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Ethanolic extract of *Aloe arborescens* stimulates neonatal rat calvarial cells proliferation, migration and osteogenic differentiation

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Abstract

The medicinal plant *Aloe arborescens* Miller has chemical compounds that could stimulate the activity of bone-forming cells, but no studies have been found in this regard. We evaluated the effects of different dilutions of aqueous (1/10; 1/100; 1/1000) or ethanolic (1/1000; 1/2000; 1/5000) extracts of parenchyma from *A. arborescens* on the viability, proliferation, migration and osteogenic differentiation of primary cell cultures from neonatal rat calvaria. In none of the conditions studied did cell viability decrease ($p \leq 0.01$). Furthermore, the 1/5000 ethanolic extract dilution showed a positive effect on cell viability at 48 h and 72 h and the latter was correlated with a 27 % ($p \leq 0.01$) increase in cell proliferation. Ethanolic extract significantly stimulated cell migration and cultured mineralization with respect to control, showing the maximal effect at a dilution 1/5000. Together, the results show that the *A. arborescens* extracts do not have toxic effects. In addition, ethanolic extract stimulates proliferation, migration and osteogenic differentiation of rat calvarial cells, suggesting a potential bone anabolic action.

Keywords: osteoblast, proliferation, migration, osteogenic differentiation.

Introduction

Bone alterations and loss of bone mass have a great impact on world society. Older age, hormonal disorders, high impact diseases such as diabetes and celiac disease among other factors are frequently associated with deficits in bone formation (Boonen et al., 1997; Kurra, Fink, and Siris, 2014; Gonnelli, Caffarelli, Giordano, and Nuti, 2015; Schacter and Leslie, 2017). Bone-forming cells, osteoblasts, originate by differentiation from mesenchymal stem cells, in response to a number of local and systemic factors (Garg et al., 2017). The activity and function of these cells is crucial in the maintenance and regeneration of bone mass, the quality of the bone formed and the functioning of the skeletal system (Neve, Corrado, and Cantatore, 2011). The main function of osteoblasts is to synthesize and secrete several proteins that integrate the organic extracellular matrix (such as cytokines, collagen and growth factors, among others) and convert the extracellular matrix into bone by mineralization (Garg et al., 2017). Although there are numerous therapeutic approaches to stimulate bone tissue repair and regeneration, these are often insufficient. Therefore, efforts to find alternative therapies to stimulate osteogenesis should be increased (Sun, 2008; Lyritis, Georgoulas, and Zafeiris, 2010).

The plant kingdom is a great reservoir, not fully explored, of biologically active compounds of therapeutic application (Baerheim Svendsen and Scheffer, 1982; Garro and Pungitore, 2015; Malongane, McGaw, and Mudau, 2017). The World Health Organization (WHO) proposes to increase research aimed at examining the potential benefits of medicinal plants use in different pharmacological approaches for disease treatment (WHO, 2004). There is a growing interest in the study of natural products derived from plants for treatment of bone conditions (Abdul Jalil, Shuid,

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and Muhammad, 2013; Dietz, Hajirahimkhan, Dunlap, and Bolton, 2016; Lambert, Hu, and Jeppesen, 2017; Wu et al., 2017; Basu, Masek, and Ebersole, 2018). This is, in part, because they offer the possibility of longer exposure times and fewer secondary complications compared to synthetic drugs (An et al., 2016a, 2016b). In this sense, *in vitro* and *in vivo* studies showed that several compounds of vegetable origin such as flavonoids, steroids, anthraquinones, polyphenols, phenolic acid, terpenoids, coumarins and glycosides stimulate the proliferation and/or differentiation of osteoprogenitor cells (Horcajada and Offord, 2012; Rufus, Mohamed, and Shuid, 2013; Srivastava, Bankar, and Roy, 2013; Tou, 2015; An et al., 2016a; Kong et al., 2018).

Aloe arborescens Miller is a species belonging to the genus *Aloe* (family Asphodelaceae). This plant, native to South Africa, has been exported to countries around the world for ornamental and medicinal use. It grows in predesert and desert areas, arid, with a temperate and warm climate, but it develops very well in places near the sea, and is widely cultivated as a material source for cosmetic, medicinal and food uses (Bastian et al., 2013). Its commercial growth started more recently in Israel and China (Smith, Klopper, Figueiredo, and Crouch, 2012). The leaf gel of *A. arborescens* is widely used as a component of many tonics and medicinal and cosmetic products. Chromatographic detection and separation methods have demonstrated the presence of phenolic compounds such as anthraquinones, anthrones, pyrones, chromones and coumarins; polysaccharides (arboran A and B); lectins and a carboxypeptidase enzyme in the leaf gel of *A. arborescens* (Nazeam, Gad, El-Hefnawy, and Singab, 2017). This species of *Aloe* has shown therapeutic properties as a neuroprotective, anticancer, immunomodulatory, antidiabetic, anti-inflammatory, antioxidant, antiulcer, antimicrobial, antifungal and wound-healing agent (Bastian et al., 2013; Singab et al., 2015; Abbaoui, Hiba, and Gamrani, 2017). Several compounds found in *A. arborescens* (anthraquinones, polyphenols, phenolic acid, coumarins, glycosides) have been shown to stimulate the activity and function of osteoblasts *in vivo* and *in vitro* (Rufus, Mohamed, and Shuid, 2013; An et al., 2016a, 2016b). Another widely studied aloe species, *A. vera*, has shown positive effects on osteoblasts (Kresnoadi, 2012; Kresnoadi et al., 2017; Machmud, Jubhari, and Ardiansyah, 2019). However, for *A. arborescens,* whose characteristics and chemical composition are markedly different from those of *A. vera* (Lucini et al., 2015; Fan et al., 2019; Andrea et al., 2020) there are no known reports on its effect on osteoblast activity and function.

In the present work, it was hypothesized that the *A. arborescens* plant would be a useful natural tool for the design of new therapeutic alternatives applicable in bone physiology. In order to address this, we studied the effect of extracts from *A. arborescens* on the activity and function of primary osteoblasts from neonatal rat calvaria.

