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ORIGINAL ARTICLE

# Evaluation of the Antioxidant and Osteoinductive Potential of the Leaf Extract from *Dysphania ambrosioides* L.

Jessica Maria de Melo Almeida[1], Priscilla Guimarães Silva Vasconcelos[2], Rennaly de Freitas Lima[2], Carolina Medeiros de Almeida Maia[2], Fernanda Ginani[3], Carlos Augusto G. Barboza[3], Hugo Alexandre de Oliveira Rocha[4], Ana Claudia Dantas de Medeiros[1], Edja Maria Melo de Brito Costa[2]

[1]Department of Pharmacy, State University of Paraiba, Campina Grande, PB, Brazil.
[2]Department of Dentistry, State University of Paraiba, Campina Grande, PB, Brazil.
[3]Department of Morphology, Federal University of Rio Grande do Norte, Natal, RN, Brazil.
[4]Department of Biochemistry, Federal University of Rio Grande do Norte, Natal, RN, Brazil.

**Corresponding author:** Priscilla Guimarães Silva Vasconcelos **E-mail:** priscillags@hotmail.com.br

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**ABSTRACT**

**Objective:** To analyze the antioxidant activity of an alcoholic extract from the leaves of *Dysphania ambrosioides* L. and investigate its influence on *in vitro* differentiation of MC3T3-E1 pre-osteoblastic cells. **Material and Methods:** The content of the extract's flavonoids, tannins, and polyphenols was determined by spectrophotometry. The antioxidant potential was evaluated through tests of total antioxidant capacity (TAC), reductive activity, sequestration of superoxide radicals, and copper chelation. Cell viability and proliferation were evaluated by reduction of tetrazolium (MTT). Osteoblastic differentiation was assessed by alkaline phosphatase activity, quantification, and matrix mineralization using von Kossa stain. **Results:** The phytochemical screening revealed that the extract has a high tannin content (43.27% catechin equivalents), with polyphenols and flavonoids accounting for 6.57% and 6.24%, respectively. The extract demonstrated antioxidant potential, particularly for the tests that evaluated TAC (120mg ascorbic acid equivalents) and copper chelation (79.2% of chelation relative to EDTA). The extract (150 µg/mL) increased the proliferation of MC3T3 cells by 251% in the first 24h. None of the osteoblastic differentiation tests demonstrated an influence on the treatments with the extract. **Conclusion:** The alcoholic extract of *D. ambrosioides* proved to be a potent antioxidant but did not exhibit osteoinductive potential.

**Keywords:** Antioxidants; Cell Differentiation; Plant Extracts.

## Introduction

The regeneration capacity of the bone is a long process [1]. However, some compounds, such as the ones with antioxidant properties, have the potential to exert a positive influence on bone metabolism since bone physiopathologies are associated with oxidative stress [2]. The capacity to generate an osteoinductive potential is important in several areas of dentistry, such as in surgical or periodontitis cases. This chronic inflammatory disease is highly associated with oxidative stress, which causes progressive bone loss [3,4].

The osteoinductive potential of compounds can be tested using pre-osteoblastic cell cultures. In this context, pre-osteoblasts cells - MC3T3 offer methodological advantages, as the differentiation of these cells into mature osteoblasts is easily identified by markers of osteoblastic metabolism, such as alkaline phosphatase (ALP) and the degree of mineralization of the extracellular matrix (ECM) [5,6].

The search for compounds derived from natural plants has gained attention over the years due to the potential therapeutic action for treating various disorders. However, a myriad of molecules has yet to be explored. In this aspect, traditional folk wisdom has guided scientific investigations that seek to ensure the population's safe, effective use of medicinal plants and stimulate their use as therapeutic alternatives and isolate compounds for the development of plant-based drugs [7]. *Dysphania ambrosioides* L., or *Chenopodium ambrosioides*, is known as wormseed, Jesuit's tea, Mexican tea, Payqu, Epazote, or Mastruz. Based on widespread knowledge, this plant is widely used by the population as an anti-parasitic, expectorant, oral infection treatment and wound-healing agent, and for the repair of fractures. For that, booth aerial parts and roots of the plant have been used in different preparations such as infusions, decoctions, and maceration [8-10].

