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Marine green macroalgae: a source of natural compounds with mineralogenic and antioxidant activities

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Abstract :

Marine macroalgae represent a valuable natural resource for bioactive phytochemicals with promising applications in therapeutics, although they remain largely under-exploited. In this work, the potential of two marine green macroalgae (*Cladophora rupestris* and *Codium fragile*) as a source of bioactive phenolic compounds was explored, and antioxidant, mineralogenic, and osteogenic activities were evaluated. For each species, a crude hydroalcoholic extract (CE) was prepared by solid/liquid extraction and fractionated by liquid/liquid purification into an ethyl acetate fraction (EAF) enriched in phenolic compounds and an aqueous fraction (AF). Antioxidant activity, assessed through radical scavenging activity and reducing power assay, was increased in EAF fraction of both species and closely related to the phenolic content in each fraction. Mineralogenic activity, assessed through extracellular matrix mineralization of a fish bone-derived cell line, was induced by EAF fractions (up to 600 % for *C. rupestris* EAF). Quantitative analysis of operculum formation in zebrafish larvae stained with alizarin red S further confirmed the osteogenic potential of EAF fractions in vivo, with an increase of more than 1.5-fold for both *C. fragile* and *C. rupestris* fractions, similar to vitamin D (control). Our results demonstrated a positive correlation between phenolic fractions and biological activity, suggesting that phenolic compounds extracted from marine green macroalgae may represent promising molecules toward therapeutic applications in the field of bone biology.

Keywords : Ulvophyceae, Phenolic compounds, Antioxidant activity, Mineralogenic activity, Proliferative activity

Introduction

A priority in biotechnological innovation in the last decade has been the exploration of natural resources towards the discovery of new and promising bioactivities, e.g. with therapeutic potential or industrial applications (reviewed in Donia & Hamann 2003; Imhoff et al. 2011; Bonifácio et al. 2014). Surfactants for biomaterials are of particular interest to improve biocompatibility and efficacy and, in this regard, phytochemicals were found to have antioxidant capacities and also to promote osteoblast proliferation and differentiation (Habauzit & Horcajada 2007; Woo et al. 2010; Karadeniz et al. 2014, 2015), which make them suitable for applications related to bone regeneration or increased bone density (Watson & Schönlau 2015; Córdoba et al. 2015a,b). Marine macroalgae, beside their role in the provision of alginates, carrageenans, fibers and minerals for agrifood (Marsham et al. 2007; Mouritsen 2013; Stiger-Pouvreau et al. 2016) also represent a valuable source for bioactive phytochemicals (reviewed in Imhoff et al. 2011; Stengel & Connan 2015). While macroalgae are largely under-exploited in Europe, they are commonly harvested in Brittany for industrial applications such as cosmetic, food, thalassotherapy and medicinal products (Bourgougnon & Stiger-Pouvreau 2011). Brown algae are by far the most harvested species (97.2%) followed by red (2.7%) and green (0.06%) algae. Among the green algae, species of the genus *Ulva* and *Enteromorpha* are mainly valorised in Brittany (Chambre syndicale des algues et végétaux marins, CSAVM, com. pers.). Phenolic compounds from marine and terrestrial plants exhibit a wide range of chemical structures (Ajila et al. 2011; Singh & Bharate 2006) and a large spectrum of biological activities (Ksouri et al. 2012; Li et al. 2011). For example, they showed anti-diabetic (Lee & Jeon 2013), anti-microbial (Eom et al. 2012), anti-oxidative (Le Lann et al. 2008; 2012; Andrade et al. 2013; Tanniou et al. 2013; 2014; Stiger-Pouvreau et al., 2014 ; Surget et al., 2015), photoprotective (Surget et al. 2015), anti-tumoral (Deslandes et al. 2000; Zubia et al. 2009; Montero et al. 2016), anti-inflammatory (Kang et al. 2013; Kim et al. 2009) and radioprotective effects (Liu et al. 2011). Properties of bioceramic-based medical scaffolds have been successfully improved upon supplementation with phenolic compounds from marine origin, in particular toward increased bone tissue regeneration (Yeo et al. 2012; Córdoba et al. 2015a,b). Phlorotannins, i.e. phenolic compounds from brown seaweeds, extracted from *Ecklonia* brown algae species have also been shown to increase alkaline phosphatase (ALP) activity, mineralization, total protein and collagen synthesis in human osteosarcoma cells (MG-63 cells) (Ali & Hasan 2012; Ryu et al. 2009; Yeo et al. 2012; Karadeniz et al. 2015), suggesting that they may act as regulators of osteoblast differentiation

and osteosarcoma differentiation, with an implication of phenolic compounds in the mitogen-activated protein kinase (MAPK) pathway together with enhancing collagen and mineralization in the non-cytotoxic concentration range (Ryu et al. 2009). In the context of the biotechnological valorisation of marine algae in Europe and the discovery of new bioactive molecules as potential biomaterials for regenerative medicine, this work intended to get insights into the osteogenic and antioxidant capacities of the phenolic compounds extracted from two green seaweeds, the native *Cladophora rupestris* and the introduced *Codium fragile*. In that sense, crude extracts and semi-purified fractions were evaluated for total phenolic content, anti-oxidative activity and mineralogenic and proliferative effects, in this case using a bone-derived cell line from gilthead seabream (*Sparus aurata*) capable of extracellular matrix mineralization (Pombinho et al. 2004). The most active fractions were then tested *in vivo*, using the zebrafish as a model system to assess mineralization (Laizé et al. 2014).

Material and methods

Regular reagents and materials

Solvents and chemicals (analytical grade) were from Carlo Erba Reagent and Sigma-Aldrich, respectively, unless otherwise stated. Cell culture reagents and plastic consumables were from Invitrogen and Sarstedt, respectively.

