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# Screening natural resources for mineralogenic and osteogenic bioactivities using *in vitro* and *in vivo* fish systems


**Faculty of Sciences and Technology**

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Master in Marine Biology

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**2016**

Declaro ser o autor deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

I declare to be the author of this work, which is original and unpublished. Authors and studies reviewed are properly cited in the text and contained in the included references list.

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(Marco Tarasco)

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# Abstract

Bone disorders affect millions of people worldwide, and available treatments have only a limited efficacy and/or bring undesirable side effects. There is therefore the need for novel compounds with bone anabolic properties. Due to its technical advantages, fish have been successfully used in biomedical and pharmaceutical research to screen for osteogenic compounds. The aim of this work was to evaluate mineralogenic/osteogenic performance of semi-purified fractions and purified molecules from terrestrial plants (cardoon and eucalyptus) and cyanobacteria. VSa13 cell line established from gilthead seabream (*Sparus aurata*) vertebra and capable of *in vitro* mineralization was used to assess the mineralogenic potential of the extracts, while zebrafish operculum was used to develop and establish a reliable *in vivo* model to screen molecules for osteogenic activity. Extracts from cardoon, eucalyptus and some cyanobacteria exhibited some moderate mineralogenic effect while triterpenic acids purified from eucalyptus strongly increased mineral deposition. Pro-mineralogenic compounds were further analysed for their bone anabolic action on zebrafish operculum and lipophilic cardoon extract, ursolic acid, oleanolic acid and ethyl acetate fraction of cyanobacteria strain #13 were found to promote an osteogenic effect. While the osteogenicity of ursolic acid has already been reported in mouse, the action of oleanolic acid and cyanobacteria extract on bone formation is revealed here for the first time. The use of a double transgenic line expressing fluorescent proteins under the control of *osterix/sp7* and *osteocalcin/oc2* promoters to get insights into mechanisms underlying the osteogenic activity of natural compounds has been explored. *In vitro* and *in vivo* data generated within the scope of this work have not only demonstrated the potential of natural resources to provide molecules with mineralogenic/osteogenic activity that may be used in pharmaceuticals or nutraceuticals but also the suitability of fish systems to screen for these molecules.



## Keywords

Natural resources; Screening system; Teleost fish; Mineralogenic cell line; Developing operculum.

# Resumo

O tecido ósseo é um tipo especializado de tecido conjuntivo constituído por células e por uma matriz extracelular, que possui como característica única a capacidade de promover a sua mineralização,, o que confere a este tecido uma extrema dureza, permitindo-lhe desempenhar importantes funções quer de suporte mecânico aos músculos, possibilitando o movimento, quer de proteção de órgãos internos, constituindo também uma reserva de iões de cálcio e de fosfato. As patologias mais importantes que afectam o esqueleto incluem doenças metabólicas, que afectam o crescimento através de alterações na formação e remoção óssea, fracturas, deformações várias,, infecções bacterianas e tumores. Entre as doenças metabólicas que afectam milhões de pessoas em todo o mundo e que resultam do desequilíbrio entre a atividade osteoblástica (formação óssea) e a atividade osteoclástica (reabsorção óssea) estão a osteoporose, a osteopetrose, o raquitismo e a doença óssea de Paget. No entanto os tratamentos usados são ineficazes e/ou estão associados a efeitos secundários indesejáveis existindo a necessidade de descobrir novos compostos com a capacidade de reverter os sintomas dessas patologias no osso. Os recursos naturais representam uma fonte valiosa de moléculas bioativas, em particular de compostos osteogénicos. A análise em grande escala de extratos ou moléculas com potenciais efeitos osteogénicos requer o uso de ferramentas *in vitro* e *in vivo* optimizadas Organismos aquáticos como o peixe-zebra (zebrafish) têm sido utilizados com sucesso nas investigações biomédica e farmacêutica devido às suas vantagens técnicas em relação a modelos animais mais clássicos, como o ratinho. Por exemplo o peixe-zebra possui um ciclo de vida mais curto, gera um elevado número de descendentes, tem o genoma sequenciado, os embriões são transparentes e desenvolvem-se externamente. Além disso, os peixe-zebra adultos também podem ser facilmente visualizados e manipulados experimentalmente e existem mutantes/transgénicos que permitem a análise da expressão de multiplos genes através de microscopia de fluorescência. O objectivo principal deste trabalho é avaliar a capacidade mineralogénica/osteogénica de fracções semi-purificadas e de moléculas purificadas de plantas terrestres, como o cardo ou o eucalipto, e de cianobactérias. A linha celular VSa13 obtida a partir de vértebras de dourada (*Sparus aurata*), que possui a capacidade de mineralização *in vitro*, foi usada para analisar o potencial mineralógenico dos extratos usados, e o opérculo do peixe-zebra foi usado para desenvolver e estabelecer um modelo *in vivo* adequado para analisar os efeitos de moléculas com atividade osteogénica (através do uso do calcitriol). Os nossos resultados demostraram a existência de um efeito mineralogénico moderado usando concentrações não tóxicas de extratos de cardo, de

eucalipto e de algumas cianobactérias, enquanto que ácidos triterpénicos purificados a partir de amostras de eucalipto (como por exemplo o ácido betulónico, ursólico e oleanólico) aumentam até 6 vezes a deposição de mineral na matrix extracelular da linha celular VSa13. Os nossos resultados mostraram ainda que *in vitro*, a cinaropicrina, uma lactona sesquiterpénica abundante nos extratos de cardo, promovia um efeito anti-mineralogénico, o que foi mais tarde comprovado utilizando o nosso modelo *in vivo*. O efeito de compostos pro-mineralogénicos foi posteriormente analisado através do estudo do opérculo do peixe-zebra e os nossos resultados demonstraram que os extratos lipofílicos do cardo, como o ácido ursólico, o ácido oleanólico e a fracção de acetato de etílo da espécie #13 de cianobactérias, possuem efeitos osteogénicos. Apesar da capacidade osteogénica do ácido ursólico já ter sido analisada e comprovada por outros grupos usando um modelo animal de ratinho, a ação do ácido oleanólico, da cinaropicrina e de extratos de cianobactérias na formação óssea nunca tinha sido descrita. O uso de uma linha de duplos transgénicos que expressam uma proteína fluorescente sob o controlo dos promotores do *osterix/sp7* ou da *osteocalcina/oc2* tem sido determinante para tentar perceber os mecanismos responsáveis por esta atividade osteogénica e sendo a mesma abordagem atualmente aplicada ao estudo do efeito dos ácidos triterpenicos. Os dados gerados a partir das ferramentas *in vitro* e *in vivo* utilizadas neste trabalho não só demonstraram o potencial de recursos naturais (terrestres ou marinhos) para a descoberta de moléculas com efeito mineralogénico/osteogénico potencialmente relevantes para as indústrias farmacêutica ou alimentar, como também evidenciaram a importância do uso de animais modelos como o peixe-zebra para a análise do efeito destas moléculas.

## Palavras-chave

Recursos naturais; Métodos de “screening”; teleósteos; linha celular mineralogénica, opérculo.

# Table of contents

# 1 Introduction

## 1.1 Bone, a major connective tissue of the vertebrate skeleton

The most important function of vertebrate skeleton is to provide structural support to the body, but it also serves as a mineral reservoir (calcium and phosphate storage), as a source for blood cells (i.e. hematopoiesis in the bone marrow) in mammals, as a protection for internal organs and as an anchor point for muscular tissue to sustain locomotion.[1,2] Among the different tissues forming the skeleton, bone is a dense connective tissue composed of a mineralized and vascularized collagen matrix and containing four different types of cells: osteoblasts, bone lining cells, osteocytes and osteoclasts (**Figure 1.1**).[3] Osteoblasts account for 4-6% of the total resident cells and are in charge of bone formation. Bone lining cells are quiescent flat shaped osteoblasts that cover the bone surfaces preventing the direct interaction between osteoclast and bone matrix when bone resorption should not occur. Osteocytes cells are buried in the mineralized bone matrix representing 90-95% of the total cell content, they coordinate the response of bone to mechanical loading and biological signals.[4] Osteoclasts are multinucleated cells with an important role in bone resorption and bone remodelling.[5,6] The origin of bone go back to approximately 420 million years ago, when the Osteichthyes (bony fish), the first organisms exhibiting an ossified endoskeleton with an hard matrix of calcium phosphate, appeared in vertebrate evolution.[7]

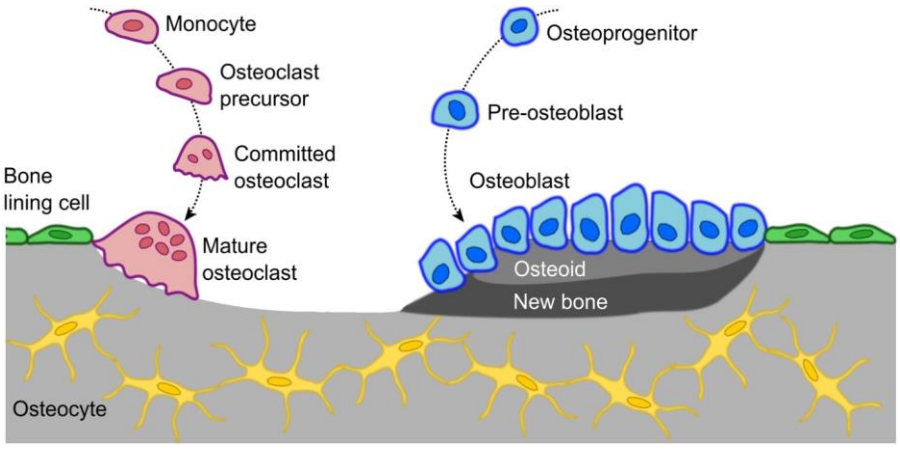


**Figure 1.1.** Schematic representation of bone cells. Osteoblast (blue cells) are the cells in charge of bone formation. Haematopoietic cells of the monocyte/macrophage lineage differentiate to mature osteoclast (purple cells) and resorb bone. Bone lining cell (green cells) are quiescent flat shaped osteoblast that cover the bone surface to protect it when resorption should not occur. Osteocytes (yellow cells), which are embedded within mineralized bone, communicate via ramification of dendritic processes and orchestrate the spatial and temporal recruitment of osteoblast and osteoclast. Adapted from Nicholls et al.[8]

## 1.2 Bone formation and remodelling

Bone is formed through different processes of ossification, the most common being endochondral and intramembranous ossification (**Figure 1.2**). In the course of endochondral ossification bone forms through the replacement of a cartilaginous anlage by an osteoid and subsequent mineral deposition, while intramembranous ossification occurs directly from mesenchymal cell condensations without the formation of a cartilage matrix.[7]

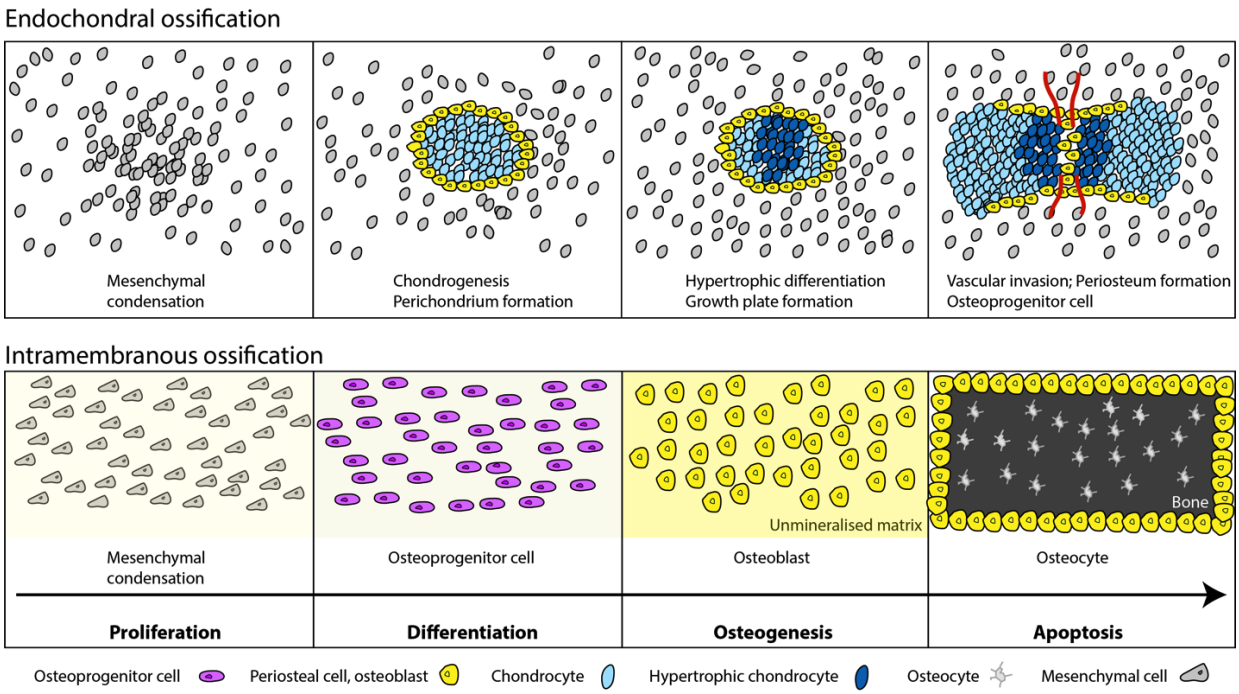


**Figure 1.2.** Representation of endochondral and intramembranous ossification processes. **Endochondral ossification** begins with the condensation of mesenchymal cells and the differentiation of central cells in chondrocytes (light blue) that later become hypertrophic (dark blue). Progression to the mature growth plate and formation of a centre of ossification accompanies the development of the perichondrium (yellow), vascular invasion (red) and the differentiation of undifferentiated cells of the periosteum into osteoblast (yellow). During **intramembranous ossification**, undifferentiated mesenchymal cells condense and differentiate into osteoprogenitor cell (pink), which will originate mature osteoblast (yellow) responsible for the deposition and mineralization of bone matrix. Osteoblasts either die by apoptosis or are entrapped in the bone matrix, becoming osteocytes (grey). Adapted from Ornitz & Marie.[14]

Bone is a dynamic tissue that is constantly remodelled to (1) reshape in order to adapt to mechanical loading, (2) heal after fractures or (3) participate in calcium homeostasis.[9] Bone remodelling is a physiological process in which old or damaged bone is removed by osteoclasts and replaced by new bone formed by osteoblasts (**Figure 1.3**). An imbalance between bone resorption and bone formation may occur under certain pathological conditions, which leads to

abnormal bone structure and the development of skeletal disorders that can alter the quality of life.[10] Bone remodelling is carried out by an anatomical structure (the basic multicellular unit) which requires the coordination of the major bone cells type and occurs in four distinct phases. When the osteocytes detect a micro damage or a bone deformation, they transmit signals to recruit osteoclast precursor to the specific bone site. Osteoclast precursors (i.e. mononuclear cells of the monocyte/macrophage lineage) get attached to the bone matrix and differentiate into active osteoclast (multinucleated giant cells) in response to elevated concentrations of the macrophage colony-stimulating factor and RANK ligand, starting the resorption process.[11,12] During this phase, pre-osteoblasts are also recruited from the bone marrow or from nearby capillaries, and progressively differentiate and start to produce the osteoid, contributing to bone formation and ensuring the equilibrium between bone removal and formation. At the final stage, the osteoid becomes mineralized concluding the bone remodelling cycle.[13]

## 1.3 Human bone disorders

Bone disorders will originate from an incorrect bone remodelling process. In human, the most common disorders are the Paget's disease of bone (PDB), osteopetrosis, rickets, osteopenia and osteoporosis. PDB is characterised by a primary increase of osteoclast bone resorption with a secondary marked increase in osteoblast activity and new bone formation, resulting in an abnormal trabecular bone.[15] Osteopetrosis refers to a rare heritable bone remodelling disorder that is characterized by increased bone density caused by a defect in bone resorption by osteoclasts.[16] Rickets disease is a nutritional vitamin D deficiency that results in enhanced bone resorption and increased bone turnover as a consequence of defective mineralization or calcification of bones.[17] Osteopenia and osteoporosis are the most common bone diseases, affecting around 200 millions of people worldwide[18] and are characterised by low bone mass and structural deterioration of the bone, causing bone fragility and increasing susceptibility to fractures.[19] Nowadays there is still no cure for osteoporosis (and also for other bone disorders) but several treatments and drugs have been developed to strengthen bones or slow down bone resorption, increasing the quality of life of aged people.[20,21] Several strategies have been proposed to prevent/limit osteoporosis effects, e.g. follow an active physical activity and take up calcium and vitamin D supplements[22], and several drugs are available to treat osteoporotic people with fracture risks. Commonly used anti-osteoporotic drugs are (1) bisphosphonates (e.g. Fosamax and Actonel), synthetic compounds with anti-resorptive activity able to decrease bone

turnover and increase bone mineral density[23], (2) RANKL inhibitors (e.g. Denosumab, a human monoclonal antibody) to block osteoclast differentiation, activation and survival and therefore decrease bone resorption[24], (3) selective estrogen receptor modulators (e.g. Raloxifene and Bazedoxifene), synthetic molecules that bind to the estrogen receptor thereby acting as estrogen agonists on bone[25] and (4) strontium ranelate (e.g. Protelos), a molecule with anti-resorbing and bone-forming effects, a dual mechanism that results in an improvement in bone microarchitecture and bone strength[26].

