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Green propolis extract promotes *in vitro* proliferation, differentiation, and migration of bone marrow stromal cells

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ABSTRACT

Background: Propolis is a resinous material extracted from bee glue with a complex chemical composition. The unique biological properties of propolis have led to its use in alternative medicine and as a nutritional supplement. Recent research shows that propolis could affect the immune system by decreasing the production of inflammatory cytokines and potentiating an effect on resident stem cells. The exact mechanism, however, is unknown. The goal of this study was to demonstrate whether green propolis extract affects any characteristic properties of mesenchymal stromal cells (MSCs)*in vitro*.

Methods: The cytocompatibility of propolis extract and the proliferation of bone marrow mesenchymal stromal cells (BMMSCs) in the presence of propolis was evaluated by live/dead cell staining and MTS viability assay over a period of 3 days. Also, we evaluated the effect of propolis extract on trilineage differentiation and migration capacity of undifferentiated and differentiated BMMSCs.

Results: Relative to the control, propolis extract resulted in a significant and linear increase in the proliferation of MSCs and inhibited the osteogenic differentiation of BMMSCs, while there was a potentiation of chondrogenesis and adipogenesis. Finally, in relevance to wound healing, an *in vitro* scratch assay demonstrated that the migratory potential of differentiated BMMSCs was enhanced in the presence of propolis.

Conclusion: We have demonstrated that propolis extract was not toxic to BMMSCs ($< 400\,\mu g/ml$), supported their proliferation, potentiated chondrogenic and adipogenic differentiation processes, and supported cell migration*in vitro*. Most interestingly, there was a down-regulation of osteogenesis. These data support the use of propolis extract for enhanced cell proliferation and tissue regeneration; however, it warrants further investigation.

1. Introduction

Natural extracts represent a promising source of alternative medicine for many degenerative diseases. Their effect is mediated by different factors, including their capacity to recruit stem cells, to increase their proliferation, to migrate, and to enhance the differentiation of endogenous stem cells [1–3]. Propolis is classified as a natural extract from honeybee glue. It has been proven to have several positive biological effects, such as being anti-inflammatory, anti-ulcerative, antibacterial, anti-oxidant, anti-tumor, and immunomodulatory, which favors its use in alternative medicine [4–13].

Propolis extracts exhibit complex chemical compositions and have been reported to contain more than 300 organic and inorganic compounds [14,15]. The major chemical components of Brazilian green propolis extracts are prenylated phenylpropanoids, including caffeic acids, cinnamic acids, P-coumoric acid, ferulic acid, and their derivatives. Caffeic acid phenethyl ester (CAPE) is one of the active components of propolis, which has been purified and tested *in vitro* and *in vivo*. CAPE has been shown to be effective for bone and cartilage regeneration [16–18], and some research has shown it to have an anticancer effect [19,20]. Studies have also demonstrated that ferulic acid has a significant effect on nerve regeneration [21,22] and neovascularization [23]. Cinnamic acid and its derivatives have been shown to protect against diabetes [24]. Recently, 9 compounds in the brazilian green propolis were found to possess free radical scavengers. Caffeoylquinic acids and artepillin C exhibited strong antioxidant activity [25]. All of

Abbreviations: CAPE, caffeic acid phenethyl ester; BMMSC, bone marrow mesenchymal stromal cell; MSC, mesenchymal stromal cell; PLF, periodontal ligament fibroblasts; NO, nitric oxide; PBS, phosphate buffered saline; HBSS, Hank's balanced salt solution HBSS; PI, propidium iodide; GAG, glycosaminoglycans

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these components render propolis extracts with a unique bioactive effect. Due to the significant variation in composition, the exact biological function of propolis *in vivo* is still under investigation.

In vitro, propolis extract has been evaluated for its cytotoxicity and regenerative capacity using stem cells derived from dental pulp, exfoliated deciduous teeth [26–28], odontoblast, osteoclasts [29,30], and fibroblasts derived from skin and periodontal ligament [31–33]. Cytotoxicity of propolis has also been tested on macrophages [34–36]. In another study, CAPE was shown to enhance the proliferative capacity of umbilical cord blood-derived hematopoietic stem cells *in vitro* [37].

In addition, propolis has been found to have a protective effect against apoptosis, as it decreased the apoptosis of periodontal ligament fibroblasts (PLF) without any cytotoxicity. These data suggested that propolis could serve as a beneficial storage medium for an avulsed tooth, as it could increase the viability and the physiological capability of PLF [38].

At the site of injury, macrophages are responsible for the induction of inflammation through the production of inflammatory cytokines and nitric oxide (NO), and they participate in tissue regeneration through the production of regulatory cytokines, such as IL10 [39,40]. Propolis was shown to have an inhibitory effect on inflammatory cytokines, such as IL12, IL6, GM-CSF, IFN-ɣ, IL-1β, TNF-α [41–43], and a stimulatory effect on the regulatory cytokines IL4, IL10, and TGF-β [42,43]. Moreover, it has an inhibitory effect on the chemotaxis of CXCL2/MIP-2 and, hence, could potentially inhibit neutrophil migration [44].