Works published in the last decade using animal models have shown that the extract obtained from this plant has prevented bone loss in collagen-induced arthritis, induced bone repair in a rodent model, and exhibited anti-resorptive properties in a ligature-induced periodontitis model in rats [10-13]. However, little is known about the effects of these phytochemical molecules on osteoblastic cell activity, as no studies are currently published in the literature. Considering the potential of *D. ambrosioides* L. in the bone repair process, this *in vitro* study investigated its antioxidant activity, its influence on the differentiation of MC3T3 pre-osteoblasts, and its effect on bone matrix mineralization. Additionally, a phytochemical analysis of the leaf extract was performed.

## Material and Methods

### Vegetal Material

The material was collected in the municipality of Lagoa Seca, state of Paraiba, Brazil (7°10′15″ S, 35°51′14″ W). A voucher specimen was deposited in the Manuel de Arruda Câmara Herbarium (nº 042/ACAM) at the State University of Paraíba, Campina Grande, Paraíba. The leaves were dehydrated in a hothouse, where air circulated and ground.

### Acquisition of the Extract

The ground leaves were first submitted to a maceration process and remained at rest for five days in a percolator containing 98% (v/v) ethyl alcohol, followed by filtration. The extract was then vacuum-evaporated and freeze-dried.

### Phytochemical Analysis

The content of flavonoids, tannins, and polyphenols of the alcoholic extract from the leaves of *Dysphania ambrosioides* L. was determined using a spectrophotometer (UV mini – 1240, Shimadzu Corp., Kyoto, Japan). Determination of total polyphenol content [14], total flavonoid content [15], and condensed tannin content were performed [16].

*In Vitro* Antioxidant Activity

Total antioxidant capacity (TAC), reduction activity, sequestration of superoxide radicals, and copper chelation were performed to evaluate the antioxidant activity. The TAC test enables the evaluation of the ability of a sample to donate electrons to oxidized compounds with reactive potential. The assay is based on reducing molybdenium+6 from an ammonium molybdate solution to molybdenium+5, which forms a complex with phosphate under acid pH and a temperature of 95 °C. This phosphomolybdenum complex has a greenish color that can be monitored spectrophotometrically at 695 nm [17]. Antioxidant potential was determined concerning the ascorbic acid standard based on the slope equation applied to the calibration curve ($r^2 = 0.98$): $y = 35.89\ x - 0.0355$. Masses of 0.1 mg and 1 mg were used for this test. It was calculated that 1 g of sample has TAC equal to 120 mg of ascorbic acid. The plotting of the calibration curve enabled the determination of the mass of ascorbic acid at which the most excellent absorbance was obtained, which was then defined as the reference.

The reduction activity evaluates the potassium ferricyanide reduction to potassium ferrocyanide, which generates a greenish blue color (Prussian blue) monitored at 700 nm in the presence of iron chloride. This assay used masses of 0.05 mg, 0.01 mg, 0.025 mg, 0.5 mg, and 1 mg. The reduction power of the samples was quantified using the methods described elsewhere [18,19].

The copper chelation method is based on the sample's chelation capacity of copper ions. This test is performed with a violet reagent, which binds to free copper ions in the solution, generating a violet color monitored at 632 nm [20]. Different sample masses (0.05 mg, 0.01 mg, 0.025 mg, 0.5 mg, and 1 mg). The determination of the percentage of chelation of copper ions by the sample was established by comparison to the blank, which represents 0% chelation (maximum absorbance for violet monitored at 632 nm). For such, the following formula was used: (Ab-Aa / Ab) x 100, in which Ab is the absorbance of the blank, and Aa is the absorbance of the sample.

The superoxide radical sequestration assay is based on the capacity of a sample to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) to formazan by the superoxide radical in the riboflavin-light-NBT system at pH 7.4 and room temperature. Formazan causes the occurrence of a change from a pale-yellow color to purple, which is accompanied by spectrophotometry at 560 nm. For such, the method described by Dasgupta and De [21] was followed, and different sample masses were evaluated (0.05 mg, 0.1 mg, 0.25 mg, and 0.5 mg). The percentage of superoxide radical sequestration was established based on the data obtained for the sample, control (which represents 100% reduction due to the absence of antioxidant) and the blank (which represents 100% sequestration), using the following formula: % sequestration = (Ac – Aa) / (Ac – Ab), in which Ac is absorbance of the control, Aa is absorbance of the sample and Ab is absorbance of the blank.