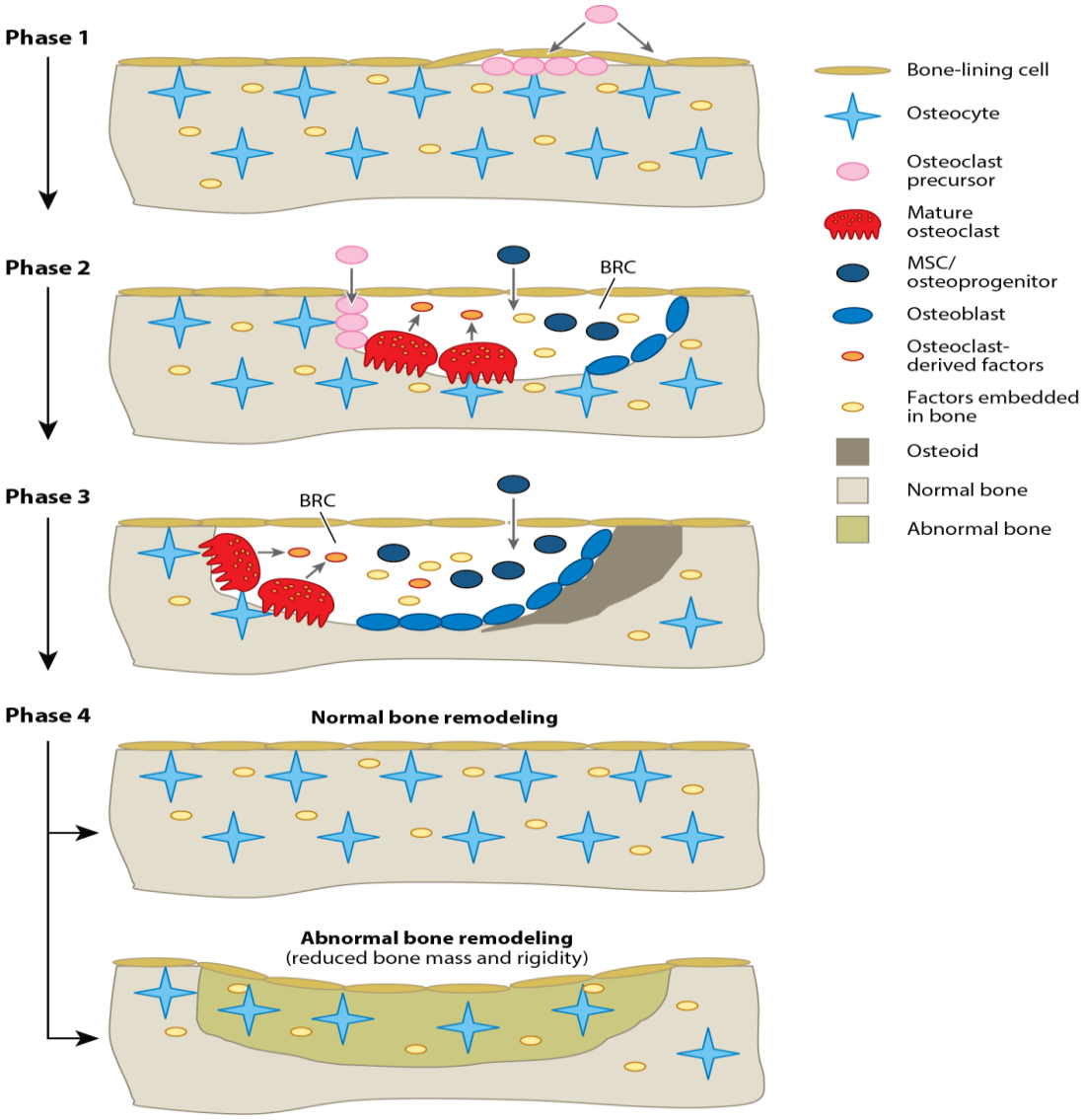


**Figure 1.3.** Schematic representation of bone remodelling. The remodelling process consists of four major distinct phases. **Phase 1:** Osteocytes transmits signals to recruit osteoclast precursors (mononuclear cells of the monocyte/macrophage lineage) at sites where bone remodelling should occur. **Phase 2:** Osteoclast precursors fuse into multinucleated active osteoclasts and initiate bone resorption. Mesenchymal stem cells (MSCs) and osteoprogenitors are recruited simultaneously. **Phase 3:** Osteoprogenitors differentiate into active osteoblasts and initiate osteoid production. **Phase 4:** Osteoid undergoes mineralization, ending bone remodelling process. In a normal process, bone mass and strength remains the same after each remodelling cycle while in certain pathological conditions, such as osteoporosis, bone mass and strength may be gradually reduced. *BRC*, bone remodelling compartment. Adapted from Feng & McDonald.[13]

All the pharmacological treatments mentioned before are approved to be used in osteoporotic patients showing good results regarding increase in bone formation. However, they also have undesirable side effects such as gastrointestinal and oesophageal irritations, risk of atrial fibrillation, osteonecrosis of the jaws, cases of atypical femoral subtrochanteric/diaphyseal fractures, risk of thrombotic events, nausea, diarrhea and others.[27] Consequently, there is a need to discover novel molecules and treatments that could successfully play an important role in osteoporosis treatments, showing an increase in bone formation with reduced side effects.

## 1.4 Natural products as source of novel molecules

Throughout the ages humans have relied on natural products not only as nutrients but also as medicines for the treatment of a wide spectrum of diseases. Plants have formed the basis of sophisticated traditional medicine systems, with the earliest records dating from around 2600 BC, documenting the uses of approximately 1000 plant-derived substances in Mesopotamia.[28] During the last century, with the discovery and establishment of new scientific tools such as PCR, bioinformatics, DNA genome sequencing, cell cultures facilities but also with the development of oceanographic sampling that allowed the discovery of new resources from the ocean, the scientific world has seen a growing interest in the exploration of natural resources toward the discovery of molecules with biomedical applications.[29,30] Natural products have been, and will always be, important sources of new pharmaceutical compounds. Crude extracts (or substances) extracted from any of these sources typically contains novel, structurally diverse chemical compounds that may have important contribution in medicine areas such as: anti-inflammatory, antibacterial, antifungal, antiparasitic, antiviral, anticancer and neurological, immunological, cardiovascular and metabolic related diseases.[31,32] Particularly in the last decade, there is a growing interest to find new drugs with osteogenic or mineralogenic activity with the potential to treat the many skeletal disorders that affect today millions of people.

Despite competition from other drug discovery methods, i.e. chemical synthesis, natural resources are still providing new clinical candidates and drugs that do not exhibit the undesirable side effects of synthetic drugs. Natural products not only complement synthetic molecules but they also exhibit drug relevant features unsurpassable by synthetic compounds.[33] This concept is based on the fact that chemical agents produced by living organisms (particularly the secondary metabolites) have evolved throughout millenniums under an evolutionary pressure, and are therefore more likely to have a specific biological activity than “randomly” assembled, man-made synthetic chemicals.[32]

## 1.5 Drug screening

Drug development is a complex process - identification of candidate molecules, synthesis, animal testing of therapeutic efficacy, drug development, clinical trials, approval, manufacturing and marketing - that can take 12-15 years.[34] Screening for compound toxicity has become a routine practice in drug discovery and development. In general, cytotoxicity endpoints such as membrane integrity, cellular metabolite content, mitochondrial and lysosomal functions are used in conjunction with cell lines for the screening of organ-specific toxicity.[35] Characterizing the biological activity and the pharmacological effects of a compound is the most important step to resolve in drug discovery after having determined drug properties. In the context of screening for compounds with anti-osteoporotic effects, endpoints such as bone mineral density, ratio cortical vs trabecular bone, fractures reduction and biomarkers of bone turnover should be evaluated.[36,37] Validation techniques regarding extracts properties and pharmacological effects range from *in vitro* tools through the use of whole *in vivo* animal systems, to modulation of a desired target in disease patients. While results from each approach (i.e. *in vitro* or *in vivo*) can provide useful information, confidence in the observed outcome is significantly increased by a multi-validation approach in a complementary system.[34]

## 1.6 *In vivo* screening systems

A large variety of mammalian species have been used in bone-related research and drug discovery. Rodents (mouse and rat) due to their high definition and experimental reproducibility as biological, genetic and immunological models, are by far the most commonly used animal in fundamental research.[38] Recently, there has been an increasing interest in the use of teleost fish as an alternative model to complement traditional mammalian systems in biomedical research and drug discovery. In fact, fish, and in particular zebrafish (*Danio rerio*, a small freshwater fish from tropical regions of South and Southeast Asia), possess many advantages that allow quick and less expensive large scale and high-throughput screening for new molecules with pharmacological effects. Zebrafish has a rapid and external embryonic development and 200-300 eggs can be produced every week by a single female. Furthermore, embryos and larvae can be easily accommodated in 96-well plates and they are able to absorb chemicals directly from the water and any skeletogenic/osteogenic effect is easily detected since body at these developmental stages is transparent and allow the visualization of mineralized tissues after proper staining.[39] Additional features such as robustness, high fecundity, small size and short

generation time have reinforced the qualities of zebrafish to become a good laboratory model. In addition, 70% of human genes have a zebrafish ortholog and zebrafish genome have been almost totally sequenced and annotated[40] allowing the development of mutant lines mimicking human disorders, in particular skeleton/bone disorders.[41] Finally, despite few differences (e.g. the presence of acellular/anosteocytic bone in advanced teleosts[42]), molecular mechanisms, pathways underlying the formation and development of skeletal structures are remarkably similar in teleost fish and mammals.[43,44]

Various fish *in vivo* tools are available to study drugs affects (osteogenic or osteotoxic activities) (**Figure 1.4**) and zebrafish mutants modeling human skeletal disorders (e.g. osteogenesis imperfecta, osteoporosis, etc.) are available to contribute to the discovery of therapeutic molecules capable of rescuing these pathologies. Increasing of mineralization/osteogenic activity can be study through morphological image analysis of teleost caudal fin rays regeneration or operculum development[41]. Several zebrafish transgenic lines (expressing fluorescent proteins) are also available for skeletal marker genes such as *barx1, col2, col10, oc2, osx, runx2, sox9* and *sox10*, providing useful tool to study levels and sites of gene expression and get insights into mechanisms underlying molecule osteogenic activity.[45–47]

## 1.7 *In vitro* screening systems

Several *in vitro* systems are available to screen molecules bioactivities such as mineralogenic vertebra-derived cell lines and mineralizing scales that after proper staining can be used to detect mineralization increment.[41] Primary cultures or cell lines established from osteoblast or osteoclast, provide valuable information about processes involved in skeletal development, bone formation and bone resorption. Well established *in vitro* models can be used to multi-validate pharmacological activities from *in vivo* system or vice versa.[48] Several bone-derived cell lines of mammalian origin are available to screen molecule libraries and natural extracts for osteogenic activity.[49–51] However, very few have been established for teleost fish.[52] The first cell line of fish origin was developed by Wolf and Quimby (1962) from the rainbow trout, *Salmo gairdneri* and since then, numerous other cell lines derived from different species and tissues have been described. Most fish lines have been extracted from different tissues such as skin, gill, heart, liver, kidney, spleen, swim bladder, brain and ovary, providing important contributions as *in vitro* systems in immunology, toxicology, ecotoxicology, endocrinology, virology biotechnology and biomedical research.[53] Particularly the cell lines VSa13 and VSa16, derived from *Sparus aurata* vertebrae[54] have been used to study extracellular matrix mineralization effect of various

molecules, such as retinoic acid[55,56], polyunsaturated fatty acids[57], and vanadate.[58] Teleost elasmoid scales can be used to study cell-cell and cell-matrix interactions but also drugs effects on bone formation/resorption since they can be cultured *in vitro* as a bone unit. Among other characteristics (they are easily accessible, translucent and regenerate fast), elasmoid scales contain both osteoblasts and osteoclasts that cohabit around the mineralized matrix resembling *in vivo* conditions.[59–61]

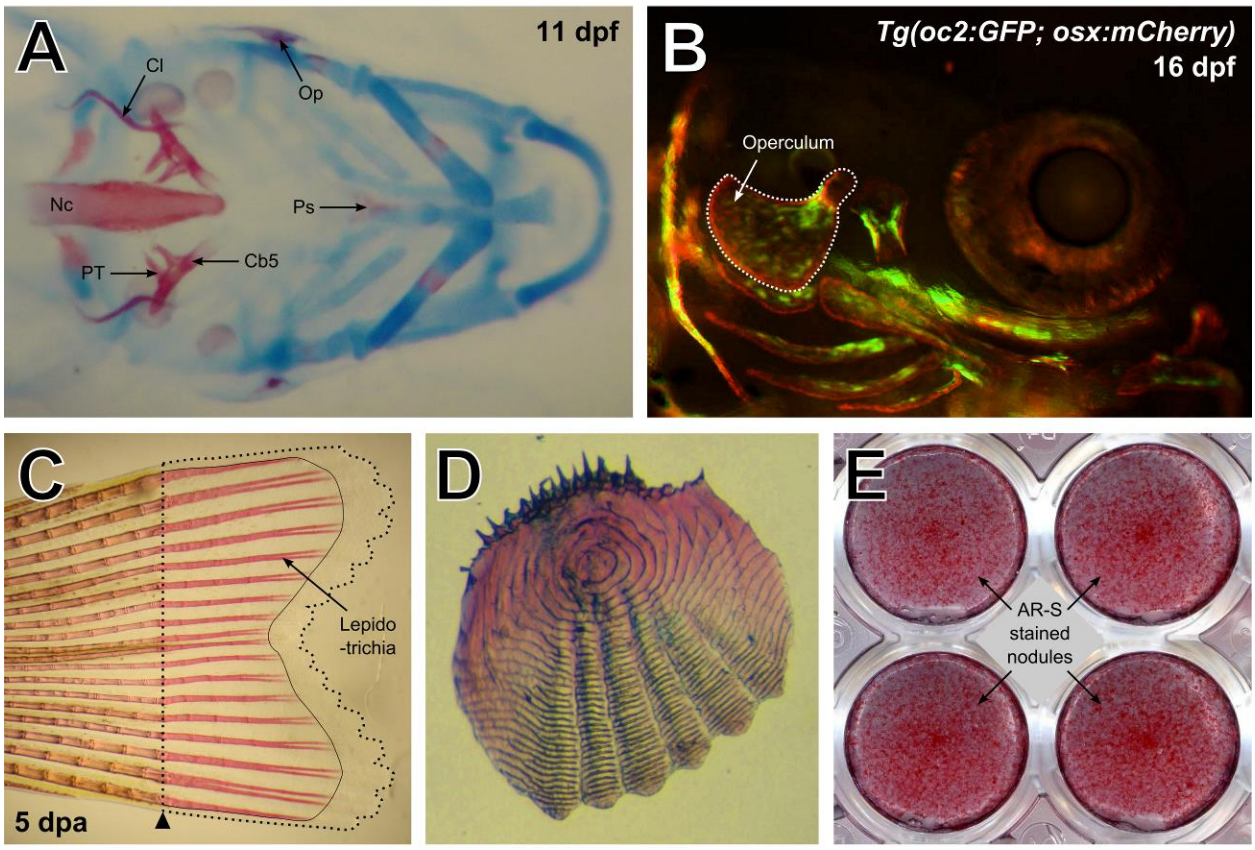


**Figure 1.4.** Fish systems available to study or screen drugs affects (osteogenic or osteotoxic activities). **(A)** Ventral view of whole-mount (alizarin red S - alcian blue) stained zebrafish larvae at 11 days post-fertilization (dpf). Calcified structures appear in red; notochord (Nc), operculum (Op), parasphenoid (Ps) cleithrum (Cl), ceratobranchial 5 (Cb5) and pharyngeal teeth (PT). **(B)** Lateral view of Tg(*oc2:GFP; osx:mCherry*) transgenic zebrafish at 16 dpf. **(C)** Lateral view of AR-S stained regenerating caudal fin of a juvenile zebrafish. Black triangle indicates the amputation plan; Dotted black line indicates regenerated area; Solid black line indicates area of new bone formation. **(D)** AR-S-stained elasmoid scale from the dorsal region of a juvenile gilthead seabream (*Sparus aurata* L.). **(E)** AR-S stained mineral nodules deposited within the extracellular matrix of gilthead seabream VSa16 cell line (osteoblast-like cells). Adapted from Laizé et al.[41]

# 2 Objectives

The aim of this work is to evaluate the mineralogenic/osteogenic performance of extracts (semi-purified fractions and purified molecules) from terrestrial plants (cardoon and blue gum/eucalyptus) and different strains of cyanobacteria (**Figure 2.1**). VSa13 cell line established from gilthead seabream *Sparus aurata* vertebra and capable of *in vitro* mineralization will be used to test mineralogenic potential, while zebrafish operculum (*Danio rerio*) will be used to develop and establish a reliable *in vivo* model to screen molecules for osteogenic activity.

Cardoon (*Cynara cardunculus* L.var. *altilis*) and eucalyptus (*Eucalyptus nitens*) have already been widely explored in terms of pharmaceutical possible activities and both plants were shown to produce molecules with pharmaceutical potential. In eucalyptus bark, lipophilic extracts contain high amounts of triterpenic acids such as betulonic, betulinic, ursolic, and oleanolic,[62,63] that have been recognized as promising compounds for the development of new bioactive agents.[64,65] For example, oleanolic and ursolic acids show anti-tumoral[66] and anti-angiogenic[67] properties; betulinic acid is also known for its anti-tumoral properties[68] and as a precursor for anti-HIV drugs, such as bevirimat.[69,70] In cardoon, cynaropicrin is the most abundant sesquiterpene lactone[71] and has shown great activities such as anti-tumoral[72], anti-proliferative and anti-inflammatory properties.[73,74]


**Figure 2.1.** Semi-purified fractions and purified molecules tested within the scope of this work were obtained from **(a)** the bark of the blue gum *Eucalyptus nitens* (image retrieved from www.pinterest.com), **(b)** the leaves of the cardoon *Cynara cardunculus* L.var. *altilis* (image retrieved from www.en.hortipedia.com) and **(c)** different strains of cyanobacteria (image adapted from Sihvonen et al.[80] Extracts prepared from cardoon and eucalyptus were kindly provided by Fátima Duarte (CEBAL - Centro de Biotecnologia Agrícola e Agro-alimentar do Alentejo, Beja) and extracts prepared from cyanobacteria by Vitor Vasconcelos and João Morais (CIIMAR - Centro Interdisciplinar de Investigação Marinha e Ambiental, Porto).

Cyanobacteria are a group of photosynthetic prokaryotes and are among the most successful and oldest life forms present on earth.[75] They inhabit almost all the habitats in earth and they have long been known for their ecological and agricultural impact; that is, as the primary colonizers of an ecosystem, their ability to fix atmospheric nitrogen and solubilize phosphates.[76] Numerous studies have been recently initiated to better understand intrinsic bioactivities, and anti-cancerous[77], anti-bacterial[78], and anti-HIV[79] activities have already been discovered.

It is worth to mention that although some of the compounds tested in this work have shown mineralogenic/osteogenic activity in mammalian systems, none of those have been tested in fish system or even examined yet.

## 2.1 Screening fish systems

VSa13 cell line derived from vertebra of the gilthead seabream (*Sparus aurata*) (**Figure 2.2A**) and capable of *in vitro* mineralization[54] will be used to assess mineralogenic activity of selected extracts, fractions and molecules but also cytotoxicity and proliferative potential. Mineral deposition in extracellular matrix of VSa13 cells occurs within 3 weeks and is easily detected through alizarin red S staining and quantified by spectrophotometry.[54] Zebrafish (*Dario rerio*) larvae undergoing skeletogenesis will be used to assess osteogenic activity of selected extracts (**Figure 2.2B**).