Biological material

Samples of the green seaweeds *Codium fragile* (Suringar) and *Cladophora rupestris* (Linnaeus) were collected in rockpools of Porsmeur bay (Lanildut, France, 48°28'55.67"N, 4°46'14.76“O). Both species are two Ulvophyceae macroalgae. *C. fragile* belongs to the Bryopsidales order and the Codiaceae family and is an introduced species in Europe, with a worldwide spread. On the contrary, *C. rupestris*, is a native species in Brittany, widely distributed in temperate and cold temperate Atlantic Ocean, and belongs to the Cladophorales order and the Cladophoraceae family. After collection and removal of epiphytes, seaweeds were washed with deionized water, frozen, freeze-dried (Christ Beta 1-8 LD freeze dryer), grinded into powder and stored in the darkness at room temperature to limit the phenolic compounds degradation, as described in Le Lann et al. (2008).

Extraction and liquid-liquid semi-purification process

Fifteen grams of dry powder were macerated for 2 h at 40ºC in 150 mL of hydroethanolic solvent (1:1 distilled water/100% ethanol mixture) under agitation (200 rotations per min) and in the dark. After centrifugation (3 000 rpm, 15°C, 10 min), supernatant was collected and pellet was resuspended in 150 mL of hydroethanolic solvent and macerated for an additional 1 h. This last step was repeated once more and the three supernatants were pooled and passed through fiberglass. Ethanol was removed at 40°C by vacuum evaporation and distilled water was added to obtain a 100-mL crude extract (CE). Crude extract was separated into two semi-purified extracts, an aqueous fraction (AF) and an ethyl acetate fraction (EAF) using a protocol adapted from Stiger-Pouvreau et al. (2014) to concentrate polyphenols in EAF. Briefly, lipidic compounds and chlorophyllic pigments were removed through several washes with dichloromethane then, proteins and carbohydrates were precipitated using pure acetone and 100% ethanol. Purified extract was then divided into ethyl acetate and aqueous fractions.

Total phenolic content

Total phenolic content (TPC) was determined by spectrophotometry using Folin-Ciocalteu assay (Sanoner et al. 1999) modified according to Le Lann et al. (2008). Wells of a 96-well plate were successfully filled with 20 µL of extracts (dilution ranging from 1 to 0.01 $g.L^{-1}$), 130 µL of distilled water, 10 µL of Folin-Ciocalteu reagent and 40 µL of sodium carbonate solution (200 $g.L^{-1}$). After agitation, plate was incubated at 70°C for 10 min then placed on ice to stop the chemical reaction. Absorbance was measured at 620 nm using a Multiskan MS plate reader (LabSystems) and phenolic content was determined using a standard curve of gallic acid ranging from 0 to 200 µg/mL. Results are expressed as mg of gallic acid per g of dried extract (DW). All measurements were performed in triplicate.

Antioxidant assays

DPPH radical scavenging activity Radical scavenging activity of extracts/fractions and positive controls was determined using the DPPH (2,2-diphenyl-1-picrylhydrasyl) assay adapted from Le Lann et al. (2008) and Zubia et al. (2009). This assay tests the capacity of samples to scavenge the synthetic radical DPPH, but is independent of sample polarity and highly reproducible (Huang et al. 2005). Briefly, 100 µL of extract/fraction, positive control (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid or Trolox) or negative controls (distilled water and 100% ethanol) were added to 100 µL of DPPH solution (36.9 $mg.L^{-1}$) in a 96-well plate. Several dilutions of extracts/fractions and positive controls (ranging from 0.005 to 1 $g.L^{-1}$) were tested. Plate was incubated for 1 h in the dark and absorbance was measured at 540 nm. Radical scavenging activity of algal extracts was calculated as previously described in Le Lann et al. (2008) and Surget et al. (2015). For each sample, a curve of extract concentration against % of DPPH inhibition was generated to determine the concentration of extract needed to cause a 50% reduction of the initial DPPH concentration (IC_{50} in $g.L^{-1}$). A high IC_{50} is indicative of a weak radical scavenging activity and *vice-versa.* All measurements were performed in triplicate.

Reducing power Reducing power (RP) of seaweeds extracts was determined following the method in Zubia et al. (2009). RP assay implies an electron transfer and tests the capacity of antioxidants to reduce Fe^{3+}/ferricyanide complex to the ferrous form (Huang et al. 2005). A volume of 25 µL of extracts/fractions, negative controls (water and ethanol) or positive controls (Trolox) was mixed with 25 µL of sodium phosphate buffer (0.2 M; pH 6.6) and 25 µL of potassium ferricyanide (1%; w/v). Mixture was incubated for 20 min at 50°C. After cooling down on ice, 25 µL of 10% (w/v) trichloroacetic acid, 100 µL of distilled water and 20 µL of 0.1% (w/v) $FeCl_3$ were added and mixture was incubated at room temperature for 10 min. Absorbance was measured at 620 nm. Capacity of extracts/fractions to reduce Fe^{3+} was determined from EC_{50} in $g.L^{-1}$ (calculated by interpolation of a regression linear curve) and corresponds to the effective concentration of the sample to obtain an optical density equal to 0.5. All measurements were performed in triplicates.

Cultures of bone-derived cell line VSa13

Cell culture maintenance Gilthead seabream bone-derived cell line VSa13 (Pombinho et al. 2004) was used to evaluate mineralogenic and proliferative capacities of seaweeds extracts. Cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS (fetal bovine serum), 1% penicillin–streptomycin, 1% L-glutamine and 0.2% fungizone, and incubated at 33°C in a 10% CO_2-humidified atmosphere (Marques et al. 2007). Cells were sub-cultured 1:4 twice a week using trypsin-EDTA solution (0.2% trypsine, 1.1 mM EDTA, pH 7.4).

Cell exposure to seaweeds molecules Dry extracts/fractions from green macroalgae were dissolved in distilled water, 50% ethanol (in distilled water) or 100% ethanol according to their polarity to prepare 1000X stock solutions. CE, EAF and AF stock solutions were added to the culture medium to achieve concentrations of 0.05, 0.5, 5, 50, 100 or 250 $\mu g.mL^{-1}$ for EAF and 0.5, 5, 50 or 100 $\mu g.mL^{-1}$ for CE and AF. Medium supplemented with extracts/fractions was 0.2-mm filtered before applied to the cell culture.