In vivo, the oral administration of propolis enhanced the healing of the fractured femur in a rat model, as evaluated by radiology, histology, and bone mineral density [45]. These effects were proposed to be due to the antioxidant effect of propolis. This was further confirmed by measuring the plasma levels of endogenous antioxidants, superoxide dismutase, myeloperoxidase, and glutathione, which were found to be significantly decreased after propolis administration [45]. Another study by Uçan et al. [46] reported that intraperitoneal injection of CAPE, an active component of propolis, in a rat calvarial defect model enhanced the efficiency of bone regeneration.

To our knowledge, there are no reports in the literature that describe the effect of propolis extract on bone marrow-derived mesenchymal stromal cells (BMMSCs). Since bone marrow is one of the preferred sources of mesenchymal stromal cells (MSCs), in this study, we investigated the effect of propolis extract at different concentrations towards proliferation, cytotoxicity, and tri-lineage differentiation of BMMSCs *in vitro*. In addition, we tested tissue regeneration potential of propolis *in vitro* by measuring its effect on the migration capacity of undifferentiated and differentiated MSCs (osteocyte, chondrocyte, and adipocyte).

2. Materials and methods

2.1. Bone marrow mesenchymal stromal cells

The isolation and characterization of BMMSCs is as previously described [47,48]. Briefly, heparinized bone marrow aspirate from goat sternum was obtained according to the protocol approved by the Institutional Animal Care and Use Committee, University of Tennessee (protocol no. 5027). The goats (n = 2) were sedated with detomidine HCl (0.015–0.026 mg/kg bwt IV). The skin at the site of harvest was desensitized by instilling 2% mepivacaine HCl subcutaneously. Ten milliliters of bone marrow were collected from the region between the 4th-5th sternebra under ultrasound guidance and while administrating heparin (300 IU/ml). Flunixin meglumine (1.1 mg/kg bwt IV) was injected to provide analgesia after the bone marrow aspiration. The aspirate was diluted 1:4 with phosphate buffered saline (PBS), layered over Ficoll, and centrifuged for 20 min at 400 *g*. The cells containing the mononuclear fraction at the interface of the PBS and Ficoll were aspirated and washed in PBS by centrifuging for 10 min at 200 *g*. The cell pellet was then suspended and plated on tissue culture polystyrene coated flasks in complete growth media composed of Dulbecco's modified Eagle medium F-12 (DMEM F12), containing 10% fetal bovine serum (Hyclone, Logan, UT, USA) and 1% penicillin/streptomycin solution in 37 °C in the presence of 5% CO_2 (passage 0). Adherent cells were harvested by 0.25% trypsin after they reached 80–90% confluency. Cells were re-plated for further expansion or used in the *in vitro* assays. BMMSCs between passages 3 to 6 were used in all the experiments described below. All experiments were carried out with 2 independent cell lines generated from each of the goats and each *in vitro* assay was performed in triplicate. This resulted in 2 biological replicates and 3 technical replicates used in this study.

2.2. Preparation of the propolis extract solution

Green propolis extract was commercially obtained (Pharma Nectar, Brazil). The extract used in this study was obtained from Baccharis dracunculifolia, a Group 12 Brazilian propolis. Its chemical composition is as described by the manufacturer (Lot# PC0214.1 V). Based on the information from the manufacturer, green propolis extract did not contain any preservatives or artificial ingredients.

The stock solution was prepared by dissolving 8 mg of green propolis extract into 40 ml Hank's Balanced Salt Solution (HBSS) and 160 µl of ethyl alcohol. The solution was then kept in a 37 °C water bath for 30 min to ensure the powder was completely dissolved. It was then filtered using a 0.2 µm syringe filter. This was the stock solution of 100% concentration.

Specific concentrations (10%, 20%, and 40%) of propolis were prepared from the 100% stock solution in which the propolis stock was diluted with the complete growth media. The amount of propolis in each concentration of the working solutions was 200 µg, 400 µg, and 800 µg for 10%, 20%, and 40% concentrations, respectively. Complete growth media without propolis was used as a control and is referred to as containing 0% propolis solution.

2.3. Cell proliferation assay

BMMSCs were seeded in 24-well tissue culture polystyrene-coated plates at a density of 5×10^3/well in the presence of the complete growth media containing 0%, 10%, 20%, and 40% propolis. Cell proliferation was measured at 2 time points, days 1 and 3, using CellTitre 96® Aqueous nonradioactive (MTS) assay (Promega, Madison, WI) according to the manufacturer's instructions. Briefly, MTS reagent was added to the cells and incubated for 3 h at 37 °C in 5% CO_2. The absorbance of the formazan complex that formed was measured at 490 nm. Wells containing the same media without any cells were used as blank controls.