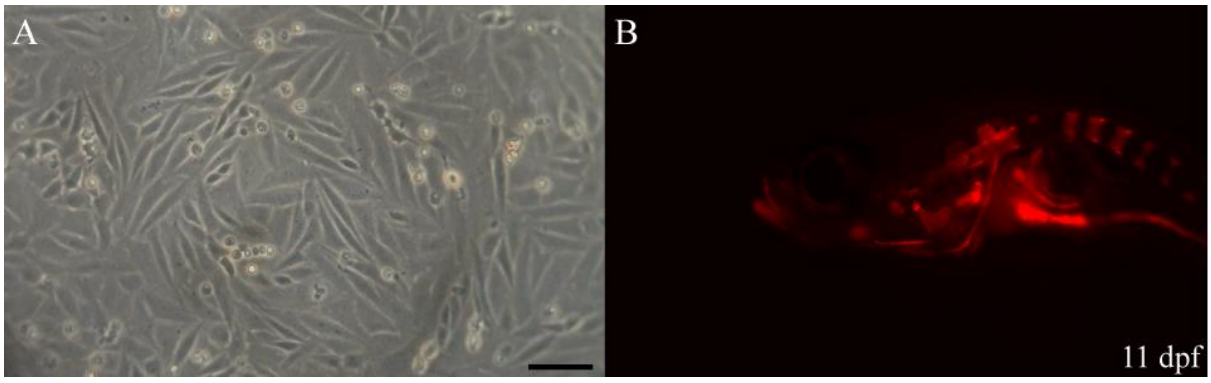


**Figure 2.2. (A)** Micrograph of vertebra-derived gilthead seabream VSa13 cell culture at confluence, in DMEM supplemented with 10% FBS. The bar represent 100 µm. **(B)** Zebrafish larvae undergoing skeletogenesis at 11 dpf. Coloration in red (alizarin red s stain) is specific for calcified structures.

Larvae will be exposed chronically for several days (to be determined within the scope of this work) to the extracts at concentrations selected from *in vitro* assays. Changes in the area of the operculum will be determined through morphometric analysis of alizarin red fluorescence pictures as system tool to detect increments regarding mineralization and osteogenesis. Although

both systems cannot be compared to robotic high-throughput screening machines available in major pharmaceutical factories, they still present several characteristics that allow the study of a great number of molecules. The use of multi-well plates give the possibility to test different molecules at the same time and the small dimension of the wells require little extracts quantity, reducing the costs and wastes. The experimental design can be planned easily and more than one assay can be performed at the same time.

## 2.2 Endpoints

Cytotoxicity will be first evaluated in confluent and sub-confluent cultures of VSa13 cells exposed to 5 different concentrations (chronic exposure for 9 days), then non-toxic concentrations will be evaluated for proliferative action (chronic exposure for 9 days) and mineralogenic performance (chronic exposure for 21 days). XTT assay will be used to assess cytotoxicity and proliferation endpoints while alizarin red S staining will be performed to detect extracellular matrix mineralization (**Figures 2.3**). Regarding the *in vivo* experiment, fish will be exposed to each extract/fraction/molecule (day of exposure to be determined within the scope of this work) and stained with alizarin red S staining. Coloration will be imaged and bone formation will be assessed through morphometric analysis of the operculum, eye, iris, distance snout-cleithrum and the head (exact day of image acquisition to be determined). Furthermore, fish death/survival will be determined to evaluate the toxicity of the molecules (**Figures 2.3 and 2.4**).

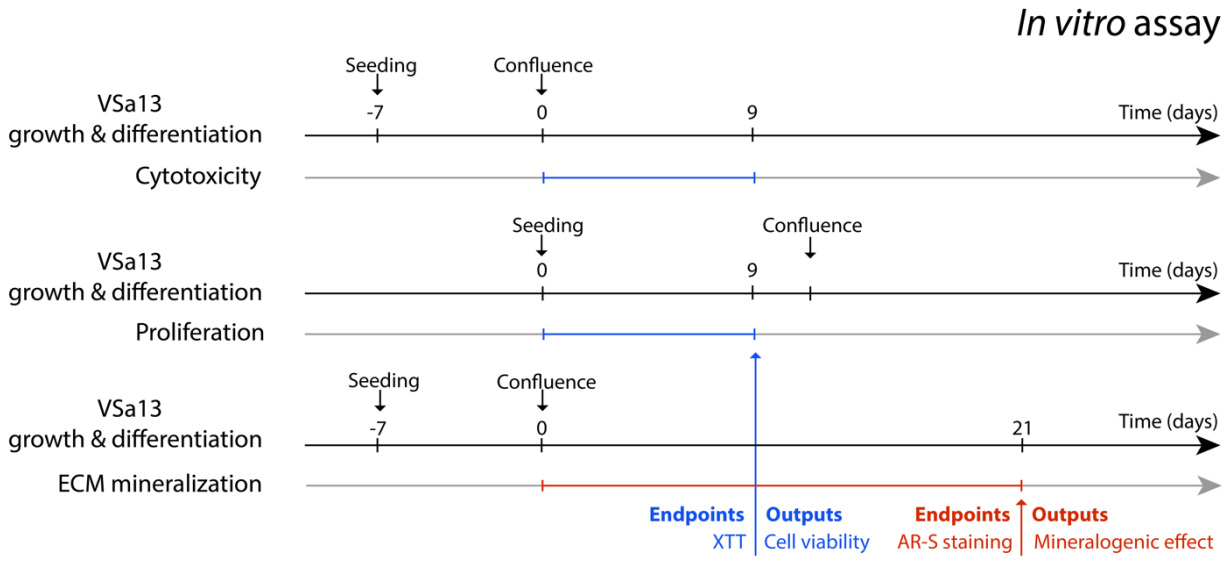


**Figure 2.3.** Experimental design of the *in vitro* assay with endpoints and expected outputs.

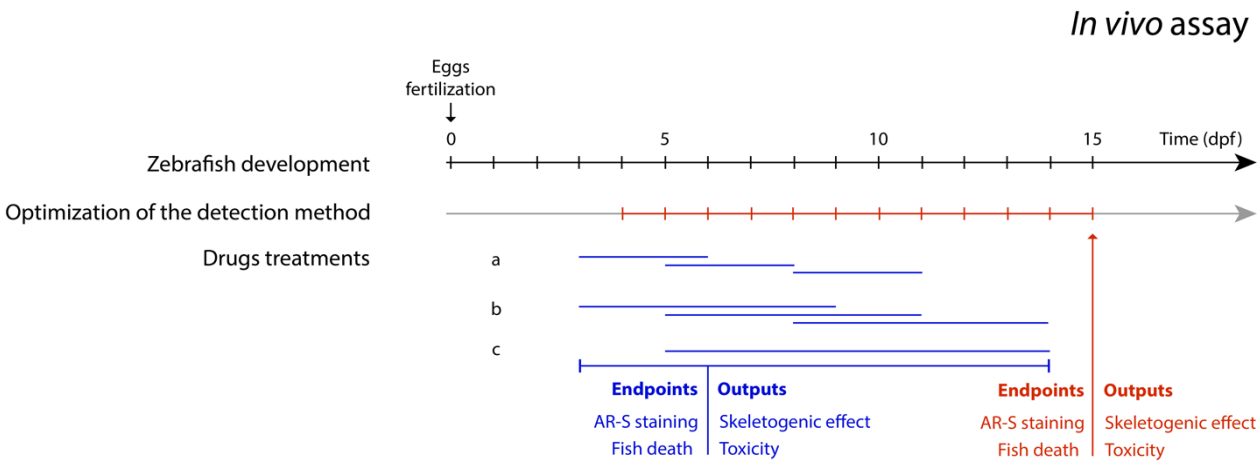


**Figure 2.4.** Experimental design of the *in vivo* assay with endpoints and expected outputs. *dpf*, days post-fertilization; *a*, 3 days of drug exposure; *b*, 6 days of drug exposure; *c*, 9 days of drug exposure.

# 3 Material and methods

## 3.1 Maintenance of the cells

VSa13 cell line - previously established from gilthead seabream *Sparus aurata* vertebra[54] - was maintained at 33°C in a 10% $CO_2$ humidified atmosphere and cultured in Dulbecco's modified Eagle medium (DMEM; Invitrogen) supplemented with 10% FBS (fetal bovine serum; Sigma-Aldrich), 1% penicillin-streptomycin, 1% L-glutamine and 0.2% fungizone (all from Invitrogen). Cell cultures at confluence were divided 1:8 every 3 days using trypsin-EDTA solution (1.1 mM EDTA and 0.2% trypsin in PBS; Invitrogen).

## 3.2 Semi-purified fractions and purified molecules

Lipophilic (dichloromethane) fractions of cardoon leaves (*Cynara cardunculus* L. var. *altilis*) and eucalyptus bark (*Eucalyptus nitens*) and purified triterpenic acids (i.e. betulinic, betulonic, ursolic and oleanolic acids) were kindly provided by Fátima Duarte (Centro de Biotecnologia Agrícola e Agro-Alimentar do Alentejo (CEBAL) / Instituto Politećnico de Beja (IPBeja), Beja). Semi purified fractions of cyanobacteria strains extracted using ethyl acetate (100%) were kindly provided by Vitor Vasconcelos and João Morais (Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR), Porto). Semi-purified fractions and purified molecules were dissolved in appropriate vehicle at a concentration of 100 or 10 mg/mL (stock) then diluted directly into culture medium or fish bath at selected concentrations.

## 3.3 Evaluation of the cytotoxicity and cell proliferation

Cytotoxicity and cell proliferation were measured upon exposure of the cells to different concentrations of the extracts using XTT Cell Proliferation kit (AppliChem). VSa13 cells were seeded in a 96-well plate (Nunc) at a density of $1 \times 10^3$ cells per well (proliferation) or $3 \times 10^3$ cells per well (cytotoxicity) and further incubated under regular growth conditions for 24 h (proliferation) or until confluence (cytotoxicity). Culture medium was supplemented with the extracts from 1000x stocks then filtered (0.2 µm) and applied to the cells. Control cells were exposed to vehicle, i.e. dimethyl sulfoxide (DMSO) or ethanol (EtOH), at a concentration of 0.1%. Treatments were applied for 9 days and renewed every 3.5 days. At the end of the treatment, 50 µL of the XTT reaction mixture and 100 µL of fresh medium were added to each

well and absorbance was determined at 460 nm and at 620 nm after 2 h of incubation using a Synergy 4 multiplate reader (BioTek).

## 3.4 Evaluation of extracellular matrix mineralization

Extracellular matrix mineralization was determined through alizarin red S staining after 21 days of exposure to the different extracts. VSa13 cell were seeded in 12-well plates (Nunc) at a density of $2.5 \times 10^4$ cells per well and incubated in regular condition until confluency. Then extracellular matrix mineralization was induced by supplemented culture medium with 50 μg/mL of ascorbic acid, 10 mM β-glycerophosphate and 4 mM $CaCl_2$. Extracts, molecules or vehicles (0.1% DMSO or 0.1% EtOH) were applied at the time of renewal of the mineralogenic medium, i.e. every 3.5 days. At the end of the treatment, medium was discarded and cells were washed 3 times with PBS. Cells were then fixed at 4 ºC for 1 h in 4% formaldehyde (prepared in PBS, pH 7.4). Fixative was discarded and cells were washed 3 times with Milli-Q water (Millipore). Cells were then stained with 40 mM AR-S (pH 4.2) for 15 min at room temperature and unbound dye was removed through multiple washes with Milli-Q water (until effluent is clear), then distained with 10% cetylpyridinium chloride (CPC; pH 7.0). Crystal-bounded stain was quantified through the measurement of the absorbance (540 nm) in CPC suspensions. All chemicals were from Sigma-Aldrich unless otherwise

## 3.5 Establishment and optimization of the zebrafish operculum as a model to assess for osteogenic activities

### 3.5.1 Ethics statement on animal experiments

All the experimental procedures involving animals followed the EU Directive 2010/63/EU and National Decreto-Lei 113/2013 legislation for animal experimentation and welfare. Animal handling and experiments were performed by qualified operators accredited by the Portuguese Direção-Geral de Alimentação e Veterinária (DGAV).

### 3.5.2 Zebrafish egg production

Sexually mature zebrafish (AB wild-type strain, and Tg(*osterix:mCherry*) and Tg(*osteocalcin:EGFP*) transgenic lines) were crossed using an in-house breeding programme.

Fertilized eggs were transferred into a 1-L container with static water conditions and the following parameters: temperature 28 ± 0.1 °C, pH 7.5 ± 0.1, conductivity 700 ± 50 µS, $NH_3$ and $NO_2$ lower than 0.5 mg/L, $NO_3$ at 5 mg/L and a photoperiod of 14-10 h light-dark. Methylene blue (0.01% stock solution) was added to reduce bacterial and fungal growth. At 5 days post-fertilization (dpf), water was renewed and larvae fed with *Artemia* (5 nauplii per mL; strain AF from INVE Aquaculture).

### 3.5.3 Exposure to calcitriol

At appropriate times, larvae were transferred to 6 well-plates (AB larvae, 15 larvae in 10 mL of water) or to plastic cups (*osx:mCherry/oc2:EGFP* double transgenic larvae, 100 larvae in 70 mL of water) and exposed to either 0.01 fg/mL of calcitriol (1α,25-dihydroxyvitamin $D_3$; Sigma-Aldrich) or to 0.1% ethanol (vehicle; Merck). Treatment medium was renewed (70% of the total volume) every day until the end of the treatment. At that time, larvae were sacrificed with a lethal dose of MS-222 (0.6 mM, pH 7.0, Sigma-Aldrich), stained for 15 min at room temperature with 0.01 % alizarin red S (AR-S) prepared in milliQ water (pH 7.4), and washed twice with water for 5 min (method adapted from Bensimon-Brito et al.[81]).

### 3.5.4 Image acquisition and morphometric analysis

AB larvae stained with AR-S were imaged using a MZ 7.5 fluorescence stereomicroscope (Leica) equipped with a green light filter ($\lambda_{ex}$ = 510-550 nm) and a black-and-white F-View II camera (Olympus). Images were acquired using the following parameters: exposure time 1 s, gamma 1.00, image format 1376×1035 pixels. Fluorescence images were analyzed using ImageJ 1.49v software.[82] For morphometric analysis, color channels of the RGB picture were split. Red channel (8-bit) was used for further analyses. Brightness and contrast were optimized to enhance the visibility of cranial bones, in particular the operculum. The minimum and maximum displayed pixel values were set to 0 and 69 respectively. The area of the head and operculum, the length between the snout to the cleithrum, and the width and height of the eye and iris, were determined using built-in tools. The areas of the eye and iris were calculated using the ellipse area formula (½ width × ½ height × π). Transgenic larvae Tg(*osx:mCherry*/*oc2:EGFP*) were imaged using a SteREO Lumar.V12 fluorescence stereomicroscope (Zeiss) equipped with GFP ($\lambda_{ex}$ = 470-440 nm; $\lambda_{em}$ = 525-550 nm) and TxRed ($\lambda_{ex}$ = 560-540 nm; $\lambda_{em}$ = 630-675 nm) filters and an AxioCam MR3 camera (Zeiss). Photographs were acquired according to the following

parameters: 16-bit black-and-white image, exposure time 120 ms (bright field) or 1 s (fluorescence), gamma 1.00, image format 692×520 pixel, binning 2x2 and 20 Z-stacks (fluorescence). For both red and green channels, Z-stacks were merged and aligned through the extended focus built-in tool of the AxioCam software. Fluorescence images (red and green channels) were 8-bit transformed in ImageJ and both the number of fluorescent pixels (pixel values from 0 to 255 after background subtraction) and the area of fluorescence were determined for each operculum using built-in tools (see example in **Figure 3.1**). The area of the head was determined from bright-field acquisitions.

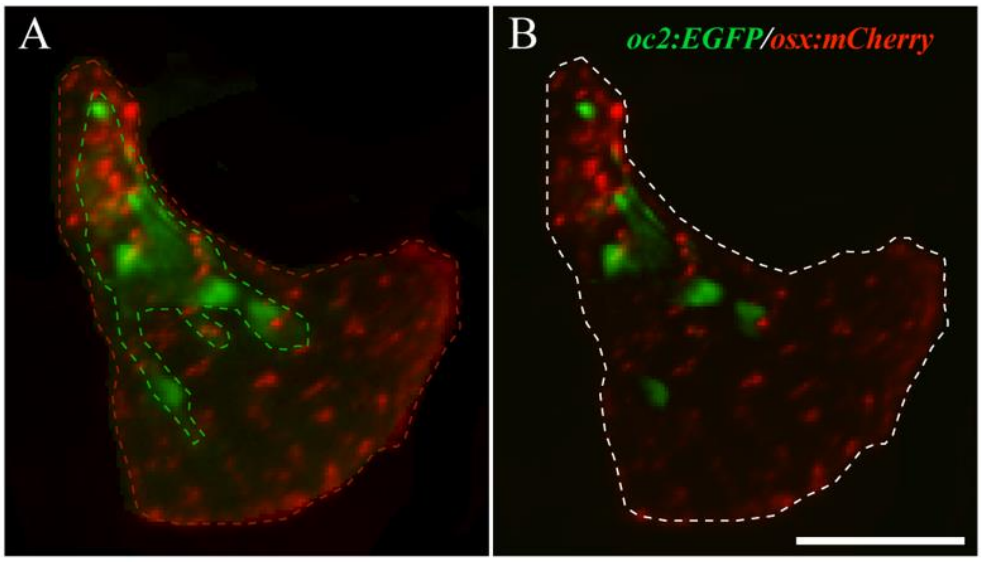


**Figure 3.1.** Morphometric analysis of the fluorescence signals in the operculum of calcitriol-treated larvae of the double-transgenic zebrafish line Tg(*osx:mCherry/oc2:EGFP*). Red (*osx:mCherry*) and green (*oc2:EGFP*) channels were merged and the following parameters were determined using built-in tools in ImageJ: **(A)** Total area of red (red dashed area) and green (green dashed area) fluorescence, and **(B)** number of red or green pixels within the operculum area after background subtraction (white dashed area). Scale bar represents 50 µm.

## 3.6 Statistical analysis

Statistical differences were determined through one-way ANOVA followed by Dunnett's multiple comparison test ($p < 0.05$). Differences between values from vehicle and calcitriol-treated larvae were analyzed through unpaired *t*-tests with Welch's correction ($p < 0.05$). The correlation between selected morphometric parameters was evaluated through a simple linear regression and the R squared. Statistical analyses were performed using Prism version 6.00 (GraphPad Software, Inc. La Jolla, CA).

