described previously (Sabokbar, Millett, Myer, & Rushton, 1994). In brief, cells were scraped in lysis buffer (1.5 M Tris/HCL (pH 7.0), 1 mM ZnCl2, 1 mM MgCl2, 1% Triton-X 100) and transferred into microfuge tubes. After centrifugation at 25 000*g* at 4 °C for 30 min, samples were transferred into a 96-well plate on ice. ALP buffer containing 100 mM diethanolamin, 0.1% Triton-X 100, and 3.7 mM pNPP was added to the samples, which were incubated for 30 min at 37 °C. Absorbance was measured via spectrophotometry at 405 nm using a FLUOstar Omega microplate reader (BMG Labtech, Ortenburg, Germany). Total protein expression was measured using a Pierce BCA protein assay kit (Thermo Scientific, Waltham, MA). Three independent experiments were conducted with three replicates per treatment.

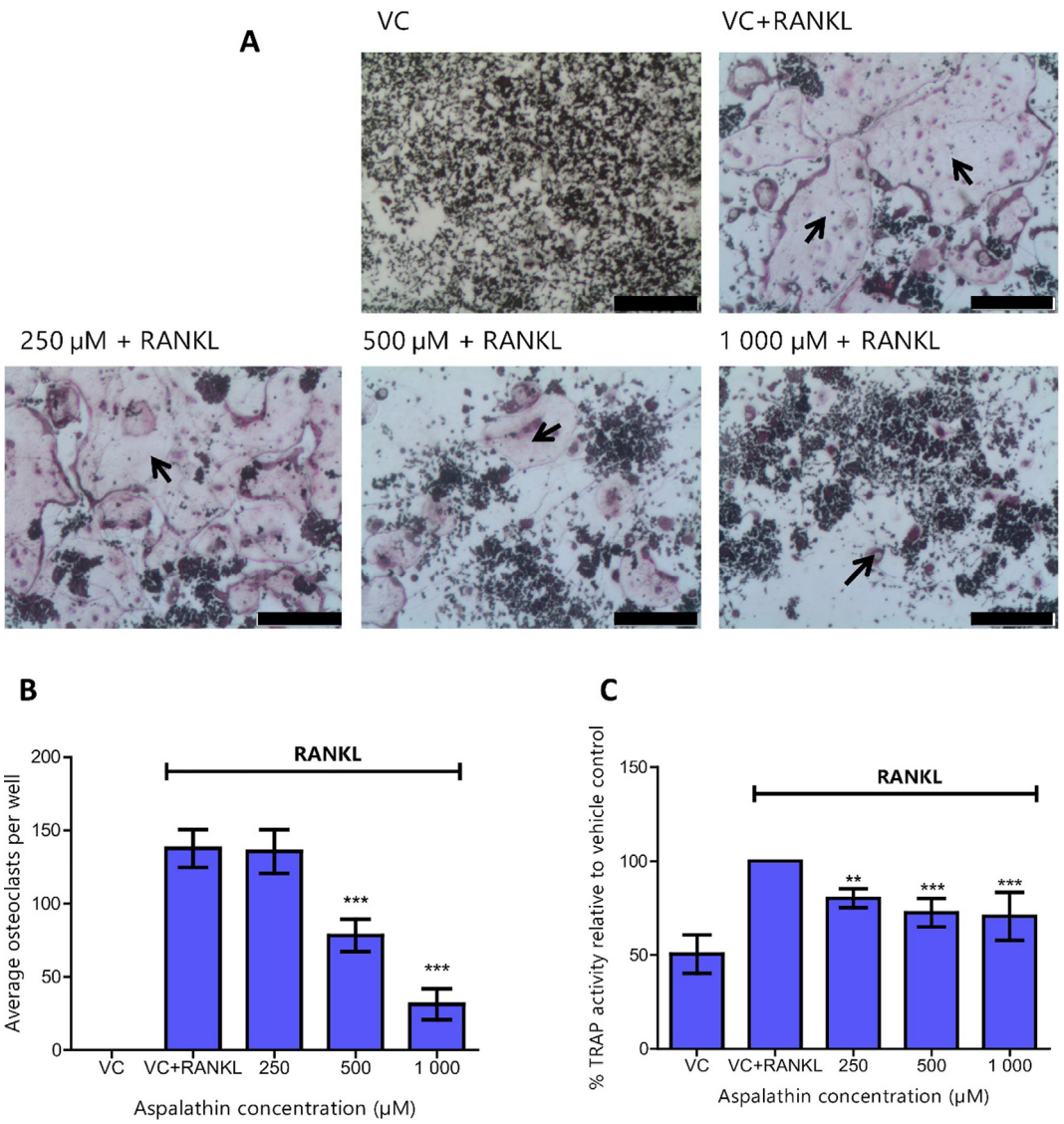

Fig. 4. Effects of aspalathin on osteoclast TRAP activity and staining. (A) RAW264.7 macrophages were treated with RANKL in the presence or absence of aspalathin as mentioned in methods for 5 days. Osteoclasts were fixed and stained for the presence of TRAP. Photomicrographs were taken of stained osteoclasts (Scale bars: 500 µM). (B) TRAP positive osteoclasts containing three or more nuclei were counted under a microscope. (C) TRAP activity in conditioned media was measured. All data is expressed as mean ± SD and is representative of three independent experiments. (VC = vehicle control. "− RANKL" = Non-RANKL exposed, "+ RANKL" = RANKL exposed. Arrows indicate osteoclasts. $^{**}P < 0.01$ vs. VC + RANKL; $^{***}P < 0.001$ vs. VC + RANKL).

2.8. Osteoclast differentiation

RAW264.7 macrophages were differentiated into osteoclasts in the presence of 15 ng/ml RANKL for 5 days as described previously (Deepak, Kruger, Joubert, & Coetzee, 2015). Briefly, cells were seeded at 5×10^3 cells/well in 96-well plates and exposed to RANKL in combination with increasing concentrations of aspalathin (0–1000 µM). Every third day, all cell culture media and factors were replaced.