Materials and methods

Materials

Ascorbic Acid 2-phosphate, β-glycerophosphate and α-MEM (1.36 mM Ca^{2+}; $CaCl_2.2H_2O$ 0.2g/L) were from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was from Natocor (Córdoba, Argentina). Neutral Red dye was donated by Paula Messina, PhD (Chemistry Department of Universidad Nacional del Sur, Bahía Blanca, Argentina). All other reagents used were of analytical grade.

Vegetable Material Collection

We worked with specimens of plants of the genus *Aloe arborescens* Miller from the city of Bahía Blanca (Bahía Blanca district, province of Buenos Aires) (Fig. 1). A reference specimen called "Blanco 2" is deposited in the Herbarium of Department of Biology, Biochemistry and Pharmacy of the National University of the South (BBB), Bahía Blanca, Argentina. The collection of the material, the post-harvest processing and the storage were carried out taking into account the general guidelines applicable to succulent plants (Mizrahi, Nerd, and Nobel, 1997) and to vegetable raw materials in the phytotherapeutic products industry (Sharapin, Rocha, and Pinzón, 2000). The leaves were collected from different adult plants chosen at random and those that were healthy and turgid (young, around 15 to 20 cm long) were selected. In the first stage of post-harvest processing, damaged parts, stained and/or with signs of attack by pathogens were discarded. The leaves were then washed with cold water and dried with absorbent paper. Next, the tips and edges of the leaves were cut and the parenchyma or gel was obtained by scraping the inside with a spatula. The plant material was mechanically disintegrated, and then it was frozen at –40 °C for 12 h and subsequently lyophilized. This procedure interrupts the degradation caused by enzymes and prevents the development of microorganisms and oxidation and hydrolysis reactions. The material obtained was stored under vacuum and protected from light at –20 °C until use.

Extract Obtaining

Aqueous extract: 1.6 g of lyophilized material was added to 1.6 ml of bidistilled water and left to stand for 2 h with intervals of shaking every 10 min during 30 sec. After centrifugation at 30,000 rpm for 5 minutes, the supernatant was collected and filtered through filters with a 0.22 μm pore diameter. Then, the aqueous extract was frozen at –20 °C until use.

Ethanolic extract: 2 g of lyophilized material were mixed with 600 ml absolute ethanol and shaken vigorously until a homogeneous suspension was obtained. It was left to rest for 7 days with shaking every 12 h for

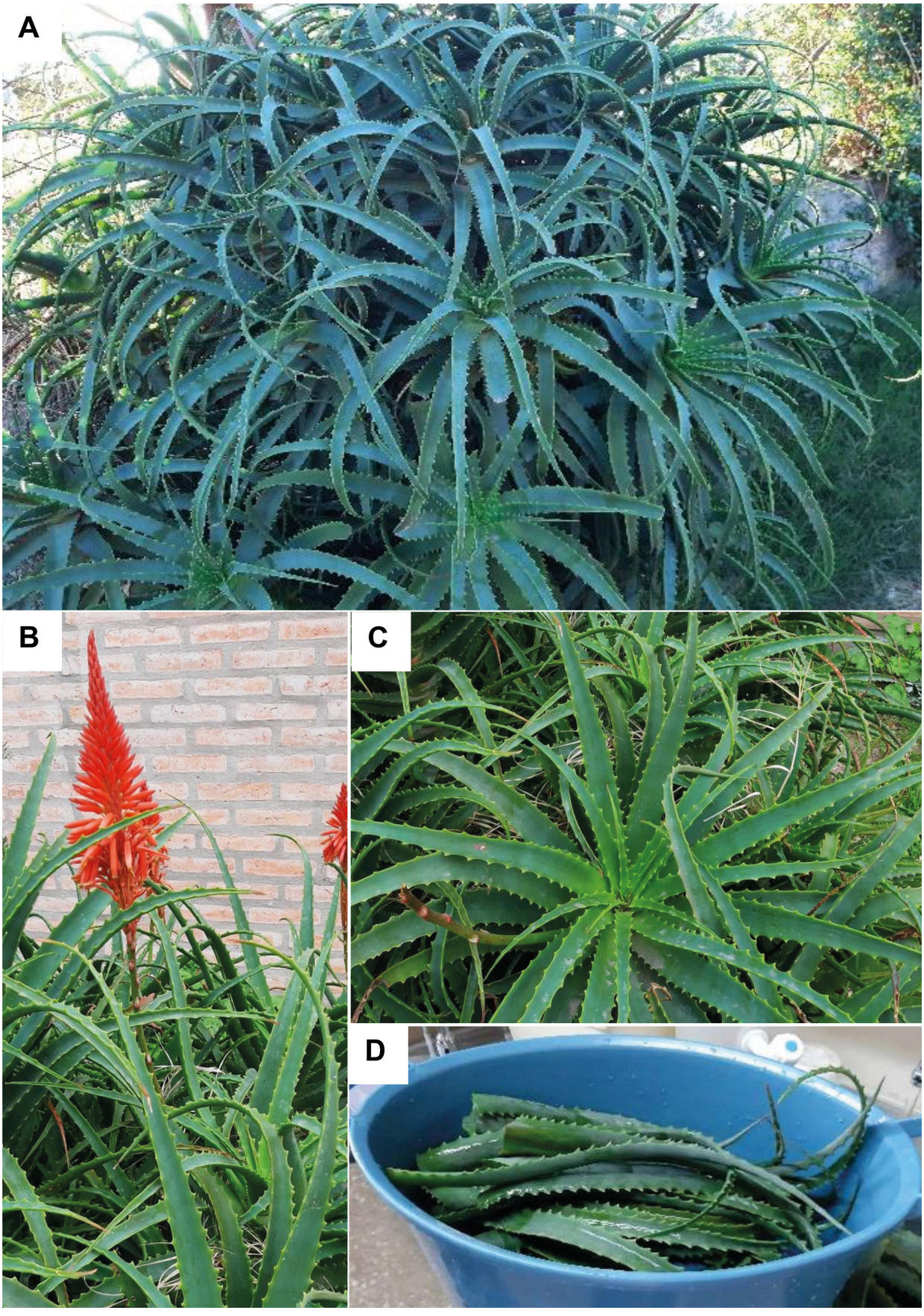

Fig. 1. *Aloe arborescens* specie. Wild specimen of *A. arborescens*-growing naturally in the Bahía Blanca town, south of the Buenos Aires province, Argentina: *A* — plant image; *B* — a plant in the state of flowering; *C* — plant leaves seen in more detail; *D* — Leaves cut from plants and washed.