Cell Viability and Proliferation Test by Reduction of Tetrazolium Dye (MTT)

Cell viability and proliferation were evaluated using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay based on the spectrophotometric determination of formazan formation

produced by viable MC3T3 cells [22]. Three MTT assays were performed at 24, 48, and 72 hours. Approximately 5 x $10^3$ MC3T3 cells were placed in each sterile 96 wells in α-MEM medium supplemented with 10% fetal bovine serum (FBS – Gibco, Thermo Fisher Scientific Inc., Waltham, MA, USA). Cells were placed in a medium without serum for 24 hours. Subsequently, the medium was aspirated, and the cells were added to the medium with 10% FBS in the absence (control) and presence of the extract at concentrations of 50 µg/mL, 100 µg/mL, 150 µg/mL, 200 µg/mL, and 250 µg/mL. Next, the MTT salt (1 mg/mL) was added to the cells, and the plates were incubated for another four hours. Ethyl alcohol A. G. (100 µL) was added to dissolve the precipitated formazan crystals, and absorbance was quantified in a 96-well plate reader at 570 nm. The assay was conducted in triplicate, and the results were obtained by comparing the treated and control cells. The relative percentage for the treatments was determined using the following formula: (Mt/Mc) x 100, in which Mt is the mean absorbance value for the treatments, and Mc is the mean absorbance value for the controls. Thus, values above 100% represent a more significant number of viable cells, and values below 100% indicate that the treatment affected cell viability.

Evaluation of Alkaline Phosphatase (ALP) Activity and Quantification

Initially, 5x$10^4$ MC3T3/mL cells were added to each well of the culture plates. After cell adhesion, the α-MEM medium was aspirated and replaced with the differentiation medium in the absence (control) or presence of the extract at concentrations of 50 µg/mL, 100 µg/mL, and 150 µg/mL. The supernatants were collected from the wells 7, 14, and 21 days after the onset of treatment and stored at -20 ºC until the assays. ALP activity was evaluated using a commercial liquiform ALP kit (Labtest Diagnóstica S.A., Lagoa Santa, MG, Brazil). In alkaline pH, the ALP present in the sample de-phosphorilizes the p-nitrophenyl phosphate reagent, releasing inorganic phosphate and p-nitrophenol. This is monitored at 405 nm, and the absorbance generated is directly proportional to the enzymatic activity of ALP. The initial absorbance reading was performed after 1 minute of reaction (A1), and another reading was done after the second minute (A2). ALP was quantified using the following formula: (A2-A1/2) x CF, in which CF is the correction factor calculated for the conditions described for Test 2764, and the result is expressed in units of ALP per liter (U/L).

Analysis of the Degree of Mineralization of the Matrix Using von Kossa Staining

The assay described by McGee-Russell [23] enables the viewing of mineralized nodules through the reaction between calcium phosphate and silver nitrate, forming silver phosphate, which is subsequently reduced to metallic silver by UV radiation. Thus, the nodules become easily identified under a microscope as black deposits on the cell layer. Each well of the three plates – one for each interval (7, 14, and 21 days) – was submitted to fixation with paraformaldehyde 4% at pH 7.4 for 20 minutes. Next, the cultures were covered with a 5% silver nitrate solution and exposed to UV radiation for 30 minutes. After rinsing in distilled water, the cultures were covered with a 5% sodium thiosulfate solution for 2 minutes to remove excess iron in the non-precipitated form and rinsed again with distilled water. The cells were counterstained with a solution of hematoxylin (1 mg/mL) for 5 minutes, rinsed with distilled water, and covered with phosphate-buffered saline. An inverted light microscope was used to photograph each well for the comparative analyses.

Statistical Analysis

The results were expressed as mean and standard deviation. Data was analyzed with GraphPad Prism software (version 8.02) using analysis of variance (ANOVA), followed by the student's t-test for the MTT assays and the quantification of ALP. A p-value < 0.05 was considered indicative of a statistically significant difference.

