Cell Proliferation

Cell Prolif., 2012, **45**, 566–576 doi: 10.1111/j.1365-2184.2012.00848.x

Inhibition of human *in vitro* osteoclastogenesis by *Equisetum arvense*

J. Costa-Rodrigues, S. C. Carmo*, J. C. Silva* and M. H. R. Fernandes*

Laboratory of Pharmacology and Cellular Biocompatibility, Faculty of Dental Medicine, University of Porto, Porto, Portugal

Received 3 April 2012; revision accepted 6 July 2012

Abstract

Objectives: *Equisetum arvense* has long been used in traditional medicines to treat different disorders, including bone pathologies. In this study a hydromethanolic extract of *E. arvense* was assessed for its effects on human osteoclastogenesis.

Materials and methods: Osteoclast precursors were maintained in non-stimulated and stimulated (presence of M-CSF and RANKL) conditions, or in co-cultures with osteoblasts. Cell cultures were treated with 0.00016–0.5 mg/ml of a hydromethanolic *E. arvense* extract.

Results: The extract did not affect spontaneous osteoclastogenesis. In osteoclast precursors committed to osteoclastogenesis (stimulated or co-cultured with osteoblasts), *E. arvense* caused dose-dependent inhibitory effect that became statistically significant at concentrations $\geq$0.004 mg/ml. This was observed using different osteoclast differentiation and activation markers. Cell response was associated with changes in relative contribution of MEK and NFkB signalling pathways, as well as PGE2 production. As there were differences in the response of osteoclast precursors maintained in the presence of inductive factors, or co-cultured with osteoblastic cells, it seems that *E. arvense* extract had the ability to modulate osteoclastogenesis, either by acting directly on osteoclast precursor cells, and/or *via* osteoblasts.

Conclusions: *Equisetum* appeared to have a negative effect on human osteoclastogenesis, which is in line with its putative beneficial role in pathophysiological conditions associated with increased osteoclastic activity, and might suggest potential utility for treatment with bone regeneration strategies.

Correspondence: M. H. R. Fernandes, Faculdade de Medicina Dentária, Universidade do Porto, Rua Dr. Manuel Pereira da Silva, 4200-393 Porto, Portugal. Tel.: +351 220 901 100; Fax: +351 220 901 101; E-mail: mhfernandes@fmd.up.pt

Introduction

Equisetum arvense, also known as horsetail, is a pteridophyte plant with aerial branched stems and regular verticilies, and a main stem around 10 mm long and 4 mm in diameter (1,2). It grows wild and widespread in the northern hemisphere, particularly in Europe, North and Central America (1). It grows in moist places in temperate climates (3). Its putative medicinal properties have been explored since times of the ancient Greeks and Romans, who used it to treat wounds (3,4). In addition, it has been included in folk remedies for arthritis, kidney troubles, bleeding ulcers, hepatitis, jaundice and tuberculosis (4,5). Among the living species of this genus, only *E. arvense* (as 'Equiseti herba') is found in several European pharmacopoeias (4).

A variety of studies has shown that *E. arvense* reveals a variety of potential pharmacological properties that might help understand its wide application in traditional phytoremedies (2–4). These include its use as antiseptic, anti-se- hypoglicemic, diuretic, anti-inflammatory, antioxidant, hepatoprotective, vasorelaxant and anti-nociceptive (1–6). In addition, it has also been observed that it can affect bone metabolism, helping in the treatment of some bone disorders, such as osteoporosis, and in healing of osteocytic tissue (3,7–10). It is thought that this characteristic of *E. arvense* might be related to its high content of silica, *E. arvense* being a plant that contains the highest known concentration of this element (11,12). This property, combined with its antiseptic activity [against several bacterial agents, including the *Staphylococcus aureus* (6), the main pathogen of bone infections (13)], makes *E. arvense* a potential, tool not only in treatment of some bone metabolic diseases but also in bone regeneration strategies.

Despite its rigid structure, bone is a very dynamic tissue, being continuously remodelled throughout life. Bone metabolism is mainly performed by co-ordinated action of two cell types, bone-synthesizing osteoblasts and bone-resorbing osteoclasts (14,15). In addition to their established roles in bone metabolic activities, it is known that there is abundant cross-talk between both cell types, affecting their differentiation stages (15,16). In this context, osteoblasts are

known to be key players in the osteoclastogenic process (16), specially by synthesis of macrophage-colony stimulating factor (M-CSF) and receptor activator of nuclear factor-κB ligand (RANKL), two important factors that promote osteoclastogenesis *in vitro* (17,18).

Although there are lines of evidence that point to potential effects of *E. arvense* on modulation of bone cell activity, this issue is far from being fully elucidated. Recently, we have reported that a hydromethanolic extract of *E. arvense* had the ability to stimulate cell viability/proliferation, ALP activity and gene expression of some osteoblastic markers, in human bone marrow cell cultures, suggesting a potentially interesting profile regarding bone regeneration and/or bone disorder contexts (19). The aim of this work was to proceed further in the characterization of the *E. arvense* modulation properties on human bone cells, by evaluating cellular and molecular effects of the same hydromethanolic *E. arvense* extract, on human osteoclast development. Thus, osteoclast precursor cells from peripheral blood were exposed to the extract of *E. arvense* in the absence or presence of osteoclastogenic factors (M-CSF and RANKL). Also, effects of the extract were tested on co-cultures of osteoclast precursors and human osteoblasts, due to their role also on osteoclastogenesis (15,16). Cell cultures were assessed for osteoclast differentiation and activity. Involvement of MEK and NFκB signalling pathways and PGE2 synthesis in the observed osteoclastogenic response was also addressed.

Materials and methods

Preparation of hydromethanolic extract of Equisetum arvense

Dried aerial components of *E. arvense* were minced into smaller particles, introduced into a glass container and compacted. Then, methanol–water (1:1) was added to completely cover the dry plant constituents and all was left for 3 days in a maceration process. Then the biological material was percolated and extracting solution was collected and evaporated in a rotating evaporator operating at 50 °C, under reduced pressure. The recovered solution was reused as extractor liquid, being added to the glass container once more. This process elapsed in a continuous way, and extract yielded between days 4 and 10 was collected. The deconcoction was concentrated to dryness under reduced pressure, and respective residue was dissolved in dimethylsulphoxide (DMSO; Merck, Darmstadt, Germany) to obtain a stock solution of 100 mg/ml that was sterilized by filtration through 0.2 μm Millipore filters, aliquoted and stored at −20 °C.

Total extract was analysed for its silicon (Si) content. Standard solutions, 10–75 ppm, were prepared by appropriate dilution of a 1000 ppm Si stock solution (Riedel de Haen) with NaCl (20 mg/ml). Absorbance of samples was determined at $\lambda = 251.6$ nm, using an atomic absorption spectrometer (GBC 904 AA) with an acetylene/nitrous oxide flame. Concentration of Si in the 100 mg/ml extract stock solution was 11.08 ± 0.81 μg/ml.