At the end of the culture period, TRAP activity analysis and TRAP staining were performed using a leucocyte acid-phosphatase kit as per manufacturer's directions (Sigma Aldrich, St Louis). For TRAP activity analysis of conditioned media, absorbance was measured at 550 nm on an ELX 800 Micro plate reader (BioTek Instruments Inc, Winooski, USA). For the staining of osteoclasts, TRAP+ cells with 3 or more nuclei were counted as osteoclasts. Photomicrographs of stained cells were taken with a Zeiss Axiocam MRc5 camera attached to a Zeiss Axiovert 40 CFL microscope (Carl Zeiss AG, Oberkochen, Germany). Three independent experiments were conducted with three replicates per treatment.

2.9. Osteoclast F-actin ring formation

RAW264.7 cells were seeded at a density of 5×10^3 cells/well on glass coverslips in 96-well cell culture plates, exposed to aspalathin and differentiated in the presence of RANKL. After 5 days of incubation, the media was discarded and the cells were fixed using 3.7% paraformaldehyde. The cells were permeabilized in PBS containing 0.25% Triton X-100, then washed in PBS. Cells were exposed to Alexa Fluor 568 (Thermo Fisher Scientific, Waltham, USA) for 40 min in the dark and subsequently washed in PBS. Lastly, the cells were incubated with 0.1 µg/ml Hoechst in PBS for 15 min (Boeyens et al., 2014). Images were immediately taken using confocal laser scanning microscopy on a Zeiss Axiovert CFL40 microscope equipped with a Zeiss Axiovert MRm monochrome camera with the following filter sets: Hoechst (Excitation: 352 nm, Emission: 455 nm); Alexa Fluor 568 (Excitation: 561 nm, Emission: 594 nm). Three independent experiments were conducted with three replicates per treatment.