3 min. Subsequently, the preparation was filtered to separate the residual material, and the ethanol was evaporated until obtaining a solid paste weighing 1.1334 grams. This was resuspended with 90 ml absolute ethanol, thus leaving an extract of 12.59 mg/ml.

Cell isolation

Calvarial cells were obtained from 3–5-day-old neonatal rats. Animals were sacrificed by fast decapitation. All procedures were carried out in conformity with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996) under protocol n° 012/2014 of Institutional Animal Care and Use Committee (CICUAE) of National University of the South, Argentina. Briefly, calvarias were incubated in phosphate buffer saline (PBS) containing 4 mM EDTA at 37 °C for two 10-min periods and the supernatants were discarded. Subsequently, calvarias were rinsed in PBS and submitted to digestion in PBS containing 200 U/ml collagenase for four 15-min periods, at 37 °C. Cells released during the first digestion were discarded and those released during the subsequent digestions were spun down, collected and combined after centrifugation for 10 min at 1500 rpm. Then, cells were cultured at 37 °C in α-MEM supplemented with 15 % FBS, 1 % penicillin and streptomycin under humidified air (5.5 % CO_2). After 24 h, the medium was replaced by α-MEM supplemented with 10 % FBS, 1 % penicillin and streptomycin and the cells were cultured until ~80 % of confluence (2–3 days). Then the cells were frozen in liquid nitrogen, at a density of 1×10^6 cells/ml, until their use.

Cell culture and treatment

Cells were thawed and seeded into 10 cm diameter glass Petri dishes and allowed to grow to 80 % confluence. Then, cells were passed at a density of 10×10^3 cells/cm^2 and cultured for 3–4 days in α-MEM supplemented with 10 % FBS, in a humidified atmosphere (5.5 % CO_2) at 37 °C. For each experiment, the cells underwent two passages, one when they were obtained before being frozen, and the other when they were counted and seeded to the various experimental conditions tested. When cells reached 80 % confluence, they were starved in 1 % FBS medium for 7–16 h before starting treatment. Treatments were performed by replacing the medium by treatment medium (α-MEM supplemented with 1 % FBS, containing the indicated amounts of extract or the vehicle used as control). When the experiment was performed in osteogenic conditions, 2 mM β-glycerophosphate and 50 μg/ml ascorbic acid-2 phosphate were incorporated to treatment medium. The control or treatment medium was renewed every 2–3 days.

Cell Viability Assay

Determination of cell viability was carried out using Neutral Red staining. It is extensively employed as a convenient and rapid assay for measuring cell viability. It is a well-known quantitative colorimetric method based on the uptake of the weakly cationic dye Neutral Red which enters into the cell by diffusion through the cell membrane. Then, the dye accumulates in the lysosomes of living cells (Repetto, del Peso, and Zurita, 2008). After treatments, cells were washed with PBS 1X and stained with Neutral Red for 2–3 h, at 37 °C. Dye excess was removed with PBS 1X and then, cells were observed under inverted microscope (Carl Zeiss, USA) and photomicrographs captured using image analyzer (Carl Zeiss, USA). Finally, the dye incorporated to cells was extracted with remover solution (50 % ethanol 96 %, 49 % deionized water, 1 % glacial acetic acid) and quantified, at 540 nm, in a spectrophotometer with plate reader.

Cell Proliferation Assay

Determination of cell proliferation was carried out using Crystal Violet staining. This is a fast, efficient, accurate, sensitive and reliable method to detect changes in both adhesion and *in vitro* cell proliferation as well as for cytotoxicity studies. The dye interacts with proteins and DNA. Thus, color intensity is directly proportional to the cell biomass and can be easily measured by spectrophotometry. Therefore, increases in the cell number of a sample result in a quantitative increase in staining. Similarly, when cells lose their adhesion, they detach from cell population, resulting in decreased staining (Geserick et al., 2009). After treatments, the cells were washed with PBS 1X, and fixed with pure methanol, for 10 minutes at 37 °C. The alcohol was then removed with distilled water and staining was started with Crystal Violet dye, which was performed for 30 minutes at room temperature. Excess dye was removed with distilled water. Stained cells were observed under inverted microscope and photomicrographs captured using image analyzer. Finally, the dye was extracted, for 30 minutes at room temperature, with Triton 0.2 %. Spectrophotometric quantification of the extracts was carried out in plate reader, at a wavelength of 590 nm.

Cell Migration Assay

Cell migration was evaluated by the wound healing assay. Simultaneously at the beginning of the treatments, a scratch or wound was made on the cell monolayers and then the advance of the cells on it was analyzed by observation under inverted microscope. The cells present in the wound or scratch were counted at different times, at the beginning (0 h) and at 8 h and 24 h of treatment. To assess the number of cells that migrated, the number

of cells counted at baseline (0 h) was subtracted from the cell count detected at 8 and 24 h.

Extracellular Matrix Mineralization Assay

Alizarin Red staining was used to evaluate the extracellular matrix mineralization in the cultures. Cells seeded in 48-well plates were cultured and treated as above for 17–25 days in osteogenic conditions. The cells were then fixed with 2% glutaraldehyde in PBS (pH 7.4) at room temperature for 10 min, washed three times with PBS, and then incubated with 2% Alizarin Red (pH 4.2) for 30 min at 37 °C. The cells were washed thoroughly with deionized water. Stained cells were observed under inverted microscope and photomicrographs captured using image analyzer. Following staining technique, evaluation of mineral distribution and inspection of fine structures by microscopy was performed. Then, for quantification of Alizarin Red staining, the samples were incubated with 500 µl of 0.1M NaOH for 5 min and O.D. (Absorbance) was measured by spectrophotometer at 548 nm.

