Food & Function

ROYAL SOCIETY OF CHEMISTRY

PAPER

Published on 10 July 2026

Cite this: DOI: 10.1039/d6fo02085b

Glucosinolate-rich broccoli microgreen extract promotes osteogenic differentiation and antioxidant responses in human stromal cells: implications for nutraceutical modulation of bone metabolism

Laura Gambari,[a] Emanuela Amore,[a] Sara Carpentieri,[a] Federica Di Padua,[b] Luca Giuseppe Dalle Carbonare,[c] Mattia Cominacini,[c] Maria Teresa Valenti[d] and Francesco Grassi *[a]

Over recent decades, interest in functional foods capable of mitigating age-related disorders and chronic inflammation-associated diseases has markedly increased. Among these, dietary organosulfur compounds, abundantly present in various vegetables, are recognized for their potential preventive effects against multiple pathological conditions. Isothiocyanates and glucosinolates (GLS) derived from *Brassicaceae* species, most notably sulforaphane and its precursor glucoraphanin, have demonstrated promising potential as adjunctive agents in the management of osteoporosis. Broccoli microgreens are especially rich in GLS; however, their potential application in osteoporosis prevention remains to be conclusively established. The present study sought to evaluate the bioactivity of a GLS-rich methanolic extract derived from broccoli microgreens cultivated under a vertical hydroponic system. Human adipose-derived stromal cells were used as *in vitro* model. Cellular viability was assessed through lactate dehydrogenase, Alamar blue, and neutral red uptake assays; expression of antioxidant genes was evaluated by real-time PCR. Osteogenic differentiation was investigated *via* Alizarin Red staining, alkaline phosphatase activity assays, and real-time PCR for osteogenic genes. Our findings indicate that broccoli microgreen extract did not exhibit any significant cytotoxicity up to 100 μM, exerted stimulatory effects on osteogenesis at 50 and 100 μM, while promoting antioxidant effect. Collectively, these *in vitro* data provide evidence that broccoli microgreen extract, characterized by high GLS content, exhibits anabolic and antioxidant activity in hADSCs, thereby supporting its potential application in future preclinical *in vivo* investigation and clinical trials targeting osteoporosis. Furthermore, the results highlight the feasibility of vertical hydroponic cultivation as a sustainable method for producing bioactive broccoli microgreen extract.

Received 28th April 2026,
Accepted 21st June 2026

DOI: 10.1039/d6fo02085b

rsc.li/food-function

Introduction

Over recent decades, the influence of food on human health has become a subject of increasing scientific and societal interest.[1–4] Particularly, the capacity to mitigate age-associated disorders and chronic inflammation-related pathologies has gained significant attention.[5–7] A growing body of evidence indicates that vegetables rich in dietary organosulfur compounds exhibit significant protective effects against diverse diseases, primarily through anti-inflammatory, antioxidant, anticancer, antiviral, antibacterial, and pro-autophagic mechanisms.[8–11]

Members of the *Brassicaceae* family are especially notable for their distinctive bioactive phytochemicals, glucosinolates (GLS).[12] Within this family, broccoli (*Brassica oleracea var. italica*) has been extensively recommended as a dietary source of health-promoting molecules and is among the most widely consumed vegetables globally.[13–16] Regular broccoli consumption has been associated with protective effects against metabolic and degenerative diseases including diabetes, atherosclerosis, cancer, cardiovascular disorders, and age-related conditions.[17–19] Reported benefits include attenuation of

[a]*Laboratorio RAMSES, IRCCS Istituto Ortopedico Rizzoli, Via di Barbiano 1/10, 40136 Bologna, Italy. E-mail: francesco.grassi@ior.it; Fax: +39-0516366090*
[b]*Department of Life Sciences and Biotechnology, Ferrara University, I-44121 Ferrara, Italy*
[c]*Department of Engineering for Innovation Medicine – University of Verona, Italy*
[d]*Department of Neuroscience, Biomedicine and Movement – University of Verona, Italy*

weight gain, improved glucose homeostasis, protection of pancreatic and hepatic tissues, prevention of low-density lipoprotein oxidation, and support for gastrointestinal and immune function.[13,20–27]

Broccoli contains a broad spectrum of bioactive compounds, including essential minerals (Ca, Na, K, P, Fe), vitamins (A, B-complex, C, K1, ascorbic acid), carotenoids, polyphenols, flavonoids (*e.g.*, quercetin, kaempferol, (−)-epicatechin, naringenin, apigenin, luteolin), phenolic acids (esters, glycosides, *p*-hydroxybenzoic acid), anthocyanins, sulfides, GLS (glucoraphanin, gluconapin, progoitrin, sinigrin), and antioxidants such as glutathione.[27,28] In addition, broccoli also provides significant amounts of dietary fiber, which contributes to their beneficial health effects.[29] However, the contribution of specific bioactive compounds to the biological activity of broccoli remains largely unexplored.[30] Mechanistic studies have identified sulforaphane (SFN) as the principal chemopreventive agent responsible for the reduced risk of cancer associated with cruciferous (including broccoli) vegetable intake shown by epidemiological investigations.[31–33] SFN is enzymatically generated from the GLS precursor glucoraphanin (GRA) through hydrolysis mediated either by endogenous plant myrosinase or by bacterial thioglucosidase within the intestinal microbiota.[34–36]

Beyond oncology, preclinical studies have suggested that SFN and GRA hold therapeutic potential in osteoporosis.[37] SFN mitigates oxidative stress, suppresses nuclear factor kappa B (NF-κB) signaling—a pivotal regulator of inflammatory cascades—and inhibits osteoclast differentiation.[38–40] Concurrently, SFN counteracts hypoxia-induced inhibition of osteoblast mineralization by reducing hypoxia-mediated apoptosis and ROS levels;[41] and rescues osteoblasts from glucocorticoid-induced apoptosis through NRF2 modulation.[42] Although GRA has not yet been evaluated in animal models of osteoporosis, *in vitro* studies indicate that it induces osteogenic differentiation in human mesenchymal stromal cells and osteocytes,[43,44] beyond its conversion to active SFN. Additional broccoli constituents, such as vitamin K1, have been associated with increased bone mineral density.[45] Nevertheless, the role of broccoli and of its constituents in osteogenesis and in osteoporosis prevention remains insufficiently substantiated.[46]

To maximize the dietary intake of phytocompounds, broccoli microgreens have attracted attention as functional food or superfood due to their shorter production cycles and higher concentrations of bioactive phytochemicals and secondary metabolites.[47–50] Microgreens are young seedlings produced from seeds of different species of plants grown using hydroponic or substrate-based systems and harvested 7–21 days after germination, when the first true leaves develop.[51] They differ from sprouts which are harvested including roots a few days after germination.[52] Notably, microgreens, similarly to sprouts, have significantly higher total GLS content (17.5 μmol per g fr. wt) compared to mature plants (8.3 μmol per g fr. wt);[24,48,53] thus, smaller intake of them can ensure the same bioactivity as substantially greater intakes of the mature plant.[54] The predominant GLS found in broccoli microgreen is GRA (>89% of total GLS),[55] corresponding to an absolute amount of 13.6 μmol per g fr. wt, very similar to what previously found in broccoli sprout extract (16.6 μmol per g fr. wt).[54,56] However, fresh cut microgreens hold limited shelf life due to their delicate tissues[53,57] and GLS intake and activity from raw food are affected by methods of storage and cooking.[57–59] Precautions to limit the loss of GLS can include: (1) limiting the time elapsed between harvesting and processing, thus lowering the hydrolysis of GLS by myrosinase released by cellular damage;[60] (2) limiting lyophilization (freeze-drying);[61] (3) optimize extraction method for achieving high recovery rates and purity of GLS.[62] Among extraction methods which can preserve GLS content, an 80% methanol (MeOH) solution was shown to effectively inactivate myrosinase, thereby preserving their concentrations during extraction.[62]

Our group has previously demonstrated that both GLS and GLS-rich extracts induce osteogenic differentiation of hMSCs.[44,63] However, whether microgreen extracts rich in GRA display anabolic activity remain unexplored. In this work, we aimed to determine if broccoli microgreen methanolic extract, grown *via* vertical hydroponic culture and at known title of GRA, stimulates osteogenic differentiation of human adipose-derived stromal cells (h-ADSCs) and modulates oxidative stress while preserving cell viability.