Cell cultures

Peripheral blood mononuclear cells. Human peripheral blood mononuclear cell (PBMC), used as osteoclast precursor cells, were isolated from blood of healthy male donors, aged 25–35 years, as described previously (20). Briefly, blood was diluted in PBS (1:1), and applied on the surface of Ficoll-Paque™ PREMIUM (GE Healthcare Bio-Sciences, Piscataway, USA). After centrifugation at 400 ***g*** for 30 min, cells were collected and washed twice in PBS. On average, in the range of 70×10^6 PBMC were obtained for each 100 ml of processed blood. Cells, seeded at 1.5×10^6 PBMC/cm^2 were maintained in α-minimal essential medium (α-MEM; Gibco – Life Technologies Ltd, Paisly, UK) supplemented with 30% human serum (from the same donor from which PBMC were collected), 100 IU/ml penicillin (Gibco), 2.5 μg/ml streptomycin (Gibco), 2.5 μg/ml amphotericin B (Gibco) and 2 mM L-glutamine (Sigma-Aldrich, MO, USA). Cell cultures were performed in the absence (base medium, BM) or presence of recombinant M-CSF (25 ng/ml) and RANKL (40 ng/ml) (21), (M+R cultures).

Co-culture of PBMC with human bone marrow osteoblastic cells. Bone marrow was obtained from patients (25–45 years old) undergoing orthopaedic surgery procedures, after informed consent. Human bone marrow osteoblastic cells (hBMC) were cultured in α-MEM (Gibco)-containing 10% foetal bovine serum (Gibco), 100 IU/ml penicillin (Gibco), 2.5 μg/ml streptomycin (Gibco), 2.5 μg/ml amphotericin B (Gibco) and 50 μg/ml ascorbic acid (Sigma-Aldrich). Cultures were maintained in 5% CO_2 humidified atmosphere at 37 °C for 10/15 days up to near confluence. Adherent cells were enzymatically detached with 0.04% trypsin and 0.025% collagenase. Co-cultures were performed as published previously (22). Shortly, the resulting cell suspension was seeded, 10^4 cell/cm^2, and incubated for 24 h in medium as described above. Then, PBMC 1.5×10^6 cells/cm^2, were added, and co-cultures were maintained in culture conditions as described above for PBMC cultures.

Exposure of cultures to Equisetum arvense extract

PBMC cultures and co-cultures of PBMC + hBMC were exposed to *E. arvense* extract at concentrations ranging between 0.00016 and 0.5 mg/ml, chosen according to

recently published data on effects of identical *E. arvense* extract on human osteoblastic cells (19). Both types of culture were maintained for 24 h, to allow for cell adhesion, and the extract was added at this stage. Cultures were incubated for 21 days at 37 °C in 5% CO_2 humidified atmosphere and were maintained in the same culture conditions as described above for PBMC cultures. Culture medium was replaced once a week and *E. arvense* extract was renewed at each medium change. As control, cultures were performed in absence of extract, but maintained in presence of the same final concentration of DMSO as extract-treated cultures. Cultures were characterized at days 7, 14 and 21 for tartrate-resistant acid phosphatase (TRAP) activity and number of TRAP-positive multinucleate cells, and at day 21 for presence of actin rings and vitronectin and calcitonin receptors. In addition, cultures exposed to lowest tested concentrations that caused statistically significant inhibitory effects on TRAP activity, and number of TRAP-positive multinucleate cells – 0.004 mg/ml – were further analysed, at day 21, by RT-PCR for expression of osteoclast-related genes and calcium phosphate resorbing ability. Finally, cell cultures were also assessed at days 7, 14 and 21 for intracellular mechanisms involved in response to *E. arvense* extract.

Characterization of osteoclastogenic response

TRAP activity. TRAP activity was determined by *para*-nitrophenilphosphate (*p*NPP) hydrolysis assay, as described before (23). After being washed twice in PBS, cells were lysed with 0.1% (V/V) Triton X-100 for 5 min. Cell lysates were incubated in 12.5 mM *p*NPP, 0.04 M tartaric acid and 0.09 M citrate (pH 4.8) for 1 h at 37 °C. After incubation, the reaction was stopped using 5 M NaOH, and absorbance of samples was determined at 405 nm in an ELISA plate reader (Synergy HT; Biotek, Winooski, USA). Results were normalized to total protein content of cultures, assessed by Bradford's method (24) and expressed as nmol/min/mg of protein.

TRAP-positive multinucleated cells. Cell cultures were washed twice in PBS and fixed in 3.7% formaldehyde for 10 min. Cells were rinsed in distilled water and stained for TRAP using Acid Phosphatase, Leukocyte (TRAP) kit (Sigma), according to the manufacturer's instructions. Then, cells were incubated for 1 h, at 37 °C, in the dark, with naphtol AS-BI 0.12 mg/ml, in presence of 6.76 mM tartrate and 0.14 mg/ml fast garnet GBC. Then, cell layers were washed and stained with haematoxilin. TRAP-positive multinucleate cells were identified and counted for each experimental condition.

Visualization of F-actin cytoskeleton, vitronectin and calcitonin receptors by confocal laser scanning microscopy. Cell layers were washed twice in PBS, fixed in 3.7% (V/V) *para*-formaldehyde for 15 min and permeabilized in 0.1% (V/V) Triton X-100 for 5 min. Cells were stained for actin with 5 U/ml Alexa Fluor® 647-Phalloidin (Invitrogen, CA, USA), and for vitronectin (VNR) and calcitonin (CTR) with 50 μg/ml mouse IgGs anti-VNR and IgGs anti-CTR (R&D Systems, MN, USA), respectively. Anti-VNR and anti-CTR detection was performed with 2 μg/ml Alexa Fluor® 488-Goat anti-mouse IgGs.

RT-PCR analysis. Cell cultures maintained in absence or presence of *E. arvense* extract, 0.004 mg/ml, were analysed by RT-PCR for expression of housekeeping gene *GAPDH*, osteoclast-related differentiation and activation factors c-myc and c-src, respectively, and for osteoclast functional genes *TRAP*, cathepsin K (*CATK*), carbonic anhydrase 2 (*CA2*) and *RANK* (25). RNA was extracted using Rneasy® Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions, and quantified by UV spectrophotometry at 260 nm. Then, 0.5 μg RNA was reverse transcribed and amplified (25 cycles) using Titan One Tube RT-PCR System (Roche, Basel, Switzerland), with annealing temperature of 55 °C. Primers used are listed in Table 1. RT-PCR products were separated by electrophoresis on 1% (w/V) agarose gel and subjected to densitometric analysis using ImageJ 1.41 software, National Institutes of Health, USA. Values were normalized to corresponding GAPDH value of each experimental condition.

M-CSF and RANKL quantification. M-CSF and RANKL quantification of culture medium from PBMC + hBMC co-cultures was performed using Human M-CSF

Table 1. Primers used on RT-PCR analysis of PBMC cultures

Gene	5′ Primer	3′ Primer
GAPDH	5′-CAGGACCAGGTTCACCAACAAGT-3′	5′-GTGGCAGTGATGGCATGGACTGT-3′
c-myc	5′-TACCCTCTCAACGACAGCAG-3′	5′-TCTTGACATTCTCCTCGGTG-3′
c-src	5′-AAGCTGTTCGGAGGCTTCAA-3′	5′-TTGGAGTAGTAGGCCACCAG-3′
TRAP	5′-ACCATGACCACCTTGGCAATGTCTC-3′	5′-ATAGTGGAAGCGCAGATAGCCGTT-3′
CATK	5′-AGGTTCTGCTGCTACCTGTGGTGAG-3′	5′-CTTGCATCAATGGCCACAGAGACAG-3′
CA2	5′-GGACCTGAGCACTGGCATAAGGACT-3′	5′-AAGGAGGCCACGAGGATCGAAGTT-3′
RANK	5′-TTAAGCCAGTGCTTCACGGG-3′	5′-ACGTAGACCACGATGATGTCGC-3′

Quantikine ELISA Kit (R&D Systems) and sRANKL (total) Human ELISA (Osteoprotegerin Ligand) (BioVendor, Laboratorni medicina a.s., Brno, Czech Republic), respectively, following manufacturers' instructions. After detection, absorbance of samples was measured at 450 nm in an ELISA plate reader (Synergy HT; Biotek). Results were expressed as ng/ml.