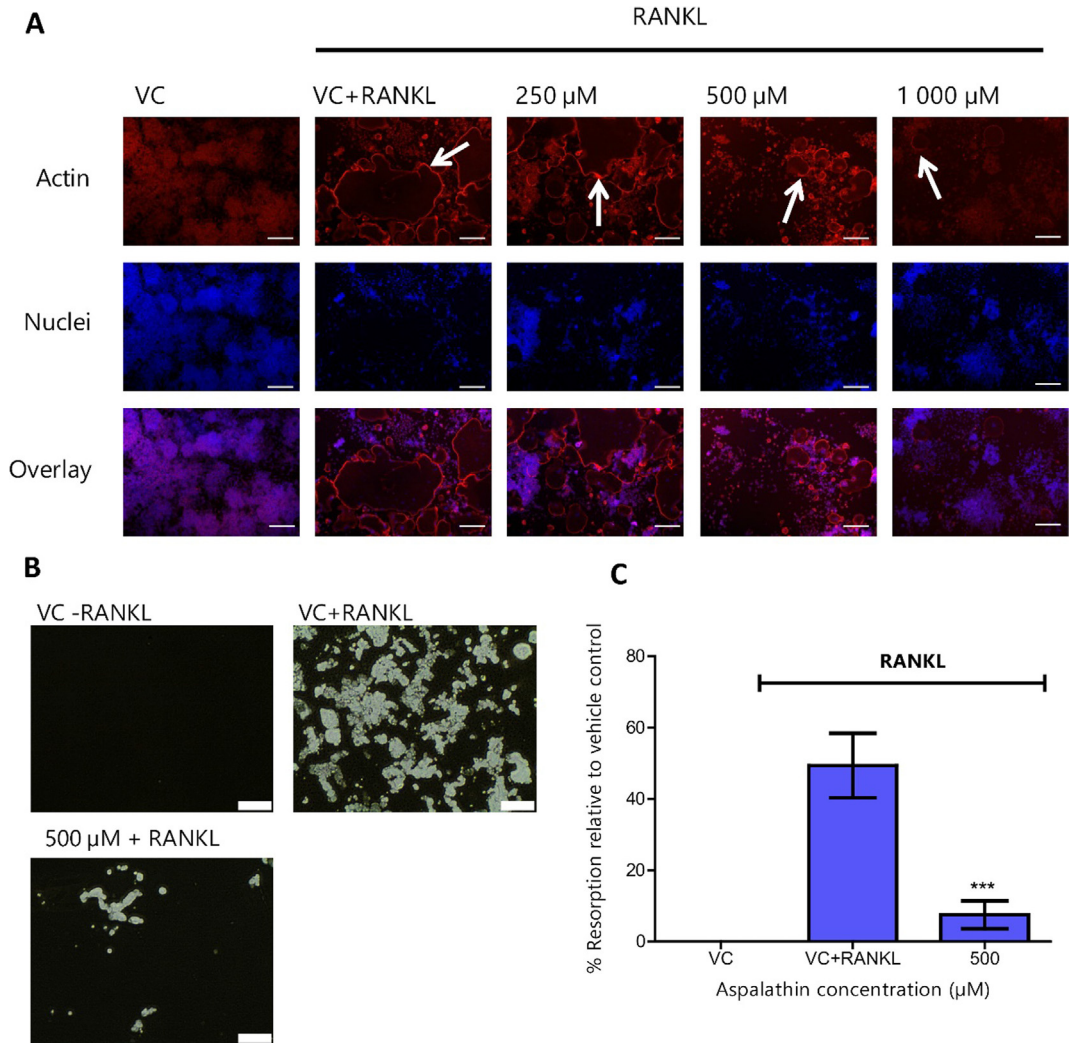

Fig. 5. Effects of aspalathin on osteoclast actin ring formation and bone resorption. (A) Fluorescence images of actin rings. RAW264.7 macrophages were treated with RANKL in the presence or absence of aspalathin as mentioned in methods for 5 days, then stained with Alexa Fluor 568 and Hoechst (Scale bars: 500 µM). (B) Photomicrographs of resorption pits. RAW264.7 cells were seeded on osteoassay plates and treated with RANKL in the presence or absence of aspalathin as mentioned in methods for 7 days (Scale bars: 200 µM). (C) Surface area of resorption. Photomicrographs were analyzed using ImageJ software and surface area of resorption was calculated. Data are expressed as mean ± SD and are representative of three independent experiments. (VC = vehicle control. ***$P < 0.001$ vs. VC + RANKL).

2.10. Bone resorption pit formation assay

RAW264.7 cells were seeded at a density of 1×10^4 cells/well in 24-well Corning OsteoAssay plates (Corning Inc., Corning, USA), exposed to aspalathin at 500 µM and differentiated as mentioned previously. After 7 days of differentiation, bone resorption on the osteoassay plates was analysed according to the manufacturer's instructions. Images were captured using a Zeiss AxioCam MRc5 camera on a Zeiss AxioCam ERc5 microscope. The percentage area covered by resorption pits was quantified using ImageJ software (Schneider, Rasband, & Eliceiri, 2012). Two independent experiments were conducted in duplicate.

2.11. qRT-PCR

For osteoblast experiments, primary murine bone marrow cells were seeded at a density of 1×10^7 cells/well in 6-well cell culture plates. Cells were exposed to osteogenic media in the presence of aspalathin (500 µM, due to the effects this concentration showed n previous experiments) for 10 or 21 days in order to study early or late markers respectively. For osteoclast experiments, RAW264.7 cells were seeded at a density of 1×10^4 cells/well in 48-well cell culture plates. To study early markers, cells were exposed to 15 ng/ml RANKL and aspalathin (500 µM) and incubated for 2 days. To study late markers, cells were exposed to RANKL and differentiated for 4 days, before being exposed to RANKL in combination with aspalathin, and incubated for a further 2 days.

