



DICTYOTA DICHOTOMA (PHAEOPHYCEAE) METHANOLIC EXTRACTS EXHIBIT ANTITUMORAL EFFECTS ON BREAST CANCER CELLS AND INDUCE OSTEOBLASTS DIFFERENTIATION

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(Received 05th July 2021; accepted 09th February 2023)

ABSTRACT. There is a growing interest in macroalgae as natural products with antioxidant and anticancer activity. In this work, we studied the anti-tumoral effect of an algal extract (AE) derived from the marine alga *Dictyota dichotoma* on human breast cancer cells (MCF-7). We also evaluated the cytotoxic effects on non-tumorigenic cells and the effects on osteoblastogenesis *in vitro*. We found that the AE contains high levels of polyphenols and anti-oxidant activity measured by DPPH and Folin-Ciocalteu methods, respectively. Using trypan blue and MTS assays we demonstrated a significant inhibition of MCF-7 cell proliferation and viability. The changes in protein phosphorylation levels were examined through Western blot analysis, finding a decrease of phosphorylated AKT (Ser473) and its target molecule BAD (Ser136). In addition, AE inhibits cell migration determined through the wound healing assay and decreases cellular adhesion at all concentrations probed. Interestingly, AE does not affect the number and morphology of normal osteoblastic human cells, indicating its selectivity. Moreover, using colorimetric methods, we found that low doses of AE increase the production of osteoblastogenesis markers. These findings indicate that *D. dichotoma* is a valuable source of bioactive compounds for its regulatory effects on processes involved in metastasis and healthy effects in osteoblasts.

Keywords: Algal extract, cell adhesion, cell migration, cytotoxicity, osteoblastogenesis.

INTRODUCTION

The research of natural products has deeply influenced the studies of various fields ranging from pharmacology to cancer medicine [1, 2]. Many constituents extracted from

marine organisms, such as algae, have been associated with a variety of biological activities [1, 3, 4]. An interesting and important property of marine drugs is the antitumor activity, and lots of algae and their metabolites have shown potent cytotoxic effects [1, 5, 6]. Consequently, these metabolites are the key to the discovery of new pharmaceutical drugs from algae as antitumor treatments. Extensive investigations of brown algal extracts evidenced anticancer potential in different tumoral cells [7]. Specifically, the macroalga *D. dichotoma* demonstrated to be a source of valuable bioactive compounds such as diterpenes and lactones with antitumoral and antiviral effects [8, 9].

AKT is a serine/threonine kinase considered a crucial therapeutic target for playing a central role in multiple interconnected cell signaling mechanisms that define cell death or survival. Disruptions in the AKT-regulated pathways are associated with cancer [10]. Phosphorylation of AKT at serine 473 is required for maximal activation of the kinase [11]. Previous studies have suggested that activated AKT could phosphorylate BAD, contributing to cell survival, otherwise apoptosis is carried out [12]. BAD can be phosphorylated at either Ser-112 or Ser-136, whereas AKT phosphorylates BAD specifically at Ser-136 [13].

It is already known that tumors have preference for specific organs, such as breast cancer that manifests predominant osteoblastic bone metastases. In patients with breast cancer, maintaining bone health is a major clinical challenge because both the disease itself and most forms of treatment exert negative effects on bone metabolism [4, 14, 15].

Cell adhesion, invasion, angiogenesis and proliferation are stages of bone metastases development and progression. Proliferation, extracellular matrix synthesis, and mineralization are different stages of osteoblast differentiation [16], characterized for the expression of different phenotypic markers. Elevated levels of alkaline phosphatase (ALP) and collagen type I expression signify that early marker of differentiation are present; meanwhile during mineralization osteocalcin appears as a late marker. Although macroalgae, especially brown and red algae, have been studied for a variety of purposes, the investigations for application in bone health are few and very recent [17, 18].

Even though anticancer agents should act exclusively against tumor cells, various chemotherapeutic drugs frequently used exhibit considerable adverse side effects. Thus, discovery of new natural drugs that specifically targets and inhibits cancer cells without damaging healthy cells is the main objective of various scientific cancer researches. In this respect, in the present work the antitumoral activity of the methanolic extracts derived from the brown alga *Dictyota dichotoma* (Hudson) J.V Lamouroux on a human breast adenocarcinoma cell line (MCF-7) was assessed. In parallel, we studied whether this algal extract (AE) has cytotoxic effects on osteoblastic non-tumoral cells (hFOB) and its effects on osteoblastogenesis.