Cytotoxicity assay Cells were seeded in 96-well plates at a density of 10^4 cells per well and further cultured until confluence. Culture medium was replaced with fresh medium containing either the vehicle (control) or algal extracts and renewed every 3-4 days. Cell viability was assessed after 9 and 18 days of exposure using the Cell Proliferation Kit XTT (AppliChem). Viability of the cells exposed to seaweeds extracts was calculated as percentage of survival in comparison with cells cultured in respective controls.

Proliferation assay Cells were seeded in 96-well plates at a density of 1.5×10^3 cells per well. After 24 h, culture medium was replaced with fresh medium containing either the vehicle (control) or seaweeds extracts and renewed every 3-4 days. Cell proliferation was determined after 9 days of exposure using the Cell Proliferation Kit XTT (AppliChem). Results are presented as percentage of cell proliferation, in comparison with respective controls.

Extracellular matrix mineralization Cells were seeded in 24-well plates at a density of 5×10^4 cells per well. Extracellular matrix (ECM) mineralization was induced in confluent cultures, by supplementing culture medium with 50 $\mu g.mL^{-1}$ of L-ascorbic acid, 10 mM of β-

glycerophosphate and 4 mM of calcium chloride (differentiation medium). Seaweeds extracts or vehicles were added to differentiation medium and renewed twice a week. After 17 days of culture, mineral deposition was revealed through alizarin red S (AR-S; Sigma-Aldrich) staining and quantified by spectrophotometry (Stanford et al. 1995). Results are expressed as percentage of ECM mineralization, relative to the respective controls.

Quantification of *in vivo* mineralization

Zebrafish larvae were exposed to increasing concentrations of EAF fractions of green macroalgae, *C. fragile* (1, 5 and 10 µg.mL^{-1}) and *C. rupestris* (5, 50 and 100 µg.mL^{-1}), and control (ethanol), from 9 to 11 days post-fertilization (dpf). Briefly, from 9 to 11 dpf, larvae were reared in a 24-well dish (2 larvae per well) placed in the dark, to avoid light degradation of the compounds, at 28.5ºC. Each well was filled with 1 mL of embryo medium (Westerfield 2007) supplemented with EAF, the most active fraction. Medium was renewed once a day and larvae were fed with *Artemia* nauplii (EG strain, INVE) twice a day. After 48 h of exposure, larvae were euthanatized with 168 µg.mL^{-1} of tricaine (MS-222; Sigma-Aldrich) and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 16 h at 4ºC. After two washes with PBS, larvae were stained with 0.01% alizarin red S (Sigma-Aldrich) solution for 30 min, washed twice with distilled water and maintained in 25% glycerol until analysis. AR-S fluorescence in zebrafish larvae was imaged under a MZ75 stereomicroscope (Leica) equipped with a green filter (excitation filter 546/10 nm, barrier filter 590 nm) and a F-view camera (Olympus). Mineralized area of the operculum was determined from the morphometric analysis of the fluorescence images using ImageJ software (National Institutes of Health) and normalized with the area of the head. Zebrafish larvae were also exposed to 1 fg.mL^{-1} of D (calcitriol; Sigma-Aldrich) as a positive control.

Statistical analysis

Results are presented as the mean of at least 3 replicates with standard deviation (SD). Homogeneity of variance was tested with the Bartlett's test at the 0.05 significance level. As the sample panel was small for the *in vitro* tests, a Kruskal-Wallis test was performed to highlight potential significant difference. In the case of significant difference between the data, non-parametric multiple comparisons (Behrens-Fisher test) were tested using the nmpc package. Statistical analysis of ECM mineralization assay and *in vivo* assay was performed with GraphPad Prism 4 (GraphPad, La Jolla, CA) using one-way ANOVA followed by

Dunnett's or Tukey's multiple comparisons tests, respectively, to determine statistical differences among groups. Differences were considered statistically significant for $p < 0.05$.

Results

Total phenolic contents and antioxidant activities

Total phenolic content (TPC) was determined in the crude extract (CE) and semi-purified fractions (EAF and AF) of *Codium fragile* and *Cladophora rupestris* (Table 1). While all the three fractions prepared from *C. rupestris* presented high phenolic contents (ranging from 15.833 ± 0.103 to 21.726 ± 0.899 mg GAE.g^{-1} DW, Table 1) with EAF showing the highest TPC, only EAF prepared from *C. fragile* exhibited a high TPC value, 10-fold higher than CE content (22.381 ± 0.206 and 2.202 ± 0.103 mg GAE.g^{-1} DW, respectively; Table 1). AF prepared from *C. fragile* exhibited the lowest TPC (0.298 ± 0.103 mg GAE.g^{-1} DW, Table 1) indicating an absence of aromatic compounds in this fraction. Antioxidant activities of each extract and those from the three positive controls (Trolox, BHA and ascorbic acid) were determined through the measurement of DPPH radical scavenging activity and reducing power, and expressed as IC_{50} and EC_{50}, respectively. In both assays, antioxidant activity was significantly higher in the ethyl acetate fractions of both species when compared to crude extracts and aqueous fractions (Table 1). Even though, EAF antioxidant activities were lower than the positive controls tested. The lowest antioxidant activity (i.e. high IC_{50} and EC_{50}) was always found in AF. Our data indicates that highest antioxidant activities were associated with EAF, which have the highest phenolic content, whereas lowest antioxidant activities were associated with aqueous fractions, exhibiting the lowest phenolic content.