At the end of incubation, total RNA was extracted using TRI-reagent (Sigma-Aldrich Inc., St Louis, USA), and reverse transcribed to cDNA using M-MuLV reverse transcriptase as per the manufacturer's instructions (New England Biolabs, Hitchen, UK). qRT-PCR was conducted using specific primers Runx2, osterix, BMP-2, and sclerostin for osteoblast experiments and for cathepsin K, c-Fos, NFATc1, and CAII for osteoclast experiments. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene primer was used as a loading control. Data was analyzed using the $2^{-\Delta\Delta CT}$ method. Two independent experiments were conducted in duplicate. Gene primer sequences are shown in Table 1.

2.12. Osteoclast differentiation in an osteoclast/osteoblast co-culture

C57BL/6 mouse pups (4 days old) were used for experiments

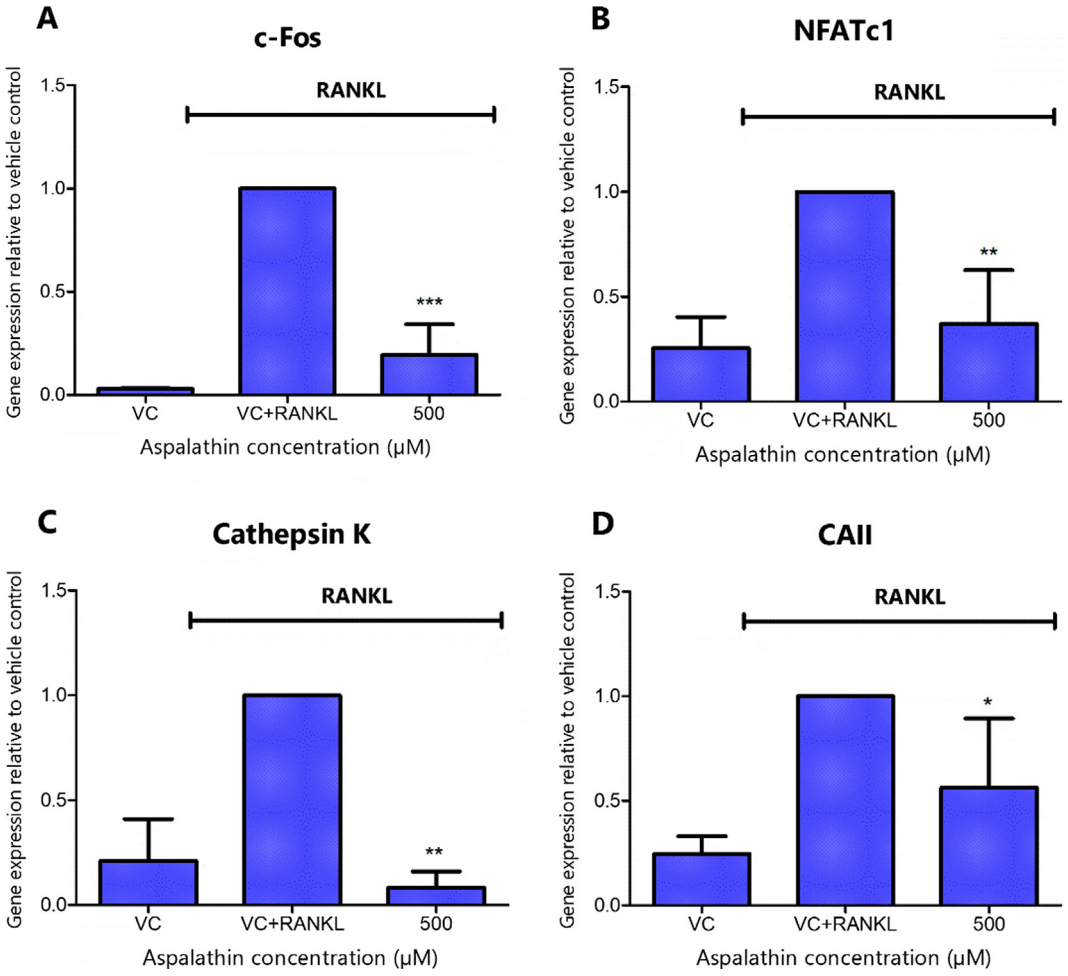

Fig. 6. Effects of aspalathin on osteoclast marker gene expression. RAW264.7 cells were exposed to RANKL and aspalathin for 48 h after which qRT-PCR analysis was conducted (A, B) or exposed to RANKL for 4 days, then exposed to RANKL and aspalathin for 48 h after which qRT-PCR gene expression analysis was conducted. (A) Gene expression analysis of c-Fos. (B) Gene expression analysis of NFATc1. (C) Gene expression analysis of cathepsin K. (D) Gene expression analysis of carbonic anhydrase II. The GAPDH gene primer was used as a loading control. Data was analyzed using $2^{-\Delta\Delta CT}$ method. Gene primer sequences are shown in Table 1. Data are expressed as mean ± SD and are representative of two independent experiments. (VC = vehicle control. *$P < 0.05$ vs. VC + RANKL; **$P < 0.01$ vs. VC + RANKL; ***$P < 0.001$ vs. VC + RANKL).