## Results

Phytochemical Analysis

The phytochemical characterization revealed a high tannin content with 43.27% catechin equivalents. Total polyphenols corresponded to 6.57% gallic acid equivalents, and flavonoids corresponded to 6.24% quercetin equivalents.

*In Vitro* Antioxidant Activity

The antioxidant activity of D. ambrosioides leaf extract was demonstrated by the TAC equivalent to 120 mg of ascorbic acid (Figure 1A) and the chelation of copper ions, relative to 79.2% of EDTA chelation (Figure 1B). Meanwhile, the percentage of superoxide radical sequestration showed a dose-dependent result (Figure 1C).

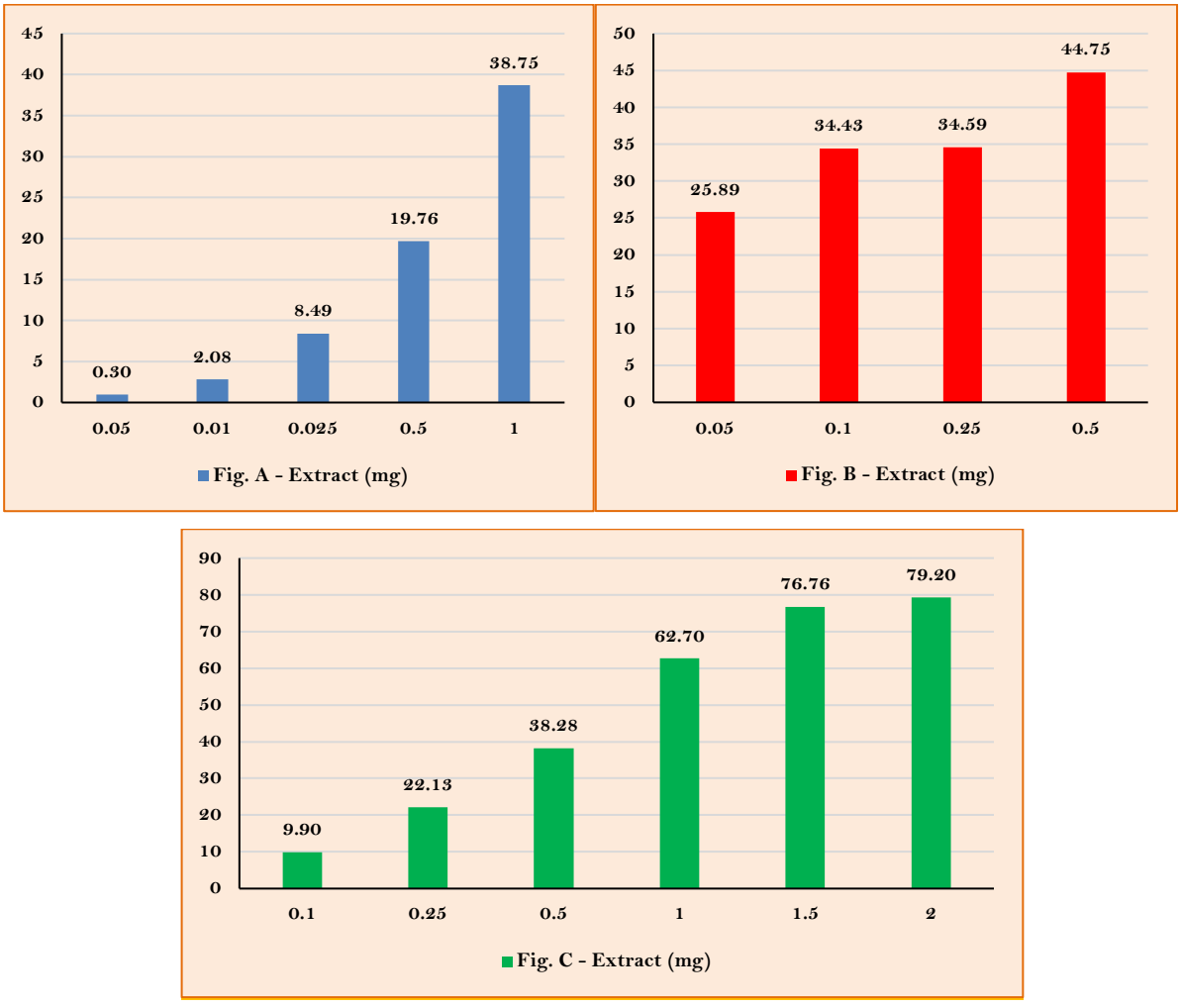



**Figure 1. Antioxidant potential of the alcoholic extract from the leaves of *Dysphania ambrosioides* L. (A) Relative percentage for reduction activity of extract; (B) Relative percentage for sequestration of superoxide radicals by extract; (C) Relative percentage of copper chelation by sample**