Calcium phosphate resorbing assay. Cells were cultured on BD BioCoat™ Osteologic™ Bone Cell Culture Plates (BD Biosciences, New Jersey, USA) for 21 days in absence or presence of *E. arvense* extract (0.004 mg/ml). Then, cells were bleached with 6% NaOCl and 5.2% NaCl, following the manufacturer's protocol and remaining calcium phosphate layers were visualized by phase contrast light microscopy. Resorption lacunae were identified and total resorbed area was quantified with ImageJ 1.41 software.

Intracellular signalling mechanisms. Cell cultures, maintained in absence or presence of 0.004 mg/ml *E. arvense* extract, were treated with different inhibitors of osteoclastogenic-related signalling pathways. U0126, a MEK pathway inhibitor, was tested at 1 and 10 μM, due to apparently contradictory effects attributed to low doses of this molecule, by different studies (26–28). PDTC, an NFκB pathway inhibitor, was used at 10 and 100 μM as the lower concentration has been previously described as IC50 for rat osteoclastic differentiation (29). Finally, indomethacin (1 μM) was tested as it blocks PGE2 synthesis, an osteoclastogenic stimulator (30,31). PBMC cultures and co-cultures of PBMC + hBMC were performed under identical experimental conditions as those described above, and were assessed for TRAP activity and number of TRAP-positive multinucleate cells.

Statistical analysis

All data presented in this work were obtained from three separate experiments, using cell cultures from six different donors. Each experiment was performed in triplicate. Quantitative data are presented as mean ± SD. Groups of data were evaluated using two-way analysis of variance (ANOVA) and no significant differences in pattern of cell behaviour were found. Statistical differences between controls and experimental conditions were assessed using Bonferroni's method. Values of $P \leq 0.05$ were considered significant.

Results

TRAP activity and number of TRAP-positive multinucleate cells

PBMC cultures performed in BM (Fig. 1a) revealed low values of TRAP activity, which increased slightly over

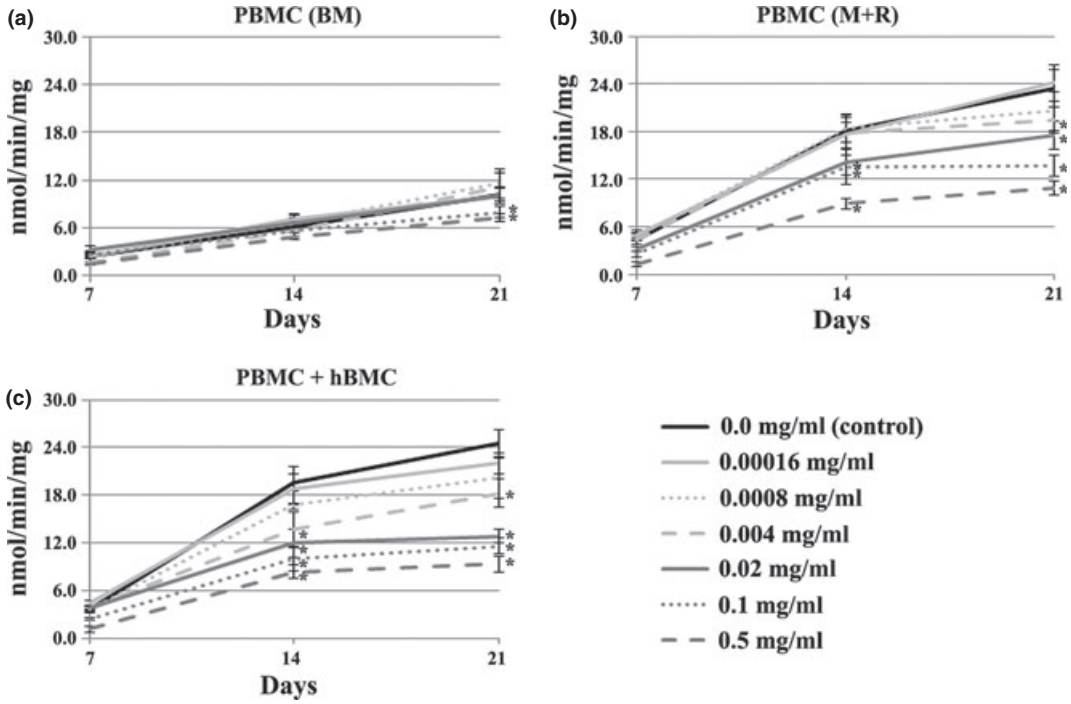

Figure 1. TRAP activity of PBMC cultures. Cell cultures, maintained in base medium (BM, a) or supplemented with M-CSF and RANKL (M+R, b) and co-cultures of PBMC + hBMC cells (c), were performed in absence (control) or presence of *Equisetum arvense* extract. *Significantly different from control cultures.

the culture period. Presence of *E. arvense* extract did not affect cell responses, except for highest tested concentrations (0.1 and 0.5 mg/ml) that elicited a significant decrease in TRAP activity.

In the presence of M-CSF and RANKL (Fig. 1b), PBMC cultures had high TRAP activity, and exposure to extract caused dose-dependent reduction in enzyme activity. Inhibitory effectsbecame statistically significant at concentrations similar to and higher than 0.004 mg/ml, ranging from 15% (0.004 mg/ml) to 54% (0.5 mg/ml) at day 21.

Exposure to *E. arvense* extract also caused dose-dependent reduction in TRAP activity in co-cultures of PBMC + hBMC (Fig. 1c). However, percentages inhibition were slightly higher than observed in supplemented PBMC cultures, at equal concentrations, varying from 25% (0.004 mg/ml) to 61% (0.5 mg/ml) by day 21.

Globally, number of TRAP-positive multinucleate cells (Fig. 2) yielded a similar pattern of response to that achieved for TRAP activity.

Presence of cells displaying osteoclastic features

Cell density and morphology remained identical at days 14 and 21 of culture (Fig. 3a). Then (day 21), cells were stained for actin, VNR and CTR (Fig. 3b). All cultures revealed presence of cells with characteristic osteoclast features. Nevertheless, relative abundance of these cells was in agreement with results obtained for TRAP determinations. Figure 3c shows representative images of this behaviour, in 21-day PBMC cultures supplemented with M-CSF and RANKL, and exposed to 0.00016 and 0.02 mg/ml of the extract.