involving primary mouse calvarial cells. In these experiments, calvarial cells were used as osteoblast precursors, and bone marrow cells were used as osteoclast precursors (Ecarot-Charrier, Glorieux, van der Rest, & Pereira, 1983). After sacrifice of the mouse pups, calvaria were removed then placed in 1 ml digestion media consisting of αMEM supplemented with 100 μg/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml fungizone, 0.1% collagenase A (Sigma-Aldrich Inc., St Louis, USA) and 0.1% dispase II (Sigma-Aldrich Inc., St Louis, USA). The calvarial cells were then extracted and suspended in αMEM supplemented with 10% FBS, 100 μg/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml fungizone. Cells were counted using trypan blue exclusion and immediately seeded for experiments.

The co-culture of osteoclasts and osteoblasts is possible because of the osteoblasts' ability to sustain osteoclast differentiation in culture (Teti et al., 1991). Freshly extracted mouse calvarial cells were seeded in 96-well cell culture plates at a density of 8×10^3 cells/well in the presence of 10 nM 1α,25-dihydroxyvitamin D_3. 1α,25-dihydroxyvitamin D_3 is added as it increases RANKL production by osteoblasts thereby increasing osteoclastogenesis in the co-culture model (Sai, Walters, Fang, & Gallagher, 2011). Cells were incubated for 24 h at 37 °C after which freshly extracted mouse bone marrow cells were seeded on top of these at a density of 2×10^5 cells/well. Cells were then exposed to increasing concentrations of aspalathin (0–1000 μM). Cells were incubated at 37 °C for 10 days with full media changes every other day (Itzstein & van't Hof, 2012). At the end of the incubation, cells were stained for TRAP as described previously. Three independent experiments were conducted with three replicates per experiment.

2.13. Statistical analysis

Data are representative of three independent experiments unless otherwise stated and are represented as mean ± standard deviation (SD). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Dunnett's post hoc multiple comparison test using Graph Pad Prism Software (GraphPad Software Inc., CA). $P < 0.05$ was regarded as statistically significant.

3. Results

3.1. Aspalathin does not significantly affect cell viability in osteoblast and osteoclast progenitor cells

The Alamar blue assay revealed that aspalathin has no significant effect on cell viability in both undifferentiated RAW264.7 murine macrophages (Fig. 1B) and primary murine bone marrow cells (Fig. 1C) at the tested concentrations (10–1000 μM). This indicates that aspalathin does not show cytotoxic effects within the tested range, and all subsequent experiments would take place within this range.