Materials and methods

Plant culture and broccoli microgreen extract production

Pre-harvest factors, including light, temperature, nutrition, water, and phenological stage are important to provide GLS-rich extracts.[64] Broccoli microgreens used in the present study were obtained under the growth conditions summarized in Table 1 ('Special extract of URIACH Broccoli', URIACH, Italy).

Broccoli microgreens were obtained according to the following methodology: microgreens were initially dried at a low temperature in order to be able to ensure a longer shelf life and to inactivate the activity of the enzyme myrosinase; then, were pulverized, recovering the fraction with particle size lower than 250 μm. Extractions were performed by adding 20 mL of

Table 1 Parameters of cultivation

Parameter	Details
Temperature	25 °C
Light spectrum	Cultipharm (cool white – warm white – red)
Photoperiod	16 h light – 8 h dark
Humidity	100% (first week) – 55% (from the second week)
Type of irrigation	ebb and flow
Planting sixth (no. of seeds/unit area)	1: a decrease in GLS synthesis occurred with an increase in the number of seeds per unit area
Timing of plant cutting	14 days
Varieties of the plant	Naxos, triton, and parthenon

80 : 20 MeOH : water (at 20 °C), exploiting MeOH for preserving GLS content, to 0.4 g of pulverized sample and sonicating for 2 h at room temperature (Vevor TH-100A; 40 kHz; 600 W). The samples were centrifuged (3300*g*; room temperature; 10 min). The supernatants were then filtered through a 0.45 μm syringe filter and injected into the HPLC system. The concentration of total GLS was determined by integration of the areas of the peaks of interest by construction of an external-standard calibration line with range 1–160 mg kg^{-1} with 250 nm reading, by using GRA as external standard. The Shimadzu LC 2040c HPLC system (SHIMADZU EUROPA GmbH, Duisburg, Germany) was coupled to a 250 × 2.1 mm C18 Reproshell ODS-1 column (Dr Maisch, Ammerbuch, Germany) with a 5 μm particle size. The flow rate of the mobile phase was set at 0.2 mL min^{-1}, while the chromatographic run, which has undergone several steps of improvement, in its final version was characterized by a linear gradient between two different mobile phases (mobile phase A: dH_2O, formic acid 99.9 : 0.1; mobile phase B: dH_2O, acetonitrile, formic acid 69.9 : 30.0 : 0.1). GLS were quantified as 8.68%.

Cell isolation and culture

In vitro cells cultures were performed using commercial hADSCs obtained from 4 different patients (Lonza, Visp, Switzerland; Lot numbers: 22TL108471, 22TL108500, 20TL027210, 19TL261894), and cultured in α-MEM 15% FBS (Thermofisher Waltham, US; Euroclone, Pero, Italy) at 37 °C, 5% CO2 and 95% O2. Medium was replaced twice per week and cells were expanded until passage 2 by using trypsin/EDTA solution 0.25% (Biochrom, Berlin, Germany).

Cytotoxicity assays

A stock solution of broccoli microgreen extract was diluted to obtain a GLS content ranging between 5 and 100 μM (Table 2), in accordance to the literature and previous experiments performed by us.[44,63] Notably, these samples contain MeOH at concentrations between 0.009% and 1.8% v/v. MeOH has been reported to be cytotoxic at concentrations above 5% v/v.[65] Therefore, to assess whether MeOH concentrations exceeding this threshold affect cell viability, two broccoli microgreen extract samples containing 4.5% and 9% v/v MeOH (corresponding to GLS concentrations of 250 and 500 μM, respectively) were prepared as shown in Table 2. Control samples treated with MeOH alone at the same concentrations present in the broccoli microgreen extract preparations were included to evaluate any direct effects of MeOH on cell viability (Table 2).

Table 2 Amount of GLS and MeOH within different dilution of broccoli microgreen extract

Samples	GLS	% MeOH
5 μM broccoli microgreen extract	5 μM	0.009%
10 μM broccoli microgreen extract	10 μM	0.2%
25 μM broccoli microgreen extract	25 μM	0.4%
50 μM broccoli microgreen extract	50 μM	0.9%
100 μM broccoli microgreen extract	100 μM	1.8%
250 μM broccoli microgreen extract	250 μM	4.5%
500 μM broccoli microgreen extract	500 μM	9%

Cells at passage 2, were seeded at a density of 4×10^4 cells per well in 48-well plates to allow the formation of a half-confluent monolayer to ensure adherence and progression to exponential cell growth. Cells were cultured in the presence or absence of broccoli microgreen extract at different GLS content for 24 and 72 hours at 5% CO_2 and 37 °C. Cells viability was assessed by analysis of lactate dehydrogenase (LDH) using a commercial Cytotoxicity Detection Kit (LDH) (Roche, Mannheim, Germany), following manufacturers' instructions and absorbance reading at 492–620 nm. Cells metabolic activity/proliferation was measured by Alamar blue (Bio-rad, Milano, Italy) by incubation of Alamar blue diluted 1 : 10 for 4 h at 37 °C, followed by absorbance reading at 570–600 nm. Cells morphology was observed at optic microscope (Kaltek, Padova, Italy). Neutral red staining (Santa Cruz Biotechnology, Dallas, Texas) was performed by incubating 1.6% of neutral red 30 min at 37 °C; after having acquired photos, neutral red solution was extracted (solution: 49% deionized water, 50% absolute ethanol, 1% glacial acetic acid; Carlo erba, Milano, Italy) and absorbance was read at 540 nm.

Osteogenic differentiation

For osteogenic assays, hADSCs were seeded at a density of 5×10^4 cells per cm^2 in 12-wells plate and cultured in osteogenic medium (α-MEM 20% FBS supplemented with 100 nM dexamethasone, 100 μM ascorbic acid and 10 mM β-glycerophosphate; Thermofisher, Euroclone, Sigma Aldrich) without phenol red for 14 days (media were replaced twice *per* week); and treated or not treated with broccoli microgreen extract at increasing content of GLS (5, 10, 25, 50, 100 μM broccoli microgreen extract). Duplicate wells were prepared for each experimental condition and for each osteogenesis functional assays. This approach was adopted to account for biological variability arising from independent cell seeding in different wells.

Osteogenesis induction was evaluated using the following approaches:

- Mineral matrix deposition evaluation, assessed by Alizarin-Red staining and subsequent spectrophotometric quantification.

- Alkaline phosphatase enzyme (ALP) enzyme activity assessment, determined by colorimetric assay.

- Expression of genes typically associated with osteoblastic differentiation, using quantitative real-time PCR.