2.4. Live-dead cell staining

The viability of BMMSCs in the presence of the complete growth media containing 0%, 10%, 20%, and 40% propolis was visualized using calcein-am and propidium iodide (PI) staining (green and red fluorescence, respectively) (Invitrogen). Cells were stained according to the manufacturer's instructions after 2 days of seeding. This was carried out to ensure all cells adhered to the polystyrene tissue culture plates and were viable. Fluorescent images were acquired using Zeiss Axiovert 40C microscope, equipped with a Canon, Powershot A620 camera, and images were evaluated with NIS-Elements™ imaging software (Nikon™).

2.5. Nuclear-cytoplasm staining

Nuclear-cytoplasm fluorescent staining was used to evaluate the morphology of BMMSCs after culturing in the complete growth media containing 0%, 10%, 20%, and 40% propolis for 7 days. For staining, cells were fixed with 4% paraformaldehyde for 10 min, and next, 5 µg of wheat germ agglutinin (WGA, Alexa Fluor 488 conjugate) was added

for 10 min at room temperature. After washing, cells were stained with 5 μg of TO-PRO-3 iodide stain (Alexa Fluor 647 conjugate) for 10 min at room temperature. Finally, cells were mounted with SlowFade Gold Antifade Reagent, and images were taken with a confocal microscope (Leica TCS SP2; Leica Microsystems©, Wetzlar, Germany) at 20X magnification.

2.6. Tri-lineage differentiation assays

Osteogenic, chondrogenic, and adipogenic differentiations were performed as previously described with slight modifications [47,49]. Briefly, 2×10^5 cells were seeded in the complete growth media containing 0%, 10%, 20%, and 40% propolis. Forty-eight hours later, when the cells reached 70% confluency, lineage-specific differentiation was induced. Cells were fed with the lineage-specific differentiation media, and the concentration of propolis extract was maintained throughout the differentiation process. For each differentiation assay, an identical number of cells without any differentiation media were used as controls.

Tri-lineage differentiation was visualized by phase contrast microscopy and cell-specific staining. The following staining techniques were used: Alizarin red staining at day 21, Alcian blue staining at day 14, and Oil-red-O staining at day 7, for osteogenesis, chondrogenesis, and adipogenesis, respectively. All images were obtained using a Zeiss Axiovert 40C microscope equipped with a Canon, Powershot A620 camera.

2.7. Migration assay

Migration of undifferentiated BMMSCs in the presence of the complete growth media containing 0%, 10%, and 20% propolis was demonstrated *in vitro* using a scratch assay. BMMSCs were seeded at a density of 20.000 cells/cm^2 to form a monolayer. Migration of differentiated BMMSCs (osteogenic, chondrogenic, and adipogenic differentiated cells) in the presence of the lineage-specific differentiation media containing 0%, 10%, and 20% propolis was also assessed. BMMSCs were induced to differentiate using the specific differentiating media for each type as mentioned above.

In all the undifferentiated and differentiated samples, one scratch/sample/well was made with a 200 μl pipette tip to ensure that a gap of 400–500 μM was created in each well. Cellular debris was gently washed with HBSS.

Each scratch assay was repeated in triplicate in two independent experiments. For gap closure, two randomly selected points along each "wound" were identified, and the horizontal distance between the two scratched edges was measured using black and white phase-contrast microscopy. Measurements were made at day 1, 2, 3, 4, 6, 8, and 10 post-wounding. Measurements taken at each time-point were compared to measurements at time 0 and then expressed as a percentage healing relative to the original size of the gap.

2.8. Statistical analysis

All data are reported as mean ± SD. The data was first evaluated for normality using a Shapiro Wilk test, and after it was confirmed to be normally distributed, data was compared using a one-way-ANOVA with Tukey's *t*-test for post hoc analysis. In all cases, a p-value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Propolis enhances cell proliferation and viability

Cell proliferation rate of previously isolated, characterized, and cryopreserved goat BMMSCs [47] was evaluated in the presence of green propolis extract *in vitro*.

Propolis concentrations used in this study were based on a dose curve, wherein the proliferation of goat BMMSCs was measured in the presence of 8, 50, 80, and 800 mgs of propolis. Cells exposed to complete growth media without any propolis (referred to as 0%) were used as controls. Results showed that all the propolis concentrations, except the 8 mg dose, resulted in a significant decrease in cell proliferation (data not shown). As the 8 mg concentration resulted in a significantly higher proliferation rate in comparison to the 50, 80, and 800 mgs concentrations, we decided to use 10, 20, 40, 50, and 60% dilutions from the 8 mg stock.

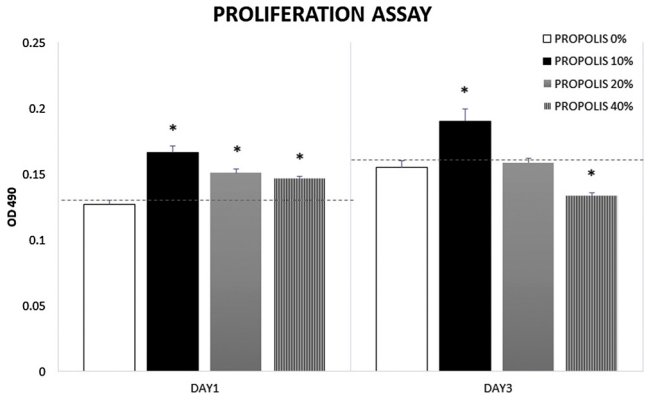

Fig. 1. Proliferation of BMMSCs. MTS assay was used to demonstrate the proliferation of BMMSCs cultured in complete growth media containing 0%, 10%, 20%, and 40% propolis over a period of 3 days. *P < 0.05 is considered significant. The dotted line represents the control value and bars above or below demonstrate changes relative to the control.