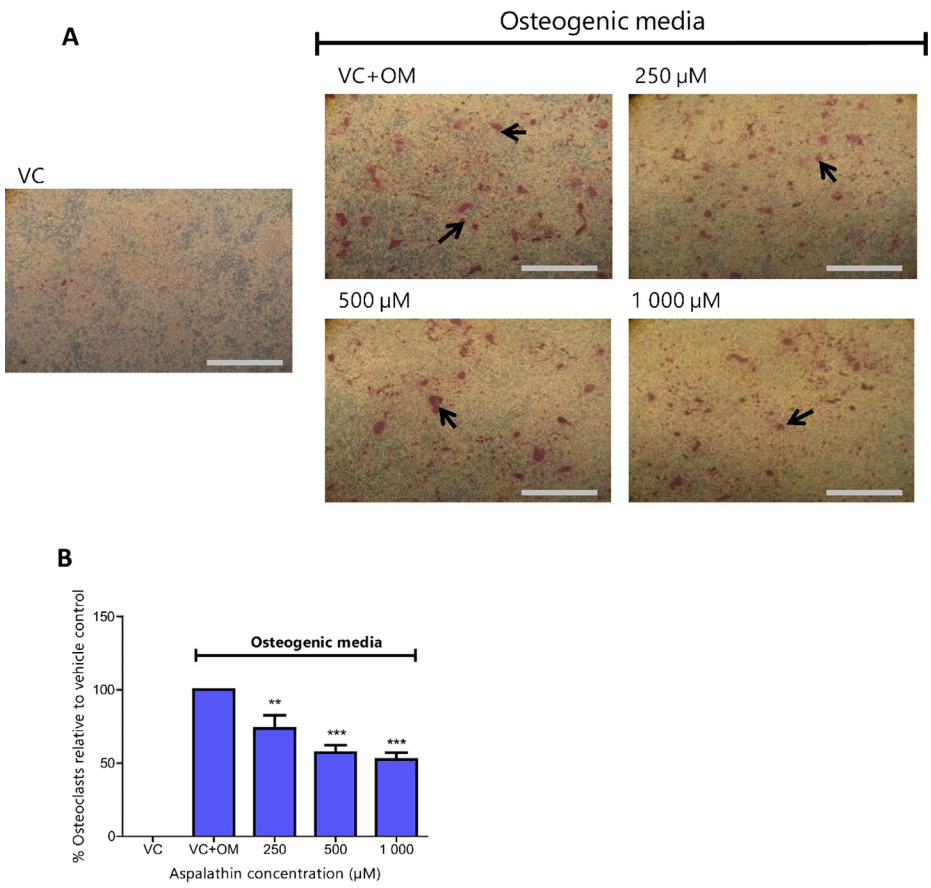

Fig. 7. Effects of aspalathin on osteoclast differentiation in osteoblast-osteoclast co-culture. (A) Primary murine bone marrow cells were seeded in co-culture with primary murine calvarial cells and treated with vitamin D_3 in the presence or absence of aspalathin for 5 days. TRAP staining was conducted as described in methods and photomicrographs were taken (Scale bars: 500 µM). (B) TRAP positive osteoclasts containing three or more nuclei were counted under a microscope. Data are expressed as mean ± SD and are representative of three independent experiments. (VC = vehicle control. OM = Osteogenic media. $^{**}P < 0.01$ vs. VC + OM; $^{***}P < 0.001$ vs. VC + OM).

3.2. Aspalathin increases osteoblast mineralization and ALP expression

Murine bone marrow cells in the presence of osteogenic media showed mineralization compared to cells in the absence of osteogenic media, as detected by Alizarin Red S Staining. Treatment with aspalathin increased mineralization with increasing concentrations (Fig. 2A), which was significantly enhanced starting at concentrations of 500 µM and above (Fig. 2B). In addition, aspalathin at 500 µM significantly increased the expression of ALP (Fig. 2C).

3.3. Aspalathin increases osteoblast formation markers

To further determine the effects of aspalathin on osteoblast differentiation, the expression of key osteoblast gene markers was determined by qPCR. Analysis of the effects of 500 µM aspalathin on gene expression of osteoblast markers revealed that aspalathin increased the expression of Runx2 (Fig. 3A) and osterix (Fig. 3B) after 10 days of culture and increased the expression of BMP-2 (Fig. 3C), while decreasing the expression of sclerostin (Fig. 3D) after 21 days of culture.

3.4. Aspalathin inhibits RANKL-mediated osteoclast differentiation

To determine the effects of aspalathin on osteoclast differentiation a TRAP activity assay as well as a TRAP stain and a cell count was conducted. Treatment with RANKL induced the differentiation of RAW264.7 murine macrophages into TRAP-positive osteoclasts. Osteoclasts formed at higher concentrations of aspalathin appeared relatively smaller, and did not stain as brightly as those exposed to RANKL alone (Fig. 4A). Cells exposed to aspalathin in the presence of RANKL showed a significant reduction in osteoclast numbers starting at 500 µM (Fig. 4B), and in TRAP activity (Fig. 4C) starting at 250 µM.

3.5. Aspalathin reduces osteoclast F-actin ring size and resorption pit formation

Treatment with aspalathin reduced the size of osteoclast F-actin rings, although there was no visible reduction in the structural integrity of the actin rings (Fig. 5A). Aspalathin at 500 µM reduced the formation of resorption pits by osteoclasts (Fig. 5B), and significantly reduced the surface area of resorption pits compared to osteoclasts exposed to RANKL only (Fig. 5C), possibly due to the reduced number and size of the osteoclasts.

3.6. Aspalathin reduces the expression of RANKL-induced osteoclast differentiation markers

In order to further investigate aspalathin's effects on osteoclast differentiation, we studied the effects of aspalathin on the expression of key osteoclast marker genes c-Fos (Fig. 6A), NFATc1 (Fig. 6B), cathepsin K (Fig. 6C), and carbonic anhydrase II (Fig. 6D). Aspalathin at 500 µM significantly reduced the expression of all tested markers, with a somewhat weaker effect on carbonic anhydrase II expression.