Alizarin red staining

hADSCs cultured for 14 days in osteogenic media with or without broccoli microgreen extract at different GLS content in duplicates, were fixed 15 minutes at RT in formaldehyde (Kaltek) 10% phosphate buffered saline (PBS, Thermofisher), washed twice with PBS, and stained with 40 mM AR-S for 20 minutes. Cells positivity at AR-S staining was observed and

captured at optic microscope (100× of magnifications), Nikon Instruments Europe BV (Amstelveen, the Netherlands). Mineral apposition was further quantified by spectrophotometric measurements at 510 nm with TECAN Infinite® 200 PRO (Tecan Italia S.r.l., Cernusco Sul Naviglio, Italy). As described in our previous studies we captured 177 readings for each well and data from 3 donors were averaged.[44,63,66]

ALP enzyme activity assay

ALP enzyme activity was evaluated in both culture medium and cell lysates at day 14 of control cells or cells treated with broccoli microgreen extract at different GLS content, using the Alkaline Phosphatase Assay Kit (Colorimetric) (Abcam, ab83369, Cambridge, UK), according to the manufacturer's instructions. At day 14 the culture medium was collected and hADSCs were lysed. With regards to cells lysis, cells were homogenized in an adequate volume of ALP Assay buffer I, centrifuged at maximum speed for 15 minutes at 4 °C, and the resulting supernatant was collected for the analysis. Both culture medium and cell lysates were analyzed in duplicate. Culture medium was assessed by reaction with *para*-nitrophenyl phosphate (*p*NPP) substrate solution (1 mM) diluted 1 : 2 while cell lysates were diluted 1 : 18. For the preparation of the standard curve, serial dilutions of *p*NPP were prepared and tested with a commercial ALP enzyme according to the kit instructions. Incubation was performed in the dark at room temperature for 60 minutes, allowing the enzyme to convert the *p*NPP substrate into the colored product *para*-nitrophenol (PNP), which absorbance was read at 405 nm, after addition of stop solution.

Real-time PCR

hADSC at day 14 of control cells or cells treated with broccoli microgreen extract at different GLS content, cultured in duplicate wells, were first lysed using 1 ml of RNA trifast solution (Euroclone); followed by a chloroform-phenol-ethanol (Sigma, Carlo Erba) extraction protocol. Then, RNA was purified from genomic DNA by treatment with DNase I (DNA-free Kit, Ambion, Austin, TX, USA) and cDNA was synthesized by using SuperScript™ VILO™ cDNA Synthesis Kit (Thermofisher) on 2720 Thermal cycler (Applied Biosystem, Life Technologies) run as following: 25 °C for 10 minutes, 42 °C for 60 minutes, 85 °C for 5 minutes and 4 °C for 30 minutes. Real-time polymerase chain reaction (PCR) analysis by using the SYBR Premix Ex Taq (TaKaRa Biomedicals, Tokyo, Japan) was performed to assess mRNA of osteogenic markers (ALP; SMAD Family member 1 – SMAD-1; WNT1-inducible-signaling pathway protein 1 – WISP1; Runt-related transcription factor 2 – RUNX-2; Collagen I – Col I; SRY-box transcription factor 9 – SOX-9; Distal-Less Homeobox 5 – DLX-5) and antioxidant markers (Nuclear Factor Erythroid 2-related factor 2 – NRF-2; NAD(P)H quinone oxidoreductase 1 – NQO1; Peroxiredoxin-1 – PRDX-1), using LightCycler Instrument (Roche) as following: one cycle at 95 °C for 10 seconds and 45 cycles at 60 °C for 20 seconds and at 95 °C for 5 seconds. Standard melting curve analyses were performed to confirm the specificity of the PCR product (at 95 °C for 10 seconds, 65 °C for 15 seconds and 95 °C in one-degree increments). PCR products were relatively quantified as compared to the housekeeping mRNA expression of glyceraldehyde-3 phosphate dehydrogenase (GAPDH) by using the comparative CT method. The sequences of the primers purchased from Life Technologies Italia are summarized in Table 3. SOX-9 and DLX-5 primers were purchased from Bio-Rad (Hercules, CA, USA); their sequences are not publicly disclosed by the manufacturer, given that are proprietary.

cDNA samples were derived using RNA independently isolated from duplicate culture wells established for each hADSC donor and experimental condition. Therefore, duplicate cDNA samples obtained from the same hADSC donor and condition

Table 3 List of primers sequences

Gene	Protein		5′-Sequence-3′	Product size (bp)	Accession number
GAPDH	Glyceraldehyde-3 phosphate dehydrogenase	FW	*CGGAGTCAACGGATTTGG*	218	NM_002046
		REV	*CCTGGAAGATGGTGATGG*		
ALP	Alkaline phosphatase	FW	*GGAAGACACTCTGACCGT*	152	NM_000478
		REV	*GCC CAT TGC CAT ACA GGA*		
SMAD1	SMAD family member 1	FW	*CACCCGTTTCCTCACTCTCC*	257	NM_005900
		REV	*TCCTCATAAGCAACCGCCTG*		
WISP1	WNT1-inducible-signaling pathway protein 1	FW	*ACACGCTCCTATCAACCCAAG*	103	NM_003882
		REV	*CATCAGGACACTGGAAGGACA*		
RUNX-2	Runt-related transcription factor 2	FW	*GGAATGCCTCTGCTGTTATG*	105	NM_001369405.1
		REV	*AGACGGTTATGGTCAAGGTG*		
COL I	Collagen I	FW	*CCTGGATGCCATCAAAGTCT*	170	NM_000088
		REV	*CGCCATACTCGAACTGGAAT*		
NRF-2	Nuclear factor erythroid 2-related factor 2	FW	*GCCCAGCACATCCAGTCA*	153	NM_006164.5
		REV	*CGTAGCCGAAGAAACCTCATT*		
NQO1	NAD(P)H quinone oxidoreductase 1	FW	*ACCTTGTGATATTCCAGTTCCCC*	108	NM_000903.3
		REV	*TGGCAGCGTAAGTGTAAGCA*		
PRDX-1	Peroxiredoxin-1	FW	*TTGAACCCCAAGCTGATAGGAA*	176	NM_002574.4
		REV	*CACAAAGGTGAAGTCAAGAGGG*		

FW: forward primer; REV: reverse primer.

do not represent technical PCR replicates of a single cDNA preparation, but rather independent biological samples that incorporate variability associated with cell culture, sample processing, RNA extraction, reverse transcription, and PCR amplification.

Statistical analysis

GraphPad Prism 7 (La Jolla, California, USA) was used for statistical analysis. Before each test, the presence of outliers was checked by ROUT (Q = 1%) test. Outliers were removed from each data set when present. D'Agostino & Pearson normality test was performed to analyze the normality of our data. We performed a Kruskal–Wallis test followed by Dunn's multiple-comparison *post hoc* test. Significance was attributed when $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****).