Statistical analysis

InfoStat software was employed. Variance Analysis (ANOVA) was used to determine differences between the average values of several statistical populations. The mean values were compared by the *post hoc* test of multiple comparisons by Bonferroni. The different letters (a-b-c-d) indicate significant differences ($p \leq 0.01$) between the groups. Means with the same letter are not significantly different ($p > 0.01$). The represented values correspond to the average ± standard deviation (SD). The total number of samples is indicated in the legend of each figure.

Results

Effect of aqueous and ethanolic extract from *Aloe arborescens* on cell viability of rat calvarial cell cultures

In order to study the effect of extract from *A. arborescens* on cell viability of rat calvarial cultures, we used the Neutral Red staining. The cells were treated with different dilutions of aqueous (1/10; 1/100;1/1000) or ethanolic (1/1000; 1/2000; 1/5000) plant extract during 24, 48 and 72 h after which viability assay was performed as indicated in Materials and Methods. Cell viability only showed a slight decrease (13%) under the treatment with ethanolic extract at a 1/1000 dilution at 48 h. In contrast, statistically significant increases in cell viability were observed with respect to the control in the cells exposed to 1/2000 dilution of ethanolic extract at all treatment times and at 72 h for 1/1000 and 1/5000 dilutions (Fig. 2). Thus, the data suggest that these *A. arborescens*

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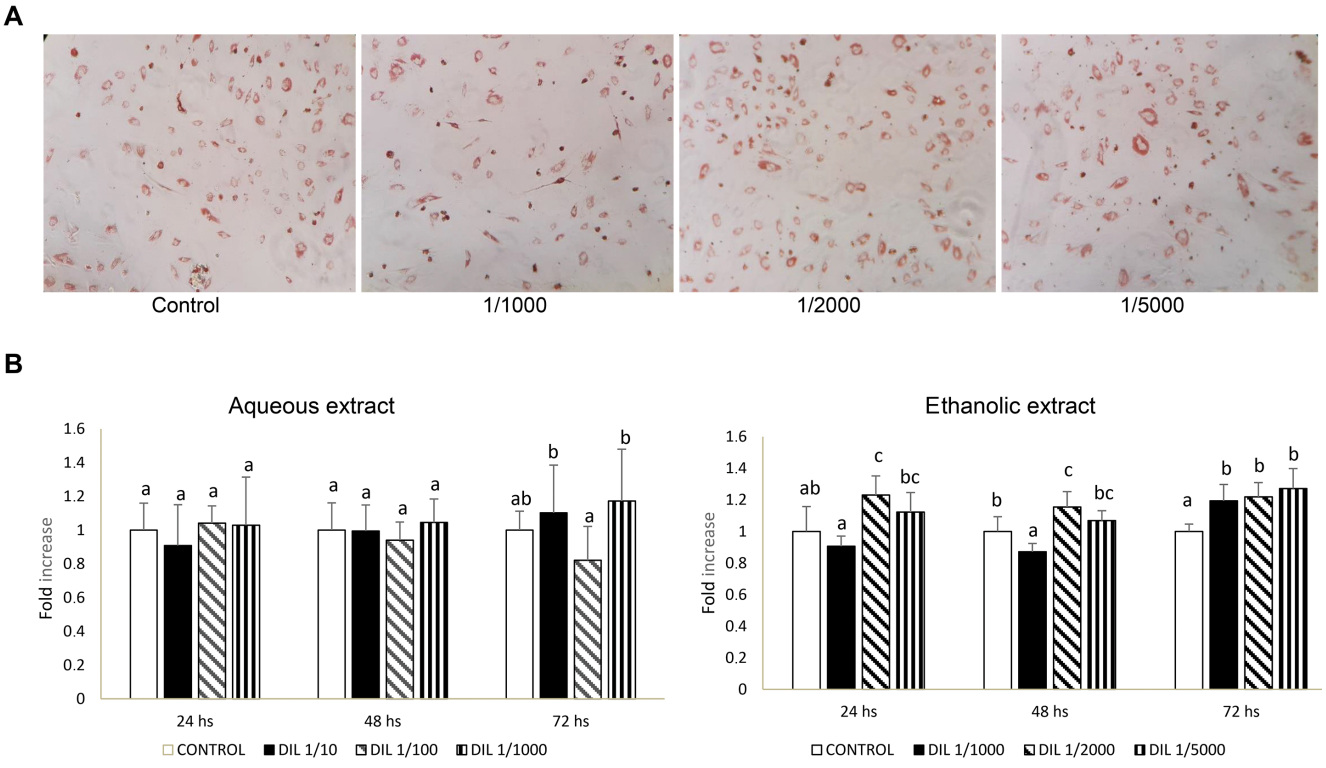

Fig. 2. Effect of *Aloe arborescens* extracts on cell viability of neonatal rat calvarial primary cultures. Cells seeded on 48-well plates were treated with vehicle (control) or the indicated dilutions of *A. arborescens* aqueous or ethanolic extract for 24, 48 and 72 h. Then, cell viability was determined by Neutral Red staining as indicated in Materials and Methods: *A* — representative images of cells stained with Neutral Red after 72 h of treatment with the indicated dilutions of ethanolic extract are shown; B — data represent the mean ± SD from at least three independent experiments, each performed with 4 wells per condition. Means with different letters indicate statistically significant differences (ANOVA — Bonferroni $p \leq 0.01$).

extracts do not cause negative effects on the viability of rat calvarial cells.

Effect of extracts from *Aloe arborescens* on cell proliferation of rat calvarial cell cultures

To assess the effect of extracts from *A. arborescens* on cell proliferation, studies were performed by staining with Crystal Violet. Cells were treated with different dilutions of aqueous (1/10; 1/100; 1/1000) or ethanolic (1/1000; 1/2000; 1/5000) plant extract during 24, 48 and 72 h and then stained with Crystal Violet as indicated in Materials and Methods. A significant increase in the staining (27 % over control) was observed in cell cultures exposed to the 1/5000 dilution of ethanolic extract for 72 h, while no significant changes were detected in the other conditions. These results suggest that none of the extracts show cytotoxicity or affect cell adhesion. In addition, the ethanolic extract stimulates cell proliferation at 1/5000 dilution (Fig. 3).