There was a significant increase in cell proliferation in the presence of 10% propolis only (0.19 ± 0.009) compared to the control (0.155 ± 0.005) over a period of 3 days ($p < 0.05$) (Fig. 1). The 20% and 40% propolis resulted in a significantly higher proliferation rate at day 1 only (0.151 ± 0.002 and 0.147 ± 0.001, respectively) relative to the control (0.12 ± 0.002) ($p < 0.05$). After 3 days, 20% propolis did not show any significant difference, whereas 40% propolis demonstrated a significantly lower proliferation (0.133 ± 0.002). This was further confirmed by the live/dead fluorescent staining of cells (Fig. 2). The presence of green fluorescent (calcein-am) and very few red fluorescent (PI) BMMSCs after 48 h of seeding in the complete growth media containing 10% and 20% propolis indicated that BMMSCs were viable and that the 10% and 20% propolis solutions were not cytotoxic. However, higher concentrations, such as 40% propolis, were cytotoxic to cells after 2 days of culture, confirming the proliferation assay data at day 3.

Cell morphology was visualized using TO-PRO-3 stain, a probe for nucleic acid detection, along with WGA specific to the cell membrane, to demonstrate the nucleus and the cytoplasmic structure of BMMSCs cultured in complete growth media containing 10%, 20%, and 40% propolis. Similar to the controls, cells showed an intact nucleus and healthy cytoplasm using fluorescence microscopy. No differences were observed between the four groups, except lower numbers of cells were observed in 40% propolis (Fig. 3), confirming the reduced proliferation and cytotoxicity.

3.2. Propolis enhances chondrogenic and adipogenic differentiation, but not osteogenic differentiation

To determine the multipotency of the BMMSCs in the presence of propolis *in vitro*, cells were induced to differentiate to osteogenic, chondrogenic, and adipogenic lineages in the presence of 10%, 20%, and 40% propolis. In this assay, lineage-specific media without propolis was used as the 0% differentiation media control for each cell type.

Subjective evaluation by Alizarin red staining showed that BMMSCs treated with all three concentrations of propolis did not show any

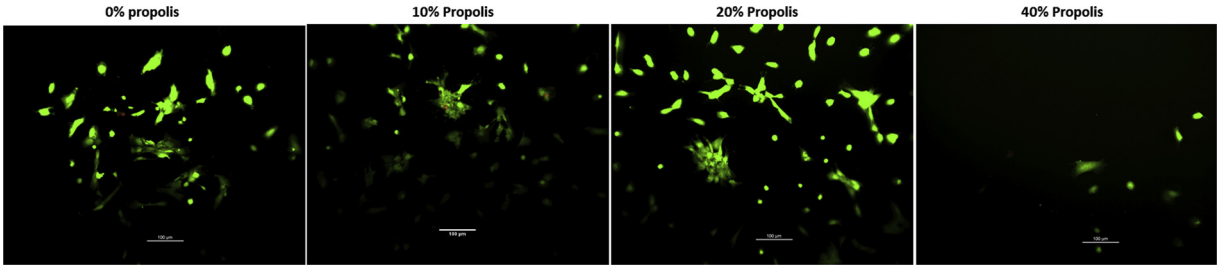

Fig. 2. Cell viability of BMMSCs. Fluorescent staining was carried out 48 h after seeding BMMSCs in complete growth media containing 0%, 10%, 20%, and 40% propolis. Cells were stained with Calcein-am, which emits green fluorescence in live cells. Lack/reduced red fluorescently stained cells by PI indicates dead cells. Scale bar = 100 µm; images were taken at 10x magnification.

mineralized nodules compared to the control (Fig. 4A), suggesting that cells failed to undergo osteogenic differentiation in the presence of propolis. However, as judged by Alcian blue staining, BMMSCs demonstrated the potential to undergo chondrogenesis *in vitro* when grown in the lineage-specific medium in the presence of 10% and 20% (Fig. 4B). Similarly, as confirmed by Oil red O staining (Fig. 4C), 10% propolis treated cells showed a higher formation of fat droplets compared to the control. On the contrary, 20% and 40% propolis showed relatively less accumulation of fat droplets relative to the control.

3.3. Propolis enhances migration of BMMSCs and promotes gap closure in vitro

An *in vitro* scratch assay was performed to mimic the *in vivo* effect of propolis on wound healing. Using this test, the undifferentiated BMMSC migration and wound gap closure was assessed in the presence of complete growth media containing 0%, 10%, and 20% propolis over a period of 10 days. Almost 85% of gap closure was observed at day 4 in all groups tested. Data was not significant at any time point (Fig. 5). Since the 40% propolis solution demonstrated a lower proliferation rate and some cell death (Figs. 1,2), it was not used in the migration assay.