Results

Effect of broccoli microgreen extract on cells viability and metabolism

Fig. 1 show analysis of cell cytotoxicity (a–c) and cell proliferation (d–f) at 24 (a and d), 72 h (b and e) and D7 (c and f) of culture. Data were obtained by respectively performing LDH assay, which detects LDH release into the culture medium by cell death and subsequent rupture of the cytoplasmic membrane; and ALAMAR blue assay, which assesses the metabolic activity of cells, by measuring the chemical reduction of Alamar blue by proliferating cells. Broccoli microgreen extract containing GLS concentrations up to 25 μM did not significantly affect cell viability or proliferation at any time point. Exposure to 50 μM and 100 μM broccoli microgreen extract did not impair proliferation; however, these concentrations induced a modest but statistically significant increase in LDH release (approximately 10–15%), observed exclusively at 24 h for 50 μM and at all time points for 100 μM. According to ISO 10993-5:2009, these increases remain below the 30% threshold and are therefore not considered cytotoxic.[67] In contrast, higher broccoli microgreen extract concentrations of 250–500 μM showed a significant toxicity as early as 24 h. Moreover, 250 μM broccoli microgreen extract showed a time dependent decrease in proliferation (ranging between 50–70%), statistically significant at 72 h and D7; while 500 μM broccoli microgreen extract showed a dramatic significant decrease in proliferation as early as 24 h (around 93%). Notably, 250 μM and 500 μM broccoli microgreen extract contained approximately 4.5% and 9% (v/v) MeOH, concentrations previously reported to be cytotoxic above 5% (v/v).[65] Control experiments with MeOH alone showed no effects on viability or proliferation at concentrations up to 0.9% (v/v). A slight but statistically significant increase in LDH release was observed at 1.8% (v/v) MeOH at 72 h and day 7, still below the threshold of cytotoxicity. Higher MeOH concentrations (4.5% and 9% v/v) induced significant increases in cytotoxicity and decreases in proliferation, but these effects were less pronounced than those observed with the corresponding broccoli microgreen extract treatments (containing 4.5% and 9% v/v of MeOH).

In a limited set of samples, we then assayed early cytotoxicity by neutral red staining assay (Fig. 2). This assay confirmed a significant drop in viability at 250–500 μM broccoli microgreen extract, only partially attributable to the presence of MeOH. Fig. 2a shows some representative images of cells stained with neutral red dye, which is retained by lysosomes only in viable cells. CTRL cells and cells treated with 0.9–1.8% v/v MeOH or 50–100 μM broccoli microgreen extract displayed a cell monolayer of fibroblastoid, confluent and viable (red) cells. 4.5% v/v MeOH and 250 μM broccoli microgreen extract showed reduced cell density with still viable cells; while 9% v/v MeOH and 500 μM broccoli microgreen extract showed a reduced number of cells which were mostly non-viable. Quantification of the red dye extracted from the cells (Fig. 2b) confirmed a significant cytotoxicity at 250–500 μM broccoli microgreen extract (between 48 and 70%); while MeOH showed a dose-dependent decrease, statistically significant and cytotoxic only at the highest concentration (40% decrease).[67]

In the light of these experiments, we identified the concentration of 100 μM as the maximum non-cytotoxic concentration of the broccoli microgreen extract in our experimental setting. Therefore, all functional experiments were performed using broccoli microgreen extract at concentrations not exceeding 100 μM.

Effect of broccoli microgreen extract on matrix mineralization

hADSCs were induced toward osteogenesis in the presence or absence of increasing concentrations of GLS within broccoli microgreen extract and were assayed by alizarin red staining, the 'gold standard' of *in vitro* functional assays for screening the ability of molecules or substances to stimulate osteogenic differentiation of cells. Experimental samples were collected after 14 days of stimulation. Fig. 3a shows representative images of cells labeled by Alizarin red at D14. Fig. 3b shows the spectrophotometric quantification of the mineral matrix produced by 3 cells donors. Data suggests a clear stimulatory effect (greater than 15%) of broccoli microgreen extract starting at concentration 25 μM of GLS, with a statistically significant dose-dependent increase (more than 50–100%) at the highest concentrations (50–100 μM broccoli microgreen extract).

Effect of broccoli microgreen extract on expression of osteogenic genes

Data on expression of osteogenic genes showed an up-regulation trend for the majority of targets (Fig. 4a, c, d and f–h): Wnt-1-inducible signaling pathway protein 1 (WISP-1), Mothers against decapentaplegic homolog 1 (SMAD-1), ALP, (d) collagen I (COL I), Distal-Less Homeobox 5 (DLX-5), SRY-Box Transcription Factor 9 (SOX-9), Runt-related transcription factor 2 (RUNX-2), RUNX-2/SOX-9. Notably, 100 μM broccoli microgreen extract induced a statistically significant up-regulation of SMAD-1; while 50 μM broccoli microgreen extract