MATERIALS AND METHODS

Collection and algal extracts preparation

Specimens of the brown alga *Dictyota dichotoma* (Phaeophyceae, Dityotales) were collected from subtidal regions in Las Grutas, Río Negro Province, Argentina (Fig. 1). That population was previously studied and identified as *D. dichotoma* by others [19, 20, 21]. Specimens are stored at the Herbarium of Universidad Nacional del Sur (BBB; Bahía Blanca, Argentina) under the codes: EJC335, EJC336 and CF347 to CF351.

The material was rinsed with distilled water and the methanolic extract was obtained as we previously reported [22]. One hundred g of dried material gave a yield of 2.46 g of methanolic extract.



Fig 1. Representative image of the brown alga *Dictyota dichotoma* (Phaeophyceae, Dictyotales) from subtidal regions in Las Grutas, Río Negro Province, Argentina.

Total phenolic content

Total phenolic content of each extract was determined spectrophotometrically using the Folin-Ciocalteu method [23]. The absorbance was measured at 517 and 760 nm in a Jenway 6715 UV–Vis spectrophotometer. Gallic acid solution was used for the calibration of the standard curve.

Antioxidant activity

The antioxidant activity was measured in terms of radical-scavenging ability according to the DPPH method and the absorbance was measured at 517 nm [24]. α -tocopherol was used as a positive control.

Cell culture

The MCF-7 human breast cancer cell line was cultured in a RPMI medium, the MC3T3-E1 cell line derived from mouse pre-osteoblasts was cultured in α -MEM and the human fetal osteoblastic cell line, hFOB, was cultured in a DMEM-F12 medium. All media were supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. For osteoblasts differentiation the cells were cultured in an osteogenic medium (4 mM β -glycerophosphate, 50 μ g mL⁻¹ ascorbic acid). All the cell lines were acquired commercially (ATCC, Manassas, VA, USA) and incubated at 37 °C and 5% CO₂.

Trypan blue uptake

The MCF-7 cells were treated with different concentrations of AE (24 h). Then, the cells were detached, collected by centrifugation and 0.04% of trypan blue was added to quantify live (unstained) or dead cells (nuclear and cytoplasmic staining), using a hemocytometer. At least 100 cells per condition were counted.

Cell viability assay (MTS).

Cellular viability was determined employing the *Cell Titer 96 Aqueous One Solution Cell Proliferation Assay* kit (Promega). The MCF-7 cells (1250 cells well⁻¹) were seeded in quadruplicate into a 96-well plate in a growth medium and incubated at the indicated concentrations of AE (24 and 48 h). MTS colorimetric assay was performed as indicated by the manufacture and the absorbance was measured at 490 nm.

Crystal Violet (CV) stain

hFOB cells were grown in a 24-well plate and treated with different concentrations of AE (24 h). Then, the cells were fixed and stained with 0.1% of CV for 30 min at room temperature and washed with distilled water. Images were recorded using a digital camera (Olympus C7070WZ) coupled to an optical microscope (Olympus BX51).

Wound-healing assay

A scratch wound (0.3–1 mm wide and approximately 1 cm in length) was applied with a sterile tip to the MCF-7 cells grown at 100% of confluence. Cell debris was removed and different concentrations of AE were added for 48 h. Images from three specific fields of the wounds were taken at 0, 12 and 24 h of incubation, using a digital camera (Olympus C7070WZ) coupled to an optical microscope (Olympus BX51). The percentage of migration was calculated by considering as 100% the gap size measured at 0 h.

Adhesion assay

Sub-confluent MCF-7 cells were incubated with a medium containing different concentrations of AE. Cells were trypsinized, counted, and seeded in triplicates in a 96-well plate (5000 cells well⁻¹). After 30 min at 37 °C, non adherent cells were removed and adherent cells were fixed (100% methanol, 10 min) and stained (0.05% CV, 15 min). Images (4x) were recorded using a digital camera (Olympus C7070WZ) coupled to an optical microscope (Olympus BX51). The number of adherent cells was counted.

Western blot analysis

Proteins of MCF-7 cell lysates were quantified by the Bradford method [25]. The Western blot protocol was performed as previously described [26].

Assays for osteoblast differentiation and mineralization

MC3T3-E1 cells were cultured in a 6-well plate (2x10⁵ cells well⁻¹) in a growth medium, which was replaced by an osteogenic medium with AE or vehicle (water) every 2–3 days. The ALP activity was determined colorimetrically using a commercial kit (Wiener Lab., Argentina) at 0, 7 and 14 days of treatment. The absorbance was measured at 405 nm and normalized with protein levels determined by Bradford Protein Assay. Collagen production evaluated with a Sirius red-based colorimetric assay and calcium deposition evidenced through Alizarin red S staining were determined after 21 and 28 days of AE treatment, following Ryu et al. procedure [27]. Images were recorded using a digital camera (Olympus C7070WZ) coupled to an optical microscope (Olympus BX51).