# 4 Results

## 4.1 Cytotoxicity and proliferative action of semi-purified fractions and related molecules

### 4.1.1 Extracts from terrestrial plants

Cytotoxic and proliferative effects of semi-purified fractions and related molecules were evaluated at 9 days (endpoint to assess toxicity and proliferation) using a wide range of concentrations, i.e. 100, 10, 1, 0.1 μg/mL for semi-purified fractions and 10, 1, 0.1, 0.01 μg/mL for pure molecules. Cytotoxicity was tested in confluent cell cultures (high cell density), while proliferative effect was assessed in sub-confluent cell cultures (low cell density). Vehicles, i.e. dimethyl sulfoxide (DMSO) or ethanol (EtOH) were tested at a concentration of 0.1% and shown not to affect cell survival (**Figure 4.1**).

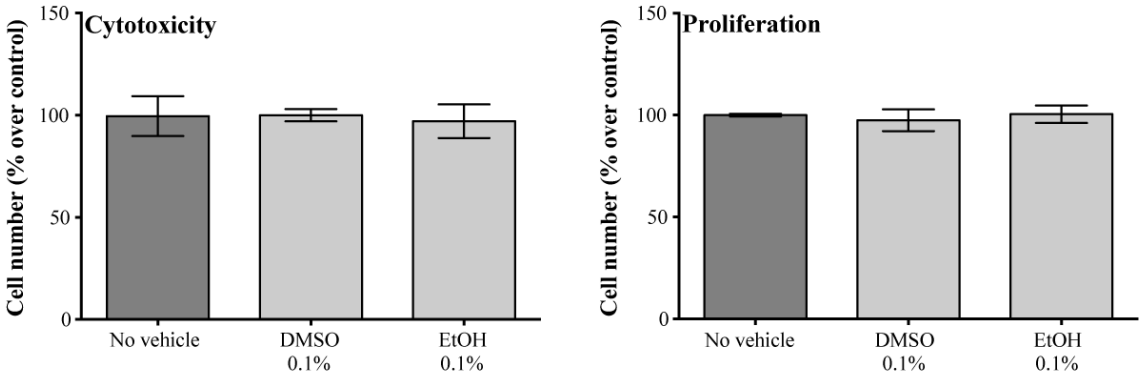


**Figure 4.1.** Cytotoxicity and proliferative effect of dimethyl sulfoxide (DMSO) and ethanol (EtOH), the vehicles used to solubilize semi-purified fractions and purified molecules. *No vehicle*, non-supplemented cell culture medium (DMEM); No vehicle value was set at 100%. *Asterisks* indicate values statistically different from the vehicle (one-way ANOVA followed by Dunnett's multiple comparison test ($p < 0.05$).

Semi-purified fractions (i.e. lipophilic fraction of cardoon extract and dichloromethane fraction of eucalyptus extract) exhibited some toxic activity at the highest concentration tested (100 μg/mL) with a survival rate below 20%, while other concentrations did not affect cell survival. While cynaropicrin, betulonic and ursolic acids affected cell survival at the highest concentration (10 μg/mL; survival rate of 65, 70 and 25%, respectively), betulinic and oleanolic acids did not show any cytotoxic effects at any of the concentrations tested (**Figures 4.2 and 4.3**). None of the semi-purified fractions and purified molecules were found to stimulate cell proliferation at any of the concentrations tested. On the contrary, anti-proliferative effects – or

most probably cytotoxic effects associated to low cell density – were observed at 100, 10 and 1 μg/mL for the semi-purified fractions (cardoon and eucalyptus), at 10 and 1 μg/mL for cynaropicrin and at 10 μg/mL for triterpenic acids (**Figures 4.2 and 4.4**).

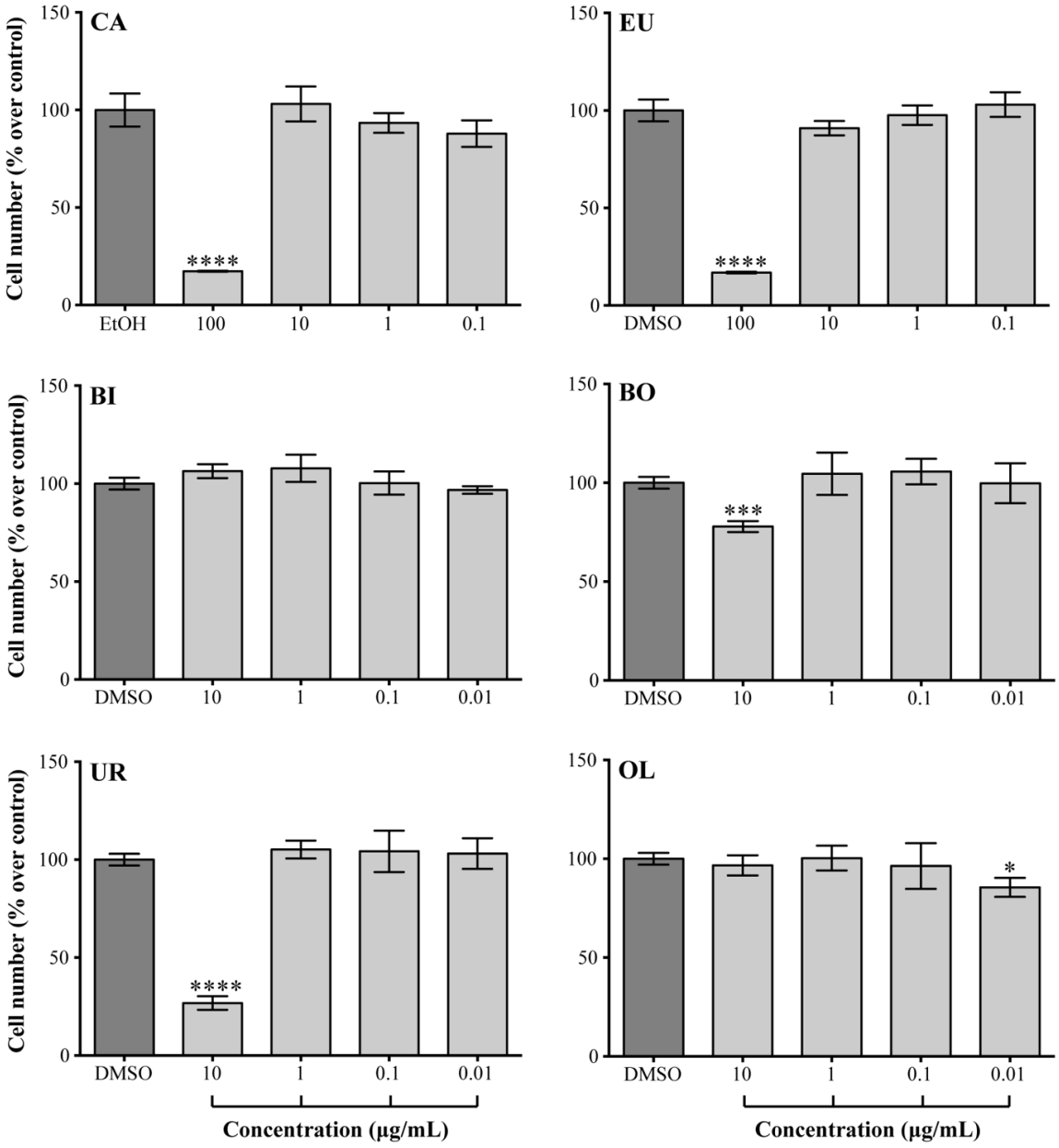


**Figure 4.2.** Cytotoxicity of semi-purified fractions (CA, EU) and purified molecules (BI, BO, UR and OL) assessed in confluent VSa13 cell cultures through the XTT cell survival assay. Value of vehicle (DMSO) was set at 100%. *Asterisks* indicate values statistically different from the vehicle (one-way ANOVA followed by Dunnett's multiple comparison test (* $p \leq 0.05$; *** $p \leq 0.001$; **** $p \leq 0.0001$). CA, cardoon; EU, eucalyptus; BI, betulinic acid; BO, betulonic acid; UR, ursolic acid; OL, oleanolic acid.

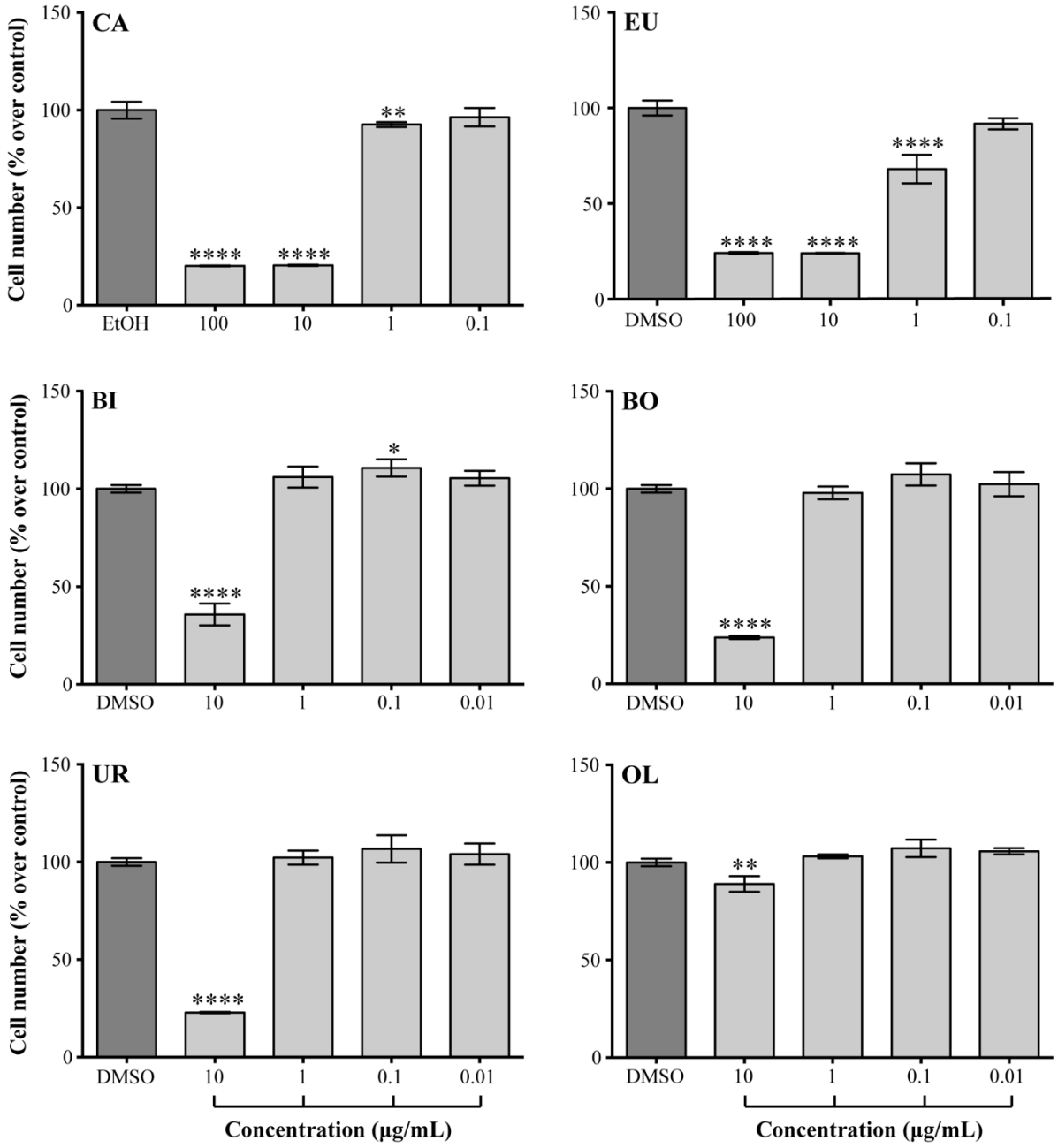


**Figure 4.3.** Proliferative effects of semi-purified fractions (CA, EU) and purified molecules (BI, BO, UR and OL) assessed in sub-confluent VSa13 cell cultures through the XTT cell survival assay. Value of the vehicle was set at 100%. *Asterisks* indicate values statistically different from the vehicle (one-way ANOVA followed by Dunnett's multiple comparison test (**$p \leq 0.01$; **** $p \leq 0.0001$). CA, cardoon; EU, eucalyptus; BI, betulinic acid; BO, betulonic acid; UR, ursolic acid; OL, oleanolic acid.

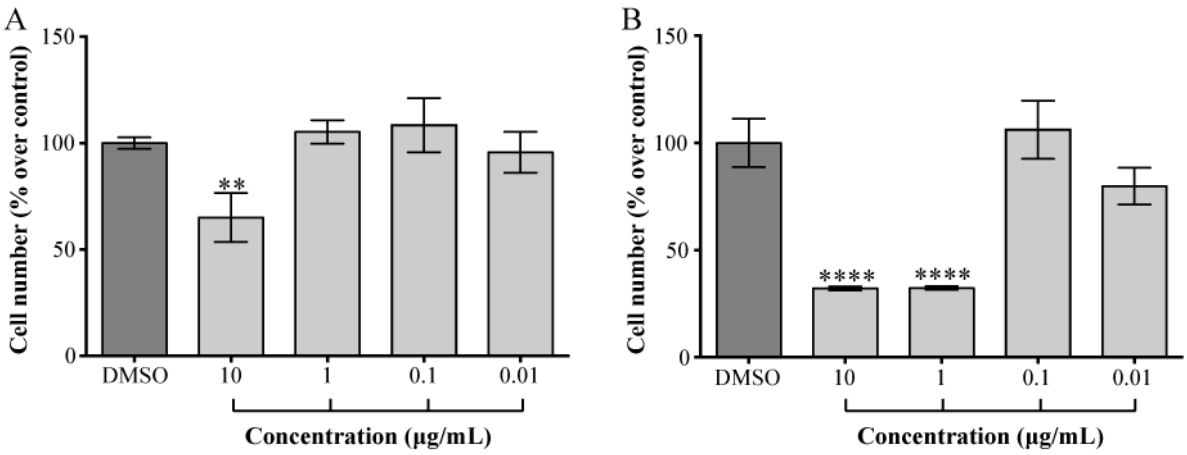


**Figure 4.4.** Cytotoxicity (**A**) and proliferative effect (**B**) of cynaropicrin assessed in confluent or sub-confluent VSa13 cell cultures, respectively, through the XTT cell survival assay. Value of the vehicle was set at 100%. *Asterisks* indicate values statistically different from the vehicle (one-way ANOVA followed by Dunnett's multiple comparison test (** $p \leq 0.01$; **** $p \leq 0.0001$).

### 4.1.2 Extracts from cyanobacteria

Because the amount of material received from our collaborators was limited, cytotoxicity and proliferative effect of ethyl acetate fractions will be evaluated in the same assay based on low density cell cultures. Among the 8 different strains of cyanobacteria, ethyl acetate fractions E9 and E10 exhibited some cytotoxicity at 10 µg/mL (survival rate of 80%) while E12 fraction affected cell survival at both 10 and 1 µg/mL (survival rate of 57% and 80%, respectively). At 0.01 µg/mL fraction E12 appeared to slightly affect cell number, although this effect may not be of biological significance since the higher concentration 0.1 µg/mL did not show any effect (**Figure 4.5**).

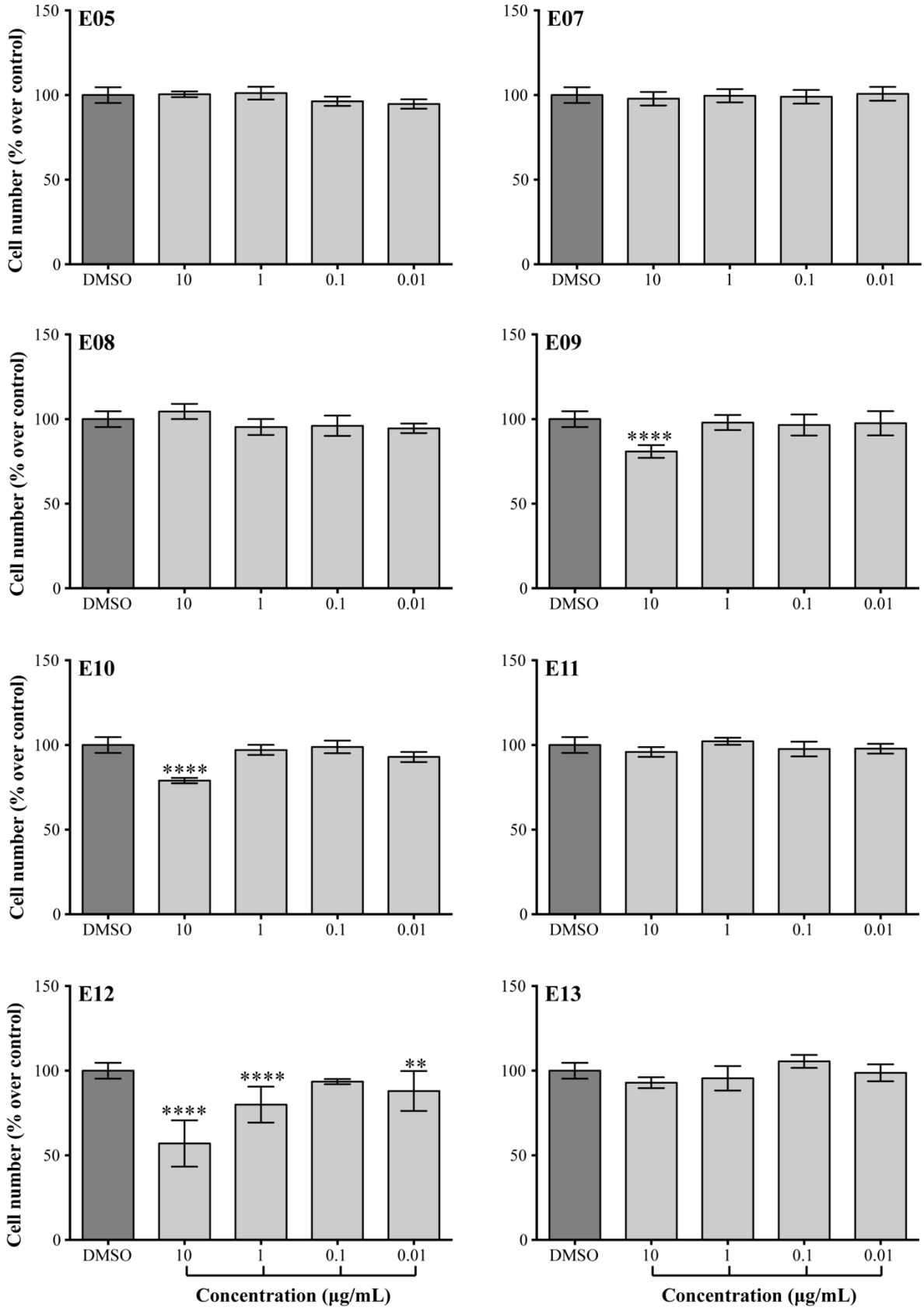


**Figure 4.5.** Proliferative effects of ethyl acetate fractions prepared from different cyanobacteria strains assessed in sub-confluent VSa13 cell cultures through the XTT cell survival assay. Value of the vehicle was set at 100%. *Asterisks* indicate values statistically different from the vehicle (one-way ANOVA followed by Dunnett's multiple comparison test (**$p \leq 0.01$; **** $p \leq 0.0001$).