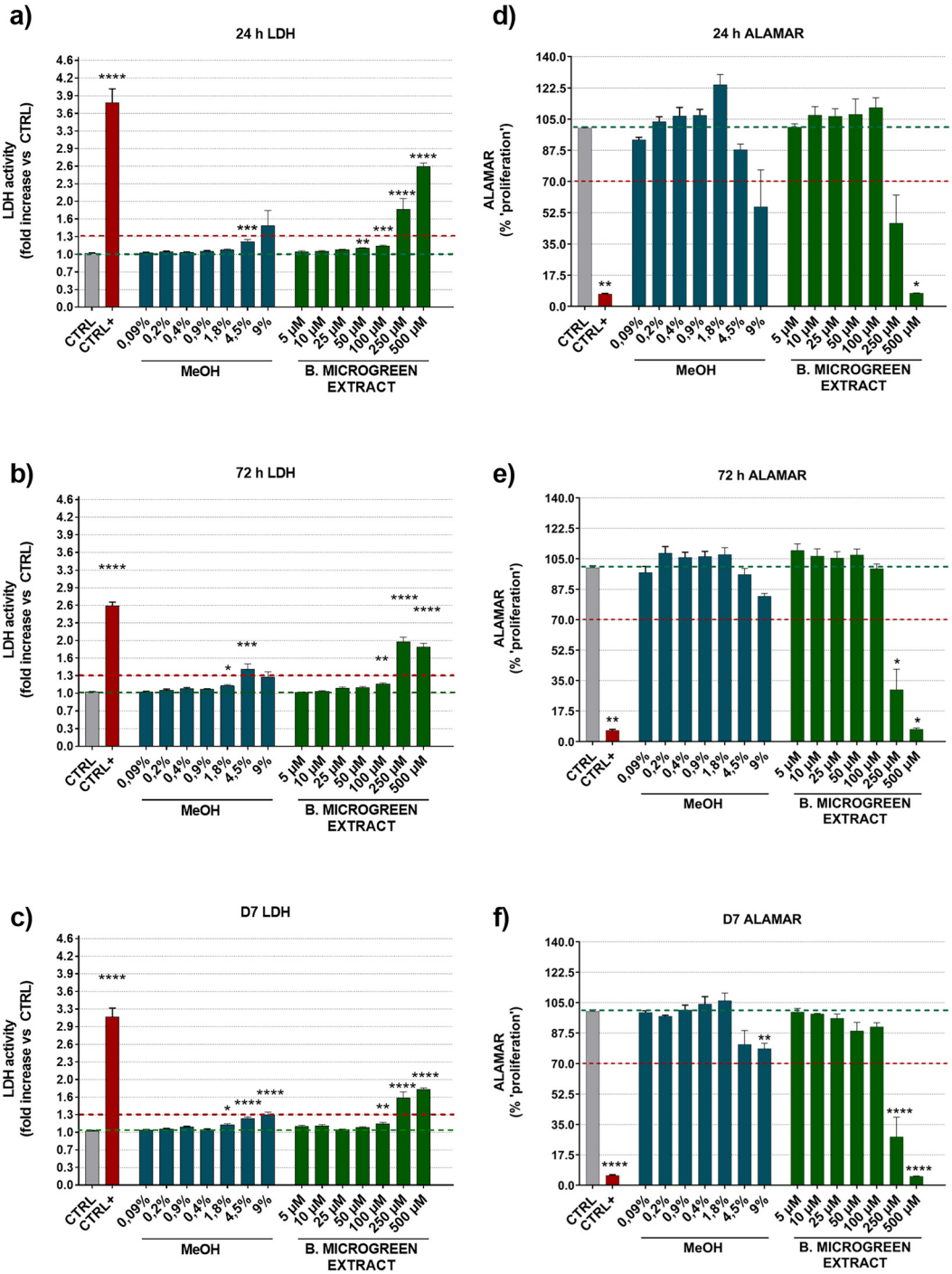

Fig. 1 Effect of broccoli (B.) microgreen extract and MeOH on cell viability (LDH) and proliferation (ALAMAR blue). Histograms showing the levels of LDH activity (a–c) and ALAMAR blue metabolism (d–f) at 24 h (a and d), 72 h (b and e), D7 (c and f) measured on control-untreated cells (CTRL, gray), cells treated with tryton X-100 to induce mortality (CTRL+, red), treated with MeOH at the % corresponding to what was present in the broccoli microgreen extract (0.09–9%, in blue), treated 5 µM–500 µM broccoli microgreen extract (green). $N = 3$ hADSC donors, technical triplicates analysis of colorimetric reaction was performed for each donor. Red line 30% increase (a–c) or decrease (d–f) *vs.* CTRL. Mean ± broccoli microgreen extract. Statistical test performed: Kruskal–Wallis test followed by Dunn's multiple-comparison *post hoc* test. *$p < 0.05$, **$p < 0.01$; ***$p < 0.001$, $p < 0.0001$.

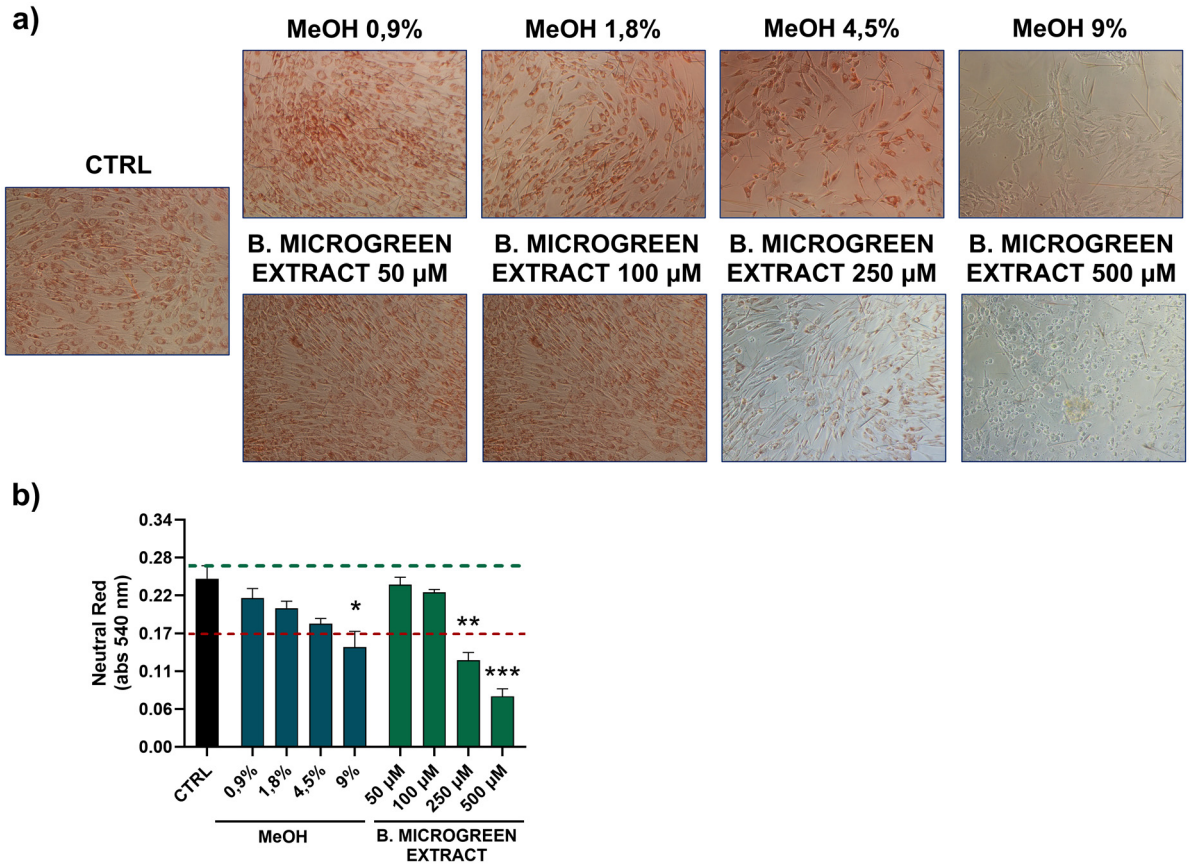

Fig. 2 Effect of broccoli (B.) microgreen extract and MeOH on early cytotoxicity (neutral red stain). (a) Representative images of neutral red stain (100× magnification). (b) Histogram showing neutral red uptake quantification at 72 h of control cells (CTRL, black) and cells treated with MeOH 0.4–9% v/v (blue) or with 50–500 µM broccoli microgreen extract (green). Red line 30% decrease *vs.* CTRL. $N = 2$ $N = 3$ hADSC donors, technical triplicates analysis of colorimetric reaction was performed for each donor. Mean ± SEM. Statistical test performed: Kruskal–Wallis test followed by Dunn's multiple-comparison *post hoc* test. *$p < 0.05$, **$p < 0.01$; ***$p < 0.001$.

induced a statistically significant up-regulation of DLX-5 (Fig. 4b and e).

Effect of broccoli microgreen extract on ALP activity

Evaluations were performed on both cell lysates and supernatants, in order to evaluate defosphorylated *p*NPP substrate abundance and ALP activity in both the intracellular and secreted fractions. Fig. 5 shows amount of *p*NPP substrate defosphorylated by ALP enzyme (a and b) and ALP enzyme activity (c and d) in both cell lysates (a and c) and supernatants (b and d). The amount of dephosphorylated *p*NPP produced in the cell lysates of broccoli microgreen extract is significantly increased compared to the CTRL for all concentrations, peaking at 350% (Fig. 5a). The amount of dephosphorylated *p*NPP produced in the supernatant shows a statistically significant increase in all broccoli microgreen extract-treated samples by about a 30% (Fig. 5D ii). ALP activity in the cell lysates is significantly increased compared to the CTRL for broccoli microgreen extract samples at all concentrations, reaching a peak of 350% relative increase (Fig. 5C). ALP enzyme activity in the supernatants show a statistically significant increase only in the 25–50–100 µM broccoli microgreen extract of about a 30% (Fig. 5D).

Effect of broccoli microgreen extract on the expression of NRF-2-dependent antioxidant genes

High levels of ROS reduce osteoblast activity and differentiation while antioxidants contribute to activate osteogenesis and mineralization either directly or by counteracting the action of oxidants.[68]

Phytochemicals belonging to the *Brassicaceae* family has shown to modulate anti-oxidant response;[69,70] however the role of these molecules during osteogenic differentiation is largely unknown. Here, to investigate whether the raw Broccoli microgreen extract retains the capacity of specific individual bioactives to trigger the activation of antioxidant response, we evaluated the expression of NFR2 mRNA and two downstream markers of NRF2 activation, NQO1 and PRDX-1. Here we found that broccoli microgreen extract induced a statistically significant but not dose response up-regulation of NRF2 (100 µM of broccoli microgreen extract; $p < 0.05$). NRF2 acts by activating antioxidant genes through binding to ARE elements on DNA, after its detachment from KEAP1, translocation into