The migration capacity of BMMSCs induced for osteogenic, chodrogenic, and adipogenic lineages was also assessed in the presence of differentiation media containing 0%, 10%, and 20% propolis. The gap closure of cells after osteogenic differentiation in presence of propolis was significantly shorter than the control until day 6 for 20% but relapsed after day 8 and became significantly longer than the control thereafter (Fig. 6A). Alizarin red staining of the cells after migration showed poor osteogenic differentiation in both 10% and 20% propolis-treated cells compared to the control (Fig. 6B), again confirming the earlier data that propolis extract inhibits osteogenesis (Fig. 4). On the other hand, 10% propolis upregulated chondrogenic lineage differentiation at 24 h, and this effect sustained until the end of the experiment at day 10. Both 10% and 20% propolis-treated cells showed significantly higher migration capacity at days 3, 6, and 10 (Fig. 7A). Alcian blue staining of the cells revealed an efficient migration of chondrocytes to close the gap in the presence of 10% and 20% propolis relative to the control (Fig. 7B). Similarly, significant effects in gap closure were consistently observed at days 1, 2, 3, and 4 in adipogenic cells with 20% propolis (Fig. 8A). At days 6, 8, and 10, both 10% and 20% propolis-treated adipocytes exhibited significantly higher migration capacity relative to the control ($p < 0.05$). Staining the adipocyte-induced cells using Oil red-O revealed significantly higher fat droplets in the presence of 20% propolis relative to the control (Fig. 8B).

4. Discussion

Green propolis extract used in this study was commercially obtained (Pharma Nectar, Brazil), and active components reported were Coumaric acid, Pinobankisin, pnocembrin, Pinobanksin-3-acetate, chrysin, Galahgin, Artepillin C, and Baccharin. Propolis belongs to group 12 of Brazilian propolis, and its botanical origin is identified as Baccharis dracunculifolia.

Many studies have tested the effect of propolis on not only grafts and tissues, but also on different cell lines *in vitro*; however, there are no reports on its effect on bone marrow-derived MSCs. Herein, we used *in vitro* experiments to evaluate the cytotoxic effect of propolis quantitatively and qualitatively. Our results revealed that propolis was not cytotoxic at concentrations lower than 400 µg/ml to BMMSCs, and in fact, it increased the proliferation rate of BMMSCs. This finding was in agreement with previously published studies, which reported that propolis could enhance the proliferation capacity of periodontal ligament fibroblast [38] and stem cells derived from exfoliated deciduous teeth [28]. However, an opposite effect was reported by Tyszka-Czochara et al., who observed that propolis exhibited an anti-proliferative effect on skin fibroblast [32].

Propolis is one of the natural extracts used for osteoarthritis due to its anti-inflammatory effect, which has been shown in an *in vivo* arthritis-induced animal model [50–53]. In the present study, as confirmed by Alcian blue staining for the glycosaminoglycans (GAG), propolis showed aggregated GAG at 10% and 20% concentrations. This effect was also observed at 50% concentration (data not shown). It has recently been shown that propolis extract could increase the extracellular matrix GAG and hyaluronic acid in a minor skin burn [54]. In

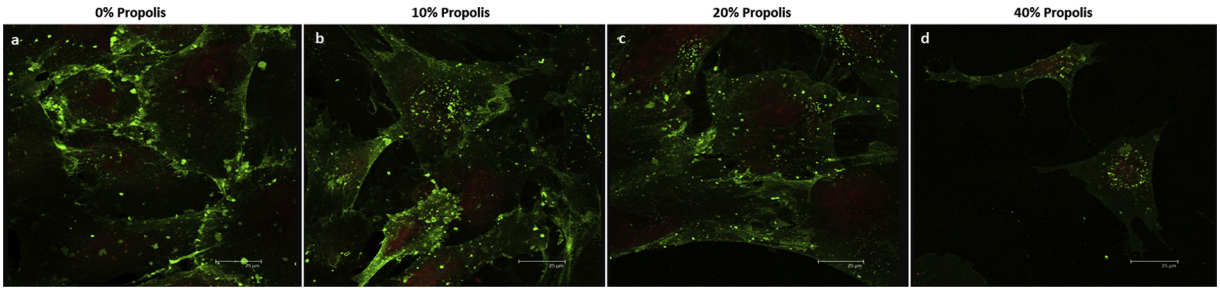

Fig. 3. Nuclear-cytoplasmic morphology of BMMSCs. Representative confocal images of cytoplasmic (WGA, green) and nuclear (TOPRO-3-iodide, red) fluorescent stains show the integrity of BMMSCs cultured in complete growth media containing 0%, 10%, 20%, and 40% propolis. Cells were stained after 7 days of seeding and proliferation. Scale bar = 25 µm.