Proliferative and mineralogenic activities of seaweeds extracts

Seaweeds extracts were further characterized for their capacity to alter cell proliferation and ECM mineralization (Figs. 1,2). Cytotoxicity of the extracts/fractions was first evaluated to determine non-toxic concentrations to be tested in subsequent assays. In that sense, cell viability was evaluated at 9 days (endpoint to assess cell proliferation) and 18 days (endpoint to assess extracellular matrix mineralization) using a wide range of extract/fraction concentrations and presented as cell survival rates. Survival rates were not concentration-dependent and always above 80% for all samples/concentrations at 9 days (Fig. 1B) and above 75% at 18 days (Fig. 2B). All concentrations were further used to evaluate proliferative and mineralogenic activities of the extracts.

Effect of seaweeds molecules on cell proliferation For both *C. fragile* and *C. rupestris* none of the extracts/fractions evidenced a pro-proliferative effect at the concentrations tested (Fig. 1A). At the highest concentrations (50 and 100 $\mu g.mL^{-1}$), the AF of *C. rupestris* presented a statistically significant negative effect of 10.7% and 13.6% respectively, compared to the control (Fig. 1A) likely indicating a toxic effect of the highest doses tested, also supported by the fact that cell survival was also negatively affected with the same doses (Fig. 1B).

Effect of the EAF fractions on ECM mineralization Alizarin red staining revealed that mineralization of VSa13 extracellular matrix was strongly increased (80% over control cultures) upon chronic exposure to the highest concentration (50 $\mu g.mL^{-1}$) of both ethyl acetate fractions (Fig. 2A). In the case of *C. rupestris* EAF, pro-mineralogenic effect increased with concentration indicating a dose-dependency. Aqueous fraction of *C. fragile* (50 $\mu g.mL^{-1}$) significantly increased mineral deposition (30% over control cultures), but no increase was observed at higher concentration indicating that this result might not be of biological significance. To further validate the pro-mineralogenic effect of ethyl acetate fractions, new EAFs prepared from the same algal powder were prepared and evaluated for their capacity to increase ECM mineralization using an increased range of concentrations 5, 50, 100 and 250 $\mu g.mL^{-1}$. Highest concentrations of *C. fragile* EAF (100 and 250 $\mu g.mL^{-1}$) were found to be toxic to the cells (data not shown) and therefore were not tested for mineralogenic activity. At 5 and 50 $\mu g.mL^{-1}$, new EAF of *C. fragile* induced mineral deposition by 50% and 370%, respectively, when compared to the control, indicating a dose dependent effect (Fig. 3A). Similarly, new EAF of *C. rupestris* stimulated ECM mineralization in a dose-dependent manner, reaching a 630% increase over the control at the highest concentration (250 $\mu g.mL^{-1}$) (Fig. 3B). These data further confirmed the mineralogenic potential of ethyl acetate fractions prepared from both green macroalgae. For the same concentrations, the effect was higher in the second assay (recent extracts).

In vivo effect of the ethyl acetate fractions on bone-mineralized area

To further study the osteogenic action of both phenolic-enriched seaweeds extracts, zebrafish larvae were exposed to ethyl acetate fractions and, *in vivo* bone formation and mineralization were assessed through the histomorphometric analysis of the operculum of exposed *versus* control larvae. After 48 h of exposure, larvae were immersed in alizarin red S, a calcium-

specific dye, to stain mineralized bone structures (Gavaia et al. 2000; Bensimon-Brito et al. 2016), which at this age (11 dpf) are mainly localized in the head. The operculum is among the first structures to develop and also straightforward to identify (Laizé et al. 2014) and its area was measured to assess EAF osteogenic activity. As expected from previous studies (Fleming et al. 2005), vitamin D (used here as a positive control) increased the mineralized area of zebrafish operculum approximately 1.5 fold over vehicle, therefore validating our experiment (Fig. 4). Ethyl acetate fractions of both *C. fragile* and *C. rupestris* also increased the mineralized area of zebrafish operculum (Fig. 4). At 1, 5 and 10 $\mu g.mL^{-1}$, *C. fragile* EAF significantly increased the mineralized area up to approximately 1.8-fold change over the vehicle (Fig. 4A). Higher concentrations were tested but were lethal to the larvae (data not shown). At 50 and 100 $\mu g.mL^{-1}$, *C. rupestris* EAF significantly increased the mineralized area of zebrafish operculum, up to approximately 1.6-fold change over the vehicle (Fig. 4B). Our data shows that both ethyl acetate fractions enriched in phenolic compounds increased the mineralized area of zebrafish operculum, at similar or higher levels than the positive control vitamin D.

Discussion

Marine macroalgae represent an under-expoited source of phenolic compounds with high potential for therapeutic or industrial applications. In this work, we explored the bioactive potential of extracts prepared from two marine Ulvophyceae algae, *Codium fragile* and *Cladophora rupestris*, focusing on antioxidant and osteogenic activities. The fractionation process proved to be efficient, since the phenolic content was higher in EAF from both species, although this process seems to depend on the species studied, since the aqueous fraction from *C. rupestris*, but not from *C. fragile*, presented residual levels of phenolic compounds. The difference in the polarity of phenolic compounds produced by each species could explain the differences occurred on each fraction. To further characterize the extracts/fractions prepared from green macroalgae, their antioxidant capacity was evaluated through two simple, fast, reliable and classical biochemical methods – the DPPH and Reducing Power tests – both measuring electron transfers (Huang et al. 2005). As antioxidant molecules can act through several and complex mechanisms (Frankel & Meyer 2000), the combination of two antioxidant tests allowed us to better highlight the *in vivo* complexity of interactions among antioxidants in foods and biological systems (Frankel & Meyer 2000). Our results revealed that independently of the assay or the species considered, EAF fractions exhibited the highest *in vitro* antioxidant activities which was also the fraction with highest

content in phenolic compounds (Table 1), in agreement with previous studies evidencing the anti-oxidative properties of phenolic compounds (Fujii et al. 2013; Tanniou et al. 2013; Stiger-Pouvreau et al. 2014; Surget et al. 2015). Since reducing power assay depends on the redox potential of the compounds present in a sample, we propose that phenolic compounds present in the marine macrophytes EAF are probably highly efficient antioxidants against free radicals (peroxyl or hydroxyl radicals) because of their low redox potential (Zhu et al. 2002).