Expression of osteoclast-related genes

PBMC and co-cultures of PBMC + hBMC were exposed to 0.004 mg/ml of *E. arvense* extract, and cultures were assessed for expression of *c-myc*, *s-src*, *TRAP*, *CATK*, *CA2* and *RANK* (Fig. 4a). PBMC cultures maintained in BM displayed low expression levels of the analysed genes, and presence of extract did not affect this behaviour significantly. In presence of M-CSF and RANKL, PBMC expressed higher levels of the tested genes, and exposure to *E. arvense* extract elicited reduction in expression of all genes (except for CA2 and RANK), between 13% and 49%. Exposure of PBMC + hBMC co-cultures to *E. arvense* extract caused a similar effect in expression of osteoclast-related genes. As observed with TRAP activity, inhibitory effects of the extract were

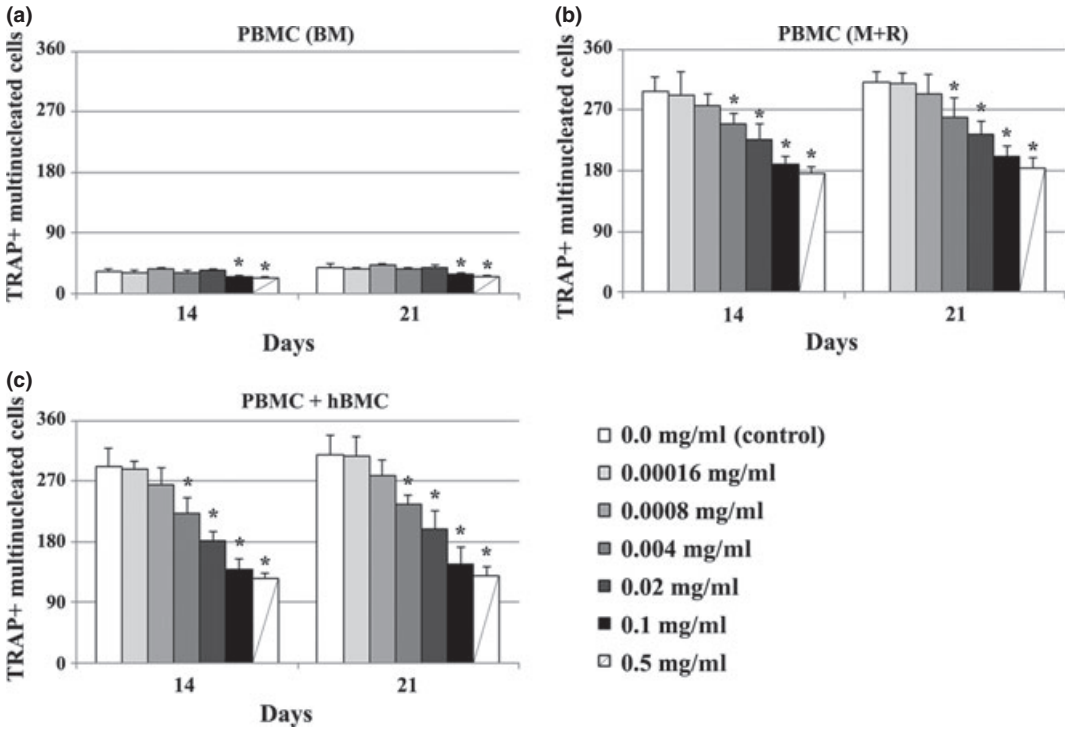

Figure 2. Presence of TRAP+ multinucleated cells in PBMC cultures. Cell cultures, maintained in base medium (BM, a) or supplemented with M-CSF and RANKL (M+R, b) and co-cultures of PBMC + hBMC cells (c), were performed in absence (control) or presence of *Equisetum arvense* extract. *Significantly different from control cultures.

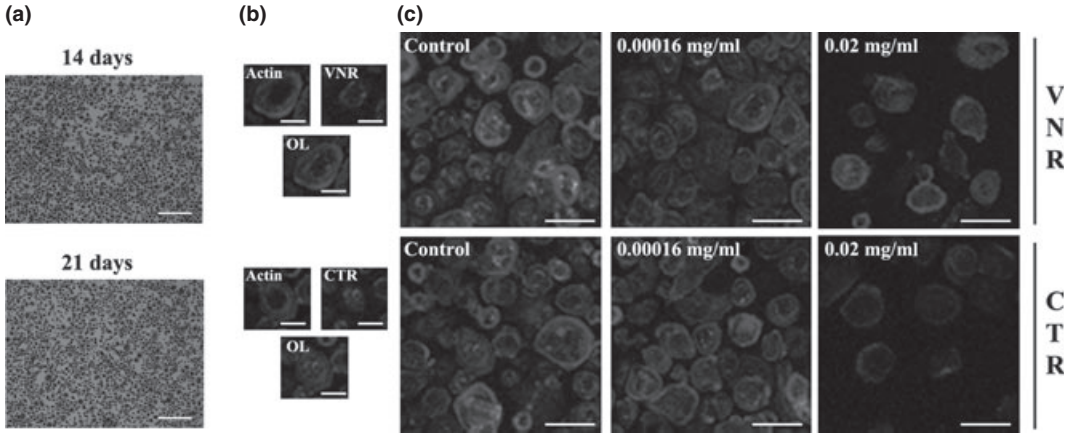

Figure 3. Staining of PBMC cultures treated with the extract. PBMC cultures (M+R) stained for nuclei and TRAP at days 14 and 21 of culture (a). CLSM visualization of PBMC cultures (21 days) supplemented with M-CSF and RANKL: b – Representative images; c – PBMC cultures: control and exposed to the extract. White bars represent 600 μm (a), 40 μm (b) and 100 μm (c).

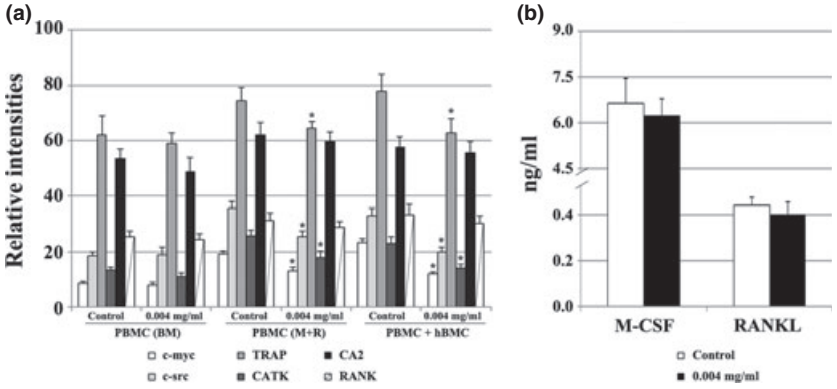

Figure 4. Analysis of the expression of osteoclast-related genes and M-CSF and RANKL. PBMC cultures (either maintained in BM or M+R) and PBMC + hBMC co-cultures, were performed in the absence (control) or presence of *Equisetum arvense* extract, 0.004 mg/ml (a). RT-PCR products subjected to densitometric analysis and normalized with value obtained for GAPDH. Quantification of M-CSF and RANKL present in culture medium of co-cultures maintained in absence or presence of 0.004 mg/ml *Equisetum arvense* extract (b). BM, base medium; M+R, M-CSF + RANKL. *Significantly different from control cultures.

slightly higher than observed for supplemented cultures, between 19% and 53%.