Statistical analysis

Statistical differences between mean were calculated by the two-tailed *t* test. Probability values below 0.05 ($p < 0.05$) were considered significant and values below 0.01 ($p < 0.01$) and 0.001 ($p < 0.001$) were considered highly significant. Quantitative data are expressed as means \pm SEM from the indicated set of experiments. The Bonferroni test was used for mean comparison.

RESULTS AND DISCUSSION

Antioxidant assays and total phenolic content

The analysis of *D. dichotoma* using water and methanol as solvents of extraction, showed an antioxidant activity 1.5-fold greater and polyphenol content 2.7-fold greater in methanolic extracts. Hence, in the present work, the methanolic extracts were selected to evaluate the antitumoral activity.

The results indicated that the total phenolic content of the methanolic extracts was $920.61 \pm 66.69 \mu\text{g GAE g}^{-1}$ dry algae and the DPPH radical scavenging activity was $37.87 \pm 5.20\%$ at the concentration of $8 \text{ mg dry algae mL}^{-1}$, which is the antioxidant activity equivalent to that found in a solution of α -tocopherol of $3.18 \mu\text{g mL}^{-1}$.

The extracts showed an IC₅₀ for DPPH activity of 35.53 mg mL^{-1} , this value was higher than the IC₅₀ of some commercial antioxidants such as α -tocopherol (IC₅₀: $4.35 \mu\text{g mL}^{-1}$, present study), ascorbic acid, BHA [28] and BHT [29], indicating a lower antioxidant activity. However, other studies in members of the same genus demonstrated higher antioxidant activity, such as *D. ciliolata* and *D. cervicornis* [30]. Regarding to polyphenol production, it cannot be considered an isolated parameter because it is governed by both extrinsic and intrinsic factors, which was previously evidenced by variations in the phenolic content of brown macroalgae [31].

Effect of AE on MCF-7 cell proliferation and cellular viability

The evaluation of MCF-7 cells morphology and number after methanolic extracts treatment indicates the absence of cytotoxicity at concentrations of 10 and $50 \mu\text{g mL}^{-1}$, whereas considerably reduction in the number of cells and rounded cells were found at 100, 200 and $500 \mu\text{g mL}^{-1}$ (Fig.2A). The trypan blue exclusion assay confirmed the cytotoxicity of AE on tumoral cells (Fig.2B). The results indicate that $100 \mu\text{g mL}^{-1}$ of AE decreases the number of tumoral cells by 18.22% ($\pm 2.73\%$) versus control, while lower concentrations do not modify the number of cells. In addition, cell viability was decreased at the same condition that decrease the cell number ($100 \mu\text{g mL}^{-1}$, 48h) (Fig.2C).

Funahashi et al. [32] confirmed that seaweeds are more effective than some chemotherapeutic agents used in the clinical treatment of breast cancer, by inducing apoptosis of tumoral cells. Similar reports demonstrated potent cytotoxic effects of other species of macroalgae on different human cancer cell lines [33, 34].

In concordance with our results, most of the studies on MCF-7 cells reported cytotoxic effect of AE $100 \mu\text{g mL}^{-1}$ or higher from different macroalgae [35, 36]. However, there is another methanolic AE (*C. surera*) which has more effective antitumoral actions on this cell line, decreasing proliferation at $20 \mu\text{g mL}^{-1}$ [22].

The reduction of MCF-7 cell viability depends on the dose of AE used and the effective concentration of cell viability inhibition of *D. dichotoma* is lower than that reported for other AE studied in human cancer cells [34].