3.7. Aspalathin reduces osteoclast differentiation in an osteoclast/osteoblast co-culture model

In order to study the effects of aspalathin on a more holistic model of a bone remodeling unit, osteoclasts and osteoblasts from murine bone marrow and murine calvarial cells respectively were exposed to increasing concentrations of aspalathin. Aspalathin reduced TRAP staining for osteoclasts (Fig. 7A) and significantly reduced TRAP-positive osteoclast numbers at all tested concentrations (Fig. 7B).

4. Discussion

Rooibos (*Aspalathus linearis*) is a popular South African herbal tea known for its naturally sweet taste and numerous health benefits (Baba et al., 2009; Marnewick et al., 2009; Mazibuko-Mbeje et al., 2019; Schloms et al., 2014). *In vitro* studies have shown that aqueous rooibos extract has the potential to increase osteoblast activity (Nash & Ward, 2016) and inhibit osteoclast formation and activity (Moosa et al., 2018). Aspalathin is a polyphenol unique to rooibos and is known to have a high antioxidant capacity (Dahlgren, 1960; Snijman et al., 2009). In this *in vitro* study, the effects of aspalathin on the differentiation and function of osteoclast and osteoblast cells were investigated. The purpose of this study was to determine whether aspalathin could be considered a viable option for research into nutritional or pharmacological treatments for the prevention of bone loss or for osteolytic diseases such as osteoporosis.

For osteoblasts, primary murine bone marrow cells from C57BL/6 mice were used. These cells contain a high number of osteoblast precursors, and osteoblast formation can be initiated in the presence of β-glycerophosphate and ascorbic acid 2-phosphate (Rauner, Sipos, & Pietschmann, 2008). Treatment with aspalathin (500–1000 μM) resulted in a significant increase in mineralisation and ALP activity when compared to the vehicle control. These results were coupled with an increase in the expression of key osteoblast markers, Runx2, osterix and BMP2 and a decrease in sclerostin expression. ALP is an enzyme responsible for assisting in the maintenance of pH during mineralisation, and is highly expressed early in the differentiation and formation of osteoblasts (Garnero & Delmas, 1998; Golub, Harrison, Taylor, Camper, & Shapiro, 1992). As such, ALP is a commonly used biomarker for osteoblast activity. Runx2 is the earliest identifiable marker of osteoblast formation and is known to upregulate the expression of ALP and osterix (Bronckers, Sasaguri, & Engelse, 2003; Lorenzo et al., 2011). The importance of Runx2 has been demonstrated using knockout mice models which show a lack of bone mineralization in the absence of Runx2 (Takarada et al., 2013). BMP-2 has also been shown to play an important role in osteogenesis (Hoffmann & Gross, 2001). Sclerostin on the other hand, is an inhibitor of osteogenesis that is secreted by osteoblasts and osteocytes, and the regulation of its expression is a major factor in the integration and control of bone remodelling (Winkler et al., 2003). Similar to our findings, Nash et al. reported that aqueous extracts of rooibos promoted ALP activity, mineralization and osteoblast regulatory markers (Nash & Ward, 2016). Earlier studies had shown that two common flavonoids found in rooibos, orientin and luteolin, also increase ALP activity and mineralization while decreasing sclerostin expression (Nash, Sullivan, Peters, & Ward, 2015). These studies may suggest that orientin and luteolin contribute to the pro-osteoblast effects of rooibos extracts. No previous studies have looked at the effects of aspalathin on osteoblast activity, even though aspalathin is the major polyphenolic compound found in aqueous extracts of rooibos (Joubert et al., 2005). Our studies suggest aspalathin may also contribute to the stimulatory effects of rooibos on osteoblast activity.