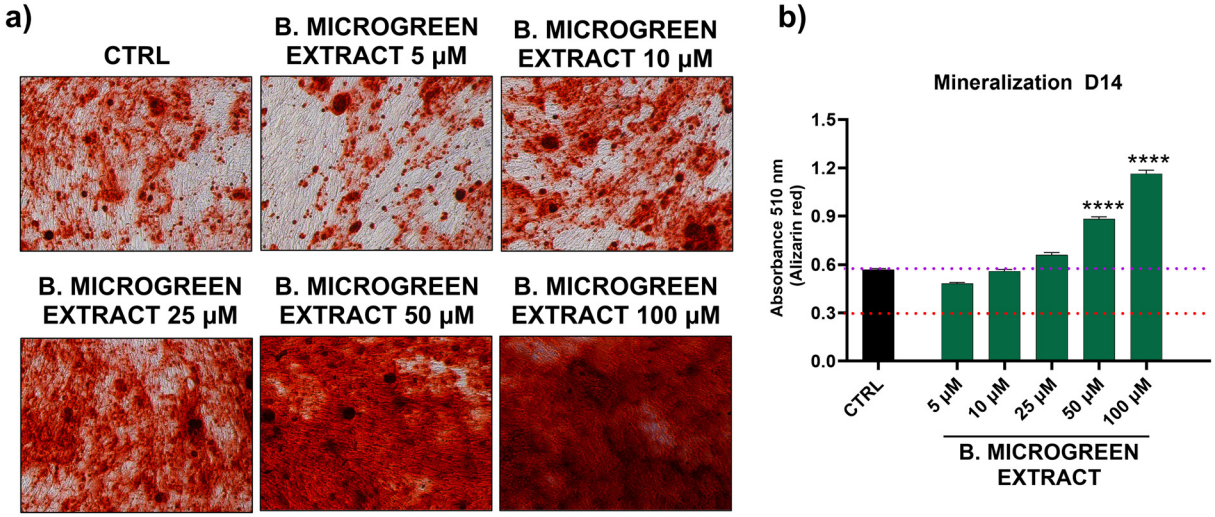

Fig. 3 Effect of broccoli (B.) microgreen extract and MeOH on mineralization. (a) Representative images of Alizarin red stain positivity (100× magnification). (b) Histogram showing mineral quantification at D14 of control cells (CTRL, black) and cells treated with Broccoli extract with 5–100 μM GLS (green). Red line: threshold of no mineralization; violet line: threshold of high mineralization. N = 3 hADSC donors. For each donor, biological duplicates were generated by seeding cells in independent culture wells, thereby accounting for biological variability associated with the seeding process. 177 readings for each sample. Mean ± SEM. Statistical test performed: Kruskal–Wallis test followed by Dunn's multiple-comparison *post hoc* test. ****$p < 0.0001$.

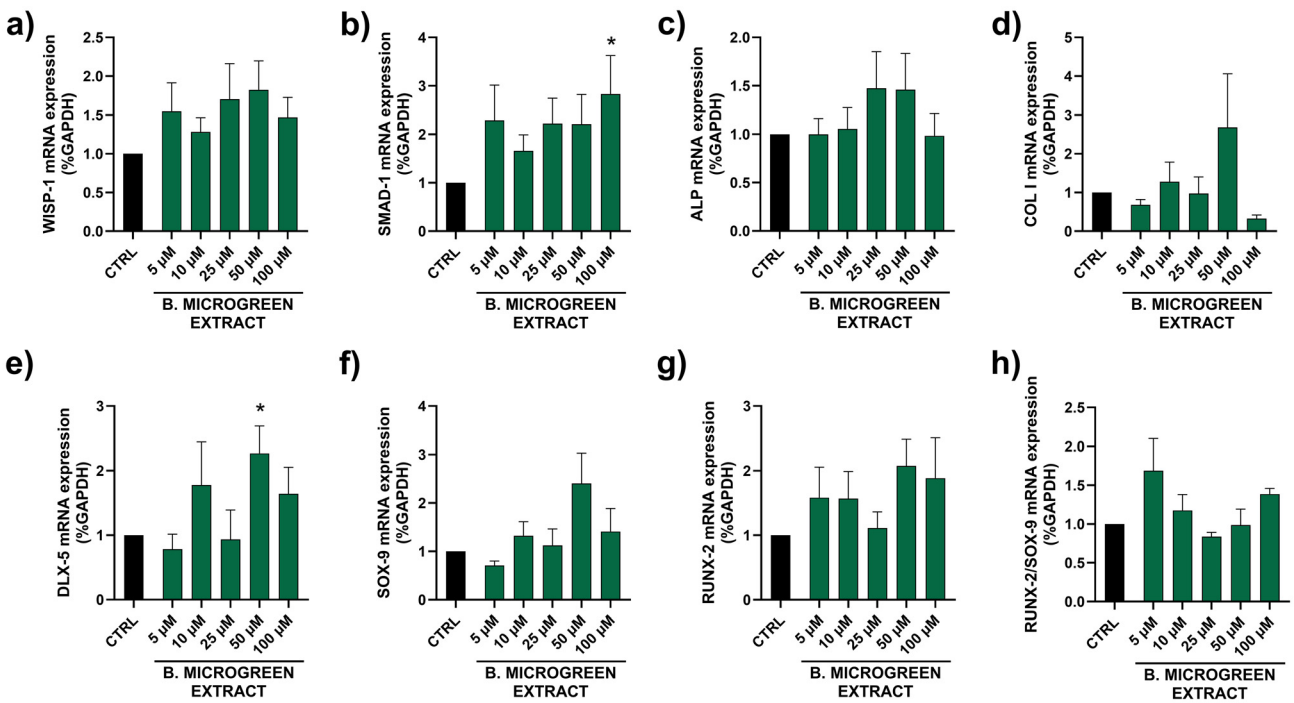

Fig. 4 Effect of broccoli (B.) microgreen extract in the modulation of osteogenic genes: histograms showing gene expression levels of osteogenic markers obtained at day 14 (D14) of control cells (CTRL, in black) or cells treated with with 5–100 μM broccoli microgreen extract (in green). (a) WISP-1, (b) SMAD-1, (c) ALP, (d) COL I, (e) DLX-5, (f) SOX-9, (g) RUNX-2, (h) RUNX-2/SOX-9. N = 3 hADSC donors. For each donor, biological duplicates were generated by seeding cells in independent culture wells, thereby accounting for biological variability associated with the seeding process and technical variability introduced during lysis, extraction, reverse transcription, and amplification (not technical replicates of amplification of each donor cDNA). Mean ± SEM. Statistical test performed: Kruskal–Wallis test followed by Dunn's multiple-comparison *post hoc* test. $p < 0.05$.

the nucleus, and association with partner proteins. Notably, broccoli microgreen extract induced the statistically significant increase of both NQO1 and PRDX-1 expression, suggesting a likely increased nuclear translocation of NRF2 protein. 5 and 50 μM of broccoli microgreen extract induced a statistically significant increase of NQO1 ($p < 0.05$). 5 and 100 μM of broccoli