Various natural compounds with antioxidant activity have been reported to influence biological and pathological processes, including bone mineralization and their therapeutic use in bone diseases, such as osteoporosis, or as nanocoatings to functionalize biomaterials by promoting cell adhesion and differentiation (Karadeniz et al. 2015; Watson & Schönlau 2015; Córdoba et al. 2015a,b). In agreement, our *in vitro* and *in vivo* data clearly demonstrated the mineralogenic and osteogenic properties of *C. fragile and C. rupestris* EAF, the fractions with higher phenolic content and higher antioxidant activity. *In vitro* assays on VSa13 cells exposed to all the extracts/fractions here purified, evidenced that only EAF of both species increased ECM mineralization, probably by promoting cell differentiation since no proliferative effect was observed. A pro-mineralogenic effect of phenolic compounds from higher plants was previously shown in mouse osteoblastic MC3T3-E1 cells by promoting calcium deposition alone (Hagiwara et al. 2011) or in combination with increased cell viability, alkaline phosphatase activity and collagen synthesis (Ding et al. 2010). Similar pro-mineralogenic effects were obtained with phlorotannins from brown algae on the human fetal lung fibroblast MRC-5 cells (Ryu et al. 2009) or human osteosarcoma MG-63 cells (Ryu et al. 2009; Yeo et al. 2012). Furthermore, the high potential of *C. fragile and C. rupestris* phenolic compounds as pro-mineralogenic phytochemicals for biomedical applications was confirmed through our *in vivo* assay based on developing zebrafish larvae. The mineralized area of the operculum (a dermal bone derived from neural crest cells; Eames et al. 2012) was increased in fish exposed to EAF fractions, at similar levels promoted by vitamin D (positive control), indicating a pro-osteogenic activity of the compounds present in both *C. fragile* and *C. rupestris* phenolic-enriched fractions, validating our *in vitro* data, and reinforcing the pro-mineralogenic activity of these fractions.

As a conclusion, the ethyl acetate fraction, which is rich in phenolic compounds, evidenced high antioxidant activity and high mineralogenic effect, promoting ECM mineralization of osteoblast-like cells *in vitro* and increasing the mineralized area of the zebrafish operculum *in vivo*. Thus, this study emphasized the biological potential of marine macroalgae and especially the use of invasive species, such as *Codium fragile*, for future biomedical

applications related to the improvement of bone status. To our knowledge, this is the first report of pro-mineralogenic activity in extracts/fraction prepared from two green macroalgae *C. fragile and C. rupestris.* Moreover, we demonstrated also the suitability of using zebrafish as a model for the screening of osteogenic and mineralogenic natural products.

Even though the identification of the active phenolic compound(s) responsible for EAF fractions activities was not the objective of this study, compounds such as bromophenols, coumarin or derivative from vanillic acids are known to be produced by green algae (Stengel et al. 2011), and could be the pro-mineralogenic compounds in these extracts. In that regard, future studies should be conducted to isolate, identify and test separately the bioactive compounds present in EAF of both Ulvophyceae *C. fragile* and *C. rupestris* in order to better understand their mechanisms of action.

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Figure legends

Table 1 Phenolic content and antioxidant activities of *Cladophora rupestris* and *Codium fragile* extracts. Phenolic content and antioxidant activities (DPPH scavenging activity and reducing power) of extracts, semi-purified fractions and commercial standards (ascorbic acid, BHA and Trolox) were assayed as described in material and methods. Results were expressed as mean values ± standard deviation, n=3. Different letters indicate significant difference between means according to Berhens Fisher test (p<0.05). The bold characters represent the most active fraction. (*mg*, milligram; *g*, gram; *L*, liter; *GAE*, Gallic Acid Equivalent; *DW*, Dried Weight extract).

Fig. 1 Effect of crude extract and semi-purified fractions of *Codium fragile* and *Cladophora rupestris* on VSa13 cell proliferation (**A**) and survival (**B**) at 9 days. Control values were determined in vehicle-treated cells and set to 100% (dotted line). Proliferation and survival data are presented as mean values ± standard deviation, n > 5. Asterisks indicate values significantly different from the control value according to Berhens Fisher test (** for p<0.01; *** for p<0.001). CE, Crude Extract; EAF, Ethyl Acetate Fraction; AF, Aqueous Fraction.

Fig. 2 Effect of crude extract and semi-purified fractions of *Codium fragile* and *Cladophora rupestris* on VSa13 extracellular matrix (ECM) mineralization (**A**) and survival (**B**) at 18 days. Control values were determined from vehicle-treated cells and set to 100% (dotted line). Mineralization and survival data are presented as mean values ± standard deviation, n = 4 (**A**) and n = 5 (**B**). Asterisks indicate values significantly different from the control value according to Berhens Fisher test (** for p<0.01; *** for p<0.001). CE, Crude Extract; EAF, Ethyl Acetate Fraction; AF, Aqueous Fraction.

Fig. 3 Effect of ethyl acetate fraction (EAF) of *Codium fragile* (**A**) and *Cladophora rupestris* (**B**) on VSa13 extracellular matrix (ECM) mineralization. Pictures of cell cultures treated with either the vehicle (V) or different EAF concentrations and stained with alizarin red S are

shown above each graph. Level of ECM mineralization in vehicle-treated cell cultures was set to 100% (dotted line). Mineralization data are presented as mean values ± standard deviation, n = 4. Asterisks indicate values significantly different from the vehicle value (one-way ANOVA followed by Dunnett's post-hoc test, * for $p<0.05$; ** for $p<0.01$). N.D. – not determined.

Fig. 4 Effect of ethyl acetate fraction (EAF) of *Codium fragile* (**A**) and *Cladophora rupestris* (**B**) on the mineralization of zebrafish operculum area. Larvae at 9 days post-fertilization were exposed for 48 h to increasing EAF concentrations, to vehicle (V) or to 1 $fg.mL^{-1}$ of vitamin D (positive control). Mineralization data are presented as mean values ± standard deviation, $n \geq 3$. Asterisks indicate values significantly different from the vehicle value (one-way ANOVA followed by Tukey's post-hoc test, * for $p<0.05$; ** for $p<0.01$; *** for $p<0.001$).