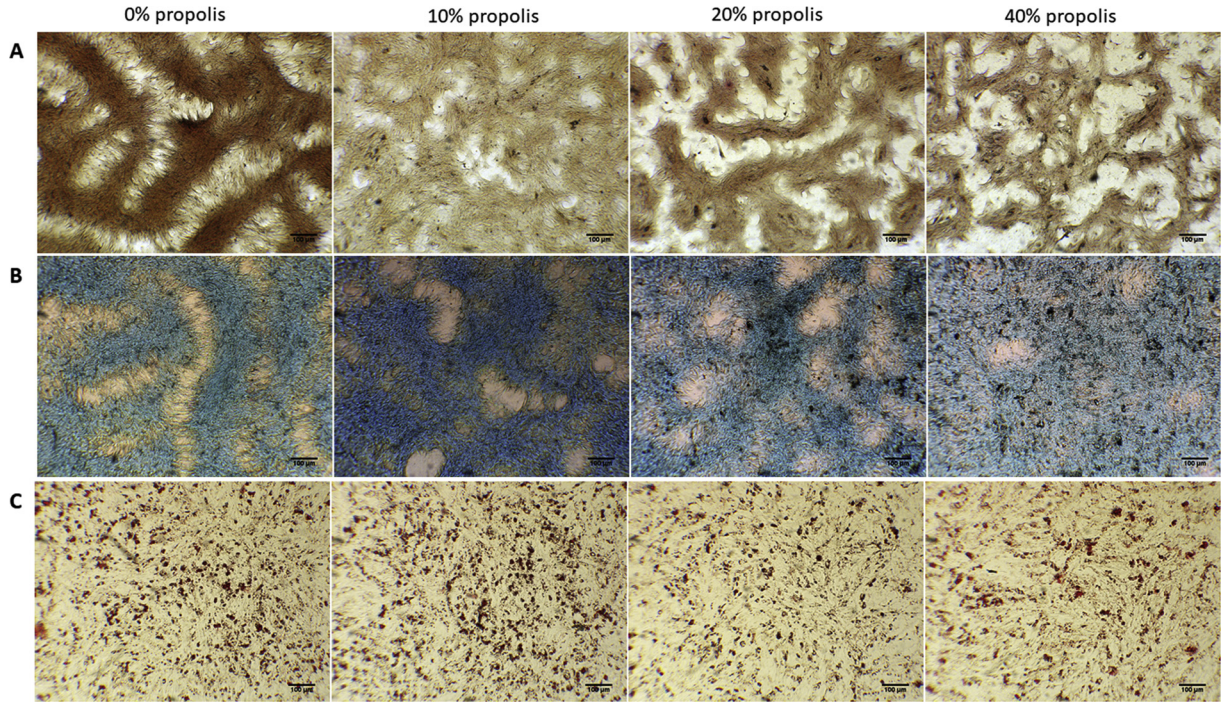

Fig. 4. Tri-lineage differentiation *i.e.* osteogenic, chondrogenic, and adipogenic differentiation of BMMSCs. Representative images show Alizarin red (A), Alcian blue (B), and Oil red O (C) staining of osteocytes, chondrocytes, and adipocytes, respectively, after *in vitro* differentiation in lineage-specific differentiation media containing 0%, 10%, 20%, and 40% propolis.

another study, propolis has been reported to increase collagen production in an animal model of diabetic wound healing *via* TGF-β1/Smad2 signaling [55]. The published studies and data reported in this study suggest that the dose of propolis used is important, and propolis extract at a defined concentration can be used as a suitable medium for the storage and transportation of osteochondral graft and as a multifunctional carrier for stem cell therapy in osteoarthritis patients.

We observed that BMMSCs showed poor osteogenic differentiation in the presence of propolis, suggesting that propolis extract may exert an inhibitory effect on osteogenic differentiation. This result is supported by Hidaka et al. [56] in which they demonstrated that propolis exerted an inhibitory effect on amorphous calcium phosphate formation. However, studies by Guney et al. [45] and Altan et al. [57] proposed a potential role for propolis in bone regeneration. It seems that this *in vivo* effect could be due to either its inhibitory effect on the osteoclast maturation as explained by Pileggi et al. [30] or due to change in the antioxidant levels at the site of the fracture as investigated by Guney et al. [45]. Our data suggest the bone regenerative effect of oral propolis may not be *via* induction of osteodifferentiation of MSCs as believed by some researchers. Future experiments to understand the lack of osteogenic differentiation observed in our study need to be initiated.