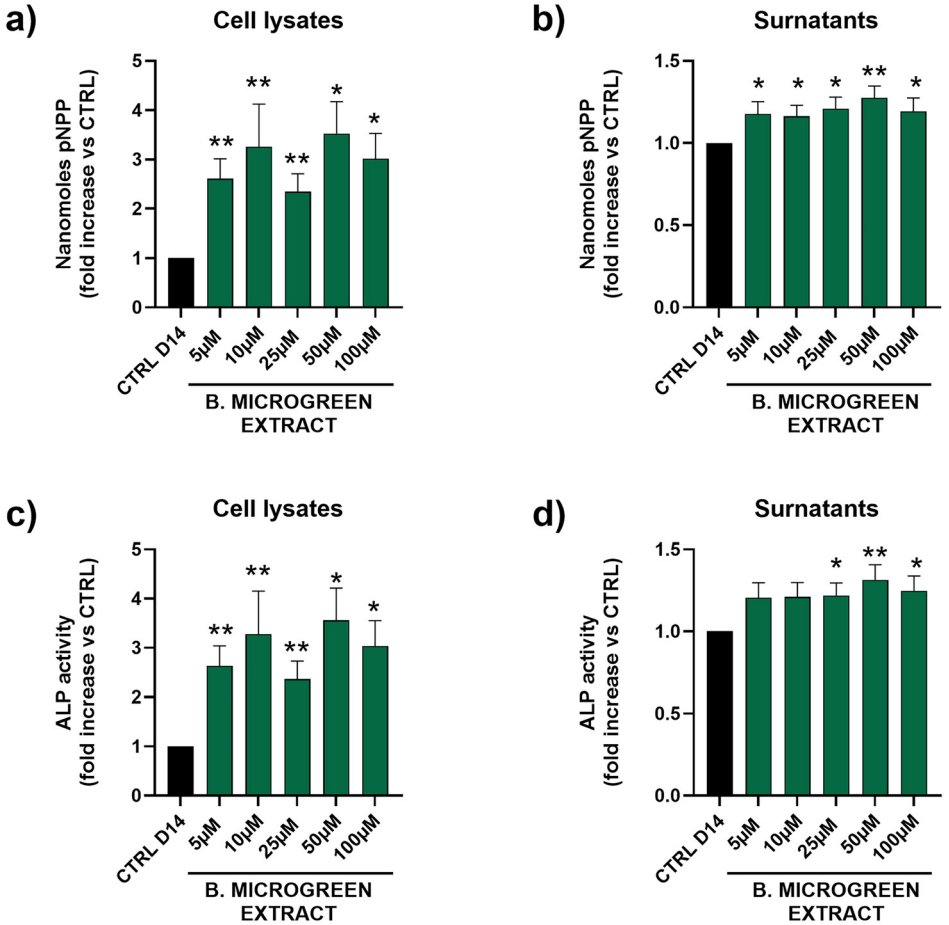

Fig. 5 Effect of broccoli (B.) microgreen extract on ALP enzyme activity: Histograms showing *p*NPP substrate defosphorylated by ALP enzyme (a and b) and ALP enzyme activity (c and d) as expressed as fold increases compared with CTRL at day 14 (D14) in cell lysates (a and c) or cell supernatants (b and d). Bars in black indicate control samples at day 0 (D0) and day 14 (D14), those in green the samples treated with Broccoli extract with 5–100 μM GRA. $N = 3$, technical triplicates analysis of colorimetric reaction was performed for each donor. Mean ± SEM. Statistical test performed: Kruskal–Wallis test followed by Dunn's multiple-comparison *post hoc* test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

microgreen extract induced a statistically significant increase of PRDX-1 ($p < 0.05$) and 25 μM of broccoli microgreen extract induced a statistically significant increase of PRDX-1 ($p < 0.01$) (Fig. 6).

Discussion

The present study demonstrates, for the first time, that a methanolic extract obtained from hydroponic cultures of broccoli microgreens, rich in GLS, exerts osteoinductive effects on hADSCs. These results provide experimental support for the hypothesis that phytochemicals derived from broccoli microgreens may promote bone formation and support further investigation of broccoli microgreen-derived compounds as candidate nutraceuticals for bone health.

Osteoporosis is characterized by deterioration of bone microarchitecture, reduced bone mass and increased risk of fracture. Within the context of the modifiable, lifestyle habits which can improve bone health in patients at risk of bone fracture, a greater dietary intake of plant-derivatives as functional foods/crops has emerged as an effective way to improve bone health.[66,67,71,72] Preclinical evidence pointed to dietary organosulfur compound as emerging players in the regulation of bone homeostasis.[9] Among them, we recently showed that *Lepidium Sativum* and *Eruca Sativa* extracts, and several GLS (GRA, benzylglucosinolate, glucoerucin), stimulates the osteogenesis of human mesenchymal stromal cells.[44,63] However, to the best of our knowledge there is no published study on broccoli microgreens or broccoli sprout extracts in pre-clinical or clinical studies related to osteogenesis or osteopenia-osteoporosis.

Edible microgreens and broccoli sprouts has gained increasing attention as a new potential functional food and germination is considered a simple and inexpensive method for improving their nutritional value.[73,74] The specific culture system represents an additional variable of potential importance for optimizing the phytochemical potential of broccoli microgreens.[24,73,75,76] The hydroponic vertical cultivation

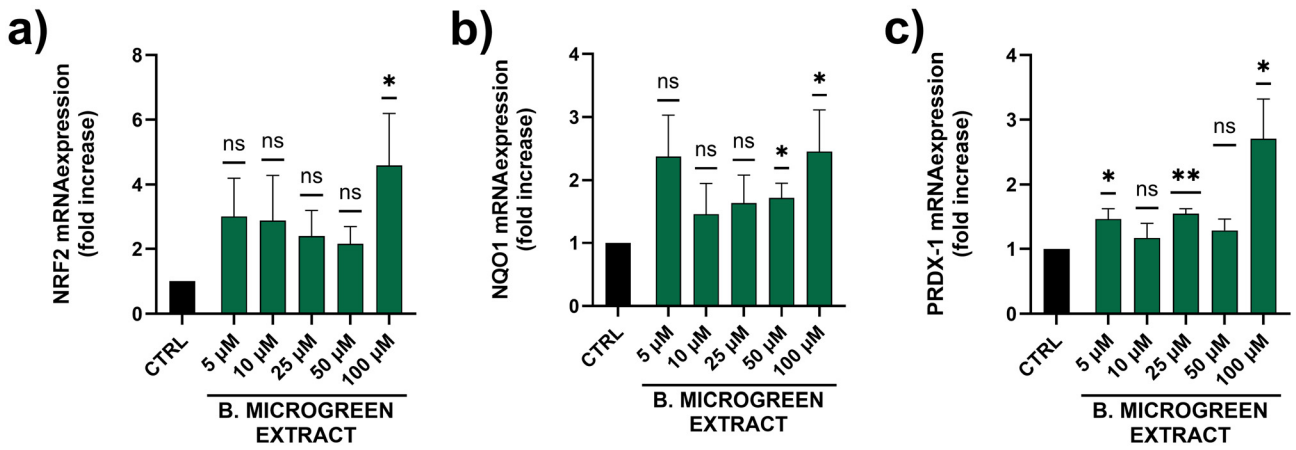

Fig. 6 Effect of broccoli (B.) microgreen extract in the modulation of antioxidant genes: Histograms showing gene expression levels of osteogenic markers obtained at day 14 (D14) of control cells (CTRL, in black) or cells treated with with 5–100 μM broccoli microgreen extract (in green). (a) NRF-2, (b) NQO1, (c) PRDX-1. N = 3 hADSC donors. For each donor, biological duplicates were generated by seeding cells in independent culture wells, thereby accounting for biological variability associated with the seeding process and technical variability introduced during lysis, extraction, reverse transcription, and amplification (not technical replicates of amplification of each donor cDNA). Mean ± SEM. Statistical test performed: Kruskal–Wallis test followed by Dunn's multiple-comparison *post hoc* test. $p < 0.05$.

employed here may also have influenced the bioactive profile of broccoli microgreen extract, as controlled environmental conditions can enhance GLS accumulation and secondary metabolite synthesis.[77,78] Indeed, the protocol used is in accordance to previous study which showed that 16 h light/8 h dark photoperiod is preferrable to growth dark conditions for increasing phytochemical composition of broccoli sprout extract,[73] especially GLS.