Effect of ethanolic extract from *Aloe arborescens* on cell migration of rat calvarial cell cultures

The effects of *A. arborescens* ethanolic extract on cell migration in rat calvarial cultures was evaluated by the scratch or wound assay. The cells were treated with different dilutions of ethanolic (1/1000; 1/2000; 1/5000) plant extract and then cell migration was evaluated at 8 h and 24 h as indicated in Materials and Methods. The ethanol extract significantly stimulated cell migration in all conditions studied, showing the greatest effect with the 1/5000 dilution (Fig. 4).

Effect of ethanolic extract from *Aloe arborescens* on mineralization of rat calvarial cell cultures

Mineralization is considered a functional *in vitro* endpoint reflecting advanced osteoblastic cell differentiation (Hoemann, El-Gabalawy, and McKee, 2009). Rat calvarial cells reach a mature osteoblast stage after long incubation times (>15 day) in osteogenic medium. Mineralization nodules can be visualized around day 15–20, or even later in such conditions (Hoemann, El-Gabalawy, and McKee, 2009; Ayala-Peña, Scolaro, and Santillán, 2013; Laiuppa and Santillán, 2016). In order to study if ethanolic extract from *A. arborescens* elicits any action in rat calvarial cell osteogenic differentiation, the extracellular matrix mineralization was assessed. Cells were treated with different dilutions of ethanolic (1/1000; 1/2000; 1/5000) extract in osteogenic medium for 17–25 days. Then mineralization was measured by determining the calcium deposits on cell cultures using the Alizarin Red organic dye, as described in Materials and Methods. Cells cultured in the presence of 1/2000 or 1/5000 dilutions of ethanolic extract showed a significant increase in calcium deposition at all treatment times studied

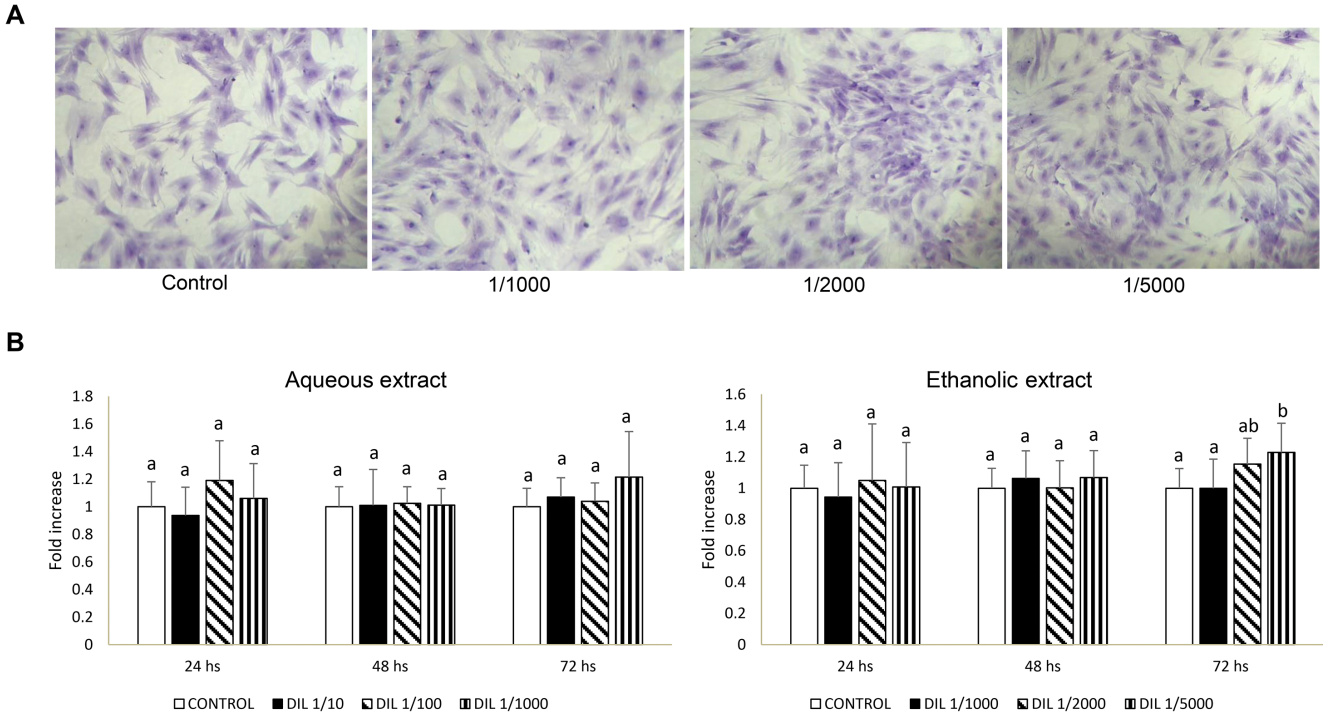

Fig. 3. Effect of *A. arborescens* extracts on cell proliferation of neonatal rat calvarial primary cultures. Cells seeded on 48-well plates were treated with vehicle (control) or the indicated dilutions of *A. arborescens* aqueous or ethanolic extract for 24, 48, and 72 h. Then cell proliferation was determined as indicated in Materials and Methods: *A* — representative images of cells stained with Crystal Violet after 72 h of treatment with the indicated dilutions of ethanolic extract are shown; *B* — data represent the mean ± SD from at least three independent experiments, each performed with 4 wells per condition. Means with different letters indicate statistically significant differences (ANOVA — Bonferroni $p \leq 0.01$).