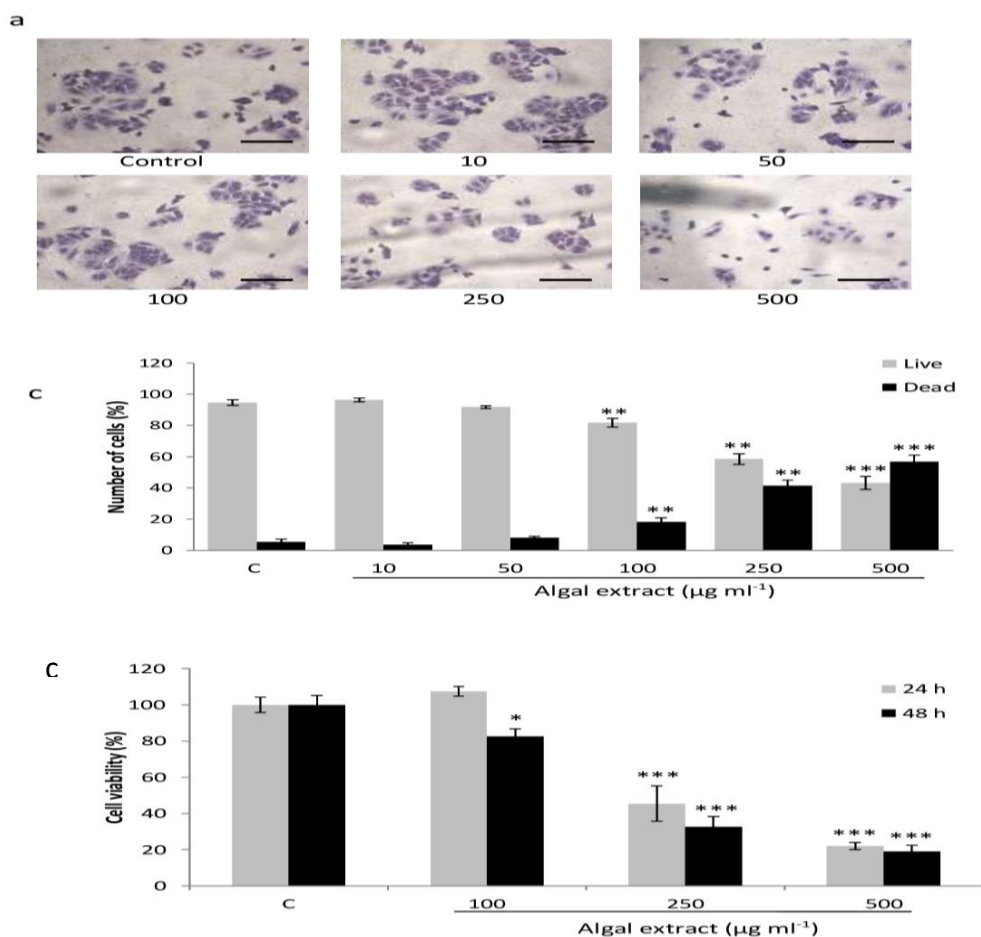


Fig. 2. AE inhibits proliferation and viability of tumoral cells. (a) Representative images of sub-confluent MCF-7 cells treated with different concentrations of AE (24 h). Scale bars, 100 µm. (b) Trypan blue assay showing the percentage of live and dead cells (24 h). (c) MCF-7 cellular viability of treated cells with different concentrations of AE (24 and 48 h) determined by the MTS assay. Representative image of the colorimetric assay and the bar graph with the percentage of cell viability are shown (24 and 48h). Results are expressed as means ± standard error of the mean (SEM). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Effect of AE on MCF-7 cell migration and adhesion

Tumor development and metastasis are pathological states associated with cellular migration. Through the wound healing assay, we evaluated the effect of AE on MCF-7 cell migration. After the scratch, the control cells migrated significantly into the cleared area and showed changes in cell morphology (Fig. 3, 24 h). At 500 µg mL⁻¹ of AE the cells occupied 12.4±3.3% of the wound compared to 36.4±1.9% of occupation of untreated cells, indicating that AE significantly inhibits the closure of the wound and cell migration. Another process that plays an important role in metastasis is the cellular adhesion. The results shown in Fig. 4 demonstrate that 24 h of AE treatment significantly

inhibits tumoral cells adhesion at all doses evaluated, with the highest inhibitory effect at $500 \mu\text{g mL}^{-1}$ ($95.8 \pm 1.4\%$ vs. control).

Most of the studies of macroalgae are focused on proliferation and cytotoxicity of tumoral cells. Less is known about other tumorigenic processes such as cellular migration and adhesion. Some studies in normal cells indicate that fucoidans obtained from brown algae affect the migration of HUVEC cells, suggesting a pro-angiogenic effect of these compounds [37]. Also, through the wound healing assay Baliano et al. [38] demonstrated a positive effect of the macroalga *Padina gymnospora* on fibroblasts migration. On the other hand, a sulfated polysaccharide extracted from brown algae showed anti-migration effects *in vitro* and also *in vivo* through the suppression of metastasis of Lewis lung carcinoma. In addition, adhesion assays demonstrated that this polysaccharide inhibits the heterogeneous adhesion on fibronectin [39]. Our previous results with *C. surera* extracts [22] indicated an inhibitory effect of AE on MCF-7 tumoral cells migration. However, up to the present, there are no other reports of studies evaluating cellular migration of tumoral cells exposed to *D. dichotoma* extracts.