Production of M-CSF and RANKL

PBMC + hBMC co-cultures, performed in absence or presence of 0.004 mg/ml *E. arvense* extract, were analysed for production of M-CSF and RANKL. For that, pro-osteoclastogenic growth factors present in culture medium were quantified (Fig. 4b). It was observed that both molecules were present in culture media from co-cultures, and that M-CSF concentration was found to be significantly higher than that of RANKL concentration. Supplementation with extract elicited slight reduction in production of both growth factors, although without statistical significance.

Calcium phosphate resorbing ability

PBMC cultures maintained in absence of exogenous osteoclastogenic stimuli elicited but few small resorption lacunae (Fig. 5a) and, thus, a low resorbed area (Fig. 5b). Exposure to 0.004 mg/ml *E. arvense* extract did not affect this behaviour. Comparatively, of presence of M-CSF and RANKL, PBMC cultures revealed a significantly higher resorbing ability, that reduced following extract treatment (around 30%). A similar response was observed for PBMC + hBMC co-cultures, with reduction of approximately 41%.

Intracellular signalling pathways

Involvement of MEK and NFκB pathways and PGE2 production of the inhibitory effect of *E. arvense*, was addressed in cultures exposed to 0.004 mg/ml extract, and assessed for TRAP activity (Fig. 6). For each of the three different cell cultures tested, cell responses were compared with that obtained in absence of any signalling pathway inhibitor.

Control PBMC cultures maintained in BM or in presence of M-CSF and RANKL were very sensitive to presence of U0126, an inhibitor of the MEK signalling

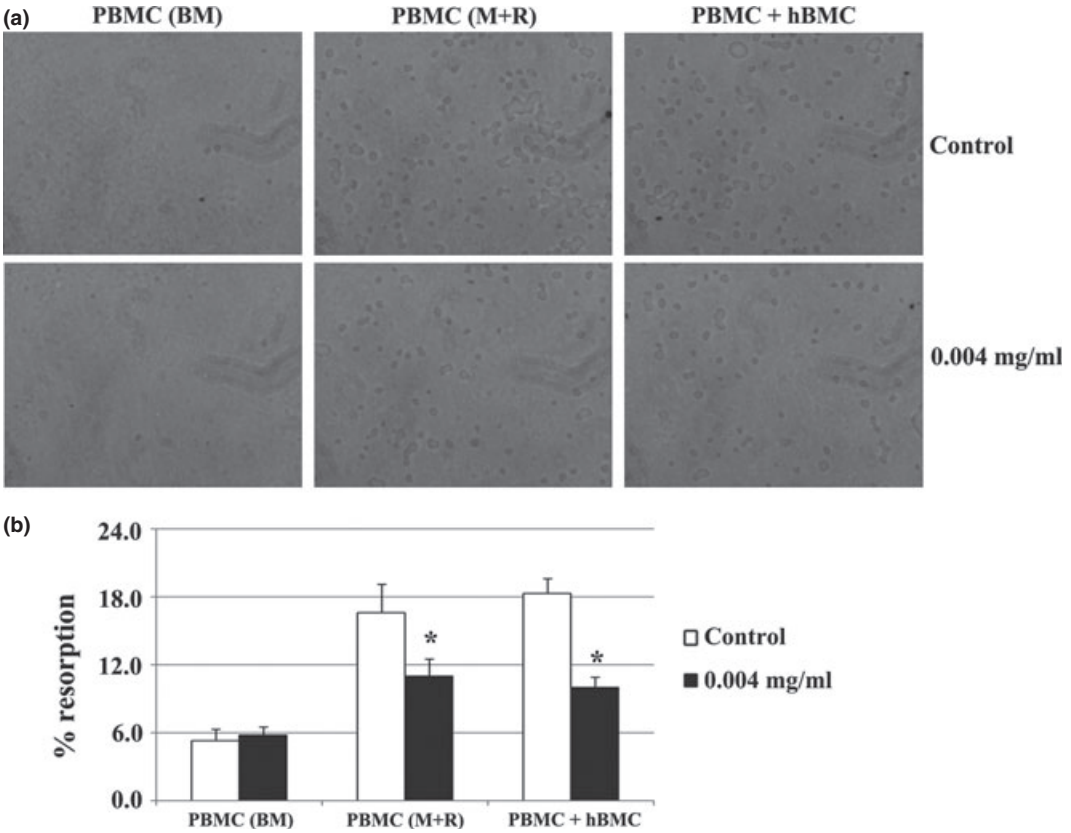

Figure 5. Calcium phosphate resorbing activity of PBMC cultures. Cells cultures, maintained in base medium (BM, a) or supplemented with M-CSF and RANKL (M+R, b), and co-cultures of PBMC + hBMC cells (c), were performed in absence (control) or presence of 0.004 mg/ml *Equisetum arvense* extract. a – Representative images of calcium phosphate layers after cell removal. b – Quantification of resorbed areas expressed as percentage of total area. *Significantly different from control cultures.

pathway; at 1 μM, significant reduction in TRAP activity was noted, and at 10 μM, inhibition was total. Moreover, full inhibition was also observed in presence of PDTC, an inhibitor of the NFκB pathway (at both tested concentrations). Indomethacin did not elicit significant changes in TRAP activity. Globally, in PBMC cultures performed in BM, presence of extract did not change this behaviour. However, in PBMC supplemented with M-CSF and RANKL, some important changes were noted. Addition of U0126, at 1 μM, did not affect TRAP activity, and, at 10 μM, only partial inhibition was achieved; also, presence of PDTC at 10 μM, did not completely abolish TRAP activity during the first 14 days of culture (although values were very low), unlike as was observed at 100 μM. Again, presence of indomethacin did not affect the cell response.

Co-cultures of PBMC + hBMC were not affected by presence of 1 μM U0126, but addition of 10 μM sharply reduced TRAP activity. Treatment with PDTC elicited concentration-dependent reduction in TRAP activity on day 14 and complete inhibition by day 21. Indomethacin caused partial inhibition of enzyme activity at day 21. Comparatively, following exposure to *E. arvense* extract, presence of U0126 at 1 and 10 μM resulted in similar behaviour. However, PDTC, at 10 μM, inhibited TRAP activity only partially, but, at 100 μM, total inhibition was observed. Indomethacin caused low levels of inhibition, specially at day 21, although slightly lower than that noted in control co-cultures.

Discussion

Traditional medicines rely mainly on use of herbal extracts to treat a wide range of disorders, including those that affect bone tissue. Indeed, there are numerous plants employed for treatment of osteoporosis and arthropathies, among others (3,7–10,32). Although use of phytomedicines might considered with care, there are cases in which use of such substances demonstrate potential use for application, this being supported both by *in vitro* and *in vivo* studies (10,32–36). Here, effects of an *E. arvense* hydromethanolic extract on human osteoclastogenesis were assessed. Three osteoclastogenic models were tested, namely, non-stimulated (base medium, absence of