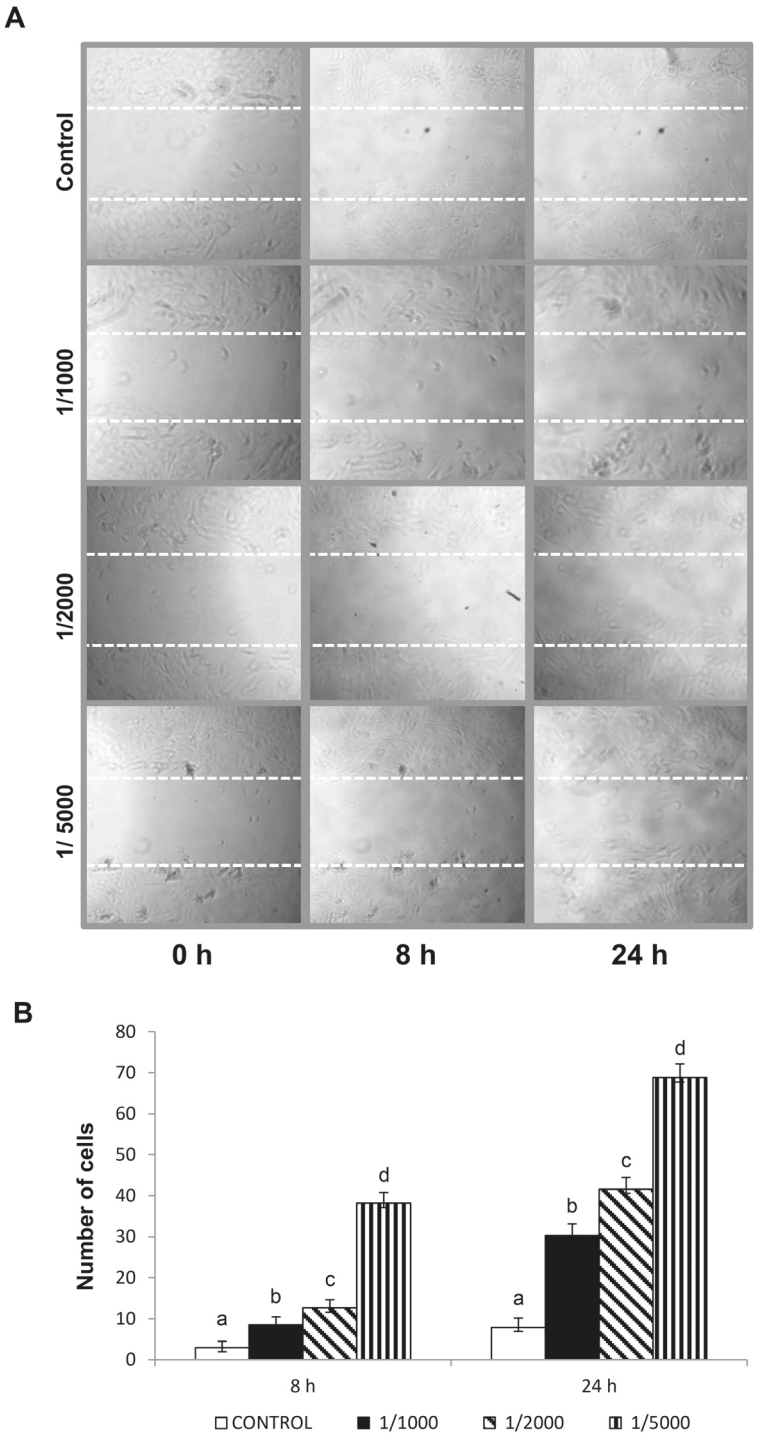

Fig. 4. Effect of *A. arborescens* ethanolic extract on cell migration of neonatal rat calvarial primary cultures. Cells seeded on 48-well plates were treated with vehicle (control) or the indicated dilutions of *A. arborescens* ethanolic extract. Then, quantification of cell migration was determined at 12 and 24 h as indicated in Materials and Methods: *A* — representative images of cells observed under the inverted microscope are shown at 0 h and at 8 and 24 h of treatment with the indicated dilutions of ethanolic extract; *B* — data represent the mean ± SD of at least three independent experiments, each performed with 4 wells per condition. Means with different letters indicate statistically significant differences (ANOVA — Bonferroni $p \leq 0.01$).