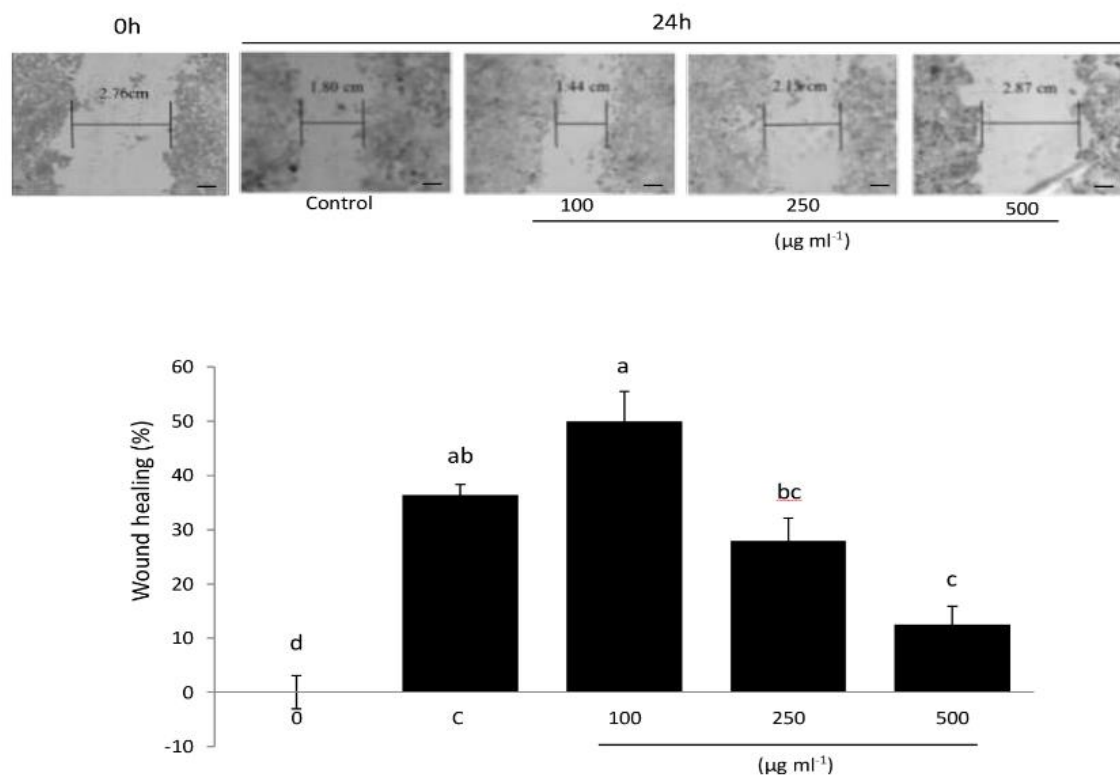


Fig. 3. AE prevents MCF-7 cell migration. Wound-healing assay evaluating the effect of different concentrations of AE on tumoral cell migration. Representative images of three independent experiments done in triplicate (magnification $40\times$). Scale bars, $100 \mu\text{m}$.

The bar graph shows the percentage of cell-covered area at 24 h versus 0 h. The Bonferroni test was used for mean comparison. Different letters indicate significant differences $p < 0.05$.