Osteoclast differentiation is marked by several key events, most notable, the expression of TRAP, the fusion and multinucleation of precursor cells and the formation of F-actin rings (Lorenzo et al., 2011; Mellis, Itzstein, Helfrich, & Crockett, 2011). These events are all necessary for osteoclasts to carry out their primary function, the resorption of bone (Bar-Shavit, 2007). Exposure to aspalathin significantly decreased the secretion of TRAP in conditioned media and decreased the number and size of TRAP stained multinucleated osteoclasts as well as the appearance of F-actin rings and resorption on bone mimetic surface. Initiation of osteoclast differentiation is controlled primarily via RANK/RANKL signalling (Wada et al., 2006). c-Fos is a key regulator in this process and forms a part of the AP-1 complex in the NFκβ activation pathway (Zamani et al., 2015). A key regulator in this pathway, NFATc1, is responsible for the control of a wide array of genetic responses during differentiation (Kim, Lee, Ha Kim, Choi, & Kim, 2008). These responses include the expression of key resorption enzymes, Cathepsin K and CAII. Cathepsin K is an important enzyme required for bone resorption by osteoclasts, and its expression is dependent on NFATc1 gene expression (Soysa, Alles, Aoki, & Ohya, 2012). CAII is one of the primary enzymes responsible for the maintenance of low pH in the sealed zone during resorption. In this present study, aspalathin was shown to inhibit the gene expression of c-Fos, NFATc1, Cathepsin K and CAII. Similar to our findings, Moosa et al. reported that aqueous rooibos extract inhibited osteoclast formation, resorption, actin ring formation and the expression of NFATc1, Cathepsin K and CAII (Moosa et al., 2018). Luteolin, a common flavonoid in rooibos, was also shown to reduce osteoclast differentiation and function *in vitro* via downregulation of the NFATc1 pathway (Lee et al., 2009). Quercetin, another flavonoid found in rooibos, has also shown potent anti-osteoclastogenic effects (Wattel et al., 2003). These polyphenols have been shown to be present in aqueous extracts of rooibos (Bramati et al., 2003), suggesting that these polyphenols may be responsible for the anti-osteoclastic effects of aqueous rooibos extracts. However, as it is the major component of rooibos extract, our results once again suggest that aspalathin may contribute to the anti-osteoclastic effects of rooibos.

Osteoclasts and osteoblasts interact with one another, meaning that any effects of aspalathin on one cell type in isolation may be different when those cells are found in a physiological system. An osteoclast/osteoblast co-culture experiment was conducted in order to estimate the effects of aspalathin on a more holistic model. This experiment is possible due to the osteoblasts' ability to sustain osteoclast differentiation in culture (Teti et al., 1991). TRAP staining analysis showed that aspalathin reduced the number of TRAP stained osteoclasts present after five days of incubation at all tested concentrations. These results are indicative that aspalathin possesses the capability to affect bone remodelling by reducing osteoclast formation in a simplistic bone cell cocultre model, although the mechanism of action is not fully understood. Aspalathin is known to have a high antioxidant capacity (Snijman et al., 2009). Ku et al. reported that aspalathin reduced reactive oxygen species (ROS) generation and nuclear factor κB (NFκB) expression in human endothelial cells (Ku, Kwak, Kim, & Bae, 2015). ROS and NFκB are crucial regulators of osteoclastogenesis (Callaway & Jiang, 2015; Wada et al., 2006), and aspalathin may target these pathways to elicit the effects seen in this present study. Indeed, in our previous studies, we have shown that aqueous rooibos extract can inhibit NFκB activation in osteoclast precursor cells (Moosa et al., 2018).

Current treatment for osteoporosis is often expensive and associated with side effects (Pandey et al., 2018). The identification of nutraceuticals with bone protective effects could offer inexpensive and safe methods to aid in the treatment of osteoporosis (Pandey et al., 2018). Taken together, our results demonstrate for the first time that aspalathin may have potential as a nutraceutical for its pro-osteoblast and anti-osteoclast effects. Recent studies demonstrated that aspalathin-enriched green rooibos tea extract improved insulin signalling and glucose uptake in obese rats (Mazibuko-Mbeje et al., 2019). This study may suggest that increasing the levels of aspalathin in the rooibos extract could enhance the biological activity of the extract. Further studies are needed to confirm the potential of aspalathin or an aspalathin enriched rooibos extract as a nutraceutical aid for bone protection.

5. Conclusion

This study showed for the first time that the phenolic compound aspalathin, found in *Aspalathus linearis* (rooibos), affects both osteoblast and osteoclast differentiation in cellular models *in vitro*. Results furthermore showed that aspalathin could reduce osteoclast differentiation in a murine osteoclast/osteoblast co-culture model. It is speculated that aspalathin is capable of shifting the balance of bone remodelling towards bone formation, and in this way could elicit bone-protective

effects. The implications of these findings are that aspalathin shows potential as a likely candidate for future treatments for diseases in which bone remodelling is unbalanced, such as osteoporosis. Further research on the precise mechanisms of actions could increase our understanding of the promising anti-osteoclastogenic and pro-osteoblast effects of aspalathin.

6. Ethics statement

The local Institutional Animal Care Committee and the local authorities approved all procedures. All animal experiments complied with the ARRIVE guidelines and were carried out in accordance with the EU Directive 2010/63/EU for animal experiments.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2019.103616.

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