## 4.2 Mineralogenic effects of semi-purified fractions and related molecules

Highest non-toxic concentrations, i.e. 1 and 0.1 µg/mL for semi-purified fractions and purified molecules, respectively, were evaluated for their effect on extracellular matrix mineralization after 21 days of exposure (endpoint to assess ECM mineralization by alizarin red S staining). Vehicles (DMSO and EtOH) did not statistically change the extent of mineral deposition when compared with control condition (culture medium supplemented with mineralogenic cocktail) (**Figure 4.6**).

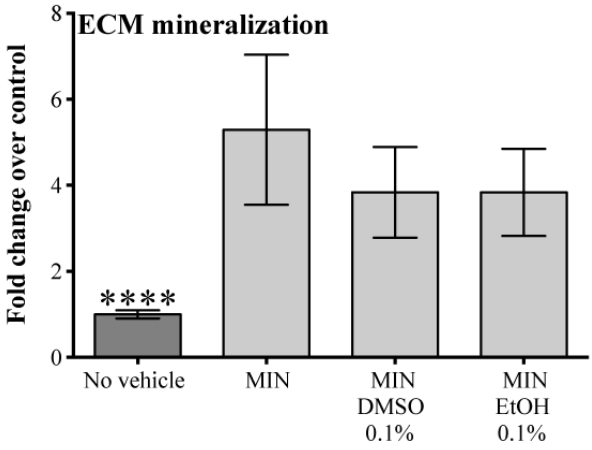


**Figure 4.6.** Mineralogenic effect of the vehicles assessed in mineralizing VSa13 cell cultures through alizarin red S staining. *No vehicle*, non-supplemented cell culture medium (DMEM); *MIN*: DMEM supplemented with the mineralogenic cocktail (ascorbic acid, β-glycerophosphate and $CaCl_2$); *DMSO*, dimethyl sulfoxide; *EtOH*, ethanol. Changes in ECM mineralization are presented as fold change over the control and *asterisks* indicate values statistically different from the vehicle (one-way ANOVA followed by Dunnett's multiple comparison test (**** $p \leq$ 0.0001).

Semi-purified fractions of cardoon (0.1 µg/mL) and eucalyptus (1 µg/mL) were found to slightly increased mineralization by 1.56 and 1.96 folds over the respective controls. Because it exhibited some apparent cytotoxicity at 1 µg/mL, cardoon fraction was tested again at 0.316 and 0.0316 µg/mL and the higher concentration (0.316 µg/mL) was found to increase ECM mineralization by 3.28 folds over the control. Regarding triterpenic acids, while betulinic acid did not affect ECM mineralization, betulonic, oleanolic and ursolic acids strongly increased mineral deposition by 4.27, 2.71 and 5.24 folds, respectively, at 1 µg/mL. At a lower concentration (0.1 µg/mL), only ursolic acid stimulated ECM mineralization (2.05 folds over the control; **Figure 4.7**).

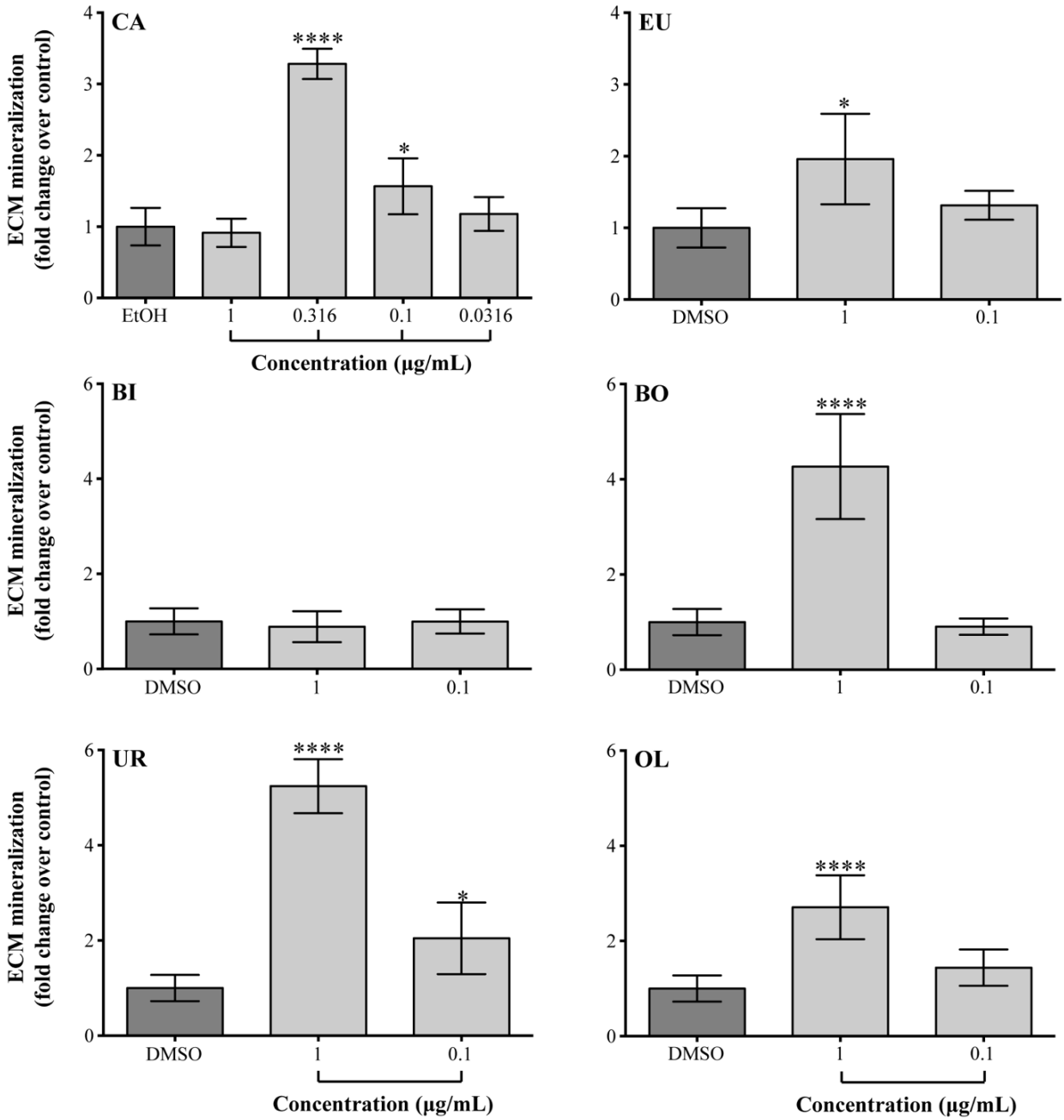


**Figure 4.7.** Mineralogenic effect of semi-purified fractions (CA, EU) and purified molecules (BI, BO, UR and OL) assessed in mineralizing VSa13 cell cultures through alizarin red S staining. Changes in ECM mineralization are presented as fold change over the control and *asterisks* indicate values statistically different from the vehicle (one-way ANOVA followed by Dunnett's multiple comparison test (* $p \leq 0.05$; **** $p \leq 0.0001$). CA, cardoon; EU, eucalyptus; BI, betulinic acid; BO, betulonic acid; UR, ursolic acid; OL, oleanolic acid.

Interestingly, cynaropicrin was shown to be anti-mineralogenic at both concentrations tested (0.1 and 0.01 μg/mL), decreasing mineral deposition by 2.13 and 2.32 folds, respectively, over the control (**Figure 4.8**).

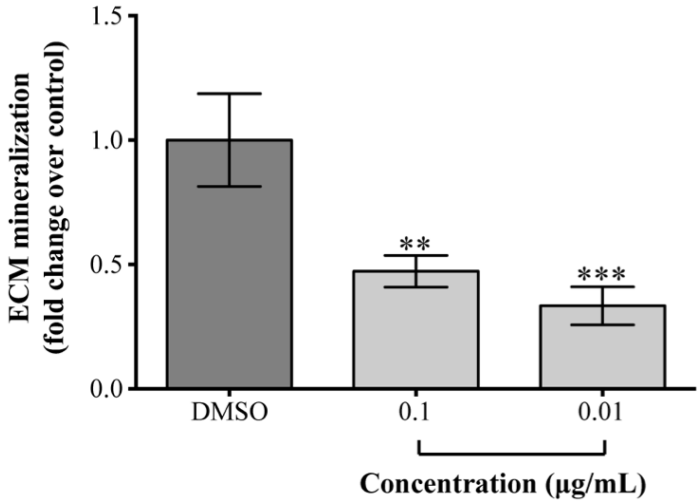


**Figure 4.8.** Mineralogenic effect of cynaropicrin assessed in mineralizing VSa13 cell cultures through alizarin red S staining. Changes in ECM mineralization are presented as fold change over the control and *asterisks* indicate values statistically different from the vehicle (one-way ANOVA followed by Dunnett's multiple comparison test (** $p \leq 0.01$; *** $p \leq 0.001$).

Mineralogenic effect of ethyl acetate fractions from cyanobacteria was tested at a concentration of 1 μg/mL, and only fractions E8, E12 and E13 were found to slightly increase ECM mineralization by 1.56, 1.58 and 1.55 folds over the control, respectively (**Figure 4.9**).

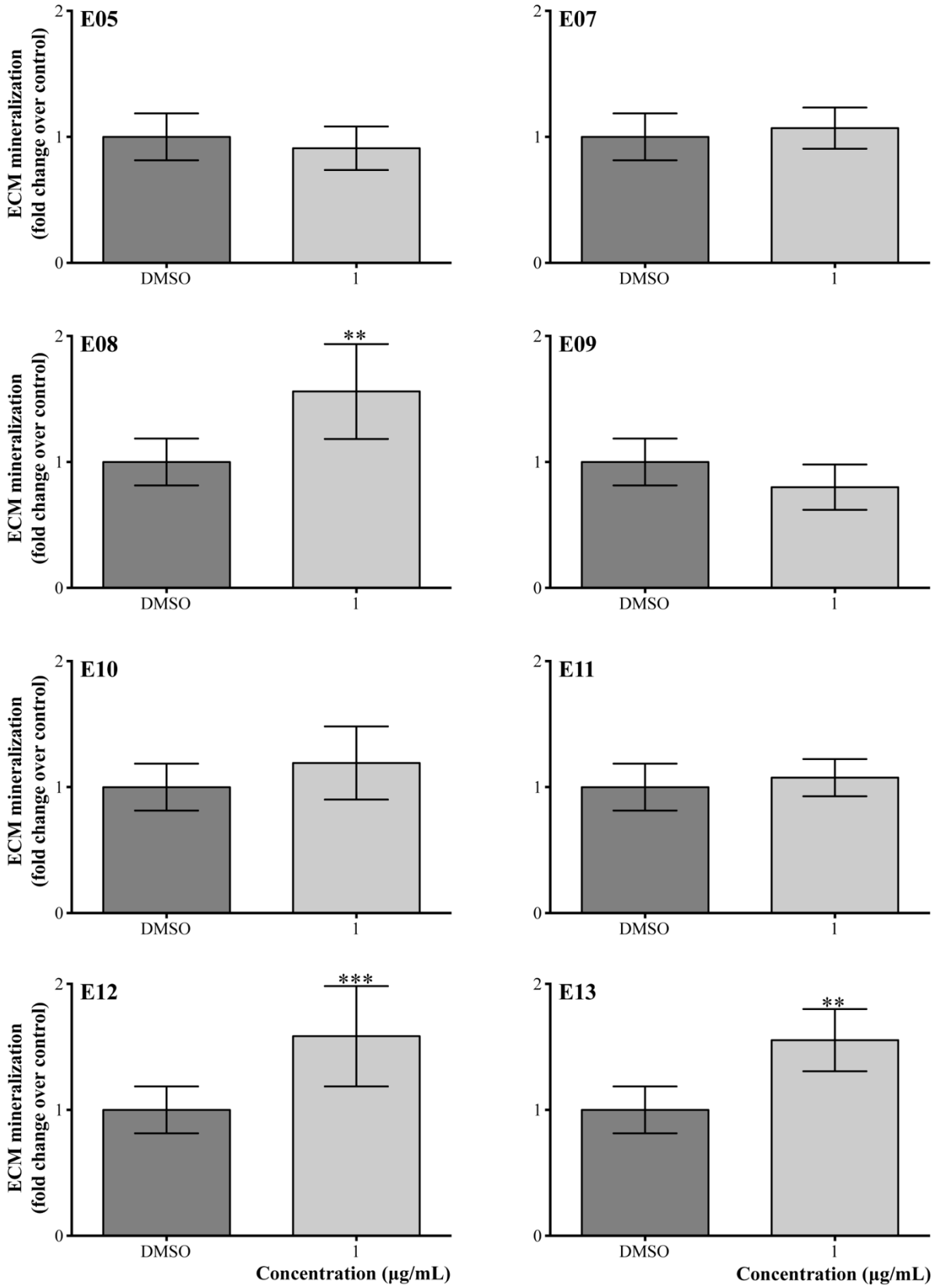


**Figure 4.9.** Mineralogenic effect of semi-purified fractions prepared from cyanobacteria assessed in mineralizing VSa13 cells through alizarin red S staining. Changes in ECM mineralization are presented as fold change over the control and *asterisks* indicate values statistically different from the vehicle (one-way ANOVA followed by Dunnett's multiple comparison test (** $p \leq 0.01$; *** $p \leq 0.001$).

## 4.3 The zebrafish operculum as a model to assess for osteogenic activities

### 4.3.1 Time course of operculum formation

Zebrafish larvae (AB line) were sampled every 24 h from 4 to 15 dpf and stained with AR-S to reveal mineralized bone structures, in particular the operculum. Fluorescence images of the lateral view of the head of each larvae were acquired (**Figure 4.10A**) and the morphometry of the different elements and structures was analyzed (**Figure 4.10B**). The area of the head (A1) and operculum (A2), the length from the snout to the cleithrum (L1), the width and height of both eye and iris (L2/L3 and L4/L5, respectively) were determined and the suitability of the different measurements to correct for inter-specimen variability in operculum area was assessed from simple linear regressions (**Figure 4.11**). While both the area of the iris and the eye and the length between snout and cleithrum exhibited a good correlation with the area of the operculum ($R^2$ of 0.88, 0.90 and 0.92, respectively), the area of the head appeared to be the most accurate parameter to correct for inter-specimen variability of the operculum area ($R^2$ = 0.94) (**Figure 20**). The time course of operculum formation was monitored from 4 to 15 dpf (**Figure 4.12A**). The operculum area was plotted in function of time either as raw data (**Figure 4.12B**) or after correction for the head area (operculum area divided by the head area, O/H) (**Figure 4.12C**) and the coefficient of variance (CV) was estimated. Averaged CV of all time points was markedly reduced upon correction for head size (51.9 ± 24.1 % *versus* 27.0 ± 9.4 %), indicating that variation in operculum area due to inter-specimen variability in sampled population could be efficiently corrected. Analysis of corrected operculum area during zebrafish development (**Figure 4.12C**), revealed four different growth phases: 3-6 dpf, 6-9 dpf, 9-13 dpf and 13-15 dpf. The operculum area increased during the first and third phase by 208 % and 85% respectively, while the other two phases were characterized by a slow growth (10 % and 2 %, respectively).

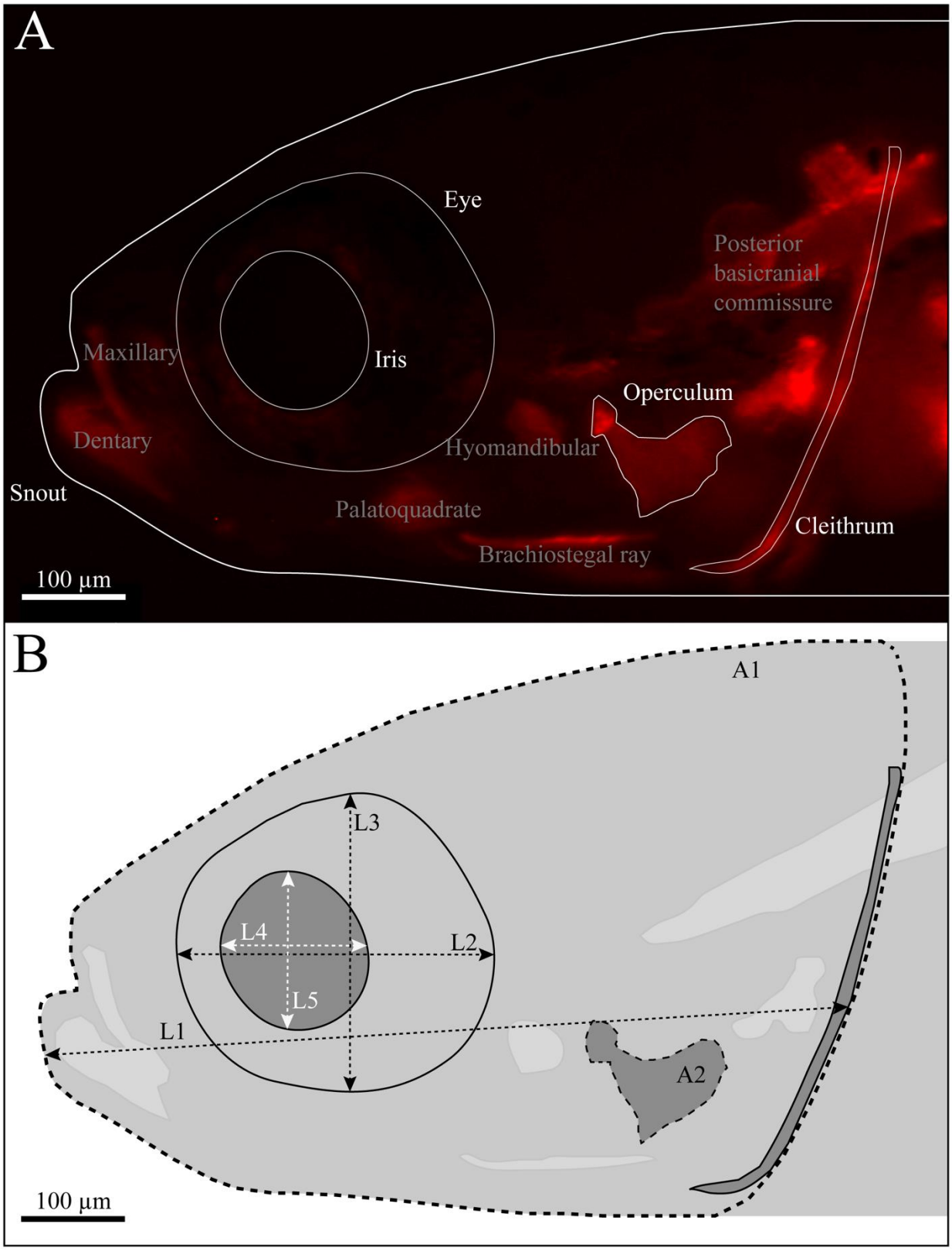


**Figure 4.10.** (**A**) Principal bone structures in the cranium of 11-dpf zebrafish after alizarin red staining. Structures used in the morphometric analysis, i.e. operculum, snout, eye, iris and cleithrum, are outlined in white, while the names of other bony structures are indicated in grey. (**B**) Schematic representation of the head structures assessed through morphometric analysis and parameters measured. *A1*, area of the skull; *A2*, area of the operculum; *L1*, length from the snout to the cleithrum; *L2*, eye width; *L3*, eye height; *L4*, iris width; *L5*, iris height.