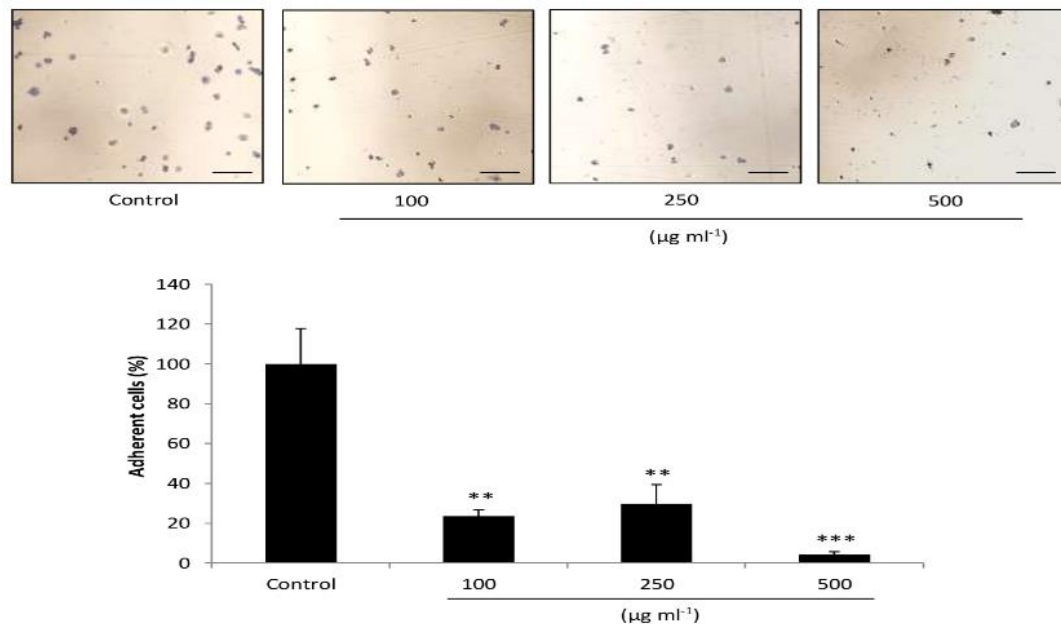


Fig. 4. AE decreases tumoral cell adhesion. The number of adherent MCF-7 cells is shown in the bar graph as mean±SEM. Significant differences are indicated as ** $p < 0.01$; *** $p < 0.001$. Representative images of three independent experiments done in triplicate (magnification 40×). Scale bars, 100 µm.

Effect of AE on cell survival pathway

To evaluate the signal transduction pathway involved in tumoral cell death, we studied the effect of AE on the phosphorylation levels of the pro-survival kinase AKT. In Fig. 5 immunoblot analysis revealed that 100 µg mL⁻¹ AE increases the phosphorylation levels of AKT at short times (5-60 min) of exposure, exhibiting a cellular defense response. However, at 24 and 48 h AE induced a decrease in AKT phosphorylation levels at serine 473, indicating an inhibition of this kinase.

Several reports indicate that natural compounds inhibit the proliferation and induce the apoptosis of human cancer cells by decreasing the levels of phosphorylated AKT. For example, flavonoid compounds such as sulfuretin and apigenin, have been proven to target AKT. Also, jaceosidin, a flavone from a traditional medicinal herb, has a marked effect on oral squamous cell carcinoma by AKT downregulation. The antiproliferative and antiinflammatory effect of luteolin via the inactivation of PI3K/AKT pathway has also been demonstrated by several studies [10].

According to the previous results of this work that clearly demonstrate that AE induces tumoral cell death in a dose-dependent manner over a range of concentrations from 100 to 500 µg mL⁻¹, we consider that the activation of AKT at short times of treatment with 100 µg mL⁻¹ suggests a cell defense response against the AE injury at early stages.

Since BAD is a target molecule of AKT kinase, we next studied possible changes in the phosphorylation status of the pro-apoptotic protein BAD at serine 136. As shown in Fig. 5 and, congruent with the above result, the Western blot analysis evidence that cell death induction by AE decreases phospho-BAD levels at long time of exposure (24 and 28 h).

The activity of the pro-apoptotic molecule BAD is regulated by the phosphorylation of two sites and it is well known that phosphorylation/ inactivation of BAD at Ser-136 is mediated by the serine/threonine protein kinase AKT-1 [40]. In MCF-7 cells, we observed a decrease of BAD phosphorylation at Ser-136 after 24-48 h of AE treatment, as a consequence of AKT inactivation, which is in agreement with the results on cell death. Altogether these results indicate that MCF-7 cells initially activate a defense response under AE aggression, whereas the programmed cell death finally starts.