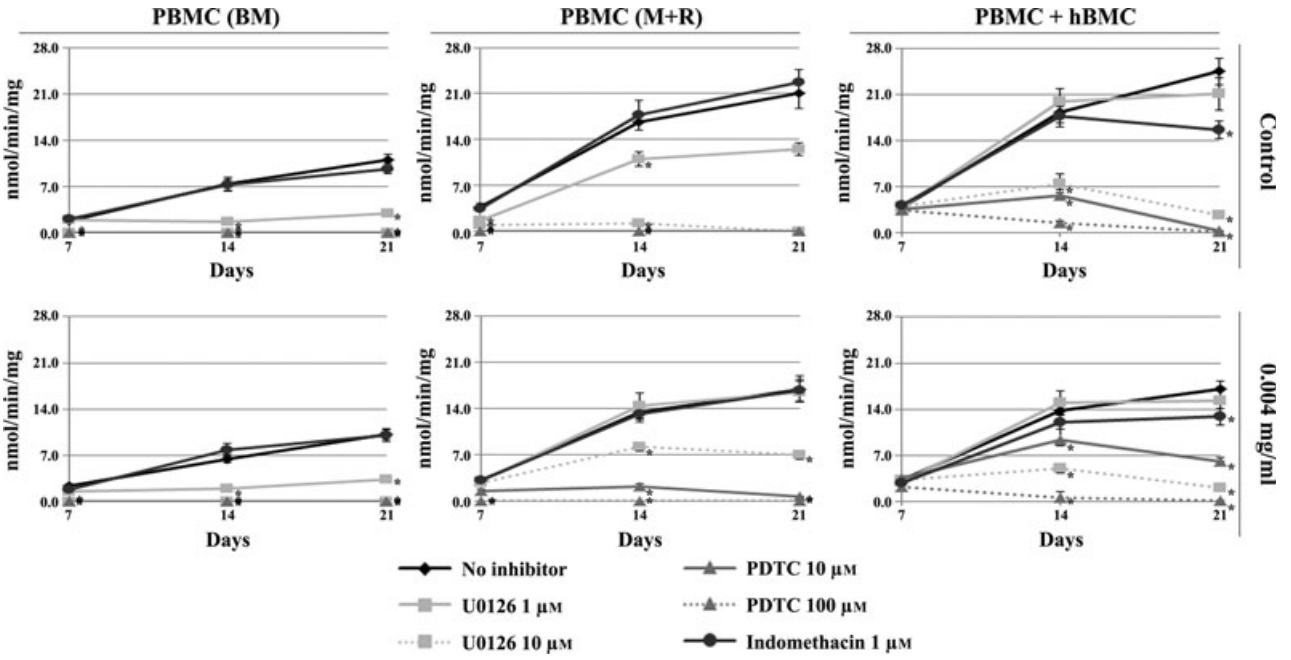

Figure 6. Involvement of MEKand NFκB pathways and PGE2 production on cellular response to E. arvense extract. TRAP activity of PBMC cultures maintained in base medium (BM) or supplemented with M-CSF and RANKL (M+R) and co-cultures of PBMC + hBMC cells, maintained in absence (control) or presence of 0.004 mg/ml *Equisetum arvense* extract. Cell cultures were treated with 1 or 10 μM U0126, 10 or 100 μM PDTC and 1 μM indomethacin. *Significantly different from control cultures.

any exogenous osteoclastogenic stimuli) and stimulated (presence of recombinant M-CSF and RANKL) osteoclast precursor cells, and co-cultures of osteoclastic and osteoblastic cells. As there is no published information regarding concentrations of *E. arvense* extract components used in the traditional medicines, this study has been conducted using a wide range of concentrations, to obtain global perspective of effects of *E. arvense* on human osteoclasts.

Results showed that *E. arvense* extract did not significantly affect spontaneous osteoclastogenesis; occurring in base medium, neither signalling pathway was involved. However, when osteoclast precursors were committed towards osteoclastogenesis (either by supplementation with M-CSF and RANKL or by presence of osteoblastic cells), clear and significant dose-dependent inhibition on osteoclastogenesis was observed. It is important to mention that total protein content (which is, with some reservations, related to cell density) of cell cultures did not significantly change by presence of the extract, except for the two higher tested concentrations (data not shown). This suggests that the observed inhibition of osteoclastogenesis might mainly be due to specific effects of the processes related to osteoclast differentiation, rather than being a non-specific toxic effect on cell density.

The present study has demonstrated that *E. arvense* can negatively modulate osteoclastogenesis. This is in line with some applications suggested for this plant in traditional medicine, particularly as treatment for bone disorders characterized by a hyperactivation of osteoclastic cells (such as osteoporosis and osteoarthritis) (3,7–10). This ability might be related to several characteristics of the plant, with regard to its composition. It is known that in life *E. arvense* accumulates high quantities of silica (11,12), a characteristic that is highly variable among different plant species (37). No other plant reveals such high concentrations of this element, which can reach values as higher as 25% dry weight (12). Despite the many beneficial properties of silica for the plants, [specially in the relief of stress (11)], this element is thought to mainly be responsible for the medicinal properties attributed to it (7,8), particularly, for treatment of bone disorders (7–9). In this context, it is important to mention that silica is found in bone tissue, where it is known to have an important role in maintenance of normal physicochemical properties of this tissue (38). Due to that, several studies have demonstrated that silica has high potential for application in numerous biomaterial-based bone regeneration strategies (39–41).

In this context, a relationship between silica content of *E. arvense* and observed osteoclastogenic response, was aimed to be established. Although some effects of silica on osteoclastogenic behaviour of cell cultures were observed (data not shown), clearly there were also fur-

ther factors of the extract contributed to the process. Detailed characterization of components of *E. arvense* extract that could modulate osteoclast differentiation and function are underway.

In addition to its high silica content, it is known that *E. arvense* also contains numerous phenolic compounds. In a previous study, it has been observed that one hydroalcoholic extract of the plant contained tannins (1), saponins (1), flavonoids [particularly isoquercetin, kaempferol glycosides, flavones glycosides and apigenin (1,4,42)] and steroids [especially β-sitosterol, campesterol and isofucosterol (1,43)]. These are natural antioxidant molecules well known for their anti-inflammatory effects [see (1,44) and refs. cited therein]. In addition, some of them have been linked to positive effects on bone metabolism and preservation of a long-term bone health (45–49). For example, in an *in vitro* study conducted with RAW264.7 cells treated with furosin (tannin), it was observed that osteoclast differentiation and function were compromised in the presence of this molecule (45). A similar inhibitory effect has been observed in osteoclast cultures performed in presence of the steroidal saponin diosgenin (47). Moreover, *E. arvense* is also rich in further molecules that can affect bone cells, such as vitamins C, E, K and some of the B group (3).

Characterization of intracellular processes involved in the observed osteoclastogenic responses revealed some important aspects, and represents, to our knowledge, the only information regarding the mechanism of action of *E. arvense* for bone diseases. In PBMC cultures supplemented with M-CSF and RANKL, presence of the extract seemed to downregulate the MEK signalling pathway. Indeed, in this condition, inhibition of TRAP activity achieved by treatment with U0126, was significantly lower than in the control. Strong dependence on the NFκB signalling pathway, and absence of involvement of PGE2 production, on the observed cell response remained identical to control.

Osteoclastogenesis observed in co-cultures of PBMC + hBMC treated with *E. arvense* extract appeared to be slightly more dependent on the MEK pathway, while NFκB pathway, although very important for the process, seemed to be downregulated in that condition. PGE2 production was also involved in osteoclast development in the co-cultures, but its relative importance appears to be slightly lower in presence of the extract. In this context, it is noteworthy to highlight that, in a previous report (50), it was observed that the flavonoid portion of an *E. arvense* hydroalcoholic extract was mainly composed of isoquercitin, an important antioxidant (1) that is known to inhibit both synthesis and release of PG-like substances (51). This property may be the main reason behind less pronounced inhibitory effects of indomethacin on co-cultures supplemented with the extract.