Cell Viability and Proliferation Tests by Reduction of Tetrazolium Dye (MTT)

All assays differed significantly from the control group ($p<0.05$) on the three tests (24, 48, and 72h), except for the 250 µg/mL - treatment (Figure 2). Based on the results, concentrations of 50, 100, and 150 µg/mL of the sample were chosen for the cell differentiation tests.

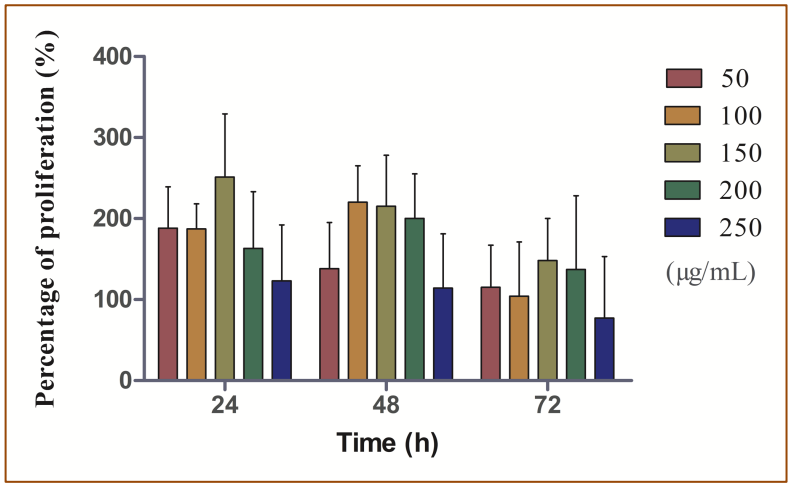
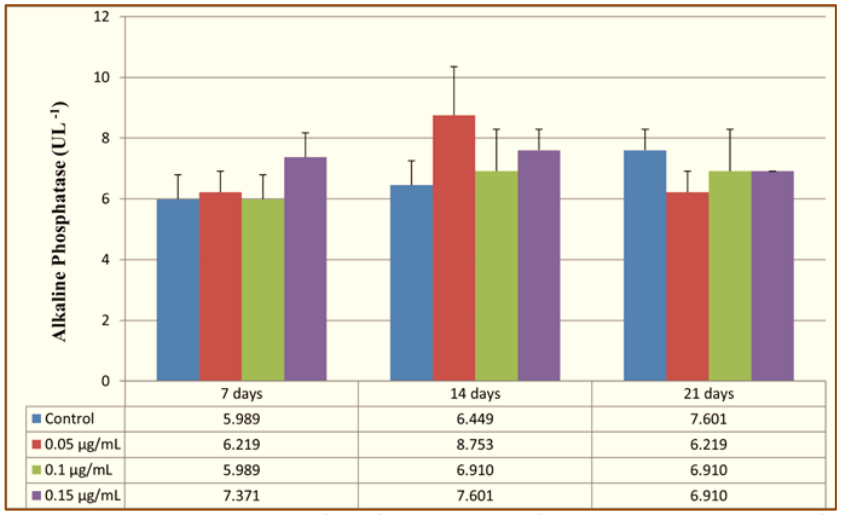



**Figure 2. Percentage of cell proliferation in each treatment (concentration of extract in µg/mL) in relation to the control for each period.**

ALP Activity and Quantification

No significant differences between the controls and treatments, between treatments on the same day or among treatments on different days were found ($p>0.05$), as stated in Figure 3.




| | 7 days | 14 days | 21 days |
|---|---|---|---|
| Control | 5.989 | 6.449 | 7.601 |
| 0.05 µg/mL | 6.219 | 8.753 | 6.219 |
| 0.1 µg/mL | 5.989 | 6.910 | 6.910 |
| 0.15 µg/mL | 7.371 | 7.601 | 6.910 |