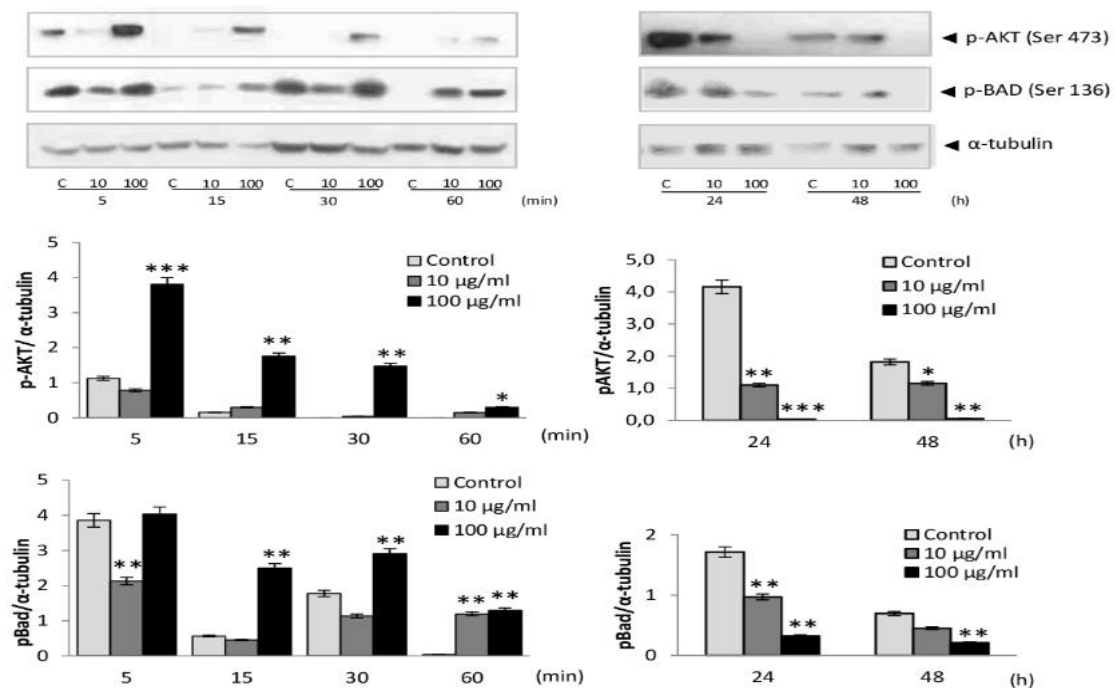


Fig. 5. AE modulates phosphorylation levels of AKT and BAD. MCF-7 cells were treated with 10 and 100 µg mL⁻¹ of AE for short (5-60 min) and long (24 and 48 h) period of time. Cell lysates were prepared and subjected to Western blot analysis using antibodies that specifically recognizes phospho-AKT (Ser 473), phospho-Bad (S136) and alpha-tubulin as loading control. The blots are representative of three independent experiments. Densitometric quantification of phosphorylated kinases and phospho-Bad normalized with loading control was performed and represented by the corresponding bar graphics. * $p < 0.05$ vs. control; ** $p < 0.01$ vs. control; *** $p < 0.001$ vs. control.

Effect of AE on healthy cells and osteoblasts differentiation

The studies of AE on non-tumoral cells would evidence its cellular selectivity. For this reason, before determining the effects on osteoblastogenesis, we evaluated the cytotoxicity of different concentrations of AE in a non-tumoral human osteoblastic cell line (hFOB), finding no significant differences in the number of cells compared to control cells (Fig.6A). The latter proves that the AE from *D. dichotoma* is not toxic to healthy cells, but can affect the viability of cancer cells. This may reveal an interesting selectivity of the investigated extracts. Namvar et al. [33] also found that methanolic extracts of three seaweed species, which inhibited the proliferation of different tumorigenic cell lines, including MCF-7, showed no toxic effects on normal Vero cell lines.

Since bone is the most common target of breast cancer metastasizes, we also focus on healthy osteoblastic cells. Thus, to advance in these studies, we evaluated the osteoblast differentiation in a preosteoblastic cell line (MC3T3-E1), cultured in an osteogenic medium with a low dose of AE ($1 \mu\text{g mL}^{-1}$). The results show that AE significantly increases the ALP activity by 28.73% ($\pm 1.28\%$) vs. control after 7 days, with no effect after 14 days (Fig.6B). In addition, treatment of osteoblasts with $1 \mu\text{g mL}^{-1}$ of AE for 21 and 28 days significantly increased the collagen fiber staining, being more effective at 28 days (Fig.6C), and alizarin red staining evidenced an increment of osteoblasts mineralization after 21 days of treatment (Fig.6D).

There are some reports of compounds derived from marine algae that have effects on bone health and osteoblast differentiation. Cho et al. [41] extracted fucoidan from brown alga *Undaria pinnatifida* and showed a significant increment in osteoblastogenesis, demonstrated by the appearance of high levels of ALP, osteocalcin and BMP-2 after fucoidan incubation. It was reported that *D. dichotoma* is one of the species of brown algae that contains fucoidan [6], which might explain our results on osteoblastogenesis. Ryu et al. [27] established that phlorotannins, tannins derived from brown algae, and its derivatives, regulate osteosarcoma differentiation by stimulating ALP activity, mineralization and collagen synthesis in human osteosarcoma cells. Up to the present, the studies about the effect of algae on osteoblast differentiation are limited and this is the first study that evaluates the selectivity and the beneficial effects of this AE on the osteogenesis process.