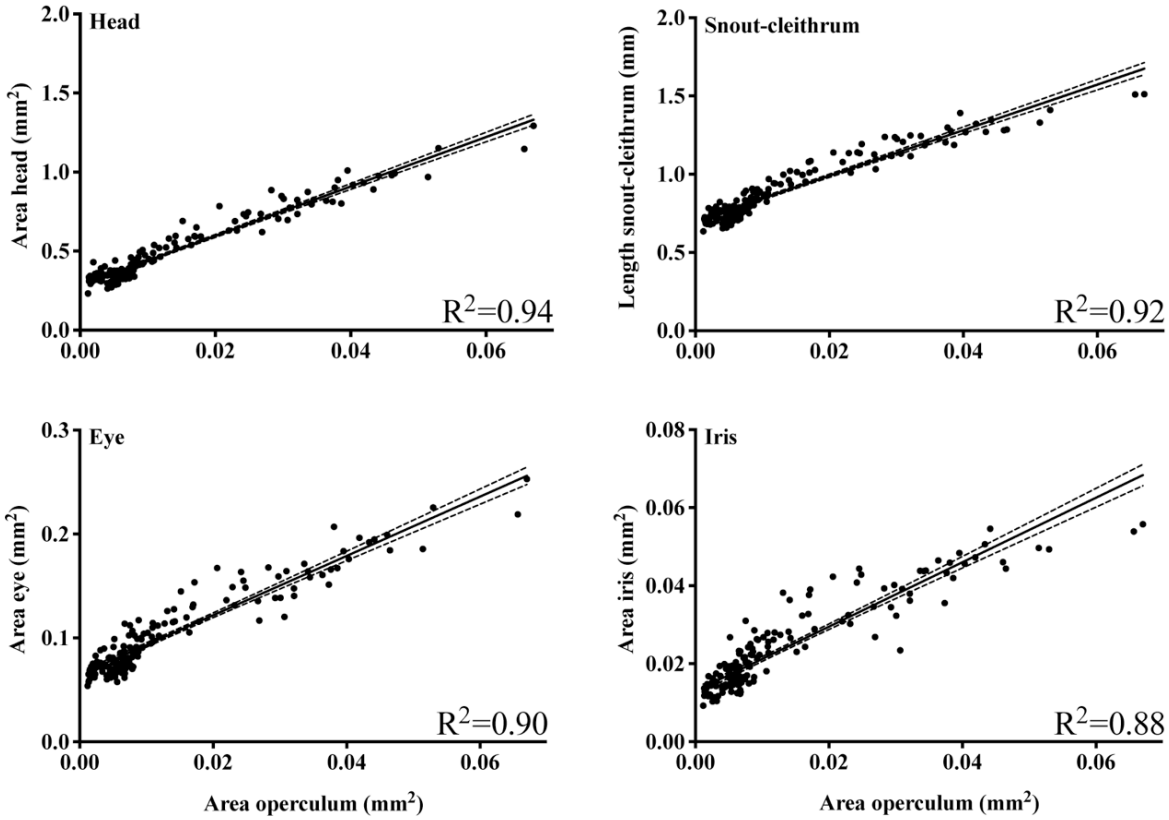


**Figure 4.11.** Correlation between the different morphometric parameters: Area of the operculum *vs* area of the head, area of the operculum *vs* length snout-cleithrum, area of the operculum *vs* area of the eye and area of the operculum *vs* area of the iris. The coefficient of linear regression, the R squared, is indicated for each correlation. Dashed lines indicate confidence intervals at 95% and solid lines indicate the best-fit of the values.

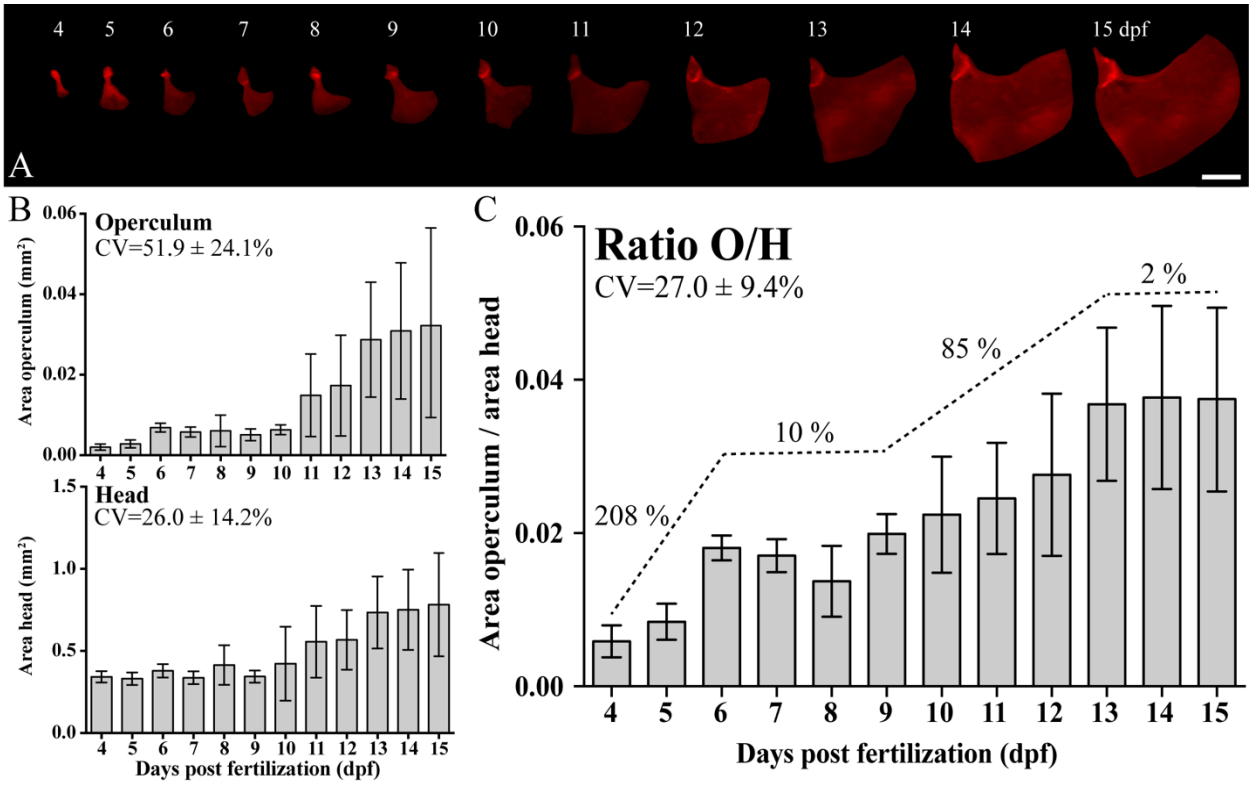


**Figure 4.12.** Time course of zebrafish operculum development. Fluorescence of the opercula stained with alizarin red was imaged from 4 to 15 days post-fertilization (**A**) and area of the operculum and head was determined from 10-15 fish for each time point (**B**). Area of the operculum was normalized with the area of the head (*ratio O/H*, **C**) and insert illustrates the different phases of operculum development with the growth increments expressed in %. The coefficient of variance (*CV*) is indicated in each graph. Values are presented as the mean ± standard deviation. White bar represents 100 µm.

### 4.3.2 Effect of calcitriol on zebrafish operculum mineralization

In order to establish a fast and reliable method to screen for the effects of organic extracts, semi-purified fractions or molecules with osteogenic activities, zebrafish larvae were treated with 0.01 fg/mL of calcitriol, a well-known osteogenic drug. Its effect on operculum formation was assessed using the morphometric analysis presented previously and compared to that of ethanol (0.1%), used as control. Larvae were exposed to calcitriol for different periods (i.e. 3, 6 and 9 days) and exposure was initiated at different developmental stages (i.e. 3, 5 and 8 dpf; **Figure 4.13**). It is important to mention that calcitriol did not affect the area of the head, the parameter used to correct operculum area (**Figure 4.14**). Exposure to calcitriol during 3 days stimulated the operculum formation/mineralization when initiated at 3 or 5 dpf, with an increase over control of 41.80 ± 7.83 % and 36.46 ± 6.37 %, respectively (**Figures 4.13A, 4.13B**). However, when the exposure started at 8 dpf no significant effects were observed in the operculum size (**Figure 4.13C**). A longer exposure to calcitriol (6 days) also increased

operculum area when initiated at 3 and 5 dpf, with an increase over control of 58.42 ± 23.73 % and 74.37 ± 21.68 %, respectively (**Figures 4.13D** and **4.13E**), while onset at 8 dpf did not significantly affect operculum size (**Figure 4.13F**). Operculum area was also increased upon exposure to calcitriol for 9 days (**Figures 4.13G** and **4.13H**) but this increase was only statistically significant when exposure was initiated at 3 dpf (90.56 ± 30.59 %). Generally, fish survival (indicated in **Figure 4.13** by n, the number of fish that survived the exposure out of the 15 larvae initially present) was affected by exposure longer than 3 days and exposure initiated after 5 dpf, supporting the suitability of a short and early exposure of the larvae.

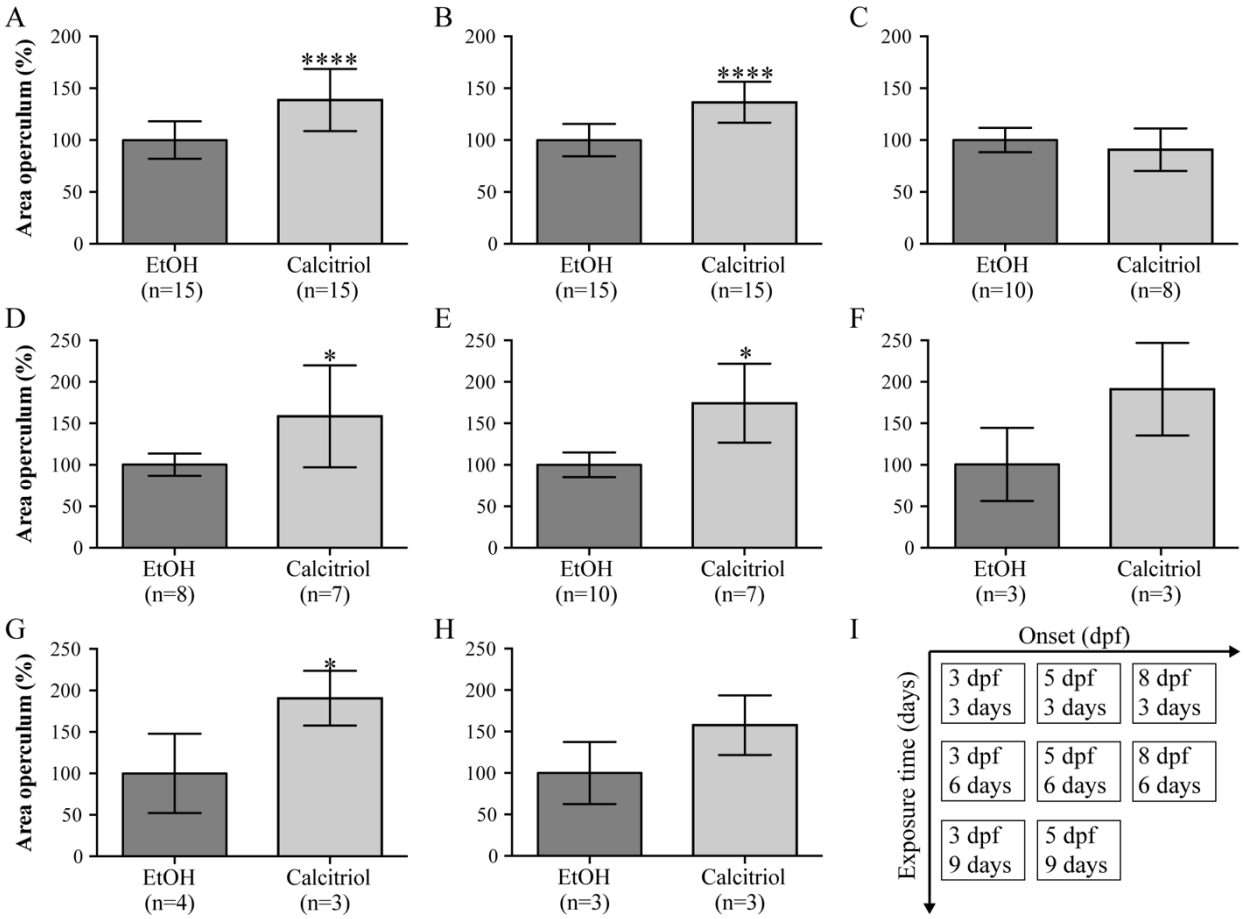


**Figure 4.13. (A-H)** Effect of calcitriol exposure (duration and onset) on the osteogenic development of zebrafish operculum. **(I)** Scheme recapitulating the different parameters of calcitriol exposure. *Light grey*, operculum area of control fish exposed to 0.1% ethanol (vehicle); *Dark grey*, operculum area of fish exposed to 0.01 fg/mL of calcitriol. Number of zebrafish larvae (n) is indicated below each column. *Asterisks* indicate values statistically different according to Student's *t* test (* $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$). Values are presented as the mean ± standard deviation.

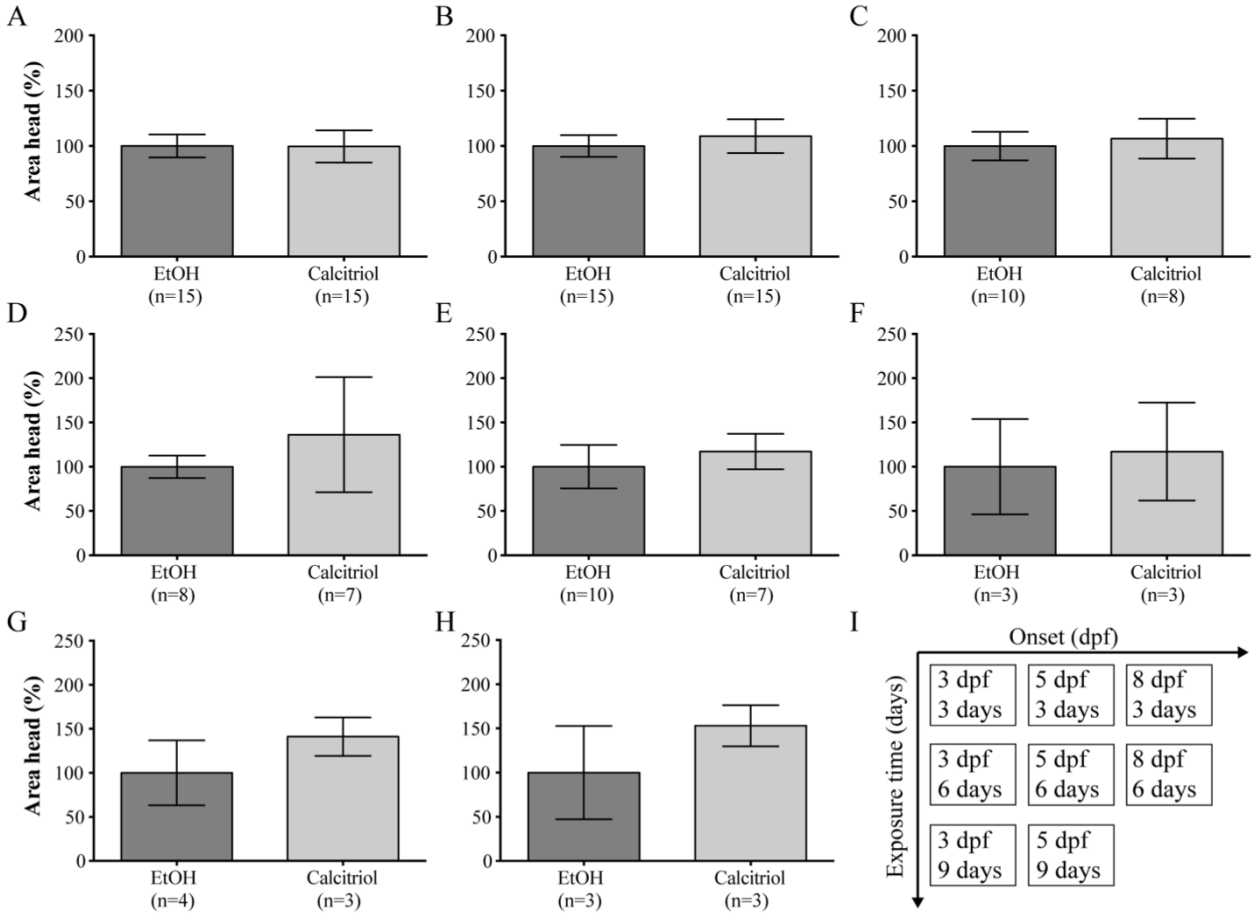


**Figure 4.14. (A-H)** Effect of calcitriol exposure (duration and onset) on the development of zebrafish head. **(I)** Scheme recapitulating the different parameters of calcitriol exposure. *Light grey*, operculum area of control fish exposed to 0.1% ethanol (vehicle); *Dark grey*, operculum area of fish exposed to 0.01 fg/mL of calcitriol. Number of zebrafish larvae (n) is indicated below each column. EtOH and calcitriol values were not significantly different (Student's *t* test, $p < 0.05$). Values are presented as the mean ± standard deviation.