**Figure 3. Quantification of alkaline phosphatase for each treatment in 7, 14, and 21 days of differentiation.**

Degree of Mineralization of the Matrix Using von Kossa Staining

The images obtained for the 7 days served as the control since the MC3T3 cells are in the maturation phase of the ECM in this period, and it is, therefore, possible only to observe cells in semi-confluence. Likewise, no mineralized nodules were found in any treatments or controls at 14 days. At 21 days, some regions were stained, indicating the presence of calcium phosphate in the medium, but no typical mineralized nodules were identified (Figures 4, 5, and 6).

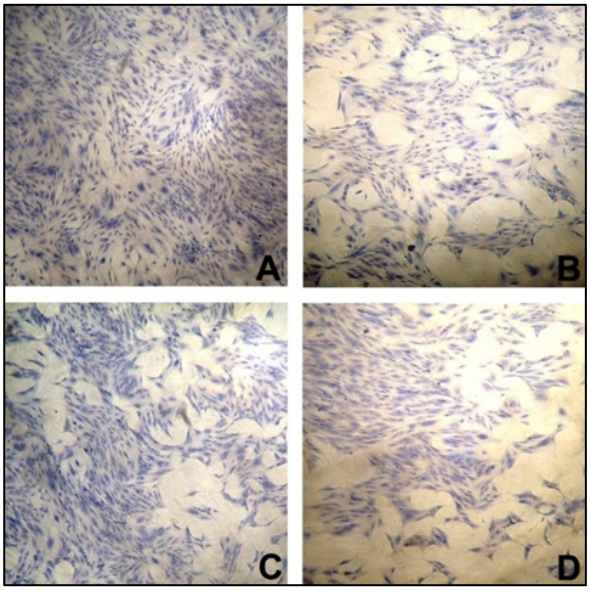



**Figure 4. Photomicrographs of MC3T3 cultures at 7 days of treatment with extract stained using the von Kossa method (magnification: 40x). Control (A); 50 µg/mL (B); 100 µg/mL (C); 150 µg/mL (D).**

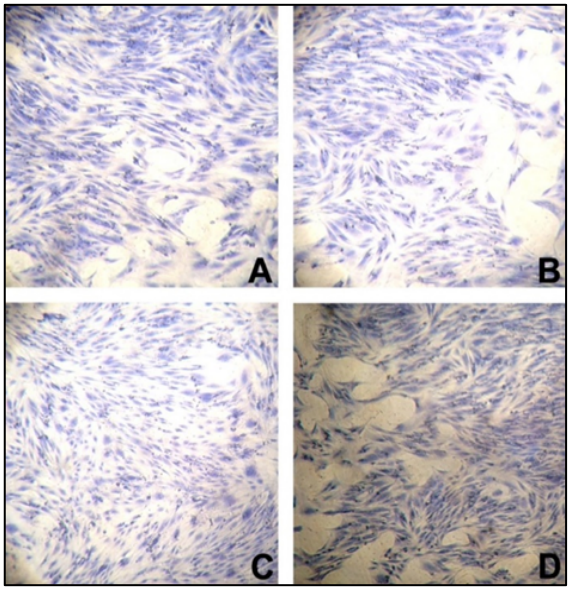



**Figure 5. Photomicrographs of MC3T3 cultures at 14 days of treatment with extract stained using the von Kossa method (magnification: 40x). Control (A); 50 µg/mL (B); 100 µg/mL (C); 150 µg/mL (D).**