Some studies suggest that the injection of differentiated cells will promote tissue regeneration [58–61]. As per our knowledge, there are no reports about the capacity of differentiated cells to migrate to the site of injury. Herein, we evaluated the effect of propolis on the *in vitro* migration potential of both undifferentiated and differentiated MSCs using scratch assay. Data shows that 10% and 20% propolis can potentiate the migration capacity of both chondro- and adipo-differentiated MSCs. The effect of propolis on migration potential of differentiated cells observed in this study is novel and has not been reported earlier. Previously, a single report by Jacob et al. [33] shows that

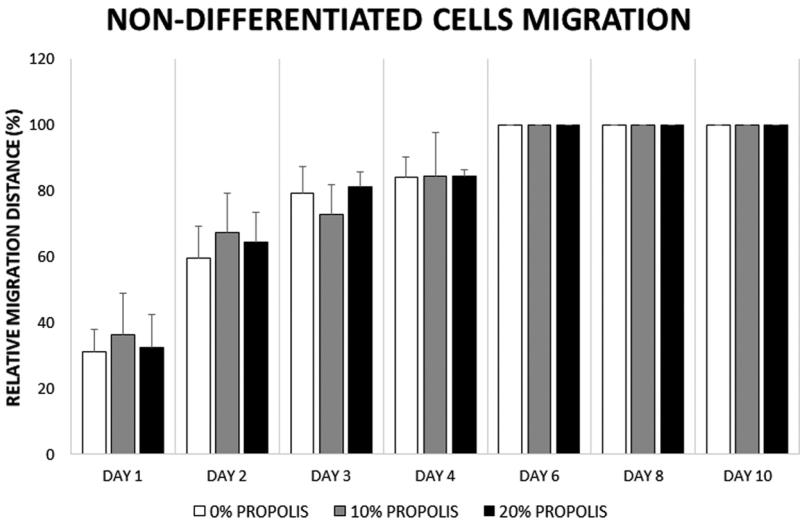

Fig. 5. Migration assay of undifferentiated (non-differentiated) BMMSCs. Quantitative analysis of the migration distance of BMMSCs cultured in complete growth media containing 0%, 10%, and 20% propolis. Results are shown as the mean ± SD of triplicates within two independent experiments. None of the data is significant.

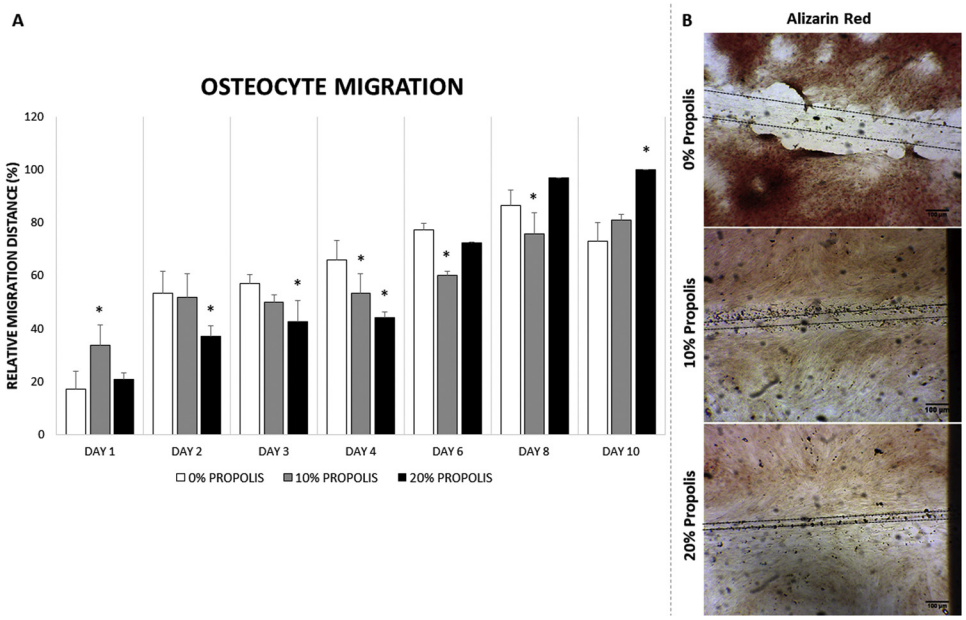

Fig. 6. Migration assay during osteogenic differentiation of BMMSCs. (A) Quantitative analysis to compare the migration of BMMSCs cultured in osteogenic differentiation media containing 0%, 10%, and 20% propolis. Results are shown as the mean ± SD of triplicates within two independent experiments. *P < 0.05 is considered significant. (B) Representative images showing the corresponding Alizarin red staining for osteogenic differentiation.

1000 µg/ml propolis has an enhancing effect on skin fibroblast migration *in vitro*.

In terms of clinical applications, future studies would determine whether propolis increases the viability and physiological health of osteochondral graft and can act as a storage media, as it did for the avulsed tooth. In addition, a cartilage defect animal model can be used to determine the effect of propolis as a carrier for MSCs to enhance cartilage regeneration.

5. Conclusions

Within the scope of this study, we have demonstrated that propolis at concentrations of ≤ 400 µg/ml was not cytotoxic to BMMSCs and enhanced their proliferation. The higher concentrations, however, hindered the proliferation and exhibited a cytotoxic effect. Most importantly, we demonstrated that propolis enhanced the chondrogenic and adipogenic differentiation processes and increased the migration potential of differentiated cells from both lineages. In line with the previous reports, our data supports the fact that propolis at a defined concentration is safe, cytocompatible, and, hence, can be used as an adjuvant therapeutic supplement which has the potential to enhance the proliferation and the tissue regeneration potential of MSCs. Due to the high degree of variability in the chemical composition of propolis extracts obtained from different geographical locations, these *in vitro* analyses should be performed prior to *in vivo* application. Most importantly, the *in vitro* system reported in this study provides an avenue to understand the mechanisms of osteogenic, chondrogenic, and adipogenic differentiation processes of MSCs in the presence of a defined dose and type of propolis extract.