Taken together, intracellular mechanisms involved in osteoclastogenic response in the presence of *E. arvense* extract appeared to involve, in all tested conditions, one or more of the tested signalling pathways. However, those, *per se*, cannot be considered to be sole mechanisms involved in the cell response, as osteoclastogenesis is a complex process that requires a network of multiple intracellular pathways (18,25). Moreover, although the overall degree of osteoclast differentiation and activation was identical in both PBMC supplemented with M-CSF and RANKL or co-cultured with osteoblastic cells, differences observed in intracellular processes suggest that *E. arvense* can affect osteoclastogenesis in different ways. As osteoblasts are key players in the osteoclastogenic process (15,16), it is tempting to speculate that the plant extract can not only act directly on osteoclast precursor cells, but also indirectly, *via* the osteoblastic cells, probably through modulation of expression of different molecules that can affect osteoclastogenesis. M-CSF and RANKL production were not significantly affected by the extract, which suggests that other mediators, in addition to these, may be involved in the process. In both situations (PBMC treated with M-CSF and RANKL and PBMC + hBMC co-cultures), an inhibitory effect on osteoclastogenesis was achieved. Recently, we have shown that the same hydromethanolic extract of *E. arvense* revealed osteogenic properties when used to supplement human bone marrow cell cultures, performed in standard cell culture plates or over hydroxyapatite disks (19). Thus, combining those observations with the ones obtained in the present work, it is tempting to suggest that *E. arvense* reveals an interesting and potentially applicable profile, when considering bone regeneration/repair strategies.

Conclusions

The present work showed that the *E. arvense* hydromethanolic tested extract could effectively reduce human osteoclast development and function, both in osteoclast precursor cell cultures and in co-cultures of osteoclastic and osteoblastic cells. Although the present results might mainly be attributed to some individual molecules, it seems more likely that the complex composition of the extract can act as a whole in the process, with possible antagonistic, additive and/or synergistic relationships among its components. To take advantage of the use of *E. arvense* derivatives as potential medicines for bone disorders, particularly those related to a high osteoclastic activity, or as a useful tool for bone regeneration strategies, a detailed understanding of the effects of the plant

on bone cells must be performed. The present data shed some new light on this subject.

Acknowledgements

Financial support was provided by Faculdade de Medicina Dentária, Universidade do Porto, Portugal. CLSM observation was performed at Advanced Light Microscopy, IBMC, University of Porto (IBMC.INEB) under the responsibility of Dr Paula Sampaio.

References

1 Do Monte FH, dos Santos JG Jr, Russi M, Lanziotti VM, Leal LK, Cunha GM (2004) Antinociceptive and anti-inflammatory properties of the hydroalcoholic extract of stems from *Equisetum arvense* L. in mice. *Pharmacol. Res.* **49**, 239–243.

2 Dos Santos JG Jr, Blanco MM, Do Monte FH, Russi M, Lanziotti VM, Leal LK *et al.* (2005) Sedative and anticonvulsant effects of hydroalcoholic extract of *Equisetum arvense*. *Fitoterapia* **76**, 508–513.

3 Stajner D, Popovic BM, Canadanovic-Brunet J, Anackov G (2009) Exploring *Equisetum arvense* L., *Equisetum ramosissimum* L. and *Equisetum telmateia* L. as sources of natural antioxidants. *Phytother. Res.* **23**, 546–550.

4 Milovanovic V, Radulovic N, Todorovic Z, Stankovic M, Stojanovic G (2007) Antioxidant, antimicrobial and genotoxicity screening of hydroalcoholic extracts of five serbian *Equisetum* species. *Plant Foods Hum. Nutr.* **62**, 113–119.

5 Oh H, Kim DH, Cho JH, Kim YC (2004) Hepatoprotective and free radical scavenging activities of phenolic petrosins and flavonoids isolated from *Equisetum arvense*. *J. Ethnopharmacol.* **95**, 421–424.

6 Radulovic N, Stojanovic G, Palic R (2006) Composition and antimicrobial activity of *Equisetum arvense* L. essential oil. *Phytother. Res.* **20**, 85–88.

7 Duke J, Bogenschutz J, du Cellier J, Duke P (2002) Handbook of Medicinal Herbs, 2nd edn, pp. 391–392. Boca Raton, FL: CRC Press.

8 Van Wyk B, Wink M (2004) Medicinal Plants of the World, pp. 136. Portland, OR: Timber Press.

9 Wichtl M (2004) Herbal Drugs and Phytopharmaceuticals, 3rd edn, pp. 195–199. Boca Raton, FL: CRC Press.

10 Putnam SE, Scutt AM, Bicknell K, Priestley CM, Williamson EM (2007) Natural products as alternative treatments for metabolic bone disorders and for maintenance of bone health. *Phytother. Res.* **21**, 99–112.

11 Currie HA, Perry CC (2009) Chemical evidence for intrinsic 'Si' within Equisetum cell walls. *Phytochemistry* **70**, 2089–2095.

12 Holzhuter G, Narayanan K, Gerber T (2003) Structure of silica in *Equisetum arvense*. *Anal. Bioanal. Chem.* **376**, 512–517.

13 DiCarlo EF, Kahn LB (2011) Inflammatory diseases of the bones and joints. *Semin. Diagn. Pathol.* **28**, 53–64.

14 Zaidi M (2007) Skeletal remodeling in health and disease. *Nat. Med.* **13**, 791–801.

15 Datta HK, Ng WF, Walker JA, Tuck SP, Varanasi SS (2008) The cell biology of bone metabolism. *J. Clin. Pathol.* **61**, 577–587.

16 Costa-Rodrigues J, Teixeira CA, Sampaio P, Fernandes MH (2010) Characterisation of the osteoclastogenic potential of human osteoblastic and fibroblastic conditioned media. *J. Cell. Biochem.* **109**, 205–216.

17 Boyce BF, Xing L (2008) Functions of RANKL/RANK/OPG in bone modeling and remodeling. *Arch. Biochem. Biophys.* **473**, 139–146.

18 Boyle WJ, Simonet WS, Lacey DL (2003) Osteoclast differentiation and activation. *Nature* **423**, 337–342.

19 Bessa Pereira C, Gomes PS, Costa-Rodrigues J, Almeida Palmas R, Vieira L, Ferraz MP *et al.* (2012). *Equisetum arvense* hydromethanolic extracts in bone tissue regeneration: in vitro osteoblastic modulation and antibacterial activity. *Cell Prolif.* **45**, 386–396.

20 Costa-Rodrigues J, Moniz KA, Teixeira MR, Fernandes MH (2012) Variability of the paracrine-induced osteoclastogenesis by human breast cancer cell lines. *J. Cell. Biochem.* **113**, 1069–1079.

21 Costa-Rodrigues J, Fernandes A, Fernandes MH (2011) Spontaneous and induced osteoclastogenic behaviour of human peripheral blood mononuclear cells and their CD14(+) and CD14(−) cell fractions. *Cell Prolif.* **44**, 410–419.

22 Costa-Rodrigues J, Fernandes A, Lopes MA, Fernandes MH (2012) Hydroxyapatite surface roughness: complex modulation of the osteoclastogenesis of human precursor cells. *Acta Biomater.* **8**, 1137–1145.