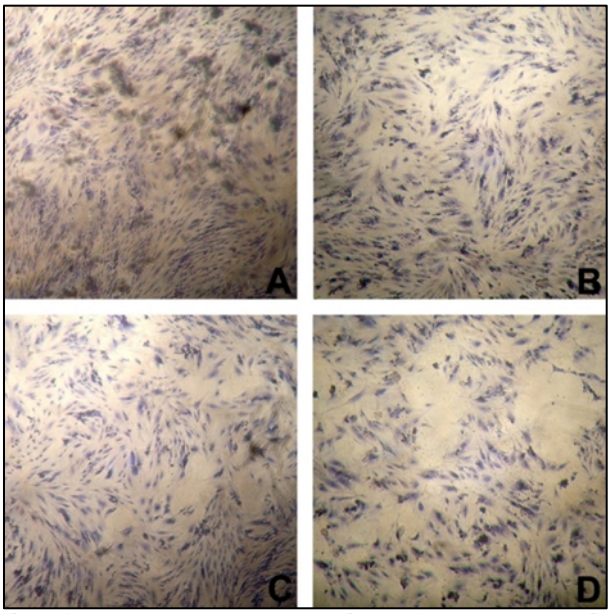



**Figure 6. Photomicrographs of MC3T3 cultures at 21 days of treatment with extract stained using the von Kossa method (magnification: 40x). Control (A); 50 µg/mL (B); 100 µg/mL (C); 150 µg/mL (D).**

## Discussion

In the present study, the tests performed on the extract from the leaves of *D. ambrosioides* indicate pronounced antioxidant activity, as demonstrated by the TAC and chelation of copper ions results. Antioxidant compounds have either direct or indirect action on bone metabolism. Such compounds affect osteoprogenitor cell recruitment and favor osteoblastic differentiation and function [24-26]. Thus, *D. ambrosioides* could have antiresorptive potential, which is an aspect that needs to be investigated.

The initial screening for determining polyphenols, flavonoids, and tannins content demonstrated that all these classes of compounds are well represented. The results underscored a high tannin content and the less

expressive content of total polyphenols and flavonoids. Flavonoids and polyphenols are vegetables' main antioxidant compounds and have been appointed as preventers of osteoporosis [26,27], and tannins are often related to healing processes [24,28]. Altogether, the phytocomplex might be responsible for the extract activities.

Besides the antioxidant potential, the anabolic potential of the *D. ambrosioides* extract was also investigated using cell culture methods. For such, it is crucial to determine the degree of toxicity and solubility of the compounds and their effect on the proliferation and morphology of the cell line. Regarding cell viability and proliferation, the 250 µg/mL treatment in the 72h period was the only group to return a value below 100%, indicating influence on cell viability. Nonetheless, other researchers have studied the toxicity potential of the D. ambrosioides extract. For instance, Carneiro et al. [13] have demonstrated safety parameters of its use in *in vivo* models, in which the extract did not cause toxicity in rats' liver, kidney, or stomach.

Accentuated cell proliferation was found in all other tested concentrations, reaching nearly triple the cell population at 150 µg/mL in the first 24 h (251% growth). Additionally, all treatments differed significantly from the control group regardless of the established time. These findings suggest that the extract can accelerate and stimulate pre-osteoblast proliferation, which is essential to the initial bone healing and remodeling phase. The concentrations of 50, 100, and 150 µg/mL were chosen for the cell differentiation assay based on MTT assay results.

The ALP enzyme is associated with the mineralization of the extracellular matrix. Still, its action is most remarkable in the second phase of osteoblastic differentiation since it is responsible for the maturation of the matrix that will subsequently be mineralized [29]. ALP promotes the hydrolysis of organic phosphates (β-glycerophosphate in the case of cell cultures), thereby increasing the concentration of phosphate ions at mineralization sites, which, together with the calcium ions in the surrounding medium, promotes the formation of calcium phosphate deposits [30]. Due to this central role and the ease of its biochemical and histological determinations, ALP has become the marker of choice in evaluating the phenotype or maturation of the development of mineralized tissue cells [29]. In the present study, the statistical analysis revealed no significant differences among treatments regardless of treatment time ($p>0.05$). Controversially, Pinheiro Neto et al. [31] noted a more excellent ALP activity during early fracture healing under the effect of a *D. ambrosioides* graft, enhancing bone regeneration.

The results indicate that the extract's action mechanism must not be directly related to osteoblastic differentiation, as no significant differences were found in the quantification of ALP between the treated cells and controls. Moreover, no mineralized nodules were found 21 days after the onset of treatment. These findings do not confirm the hypothesis of the osteoconductive potential of *D. ambrosioides*, which is based on folk medicine in communities where this plant is used topically on bone injuries to accelerate the regeneration process [7,24].