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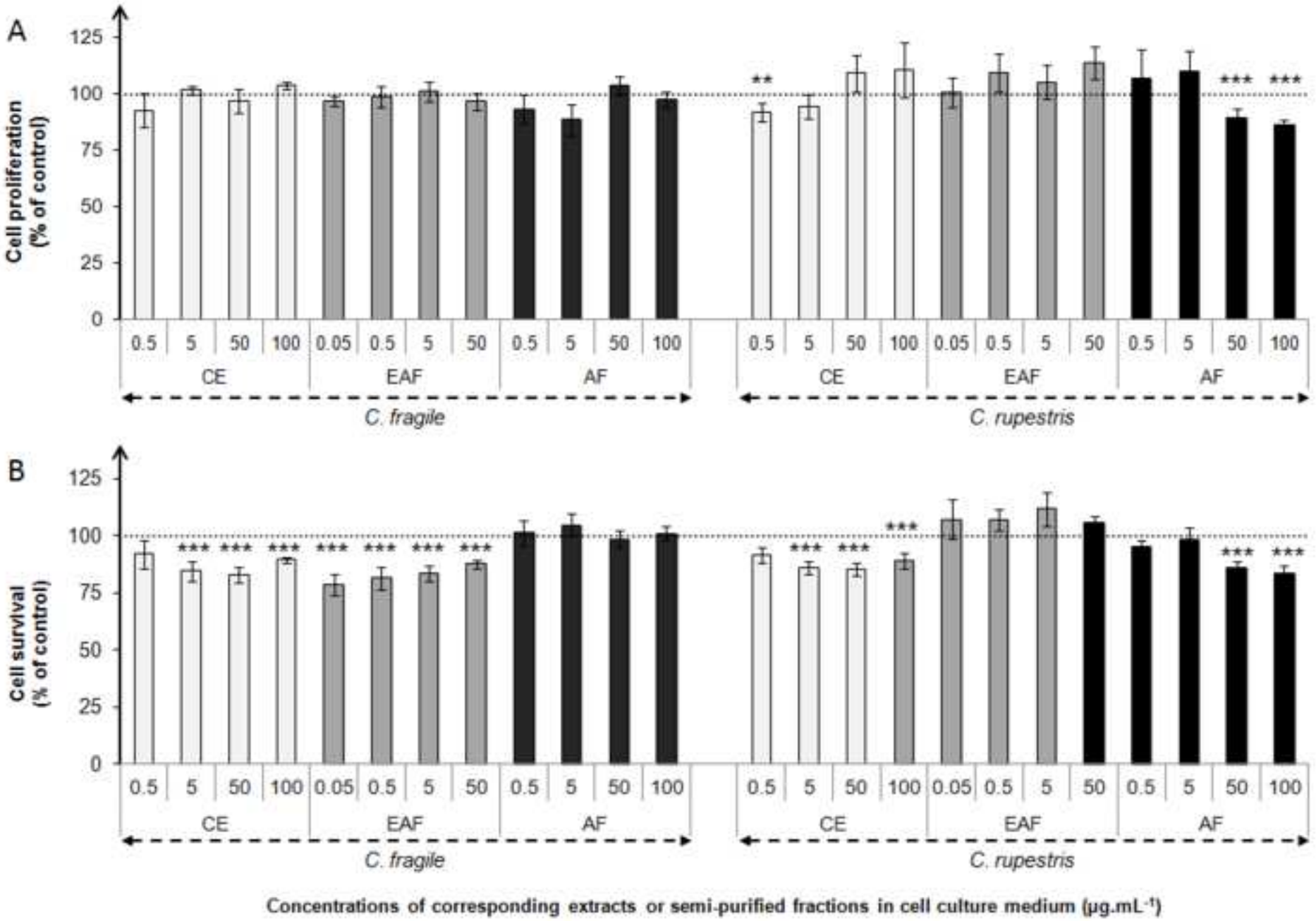