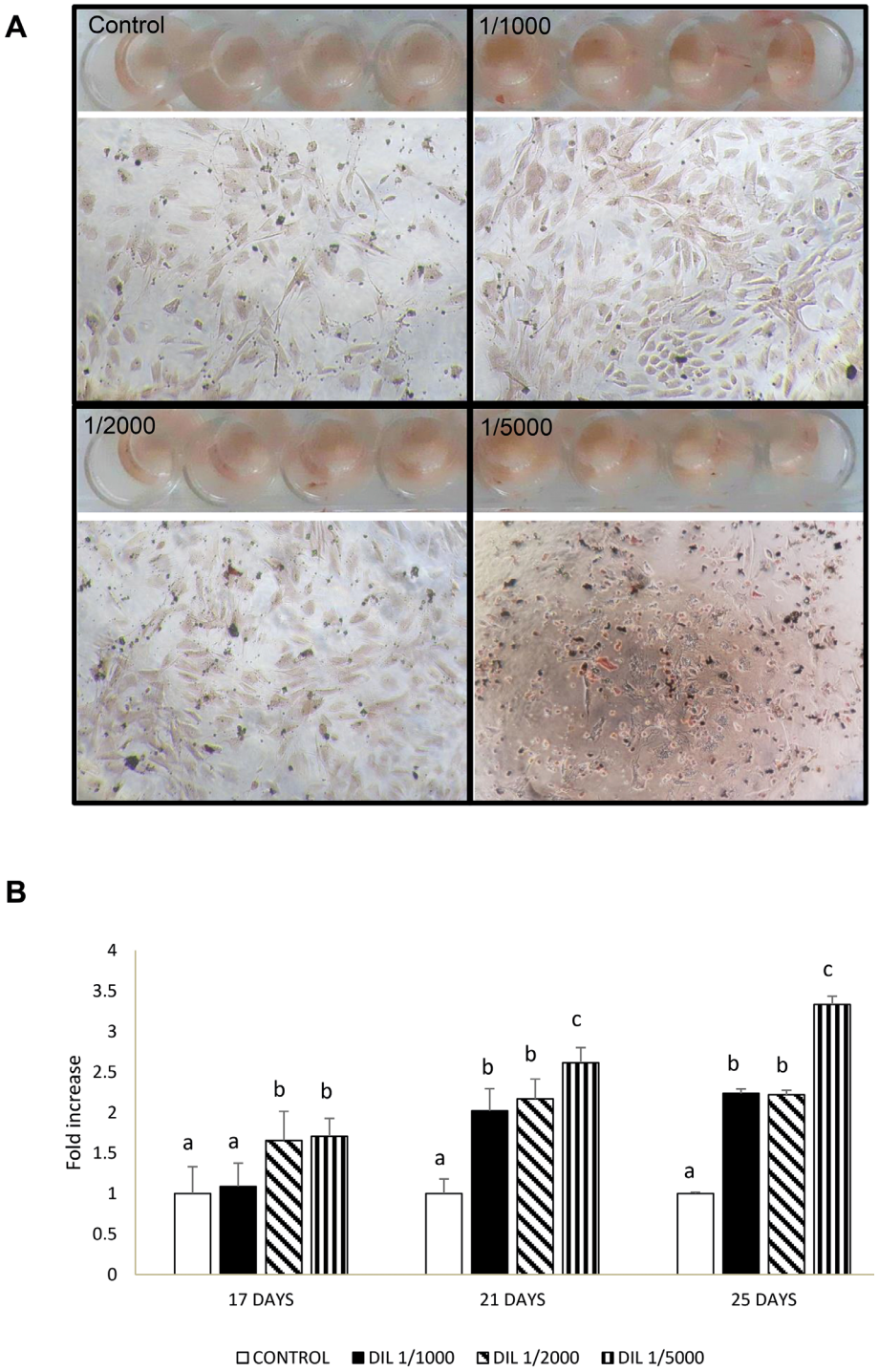

Fig. 5. Effect of *A. arborescens* ethanolic extract on mineralization of neonatal rat calvarial primary cultures. Cells seeded on 48-well plates were treated with vehicle (control) or the indicated dilutions of *A. arborescens* ethanolic extract for 17, 21 and 25 days in osteogenic medium. Then, culture mineralization was determined by Alizarin Red staining as indicated in Materials and methods: *A* — representative images of cells stained with Alizarin Red after 25 days of treatment with the indicated dilutions of ethanolic extract are shown; *B* — data represent the mean ± SD of at least three independent experiments, each performed with 4 wells per condition. Means with different letters indicate statistically significant differences (ANOVA — Bonferroni $p \leq 0.01$).

compared to control, with the greatest effect for the 1/5000 dilution at 25 days. While the 1/1000 dilution significantly increased the culture mineralization at 21 and 25 days of treatment and in a similar manner to the 1/2000 dilution at those times (Fig. 5). These results suggest that the ethanolic extract of *A. arborescens* favors osteogenic differentiation.

Discussion

In the present study, we show for the first time an evaluation of the osteogenic potential of extracts from *A. arborescens* using neonatal rat calvarial cells cultures. These cultures are a heterogeneous cell population majorly containing mesenchymal stem cells, committed osteoprogenitor cells, preosteoblasts and osteoblasts. Under appropriate conditions, these cell cultures can differentiate into osteoblasts, chondrocytes or adipocytes as it has been described (Pirih et al., 2008; Weivoda and Hohl, 2012). Under osteogenic conditions, the osteoblasts precursor cells have an osteoblast development pattern similar to *in vivo* bone formation. Thus, these cultures are a well-accepted model of osteogenesis *in vitro*. During the culture proliferative phase, the cells undergo DNA synthesis and cell division, resulting in a rapid increase in cell number until confluence. Then, proliferation is arrested and mature osteoblast characteristics begin to emerge, such as alkaline phosphatase production, conversion of procollagen to collagen, and deposition of extracellular matrix on the substrate, which is subsequently mineralized (Hoemann, El-Gabalawy, and McKee, 2009; Ayala-Peña, Scolaro, and Santillán, 2013; Laiuppa and Santillán, 2016, 2018). The conducing mechanism of osteogenesis has not been fully clarified. Although various osteogenic formulations are known, as well as the importance of each one of their constituents for induction of *in vitro* osteogenic differentiation, this has not yet been fully optimized, generating a wide interest in discovering new osteogenic active principles (Vater, Kasten, and Stiehler, 2011).

One of the criteria used to identify active ingredients of compounds at the bone level comprises the lack of toxicity and the promotion of osteoblast activity. In this sense, our results show that neither aqueous nor ethanolic extracts of *A. arborescens* are toxic to neonatal rat calvarial cells at the dilutions used, since no negative effects on cell viability or adhesion were observed. The significant increase in cell viability observed in the conditions treated with the ethanolic extract could be due to an increase in the number of cells since this extract also stimulated cell proliferation.

The stimulation of cell proliferation shown by the ethanolic extract is also a beneficial characteristic, mainly due to its potential application in the treatment of bone conditions that require an increase in the number of bone-forming cells, such as osteoporosis, osteopenia or non-healing fractures. In agreement with our results, extracts of other *Aloe* species have been shown to stimulate cell proliferation, such as *A. vera* on human fibroblasts and keratinocytes (Moriyama et al., 2016; Teplicki et al., 2018; Zago, Prado, Benedito, and Pereira, 2021) and fibroblast of a diabetic mouse model (Gharaboghaz, Farahpour, and Saghaie, 2020); *A. saponaria* on human dermal fibroblasts (Kim and Park, 2022); *A. cae* on keratinocytes (Moriyama et al., 2016). However, except for our work, the action of *Aloe* extracts on neonatal rat calvarial cells proliferation has not been studied. A very interesting report reveals that acemannan, a polysaccharide extracted from *A. vera*, stimulates the proliferation of primary rat bone marrow stromal cells (Boonyagul, Banlunara, Sangvanich, and Thunyakitpisal, 2014). In addition, *A. vera* polysaccharide has been reported to stimulate the proliferation of adipose tissue-derived stromal cells (Yao et al., 2022). Although *A. arborescens* also contains polysaccharides such as acemannan, as reported by Nazeam et al., 2017, these compounds would not be extractable by ethanol, therefore they would not be involved in the effect that we report here, but this must be demonstrated in future studies. Contrarily, antiproliferative effects for *A. arborescens* extracts have been evidenced on several human transformed cell lines (Di Luccia et al., 2013). *A. vera* extracts have also shown antiproliferative effect in neuroblastoma cells in vitro (Yonehara et al., 2015) and the *A. perryi* flowers extract has antiproliferative activity on human liver, colon, breast, lung, prostate and epithelial cancer cell lines (Al-Oqail et al., 2016). Of relevance, we have not found studies on the effect of *A. arborescens* extracts on the growth of bone tumor cells, which we visualize interesting for future investigations.