A remarkable finding from this work is the strong stimulus for osteogenic differentiation that emerges from the *in vitro* mineralization assay of hADSCs in the presence of broccoli microgreen extract. The robust and dose-dependent increase in mineralized matrix—doubling Alizarin Red staining at the highest concentration in respect to control cells—demonstrates that broccoli microgreen extract provides a potent anabolic stimulus. This was corroborated by a significant upregulation of ALP activity, both intracellular and in the culture supernatants. ALP is a widely recognized early marker of osteoblast differentiation, catalyzing phosphate liberation necessary for hydroxyapatite formation. At the level of gene expression, SMAD-1 and DLX-5 were significantly up-regulated, while RUNX-2, COL I, and WISP-1 exhibited upward trends. These transcription factors and signaling mediators play pivotal roles in osteogenesis. SMAD-1 is a downstream effector of BMP signaling that drives the transcription of RUNX-2, the master regulator of osteoblast differentiation.[79,80] DLX-5 acts upstream of RUNX-2 to promote osteogenic gene networks,[81] while WISP-1 enhances extracellular matrix deposition and mineralization.[82,83]

Thus, our results indicate that broccoli microgreen extract activates canonical osteogenic pathways, potentially through modulation of the BMP–SMAD–RUNX axis.

Notably, gene expression analysis demonstrated that Broccoli extract exerts antioxidant effects during osteogenesis by up-regulating NRF2 signaling. The relationship between NRF2 and osteogenesis remains controversial.[84] By showing activation of NRF2-mediated gene transcription without impairing osteogenic differentiation, our findings are coherent with studies reporting a positive role of NRF2 signaling during osteogenesis. However, functional assays are required to conclusively determine the role of NRF2 pathway activation in this process. Moreover, the phytochemicals involved in NRF2 activation remain to be established. GLS are not electrophilic and are therefore unlikely to activate NRF2 through the same mechanism as the isothiocyanate SFN, a well-established activator of NRF2.[85] Future studies should investigate whether NRF2 activation is mediated by GLS through alternative signaling pathways, by GLS-derived metabolites, or by other phytochemicals present in the extract. Beyond its role during osteogenesis, activation of NRF2 signaling could also be relevant in osteoporosis, where estrogen deficiency contributes to increased oxidative stress.[68]

Given the anabolic and anti-oxidative activity displayed by broccoli microgreen extract, they could be further investigated as an alternative to the use of raw broccoli (possibly overcoming some of their limitations) to counteract bone loss. Broccoli microgreen extract are GRA-rich extracts, and we speculate that GRA could have contributed to their biological activity. However, other investigations should be performed to analyze the interplay with other phytochemical constituents. In this line, the observation that the whole extract showed a slightly different efficacy profile than pure GRA from our previous studies is noteworthy. Indeed, in our previous study GRA alone increased the amount of mineralized matrix beginning at the concentration of 3.3 μM,[44] while in this work broccoli microgreen extract required concentrations over 10-times greater to induce a similar outcome. This suggests a potential phytochemical "interference" effect. While the extract contains high concentrations of GRA, it also contains a spectrum of other

phytochemicals such as polyphenols and flavonoids.[86,87] These compounds may modulate different branches of the Wnt or BMP signaling pathways, potentially explaining why concentrations below 50 μM failed to induce significant stimulation in functional assays. Interestingly, we observed a similar trend when studying *Lepidium sativum* and *Eruca sativa*. *Lepidium sativum* induced osteogenesis with lower efficacy than its major constituent in GLS, benzylglucosinolate; while *Eruca sativa* induced osteogenesis with greater potency than its major constituent in GLS, glucoerucin.[63] These data suggested a partial inhibitory effect of other constituents for *Lepidium sativum* and stimulatory effect for *Eruca sativa*. Despite this variability, it is noteworthy that bioactives derived from several *Brassicaceae*, either in the form of complex extracts or as isolated molecules (members of the GLS or ITC families), tend to deliver similar stimulative effects to the differentiation of osteoprogenitor cells. One potential unifying hypothesis for these findings is the capacity of GLS and their hydrolytic catabolites ITCs to release in the microenvironment hydrogen sulfide (H_2S), a gasotrasmitter that can stimulate osteogenic differentiation *in vitro* and bone formation *in vivo*.[44,63,88] Notably, H_2S has been reported to activate NRF2 signaling through KEAP1 persulfidation and other redox-sensitive pathways,[89] raising the possibility that sulfur metabolism contributes to NRF2 transcriptional response observed following broccoli extract treatment. Moreover, recent evidence showed that sulfide-dependent signaling operates through mitochondrial sulfide oxidation to shape intracellular redox tone and cellular fate decisions in osteoprogenitor cells, leading an osteogenic permissiveness with mechanisms involving NRF2 activation.[90] Although speculative, these observations suggest a potential link between NRF2 activation in the context of enriched H_2S stimulation and osteogenic differentiation that merits further investigation.

In addition, the contribution of SFN resulting from the enzymatic activity of gut thioglucosidases on GRA *in vivo* may provide additional benefit to osteogenesis; future studies may address this topic *in vitro* by adding myrosinase to the extracts or *in vivo* in animal models of pathological bone loss or in clinical studies targeting postmenopausal osteoporosis.

Our study holds some limitations. First, the study was restricted to short-term *in vitro* assays, which do not replicate the complexity of bone remodeling or systemic metabolism. Moreover, the use of a crude plant extract implies that numerous phytochemicals are present in the cell culture system in addition to GLS. Consequently, the observed biological effects cannot be attributed exclusively to GLS, and potential synergistic or antagonistic interactions among extract constituents may have influenced the results. Because individual bioactive components (beyond GLS content) were not quantified, it is not possible to assess correlations between specific molecules and osteogenic potency. Among potential bioactives, dietary fiber, essential components of a healthy diet, which exhibit antioxidant activity and contribute to the prevention of several chronic diseases,[91] were likely present only in negligible concentration given that the extraction procedure used in the present study was optimized for GLS recovery rather than for the isolation of fibers. Further studies employing purified GLS, their hydrolysis products, or fractionated extracts are needed to identify the compounds responsible for the observed activity and clarify their mechanisms of action.

Future implications of this study extend to the perspective of a personalized nutrition approach in patients at greater risk of developing bone fractures. Vegetables belonging to the family of *Brassicaceae*, rich in GLS and ITC, are ideal candidates for dietary regimens aiming at improving bone health. Attesting the potential impact of this hypothesis, a prospective study evaluating post-menopausal Australian women aged 70 or more suggested that a higher consumption of cruciferous vegetables (such as broccoli) is associated with lower incidence of fractures.[92] Products derived from broccoli microgreens could hold additional value in this field.

Conclusions

In conclusion, GLS-rich methanolic extracts of broccoli microgreens cultivated *via* hydroponic vertical cultivation exhibit significant osteoinductive and antioxidants properties on h-ADSC. The extract enhanced ALP activity, matrix mineralization, and expression of osteogenic genes, supporting the rationale for performing future study to assess the potential of broccoli microgreen derived products as a functional ingredient for bone health. These results contribute to the growing evidence that dietary *Brassicaceae* phytochemicals can modulate osteogenesis and suggest potential novel applications of broccoli microgreen-based nutraceuticals in the prevention or management of osteoporosis.

Conflicts of interest

Authors declare no conflicts of interest.

Data availability

The data supporting this study are available within the article.

Acknowledgements

This study was supported by URIACH Italy Srl. The funder had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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