The degree of mineralization of the ECM formed by the culture cells was reflected by the mineralized area identified with an inverted microscope after treatment with silver nitrate using the von Kossa staining method. At 21 days after the onset of treatment for cell differentiation, the control group exhibited discrete grayish spots, which may represent the beginning of nodular formation. MC3T3 cells take approximately 21 days to differentiate and can exhibit mineralized nodules beginning in the second week of differentiation. These results are in accordance with Penha et al. [11] findings, in which the authors observed histological bone mineralization and repair after 30 days of treatment with *D. ambrosioides* extract.

Even though the findings do not confirm the osteoconductive action of the extract, one must not discard the possibility that the extract is beneficial to bone health during the repair of injuries or the

maintenance/recovery of normal bone mass since the extract of *D. ambrosioides* has already shown positives results regarding the increase bone density [11,31,32]. In addition, studies have also demonstrated the protective potential of the extract. Carneiro et al. [13], for instance, showed that *D. ambrosioides* was able to reduce bone loss by 58%, marked by a reduction in the number of osteoclasts in rats with periodontitis.

Even though antioxidant activity is correlated to combat oxidative stress, which is beneficial to bone health, reactive oxygen species (ROS) in low concentrations also act as modulators of osteoclasts and osteoblasts. Morphogenetic proteins (BMBs) in osteoblasts generate ROS, vital to the activation of ALP. Moreover, ROS induces mitogen-activate kinase proteins, which are important to the osteoclastic differentiation process [33]. Thus, previous studies have associated *D. ambrosioides* antioxidant potential through the 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical method with the enhancement of bone regeneration [31].

As the treated cells in the present study were not under oxidative stress, the presence of a potent antioxidant may have negatively affected osteoblastic differentiation. Arakaki et al. [33] found that MC3T3-E1 cells incubated in the presence of N-acetylcysteine antioxidants, in which the production of ROS was significantly suppressed, mineralization was also suppressed, demonstrating that ROS play an essential role in the regulation of osteoblastic differentiation [25,34,35]. Thus, further investigations are encouraged.

Moreover, compounds can positively influence bone health in different ways. Some can promote an anabolic effect on osteoblasts, stimulating proliferation, differentiation, and mineralization, whereas others have an anti-catabolic effect, acting on the differentiation and function of osteoclasts. For instance, pro-angiogenic compounds are also helpful to the regeneration process of bone injuries [11,13,24,31]. This is explained by the fact that the regeneration of a bone injury is a complex process involving several steps. Thus, besides promoting pronounced cell proliferation, which is important to the healing of bone tissue, *D. ambrosioides* may also influence other processes that were not analyzed herein. Other analyses need to be performed focusing on antiresorptive and osteoinductive activities to demonstrate its effect on bone health, the fracture healing process, and angiogenic activity.

## Conclusion

The alcoholic extract from the leaves of *D. ambrosioides* proved to be a potent antioxidant but did not exhibit osteoinductive potential.

## Authors' Contributions

| | | |
|---|---|---|
| JMMA | https://orcid.org/0009-0009-0496-7247 | Formal Analysis, Investigation, Data Curation, and Writing - Original Draft. |
| PGSV | https://orcid.org/0000-0001-8975-7162 | Writing - Review and Editing. |
| RFL | https://orcid.org/0000-0002-2818-7036 | Writing - Review and Editing. |
| CMAM | https://orcid.org/0000-0003-4104-3448 | Writing - Review and Editing. |
| FG | https://orcid.org/0000-0003-0636-1635 | Formal Analysis and Data Curation. |
| CAGB | https://orcid.org/0000-0003-1979-9919 | Methodology, Formal Analysis, and Resources. |
| HAOR | https://orcid.org/0000-0003-2252-1221 | Methodology, Formal Analysis, and Resources. |
| ACDM | https://orcid.org/0000-0002-8682-0178 | Methodology and Resources. |
| EMMBC | https://orcid.org/0000-0002-3166-709X | Conceptualization, Writing - Review and Editing and Supervision. |

All authors declare that they contributed to a critical review of intellectual content and approval of the final version to be published.

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## Conflict of Interest

The authors declare no conflicts of interest.

## Data Availability

The data used to support the findings of this study can be made available upon request to the corresponding author.

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