Bone formation, during bone remodeling or fracture repair, requires the presence of mature osteoblasts to deposit bone at the precise site. To do this, osteoblast precursors and their progeny from resident stem cells must migrate to the bone formation site. In this regard, the stimulatory effect of the *A. arborescens* ethanolic extract on cell migration is a favorable aspect that could play a beneficial role in the treatment of metabolic bone diseases such as osteoporosis, since this could enhance the arrival of osteoblastic precursors at the site of the bone lesion or region of interest for its repair. In agreement with our results, the use of *A. arborescens* for the preparation of green-synthesized silver nanoparticles has shown to stimulate the migration of fibroblasts (Dhilip Kumar, Houreld, and Abrahamse, 2020). Other *Aloe* species have also been shown to stimulate cell migration in fibroblast and keratinocyte cultures (Moriyama et al., 2016; Fox et al., 2017; Fouché et al., 2020; Shafaie et al., 2020). However, no reports have been found on this action of *A. arborescens* in bone cells, so our finding is the first evidence. In contrast, the inhibition of colon cancer cell migration by extracts of *A. vera* and *A. arborescens* has been reported, showing *A. arborescens* to be a more effective inhibitor than *A. vera* (Lima et al., 2020).

Another desirable effect of active principles at the bone level is promoting cell differentiation, since increases in the population of mature osteoblasts would favor bone formation. Culture mineralization is considered a functional *in vitro* endpoint reflecting advanced osteoblastic cell differentiation (Hoemann, El-Gabalawy, and McKee, 2009). Bone mineralization occurs by a set of physico-

chemical and biochemical processes that facilitate the deposition of hydroxyapatite crystals both along the collagen fibrils in the extracellular matrix (Glimcher, 2006), as well as within the lumen of the matrix vesicles derived from osteoblasts (Anderson, Garimella, and Tague, 2005). In our study, we detected a significant increase in the mineralization of cultures due to the treatment with ethanolic extract of *A. arborescens*, suggesting that the extract exerts a positive effect on osteogenic differentiation, showing osteoconductive activity, since this effect was observed in an osteogenic medium. In accordance with this, the *A. vera* extract was shown to significantly increase the percentage of calcium deposition by osteodifferentiated mesenchymal stem cells in osteogenic media (Rasoulian et al., 2019). *Aloe vera* compounds such as barbaloin and acemannan, also found in *A. arborescens* (Nazeam, Gad, El-Hefnawy, and Singab, 2017), have been shown to promote osteogenic differentiation of bone marrow mesenchymal stem cells (Boonyagul, Banlunara, Sangvanich, and Thunyakitpisal, 2014; Wang, Gan, Yang, and Wang, 2022). In addition, *A. vera* polysaccharide has been reported to stimulate the osteogenic differentiation of adipose tissue-derived stromal cells (Yao et al., 2022). In contrast, emodin also present in *A. arborescens* (Nazeam, Gad, El-Hefnawy, and Singab, 2017), attenuated Ca^{2+} induced calcification through inhibiting osteoblast differentiation genes in vascular smooth muscle cells (Sapkota et al., 2019). Considering the above, barbaloin (a glycosylated anthroquinone, extractable with ethanol) could be responsible for the activity observed in our study, but this must be demonstrated. In order to achieve this, more exhaustive studies are required, such as the fractionation of the ethanolic extract with the isolation of the active principle and demonstration of its effect on bone regeneration in vivo, that should be done in future research.

Aloe arborescens is very easy to cultivate, it grows in pre-desert and desert areas, arid, with a temperate and warm climate. In addition it develops very well in places near the sea. Our results can serve as a basis for future research on the application of *A. arborescens* as a natural therapeutic resource for the treatment of metabolic bone diseases. It is relevant to consider the ecological, economic and social advantages that the medical and/or nutraceutical use of *A. arborescens* could have, since it is a rustic plant, easy to spread, handle and process and that grows in areas that cannot be used for other crops. In addition, it is important to note that plant extracts as other naturally available materials such as vitamins, carbohydrates and biodegradable polymers, among others, can act as solvents, reducing agents and/or stabilizers in the synthesis of nanoparticles for the development of bio-materials applicable in medicine (Iravani, 2011; Gopi et al., 2014; Buitrago-Vasquez and Ossa-Orozco, 2018). In this sense, the use of *A. vera* in the construction of polyurethane scaffolds with hydroxyapatite for osteogenesis and bone regeneration has been explored (Shanmugavel et al., 2014). We believe that *A. arborescens* could be implemented to improve the bioactive osteogenic potential of compounds used in bone regeneration, such as the biological or chemical synthesis of hydroxyapatite nanoparticles for their use in certain branches of medicine such as orthopedics, orthodontics and esthetics surgery. Related to this, there is a great interest in replacing hazardous materials with green chemicals that protect human health and the environment (Zhang et al., 2011; Liu, Tao, and Zhang, 2012).

In conclusion, our results suggest that extracts of *A. arborescens* have beneficial effects on the activity and function of neonatal rat calvarial cells. This, together with the ecological, economic and social advantages of *Aloe* spp. Cultivation, will be a starting point to promote the implementation of clinical studies with extracts of *A. arborescens*, as a potential bone anabolic agent for the treatment of bone degenerative diseases, including osteoporosis.

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