Competing interests

The authors declare that they have no competing interests.

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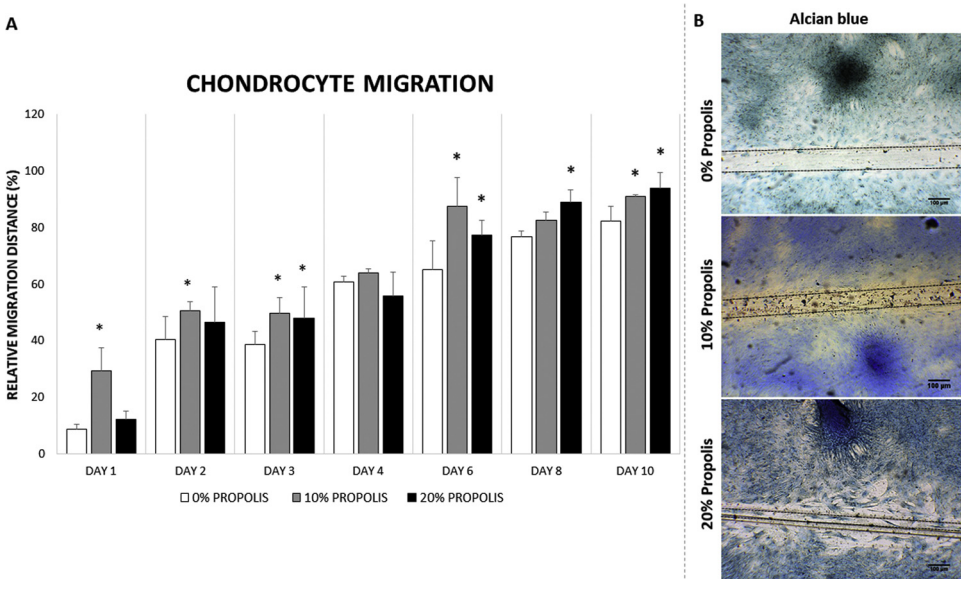

Fig. 7. Migration assay during chondrogenic differentiation of BMMSCs. (A) Quantitative analysis to compare the migration of BMMSCs cultured in chondrogenic differentiation media containing 0%, 10%, and 20% propolis. Results are shown as the mean ± SD of triplicates within two independent experiments. *P < 0.05 is considered significant. (B) Representative images showing the corresponding Alcian Blue staining for chondrogenic differentiation.

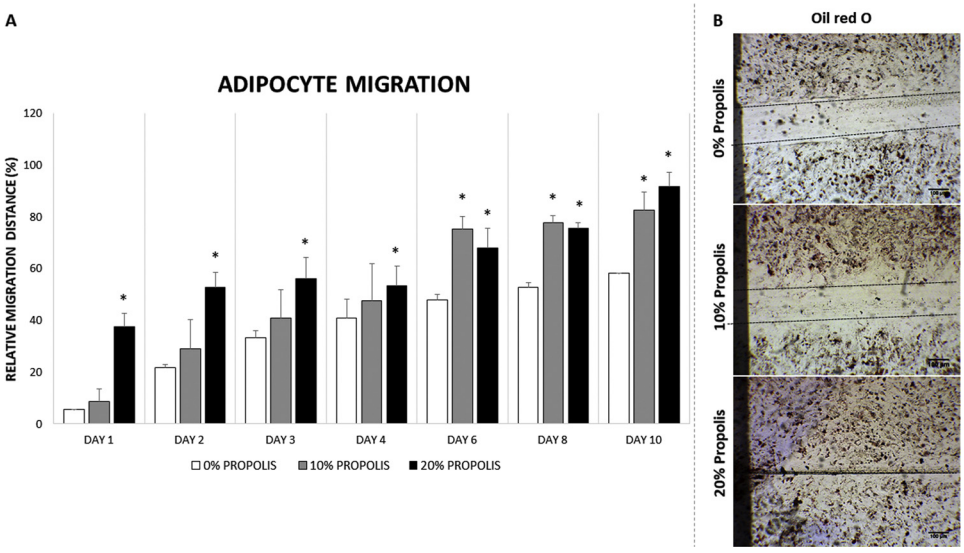

Fig. 8. Migration assay during adipogenic differentiation. (A) Quantitative analysis to compare the migration of BMMSCs cultured in adipogenic differentiation media containing 0%, 10%, and 20% propolis. Results are shown as the mean ± SD of triplicates within two independent experiments. *P < 0.05 is considered significant. (B) Representative images showing the corresponding Oil-red-O staining for adipogenic differentiation.

Authors' contributions

HE carried out the research, performed the analysis, and participated in manuscript writing. NEB performed the analysis and participated in manuscript writing and review. MD designed, supervised the research, and participated in manuscript writing and review. All authors read and approved the final manuscript.

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