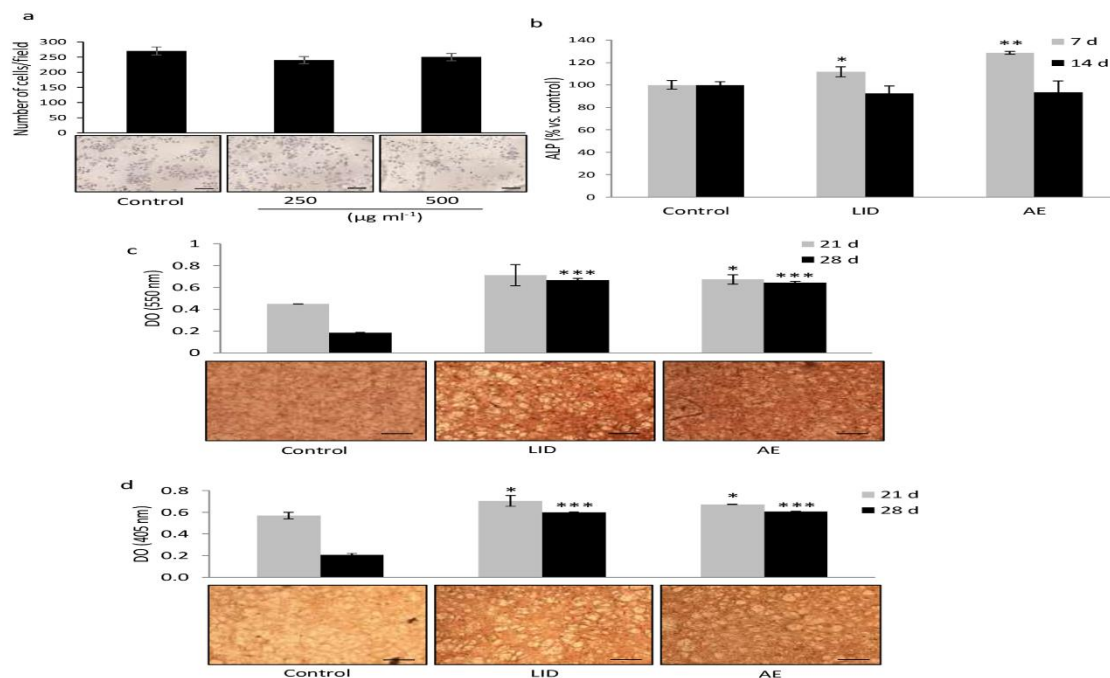


Fig. 6. AE is not cytotoxic for non-tumoral human osteoblasts and stimulates osteoblasts differentiation. (a) Effect of different concentrations of AE on hFOB cells after 24 h of exposure. Cell number is expressed as means \pm standard deviation (SD). $*p < 0.05$. (b) Effect of $1 \mu\text{g mL}^{-1}$ AE and 10^{-9} M lidandronate (LID, positive control) on ALP activity in MC3T3-E1 preosteoblasts cultured in osteogenic medium for 7 and 14 days. Sirius red (c) and alizarin red (d) staining after 21 and 28 days of treatment. Images are representative of three independent experiments ($n=3$) at day 28. Data are means \pm SD. $*p < 0.05$, $***p < 0.001$ versus control. Scale bars, 100 μm .

CONCLUSION

The macroalga *D. dichotoma* may be a source of valuable bioactive compounds useful as antitumoral preventive therapy demonstrating selective effects on metastasis processes regulation on human breast cancer cells, and for its additional healthy effects on osteoblasts. Nevertheless, additional research is required to identify the algal extract components which are responsible for its biological activity.

Conflict of Interest. The author declared that there is no conflict of interest.

Authorship Contributions. FM: methodology, investigation. CF: resources, investigation, formal analysis, conceptualization, validation, writing-review and editing. EP: Funding acquisition, supervision. SM: Funding acquisition, supervision, project administration. VL: resources, investigation, formal analysis, conceptualization, validation, writing-original draft, writing-review and editing, funding acquisition.

Financial Disclosure. This study was funded by CONICET (PIP11220130100070CO); PGI Universidad Nacional del Sur (24/234 and 24/124) and Agencia Nacional de Promoción Científica y Tecnológica (PICT 1749).

Compliance with ethical standards: The procedures were in accordance with the national laws.

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