## 4.4 Osteogenic effects of semi-purified fractions and related molecules

To assess their osteogenic effects, semi-purified fractions and purified molecules were tested *in vivo* at concentrations found to have a mineralogenic effect and using conditions previously optimized (see previous sections). Zebrafish larvae at 3 dpf were exposed for 3 days to mineralogenic concentrations and treatments were renewed every day until 6 dpf, when larvae were imaged and the corrected operculum area was determined. In all experiments, calcitriol at 0.01 fg/mL was used as a control for positive osteogenic effect. Vehicles (DMSO and EtOH) did not significantly affect the area of zebrafish operculum (data not shown). While semi-purified fraction from eucalyptus at 1 µg/mL did not exhibit any osteogenic activity, that of cardoon, also at 1 µg/mL, stimulated the area of zebrafish operculum by approximately 15% over the area of control fish. All purified molecules were tested at 1 µg/mL at the exception of betulonic acid which was toxic to zebrafish larvae at this concentration and was therefore tested at 0.5 µg/mL.

While betulinic and betulonic acids did not affect operculum growth, ursolic and oleanolic acid strongly increased the area of the operculum by 63% and 60%, respectively (**Figure 4.15**). A mixture of the 4 triterpenic acids mimicking the proportion found in crude extracts of the shining gum *Eucalyptus nitens* (i.e. BI 36.2 %, BO 26.6 %, UR 17.88 %, OL 19.32 %) was also tested *in vivo* to evaluate a possible synergic effect, but no significant effect was observed (data not showed). In contrast to the osteogenicity of cardoon extract, cynaropicrin exhibited an anti-osteogenic effect and decreased operculum area by 27% at 0.1 µg/mL and 18% at 0.01 µg/mL (**Figure 4.16**). Among the different ethyl acetate fractions from cyanobacteria, only fractions E8, E12 and E13 (those shown to increase ECM mineralization) were tested *in vivo*, and only E13 increased the area of the operculum of 23% over control fish at a concentration of 1 µg/mL (**Figure 4.17**).

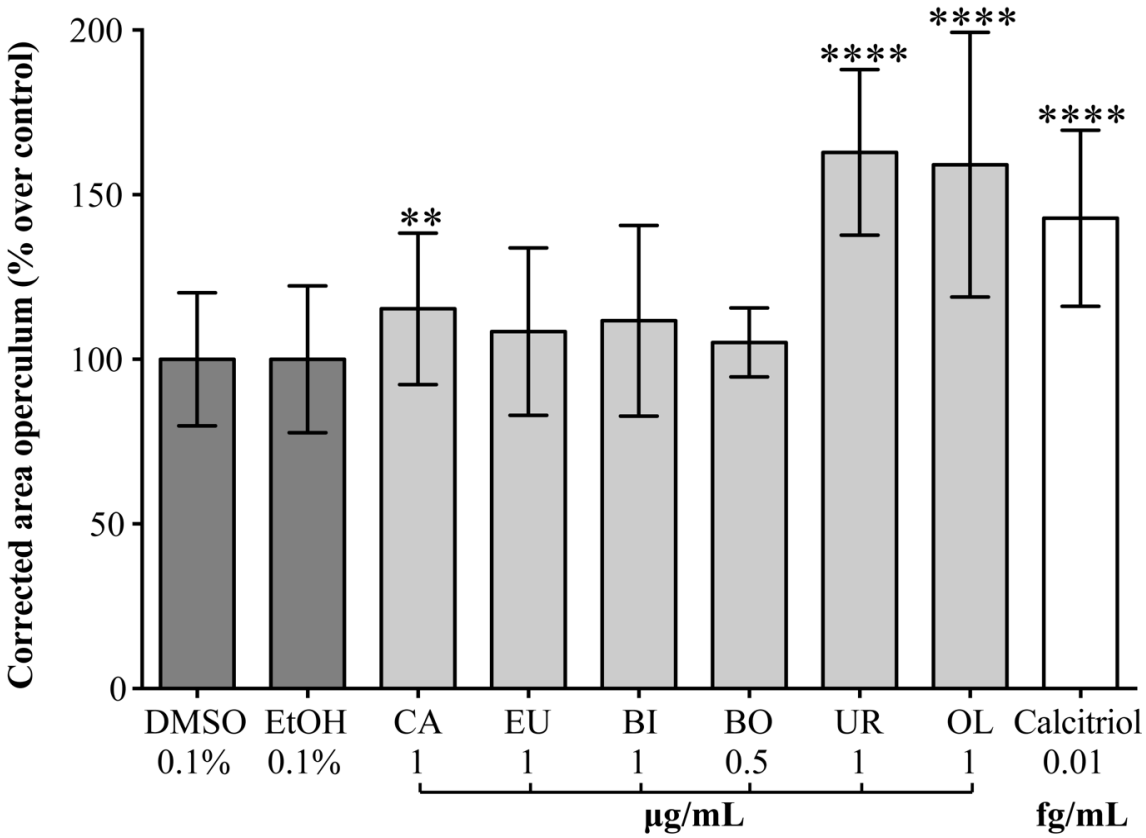


**Figure 4.15.** Osteogenic effect of semi-purified fractions (CA, EU) and purified molecules (BI, BO, UR and OL) assessed in developing zebrafish larvae through alizarin red S staining of the operculum. Changes in operculum area are expressed as percentages over the respective control (DMSO or EtOH). *Asterisks* indicate values statistically different from vehicle values (one-way ANOVA followed by Dunnett's multiple comparison test (** $p \leq 0.01$; **** $p \leq 0.0001$). CA, cardoon; EU, eucalyptus; BI, betulinic acid; BO, betulonic acid; UR, ursolic acid; OL, oleanolic acid; Vit. D, calcitriol.

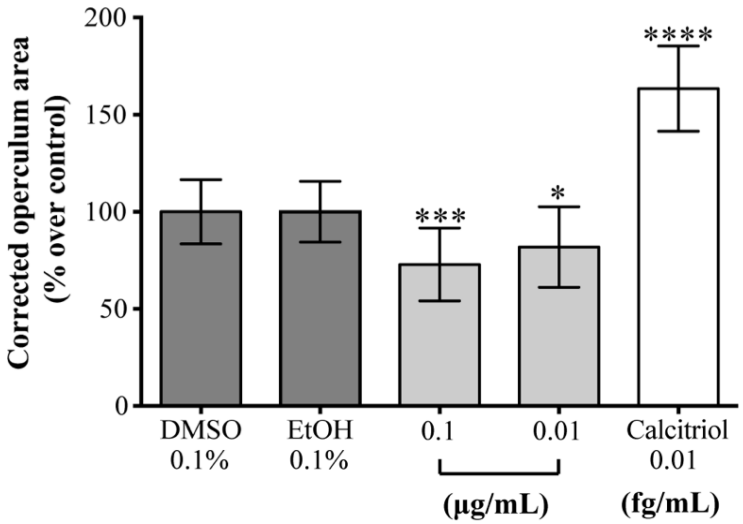


**Figure 4.16.** Osteogenic effect of cynaropicrin assessed in developing zebrafish larvae through alizarin red S staining of the operculum. Changes in operculum area are expressed as percentages over the respective control (DMSO or EtOH). *Asterisks* indicate values statistically different from vehicle values (one-way ANOVA followed by Dunnett's multiple comparison test (* $p \leq 0.05$; *** $p \leq 0.001$; **** $p \leq 0.0001$).

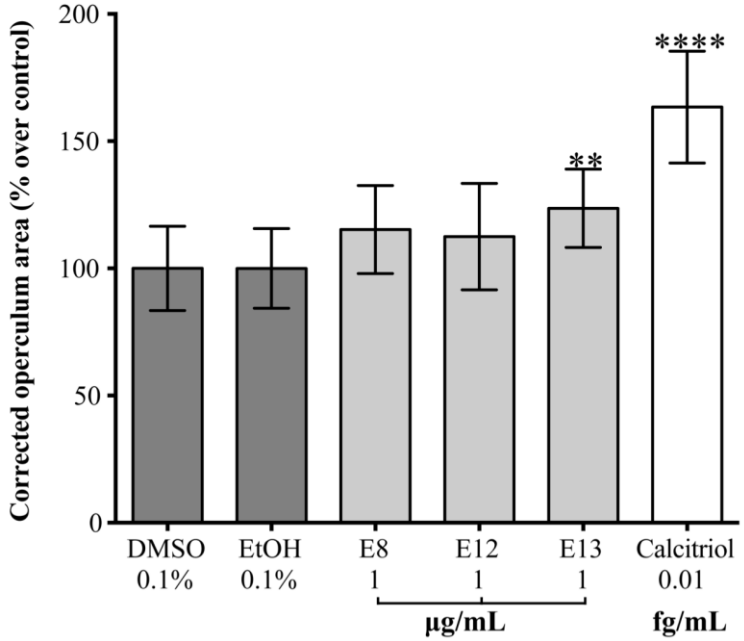


**Figure 4.17.** Osteogenic effect of cyanobacteria semi-purified fractions (E8, E12 and E13) assessed in developing zebrafish larvae through alizarin red S staining of the operculum. Changes in operculum area are expressed as percentages over the respective control (DMSO or EtOH). *Asterisks* indicate values statistically different from vehicle values (one-way ANOVA followed by Dunnett's multiple comparison test (** $p \leq 0.01$; **** $p \leq 0.0001$).

## 4.5 Tg(*osx:mCherry/oc2:EGFP*) transgenic zebrafish line to get insights into mechanisms underlying osteogenic effects

Zebrafish transgenic lines expressing green and red fluorescent proteins under the control of the promoters of two osteoblast markers – *osterix* (*osx* or *sp7*), a marker of immature osteoblasts and *osteocalcin 2* (*oc2*), a marker of mature osteoblasts – were used to get insights into the cellular

dynamics underlying the osteogenic effects of calcitriol observed during operculum formation to evaluate the applicability of this system for further molecules screenings. Double transgenic larvae Tg(*osx:mCherry/oc2:EGFP*) at 3 dpf were exposed for 3 days to 0.01 fg/mL of calcitriol or to 0.1% ethanol (control) and the effects on osteoblasts were assessed through the analysis of fluorescence images at 6 dpf (**Figure 4.18A**). Exposure to calcitriol increased the operculum area positive for *osx:mCherry* by 32.28 ± 6.76 % over the control (**Figure 4.18A** and **4.18B**), an increase similar to the one observed in AR-S-stained wild-type fish exposed to calcitriol in the same conditions (**Figure 4.18A**), suggesting that the density of immature osteoblast was probably unaltered by calcitriol. Exposure to calcitriol also increased the operculum area positive for *oc2:EGFP* (**Figure 4.18A and 4.18C**), although a stronger effect was observed (83.61 ± 10.69 % over control), indicating that the density of mature osteoblast was stimulated upon calcitriol treatment. An analysis of the number of fluorescent pixels per operculum area further confirmed the specific effect of calcitriol on mature osteoblast population (515.20 ± 164.50 % increase in calcitriol-treated fish over the control; **Figure 4.18E**), while effect on immature osteoblast population was mild (36.43 ± 13.34 % increase in calcitriol-treated fish over the control; **Figure 4.18D**).

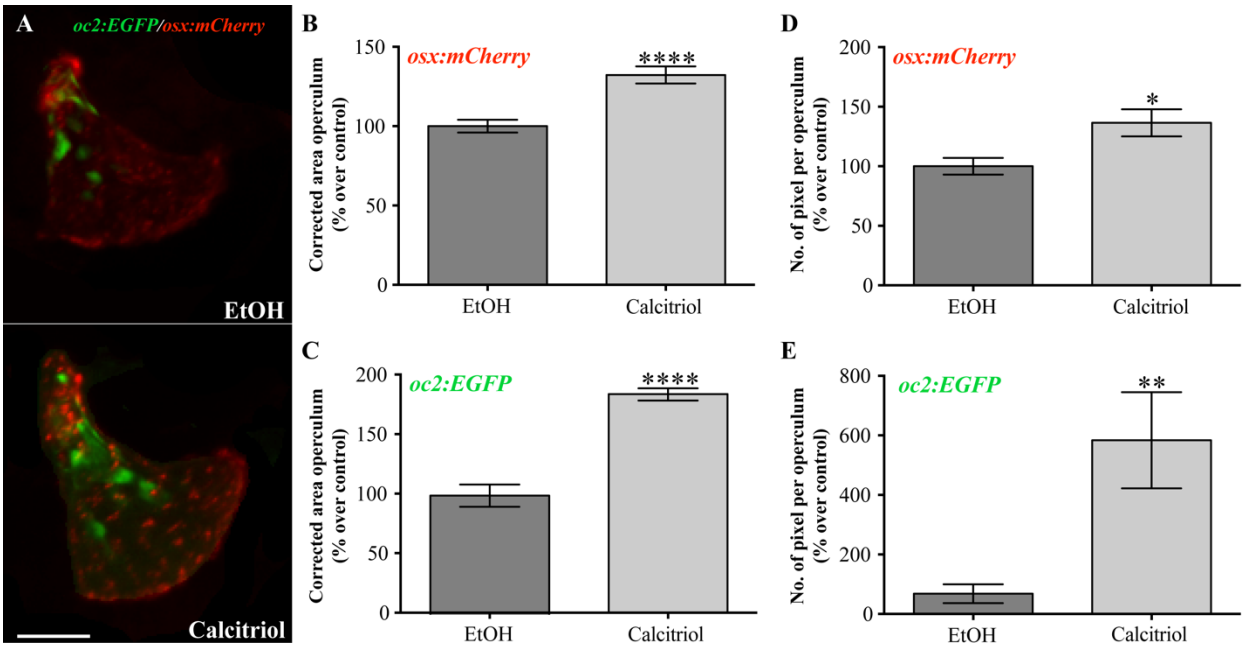


**Figure 4.18.** Effects of calcitriol exposure (from 3 to 6 dpf) on the expression of *osterix* and *osteocalcin 2* in the operculum of double-transgenic zebrafish larvae Tg(*osx:mCherry/oc2:EGFP*). (**A**) Fluorescence stereomicroscopy images of the operculum from control (0.1% ethanol; EtOH) and calcitriol-treated (0.01 fg/mL) larvae. Red (*osx:mCherry*) and green (*oc2:EGFP*) channels were merged. Scale bar represents 50 µm. (**B-C**) Corrected red and green opercular area (percentage over the control). (**D-E**) Number of red and green pixels per operculum area. *Asterisks* indicate values statistically different according to Student's *t* test (* $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$). Values are presented as the mean ± standard error of the mean.

# 5 Discussion

The purpose of this work was to evaluate mineralogenic and osteogenic activities in semi-purified fractions from several natural resources – cardoon, eucalyptus and cyanobacteria – but also to test the ability of molecules purified from these fractions – triterpenic acids and a sesquiterpene lactone – to increase extracellular matrix mineralization *in vitro* and operculum growth *in vivo*. Data related to the cytotoxic, proliferative, mineralogenic and osteogenic effects of these fractions and molecules were collected. In parallel, a methodology to assess osteogenic bioactivities was optimized with calcitriol and the suitability of a double transgenic line expressing fluorescent proteins under the control of *osterix* and *osteocalcin 2* promoters to get insights in osteogenic effects at cellular level was established.

## 5.1 The zebrafish operculum: a powerful system to assess osteogenic bioactivities

A major objective of this work was to establish a simple, reproducible and reliable zebrafish-based screening method to assess the effects of extracts, fractions or molecules for osteogenic activity. Because they are surface bones that form early during development and can therefore be easily monitored in larval stages, opercula represent a skeletal structure with a high potential for high-throughput screening. The suitability of zebrafish alizarin red-stained opercula to screen for osteogenic compounds was established and the duration and initiation of larval exposure optimized. Analysis of the complete data set revealed that the osteogenic effects of calcitriol were not statistically different, regardless of duration or onset of exposure, indicating that a short (3 days) and early (3 dpf) exposure – a situation favoured in high throughput screening methods – was appropriate, although the other conditions were also suitable. In these economically favourable conditions (i.e. short exposure synonymous of a limited use of compound and early exposure synonymous of high animal turnover), operculum exhibited the highest growth increment (208 %), while the mortality associated with the housing conditions (e.g. limited space as larvae grow and degraded water quality due to static conditions) was minimal. Increased mortality observed for longer exposures or for older larvae has been principally associated with housing conditions but it may also be related to the point-of-no-return (PNR), when zebrafish larvae, after switching from endogenous nutritional reserves to exogenous feeding (5-7 dpf[83]), are not able to feed and recover even if food becomes available[84]. PNR is associated with increased mortality and screening should therefore end before this point to minimize stress and

avoid mortality not related to molecules or extracts. Accordingly, we propose that the screening, or the study, of osteogenic bioactivities in zebrafish should be initiated at 3 dpf and larvae exposed to the compounds to be tested for 3 days.

Alizarin red staining combined with morphometric approaches demonstrated to be an easy and accurate way to detect and quantify bone mineral deposition under fluorescence conditions, as already shown in previous studies.[81] Other techniques – e.g. X-rays or micro-computed tomography – have been used to image mineralized structures in zebrafish, but their application in the context of the methodology presented here may be difficult due to technical limitations, such as acquisition time (micro-CT) or low image resolution of poorly mineralized larvae (X-ray).[85,86] This study also considered the necessity to correct for inter-specimen variability and, while several parameters were deemed appropriate, the area of the head proved to be superior to calibrate operculum area measurements, but also to be easily determined from the same set of images used to assess operculum area. Several studies have reported the use of alizarin red to screen for molecules with osteogenic and mineralogenic bioactivities in zebrafish. They assessed the mineralization of cranial bones in larvae exposed from 3 to 9 dpf[87] or the count of vertebral segments at 10 dpf[88] but none of these studies considered inter-specimen variability nor quantified accurately the osteogenic effect. They also failed to provide a high-throughput approach with long exposures, late endpoints and a laborious image acquisition, and therefore have a limited applicability for large-scale screening of molecules.