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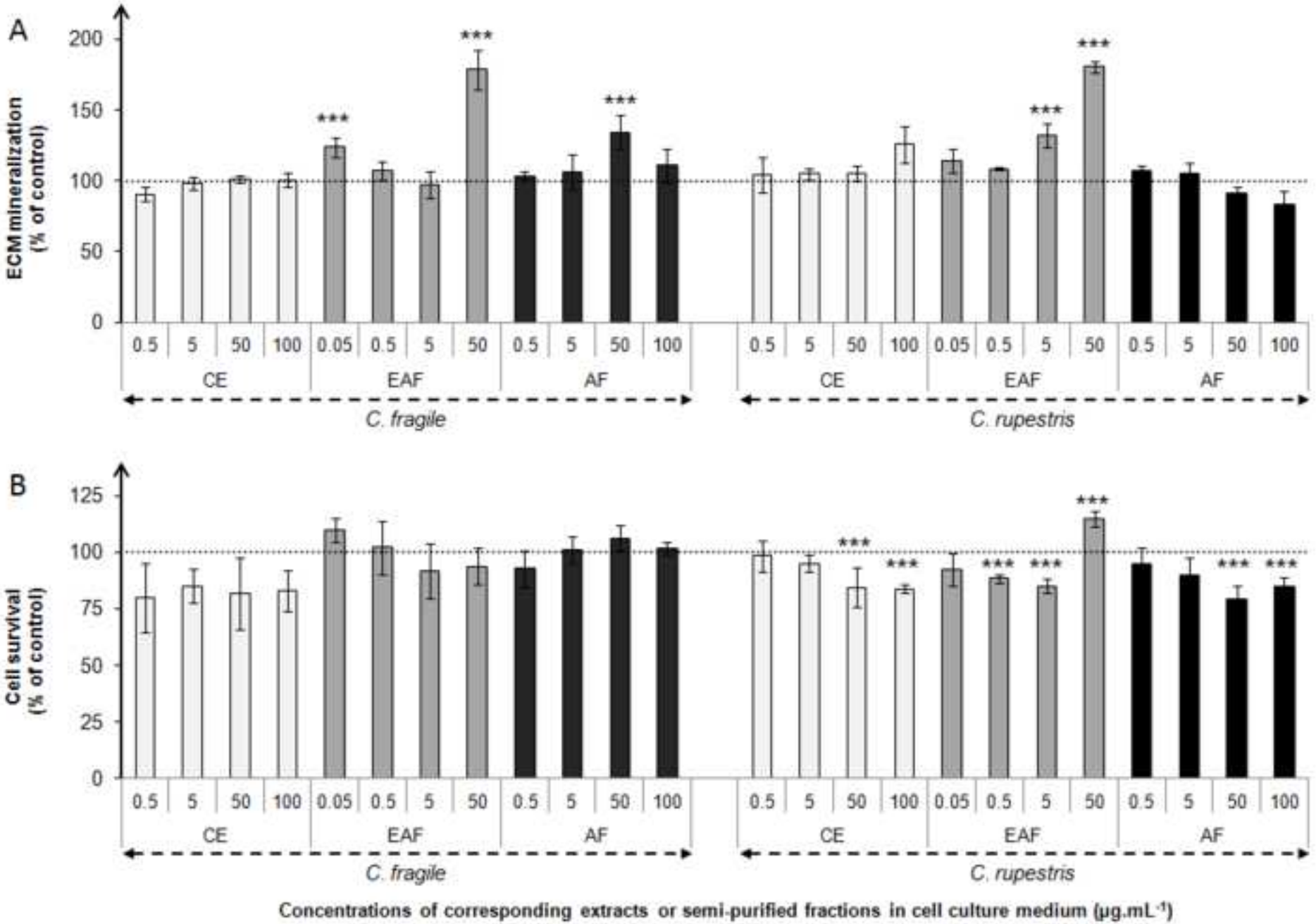

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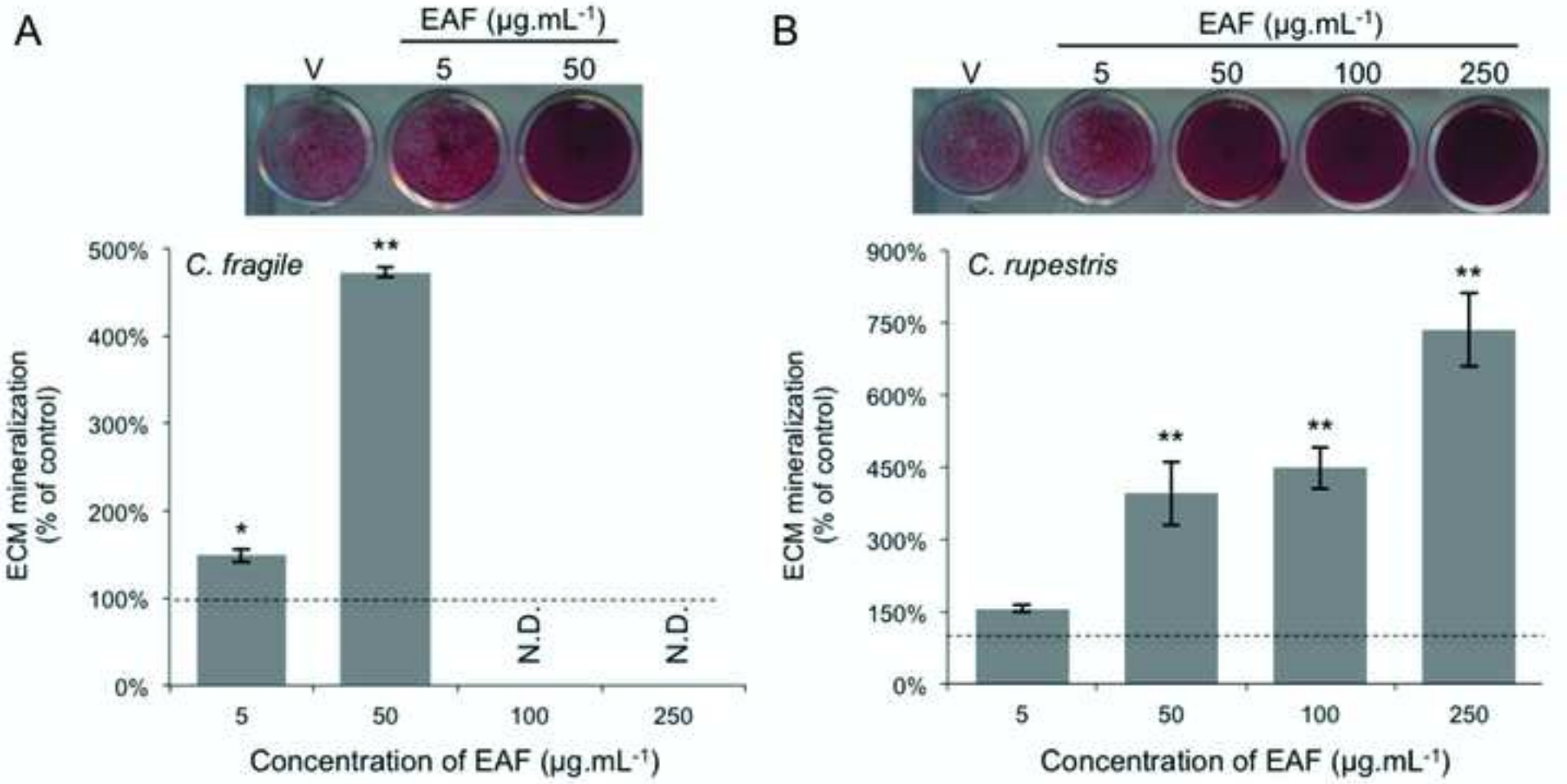