23 Costa-Rodrigues J, Fernandes MH (2011) Paracrine-mediated differentiation and activation of human haematopoietic osteoclast precursor cells by skin and gingival fibroblasts. *Cell Prolif.* **44**, 264–273.

24 Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 2448–2454.

25 Zhao Q, Shao J, Chen W, Li YP (2007) Osteoclast differentiation and gene regulation. *Front. Biosci.* **12**, 2519–2529.

26 Hotokezaka H, Sakai E, Ohara N, Hotokezaka Y, Gonzales C, Matsuo K *et al.* (2007) Molecular analysis of RANKL-independent cell fusion of osteoclast-like cells induced by TNF-alpha, lipopolysaccharide, or peptidoglycan. *J. Cell. Biochem.* **101**, 122–134.

27 Costa-Rodrigues J, Fernandes A, Fernandes MH (2011) Reciprocal osteoblastic and osteoclastic modulation in co-cultured MG63 osteosarcoma cells and human osteoclast precursors. *J. Cell. Biochem.* **112**, 3704–3713.

28 Costa-Rodrigues J, Teixeira CA, Fernandes MH (2011) Paracrine-mediated osteoclastogenesis by the osteosarcoma MG63 cell line: is RANKL/RANK signalling really important? *Clin. Exp. Metastasis* **28**, 505–514.

29 Hall TJ, Schaeublin M, Jeker H, Fuller K, Chambers TJ (1995) The role of reactive oxygen intermediates in osteoclastic bone resorption. *Biochem. Biophys. Res. Commun.* **207**, 280–287.

30 Kawashima M, Fujikawa Y, Itonaga I, Takita C, Tsumura H (2009) The effect of selective cyclooxygenase-2 inhibitor on human osteoclast precursors to influence osteoclastogenesis in vitro. *Mod. Rheumatol.* **19**, 192–198.

31 Kellinsalmi M, Parikka V, Risteli J, Hentunen T, Leskela HV, Lehtonen S *et al.* (2007) Inhibition of cyclooxygenase-2 down-regulates osteoclast and osteoblast differentiation and favours adipocyte formation in vitro. *Eur. J. Pharmacol.* **572**, 102–110.

32 Zhang Y, Leung PC, Che CT, Chow HK, Wu CF, Wong MS (2008) Improvement of bone properties and enhancement of mineralization by ethanol extract of Fructus Ligustri Lucidi. *Br. J. Nutr.* **99**, 494–502.

33 Jeong JC, Lee JW, Yoon CH, Lee YC, Chung KH, Kim MG *et al.* (2005) Stimulative effects of Drynariae Rhizoma extracts on the proliferation and differentiation of osteoblastic MC3T3-E1 cells. *J. Ethnopharmacol.* **96**, 489–495.

34 Dong GC, Chen HM, Yao CH (2008) A novel bone substitute composite composed of tricalcium phosphate, gelatin and drynaria fortunei herbal extract. *J. Biomed. Mater. Res. A* **84**, 167–177.
35 Yao CH, Tsai HM, Chen YS, Liu BS (2005) Fabrication and evaluation of a new composite composed of tricalcium phosphate, gelatin, and Chinese medicine as a bone substitute. *J. Biomed. Mater. Res. B Appl. Biomater.* **75**, 277–288.
36 Sun JS, Dong GC, Lin CY, Sheu SY, Lin FH, Chen LT *et al.* (2003) The effect of Gu-Sui-Bu (Drynaria fortunei J. Sm) immobilized modified calcium hydrogenphosphate on bone cell activities. *Biomaterials* **24**, 873–882.
37 Hodson MJ, White PJ, Mead A, Broadley MR (2005) Phylogenetic variation in the silicon composition of plants. *Ann. Bot.* **96**, 1027–1046.
38 Jugdaohsingh R (2007) Silicon and bone health. *J. Nutr. Health Aging* **11**, 99–110.
39 Pietak AM, Reid JW, Stott MJ, Sayer M (2007) Silicon substitution in the calcium phosphate bioceramics. *Biomaterials* **28**, 4023–4032.
40 Patel N, Best SM, Bonfield W, Gibson IR, Hing KA, Damien E *et al.* (2002) A comparative study on the in vivo behavior of hydroxyapatite and silicon substituted hydroxyapatite granules. *J. Mater. Sci. Mater. Med.* **13**, 1199–1206.
41 Gomes PS, Botelho C, Lopes MA, Santos JD, Fernandes MH (2010) Evaluation of human osteoblastic cell response to plasma-sprayed silicon-substituted hydroxyapatite coatings over titanium substrates. *J. Biomed. Mater. Res. B Appl. Biomater.* **94**, 337–346.
42 Graefe EU, Veit M (1999) Urinary metabolites of flavonoids and hydroxycinnamic acids in humans after application of a crude extract from *Equisetum arvense*. *Phytomedicine* **6**, 239–246.
43 D'Agostino M, Dini A, Pizza C, Senatore F, Aquino R (1984) Sterols from *Equisetum arvense*. *Boll. Soc. Ital. Biol. Sper.* **60**, 2241–2245.
44 Cetojevic-Simin DD, Canadanovic-Brunet JM, Bogdanovic GM, Djilas SM, Cetkovic GS, Tumbas VT *et al.* (2010) Antioxidative and antiproliferative activities of different horsetail (*Equisetum arvense* L.) extracts. *J. Med. Food* **13**, 452–459.
45 Park EK, Kim MS, Lee SH, Kim KH, Park JY, Kim TH *et al.* (2004) Furosin, an ellagitannin, suppresses RANKL-induced osteoclast differentiation and function through inhibition of MAP kinase activation and actin ring formation. *Biochem. Biophys. Res. Commun.* **325**, 1472–1480.
46 Nian H, Qin LP, Chen WS, Zhang QY, Zheng HC, Wang Y (2006) Protective effect of steroidal saponins from rhizome of Anemarrhena asphodeloides on ovariectomy-induced bone loss in rats. *Acta Pharmacol. Sin.* **27**, 728–734.
47 Shishodia S, Aggarwal BB (2006) Diosgenin inhibits osteoclastogenesis, invasion, and proliferation through the downregulation of Akt, I kappa B kinase activation and NF-kappa B-regulated gene expression. *Oncogene* **25**, 1463–1473.
48 Dew TP, Day AJ, Morgan MR (2007) Bone mineral density, polyphenols and caffeine: a reassessment. *Nutr. Res. Rev.* **20**, 89–105.
49 Bandyopadhyay S, Lion JM, Mentaverri R, Ricupero DA, Kamel S, Romero JR *et al.* (2006) Attenuation of osteoclastogenesis and osteoclast function by apigenin. *Biochem. Pharmacol.* **72**, 184–197.
50 Broudiscou LP, Lassalas B (2000) Effects of Lavandula officinalis and *Equisetum arvense* dry extracts and isoquercitrin on the fermentation of diets varying in forage contents by rumen microorganisms in batch culture. *Reprod. Nutr. Dev.* **40**, 431–440.
51 Chanh PH, Ifansyah N, Chahine R, Mounayar-Chalfoun A, Gleye J, Moulis C (1986) Comparative effects of total flavonoids extracted from Ribes nigrum leaves, rutin and isoquercitrin on biosynthesis and release of prostaglandins in the ex vivo rabbit heart. *Prostaglandins Leukot. Med.* **22**, 295–300.