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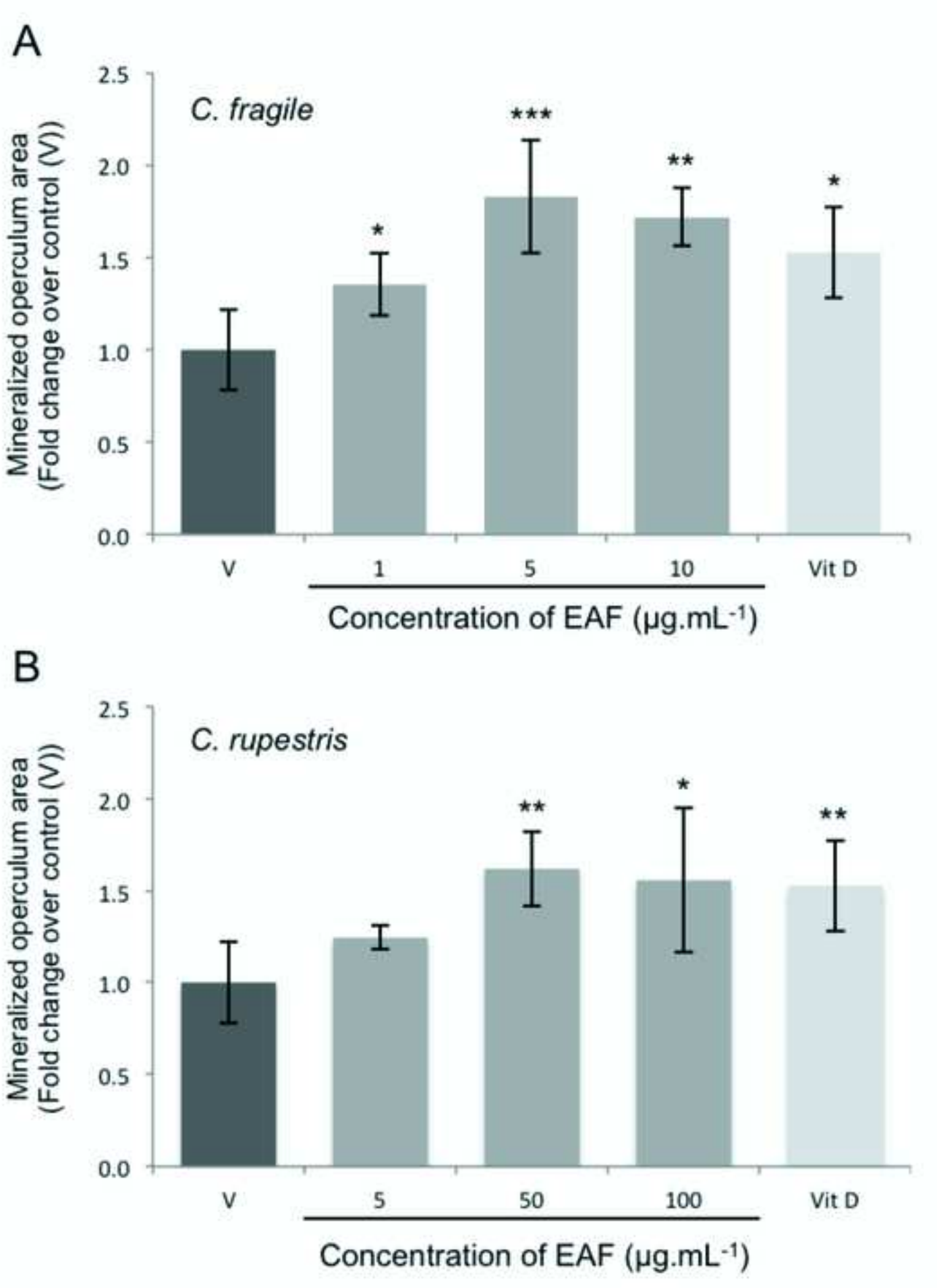

Table 1. Phenolic content and antioxidant activities of *Cladophora rupestris* and *Codium fragile* extracts. Phenolic content, DPPH scavenging activity and reducing power were determined for crude extracts and semi-purified fractions and commercial standards (ascorbic acid, BHA and Trolox). Values are expressed as mean ± SD (n=3). Different letters indicate significant difference between means according to Berhens Fisher test ($p < 0.05$). Values in bold indicate the most active fraction. *GAE*, Gallic Acid Equivalent; *DW*, Dried Weight.

		Phenolic content (mg GAE.g^{-1} DW)	DPPH scavenging activity: IC50 (g.L^{-1})	Reducing power: EC50 (g.L^{-1})
C. rupestris	CE	20.179 ± 0.179 ^{a}	1.228 ± 0.010 ^{a}	10.129 ± 0.250 ^{a}
	EAF	21.726 ± 0.899 ^{b}	**0.768 ± 0.021** ^{b}	**7.825 ± 0.618** ^{b}
	AF	15.833 ± 0.103 ^{c}	2.220 ± 0.069 ^{c}	15.125 ± 0.953 ^{c}
C. fragile	CE	2.202 ± 0.103 ^{d}	7.026 ± 0.090 ^{d}	48.082 ± 7.130 ^{d}
	EAF	22.381 ± 0.206 ^{b}	**0.303 ± 0.002** ^{e}	**5.478 ± 0.891** ^{e}
	AF	0.298 ± 0.103 ^{e}	31.815 ± 7.800 ^{f}	85.959 ± 52.100 ^{f}
Positive controls	Ascorbic acid	n.d.	0.005 ± 8.91×10^{-05} ^{g}	0.072 ± 1.58×10^{-03} ^{g}
	BHA	n.d.	0.010 ± 2.17×10^{-04} ^{h}	0.091 ± 1.24×10^{-03} ^{h}
	Trolox	n.d.	0.007 ± 1.25×10^{-04} ^{i}	0.169 ± 3.08×10^{-03} ^{i}