Calcitriol, the bioactive form of vitamin D, was used throughout this study to optimize the screening procedure but also to validate the mineralizing operculum as a suitable system to monitor osteogenic bioactivities. Calcitriol is a well-known osteogenic and anti-osteoporotic compound[27,89,90] that stimulates osteoblast growth and differentiation, and therefore bone formation *in vivo*[91,92] and mineralization *in vitro*.[93,94] Calcitriol also increases bone formation in zebrafish skeletal structures, such as in the perichordal sheath and coracoid processes, after exposure from 3 to 9 dpf (at concentration higher than 25 fg/mL)[95] and in maxilla, branchiostegal rays, hyomandibulars, entopterygoids and ceratohyals after exposure from 5 to 10 dpf (at 200 ng/mL).[96] A pro-osteogenic effect of calcitriol on zebrafish opercular development is reported here for the first time. Remarkably, the concentration used here (i.e. 0.01 fg/mL) is much lower than those previously tested,[95,96] suggesting that operculum may be more sensitive than other systems to the action of osteogenic compounds.

## 5.2 Cytotoxicity and proliferative effects

This is the first time that semi-purified fractions prepared from cardoon, eucalyptus and cyanobacteria, and molecules purified from these extracts (i.e. triterpenic acids and cynaropicrin) are tested in a fish cell line (VSa13) for their effects on cell viability and proliferation. Highest non-toxic concentrations were identified for each fraction and molecule. At the same concentrations, semi-purified fractions were less toxic than molecules purified from the same source (e.g. cardoon / cynaropicrin and eucalyptus / triterpenic acids), an observation probably related to the lower concentration of the molecules in the fractions combined with the lack of synergy in toxic effects. We also observed that, although identical or very similar in formula, triterpenic acids exhibited different levels of cytotoxicity, with ursolic acid strongly affecting cell survival at concentrations not toxic for other triterpenic acids, showing how subtle changes in molecule structure may trigger different biological responses. In this regard, mineralogenic and osteogenic effects of triterpenic acids were also found to be different (see below). None of the semi-purified fractions and purified molecules stimulated cell proliferation at the concentrations tested. On the contrary, an evident decrease of the number of cells upon exposure of sub-confluent cultures to the highest concentrations of the semi-purified fractions and triterpenic acids was observed and while it could suggest an anti-proliferative effect we believe that it is most probably related to the toxic effect observed in confluent culture and intensified by the low cell density used in this assay (i.e. $1\times10^3$ cell/well in proliferation assay *versus* $3\times10^3$ in cytotoxicity assay). A microscopic observation of the cell cultures exposed to these concentrations showed changes in cell morphology compatible with a toxic effect (data not shown). A possible explanation would be that cells at low confluency have a higher surface exposed to the toxic action of the molecules, and would be therefore more vulnerable. Several studies based on different cancer cells lines have in fact demonstrated that the level of resistance to drugs correlates with the degree of cell confluence in monolayer cultures, this phenomena has been described as "confluence-dependent resistance".[97,98] In particular, it has been proposed that an increase in cell confluence alters passive diffusion across the cells membranes, resulting in a reduction of drug intake into the cells.[98] Another study suggested that cells at high confluence create a microenvironment (decrease of medium nutrients, changes of pH, oxygen) that may conduct to the development of a drug resistant phenotype.[99,100] Finally, cancer therapeutics were found to be more effective on rapidly dividing cells than on cells with a slow or suspended growth, e.g. cells in confluent cultures.[101]. Based on the data reported here and collected from the literature, we propose to limit the evaluation of fractions/molecules cytotoxic effect to dividing

cell cultures when high-throughput and/or material availability is an issue. This principle was applied to the evaluation of cyanobacteria extracts that were available in limited amounts.

Limited data is available on cytotoxic and proliferative effects of the semi-purified fractions and purified molecules analysed in this study. While none are available in cells systems of fish origin, some data have been collected in several mammalian cell lines although most of them are cancer-related cell lines not capable of *in vitro* mineralization. The lipophilic fraction of eucalyptus extract decreased the survival of human colorectal carcinoma cell line HCT116 (seeded in 24-well plates at $8\times10^4$ cells/mL) after 2 days of exposure[102] at concentrations very similar to those reported for seabream bone cells VSa13 (i.e. 1 and 10 μg/mL of extract. Survival of human breast cancer cells MDA-MB-231 was reduced by half when exposed for 2 days to 259 μg/mL of a methanol fraction of cardoon extract[103], a value higher than the one determined in our fish mineralogenic cell system (IC50 was roughly estimated to 63 μg/mL using the formula published by Zeidan and Oran (2014)[104]). Differences could be related to the cellular context (cancer *versus* bone-derived cell lines), the duration of the exposure (2 days *versus* 9 days), the cell density in the culture ($3\times10^5$ *versus* $3\times10^3$ cells/well) and in the case of the cardoon extract, the solvent used (methanol *versus* dichloromethane). The exposure for 2 days to cynaropicrin of U937, Eol-1 and Jurkat T human leukocyte cancer cell lines (seeded at $1\times10^5$ cell/well) decreased cell viability by half at concentrations of 1, 3.46 and 0.8 μg/mL respectively.[105] Survival of rat skeletal myoblast (L6-cells) was also reduced by 50% when exposed to 0.76 μg/mL of cynaropicrin for 3 days.[106] Although the comparative analysis of the data collected for cynaropicrin in fish *versus* mammalian systems may be difficult due to variable parameters, cell viability appeared to be drastically affected by concentrations higher than 1 μg/mL.

Few studies have reported cytotoxic and proliferative data in a context of *in vitro* mineralization or using cell lines derived from bone tissues (normal or tumor-related): Betulinic[107], ursolic[108] and oleanolic[109] acids were evaluated in a murine calvaria-derived osteoblastic cell line (MC3T3-E1) known to mineralize its extracellular matrix and ethyl acetate fractions of several strains of cyanobacteria were tested in a human osteosarcoma cell line (MG-63). A comparative analysis of our data *versus* those from the literature is not easy because conditions – duration of the exposure, amount of molecules and density of cultured cells – are highly variable (the information related to the species of cyanobacteria used in this work are also not yet available). In agreement with the data collected in VSa13 cells, an exposure for 3 days of MC3T3-E1 cells (seeded at $5\times10^3$ cells/well) to betulinic acid at concentrations ranging from 0.5 to 9 μg/mL did not affect cell viability or promoted proliferative effects.[107] Similarly, oleanolic acid had no cytotoxic or proliferative effect in MC3T3-E1 cells at concentrations ranging from 0.05 to

4.5 μg/mL.[109] Ursolic acid was tested in MC3T3-E1 cells at a cell density similar to those of VSa13 cells (i.e. $1\times10^3$ cells/well) but with a shorter exposure (3 days) and was reported to arrest cell-cycle at concentrations higher than 4.5 μg/mL[108], a result compatible with an IC50 of ursolic acid between 1 and 10 μg/mL in VSa13 cells. Toxicity and proliferative effect of betulonic acid has not been tested in MC3T3-E1 cells or in any other bone-derived cells of mammalian origin, but has been evaluated in 3 prostate cancer cell lines (i.e. LNCaP, DU-145, and PC-3), where a concentration of 4.5 μg/mL was found to decrease growth by 75%, 31% and 47%, respectively.[110] Again, a different cellular context, a different duration of the exposure and probably a different density of cells initially seeded could possibly explain the different sensitivities of fish and human cell lines to betulonic acid.

Crude extracts and semi-purified fractions of several strains of cyanobacteria have been tested in a large selection of human cancer cell lines.[111] Of particular interest for this work, ethyl acetate fractions were tested on the MG-63 osteosarcoma cell line, where they promoted cytotoxicity and a high anti-proliferative effect, suggesting the presence of molecules with potential anti-cancerous activities.[112,113] In this work, ethyl acetate fraction of strain #12 and to a lesser extent of strains #9 and #10 only decreased cell viability at a concentration of 10 μg/mL while no effect was observed at a lower concentration found to affect human cancer cells, indicating that VSa13 cells, which are not cancer cells, are more resistant to cytotoxic effect of the molecules present in ethyl acetate fractions.

## 5.3 Mineralogenic action of extracts/molecules from natural resources

This is also the first time that semi-purified fractions from cardoon, eucalyptus and cyanobacteria, and purified molecules are tested in a fish cell line (VSa13) for their effects on extracellular matrix mineralization. While eucalyptus fraction and the molecules purified from the same extract (i.e. the triterpenic acids) exhibited a pro-mineralogenic effect (or no effect in the case of betulinic acid), cardoon fraction and cynaropicrin exhibited opposite mineralogenic effects, suggesting that the molecule responsible for the pro-mineralogenic effect of cardoon extract is not the cynaropicrin and remains to be identified. Also abundant in the lipophilic fraction of cardoon extract, taraxasteryl acetate is a pentacyclic triterpene[71] that could be the next molecule to be tested as a potential candidate for cardoon mineralogenic/osteogenic activity. While not mineralogenic in the context of the gilthead seabream VSa13 cells, betulinic acid stimulated ECM mineralization of the MC3T3-E1 cells at concentrations higher than 2.28 μg/mL,[107] concentrations not tested in our fish cell system (highest concentration tested was

1 μg/mL). At a concentration of 1 μg/mL, the mineralogenic potential of the other triterpenic acids in VSa13 cells was as followed: ursolic acid > betulonic acid > oleanolic acid. While this is the first time that a pro-mineralogenic effect is reported for betulonic acid *in vitro*, ursolic acid has already demonstrated its capacity to mineralize the extracellular matrix of MC3T3-E1 cells[108] at a concentration (1.14 μg/mL) and to an extent (6 fold) identical to those observed in fish cells. Mineralogenic effect of oleanolic acid has also been demonstrated, although not quantified, in MC3T3-E1 cell line at concentrations higher than 2.28 μg/mL[109]. This concentration was not tested in our *in vitro* cell system but 1 μg/mL stimulated ECM mineralization in VSa13 cells by more than 2 folds, indicating a similar pro-mineralogenic effect. Absence of mineralization in MC3T3-E1 cell exposed to 1.14 μg/mL could be related to the shorter exposure time (i.e. 14 days in mammalian cells *versus* 21 days in fish cells).

Regarding ethyl acetate fractions of cyanobacteria, only those from strains 8, 12 and 13 were found to slightly increase ECM mineralization of VSa13 cells at 1 μg/mL. As for *in vitro* mineralization, data on the osteogenic effect of fractions/molecules derived from cyanobacteria are scarce, and only two studies have reported evidences toward a possible mineralogenic effect of compounds purified from cyanobacteria. Largazole[114] was shown to significantly induce the expression of alkaline phosphatase and osteopontin genes in murine pluripotent mesenchymal precursor C2C12 through the increased expression of Runx2 and BMPs. Biselyngbyaside[115] was found to inhibit osteoclastogenesis and to induces apoptosis of osteoclasts via pathways distinct of those from other macrolides that suppress osteoclastogenesis or induce osteoclast apoptosis.

Similarities between fish and mammalian cell systems regarding *in vitro* data suggest that molecular mechanisms related to extracellular matrix mineralization may have been conserved throughout evolution from fish to human, as also suggested in other studies[116–118].

## 5.4 Osteogenic action of extracts/molecules from natural resources

The *in vivo* methodology developed and optimized within the scope of this work was successfully applied to the assessment of the osteogenic action of extracts/molecules from natural resources. The mineralogenic potential of the semi-purified fraction prepared from the cardoon was confirmed *in vivo* through an increase of zebrafish operculum size in larvae exposed to this extract. On the contrary, no osteogenic action of eucalyptus extract was observed, indicating that pro-mineralogenic molecules present in eucalyptus are probably not in sufficient amount to significantly affect operculum size and mineralization. It is worth to note that osteogenic effect of cardoon extract was weaker than its mineralogenic effect, indicating that *in*

*vivo* system might be less sensitive; in this regard, it is not totally unexpected that the weak mineralogenic effect of eucalyptus extract *in vitro* was not translated into a significant osteogenic effect *in vivo*. While cardoon is a well-known source of bioactivities such as anti-tumoral[72], anti-proliferative and anti-inflammatory properties[73,74], this study show for the first time that it may also be used to stimulate bone formation and mineralization. In line with the anti-mineralogenic effect identified *in vitro*, cynaropicrin demonstrated an anti-osteogenic effect *in vivo*. Although this work aims at identifying compounds with bone anabolic potential and not compounds decreasing bone formation, cynaropicrin could have an application in pharmaceutics aiming at treating bone disorders characterized by an excess of bone formation e.g. osteopetrosis.[13,119] *In vivo* data collected for cynaropicrin indicated the suitability of the zebrafish operculum system to detect anti-osteogenic bioactivities. For example, it could be used to evaluate the osteotoxicity of environmental pollutants or anthropogenic chemicals before released in the environment.

Ursolic acid stimulated operculum formation in zebrafish, confirming the mineralogenic potential observed *in vitro*, and in agreement with the osteogenic effect observed in mouse calvaria. [108] In this system, ursolic acid was found to increase by 7 folds the formation of new woven bone after three weeks of treatment. The similar effect of ursolic acid in both *in vivo* systems indicates that mechanisms underlying its osteogenic action were conserved throughout evolution, from fish to mammals. It also confirms the suitability of fish systems to screen for osteogenic activities as an alternative or a complement of the classical mammalian systems (e.g. rodents). Moreover, osteogenic potential of ursolic acid could be revealed in zebrafish after only 3 days of exposure (3 weeks in mouse) confirming the high-throughput potential of the operculum system. The lack of osteogenic effect by betulonic acid evidenced by *in vitro* data was further confirmed *in vivo* by its incapacity to increase operculum formation. On the contrary, the strong mineralogenic effect of betulonic acid observed *in vitro* could not be reproduced *in vivo* possibly due to its elevated toxicity at mineralogenic concentration. Lower non-toxic concentrations did not show any effect, suggesting that betulonic acid may have a very limited range of effective concentrations or a complex mechanism of action. For the first time, oleanolic acid was shown to have an osteogenic action to an extent similar to that of the ursolic acid. We propose that this similar effect could be related to the chemical structure of the two molecules that differ just for the position of a methyl group on the E loop.[66]

## 5.5 Zebrafish transgenic lines to get insights into the osteogenic effect of purified molecules

This study also aimed at testing the suitability of transgenic zebrafish lines to get insights into the mechanisms underlying osteogenic effects observed in wild-type larvae stained with alizarin red. Among the several transgenic zebrafish lines available to study bone formation and skeletogenesis,[45] Tg(*osx:mCherry/oc2:EGFP*) has already been successfully used to assess the role of immature/mature osteoblasts during *de novo* bone formation throughout zebrafish development[10,39] and caudal fin regeneration[18]. In the present report, we highlight the positive effect of calcitriol on osteoblast maturation in the developing operculum. A stimulation of osteocalcin transcription in osteoblasts upon exposure to calcitriol has already been reported *in vitro* in human and murine cell systems[122] and *in vivo* in transgenic mice[92], and has been associated with the presence of binding elements for calcitriol receptor within the promoter of osteocalcin gene[123,124]. Osteocalcin is a bone matrix protein mainly produced by osteoblasts[5,125] involved in calcium[126] binding and consequently in bone matrix formation and mineralization[127,128]. It is probably a critical intermediate in the osteogenic effect of calcitriol on zebrafish operculum, as demonstrated by data collected from osteocalcin transgenic line. Although the analyses of the fluorescent area and pixel number provided comparable results (i.e. a stimulation of osteocalcin-related signal), we believe that area measurements are more appropriate to get a fast data acquisition – synonym of high-throughput – while analysis of pixel number is more time consuming but deliver more trustworthy data. The use of transgenic zebrafish lines – Tg(*osx:mCherry/oc2:EGFP*) lines or any of the zebrafish lines available to study bone marker genes – will certainly provide new insights into the mechanisms underlying the osteogenic effect of the molecules evaluated within the scope of this work.

# 6 Conclusions and future perspectives

Cytotoxic, proliferative, mineralogenic and osteogenic activities of semi-purified fractions (cardoon, eucalyptus and cyanobacteria) and molecules purified from these extracts (triterpenic acids and cynaropicrin) have been established using fish tools already available (i.e. gilthead seabream mineralogenic cell line) or developed within the scope of this work (i.e. developing zebrafish operculum). *In vitro* and *in vivo* data generated within the scope of this work has not only demonstrated the potential of natural resources (terrestrial or marine origin) to provide molecules with mineralogenic/osteogenic activity that may be used in pharmaceuticals or nutraceuticals but also the suitability of fish systems to screen for these molecules. Among the extracts/molecules evaluated in this work, several semi-purified fractions and some triterpenic acids exhibited pro-mineralogenic/osteogenic effects while cynaropicrin had the opposite effect, demonstrating the ability of our approach to uncover molecules with anabolic or catabolic effects on bone. Finally, we have developed and optimized a methodology to accurately and rapidly assess changes in the zebrafish operculum formation that can be applied to large-scale molecule screenings and complemented with the use of bone-specific transgenic zebrafish lines to get insights in the mechanisms underlying osteogenic effects. This methodology can be used as a pipeline to test more molecules/extracts (Figure 6.1).

Future work to pursue in order to advance on the screening of natural resources for osteogenic molecules could be related to:

- Test additional concentrations of the osteogenic molecules to determine the most effective concentration;
- Further purify osteogenic fractions toward the identification of novel osteogenic molecules;
- Explore the osteogenic potential of semi-purified fractions prepared from cyanobacteria extracts using hexane, methanol and a mixture 50:50 of ethyl acetate/methanol;
- Identify mechanisms of action of the osteogenic molecules using (1) zebrafish transgenic lines to uncover molecular and cellular determinants, (2) the CIGNAL reporter array (QIAGEN) to identify signalling pathways involved in molecule activity on bone and (3) quantitative real time PCR to evaluate the expression of bone-specific marker genes;
- Investigate in zebrafish operculum system the action of osteogenic molecules on osteoblast function by evaluating alkaline phosphatase (ALP) activity through colorimetric methods;

- Evaluate the role of osteoclast activity in the osteogenic effect observed in zebrafish operculum through the use of tartrate-resistant acid phosphatase (TRAP) staining or cathepsin K (*ctsk:GFP*) transgenic line;
- Further confirm the osteogenic potential of identified molecules on *de novo* bone formation using the capacity of zebrafish caudal fin to regenerate and formed new bony rays;
- Explore commercial applications for these molecules. More tests and data will be needed to confirm the potential of these molecules to improved bone status in human patients (e.g. tests in rodents then clinical tests in human) and a collaboration with a company specialized in the different procedures leading to the marketing of new pharmaceutics will be required. An application in the field of farmed animal nutrition may be possible at a shorter term as osteogenic extracts/molecules could be used to supplement existing diets (nutraceuticals) to improve skeletal status, e.g. reduce skeletal malformations or improve bone formation in farmed fish;
- Explore the applicability of the zebrafish operculum system to evaluate osteotoxicity of environmental pollutants.

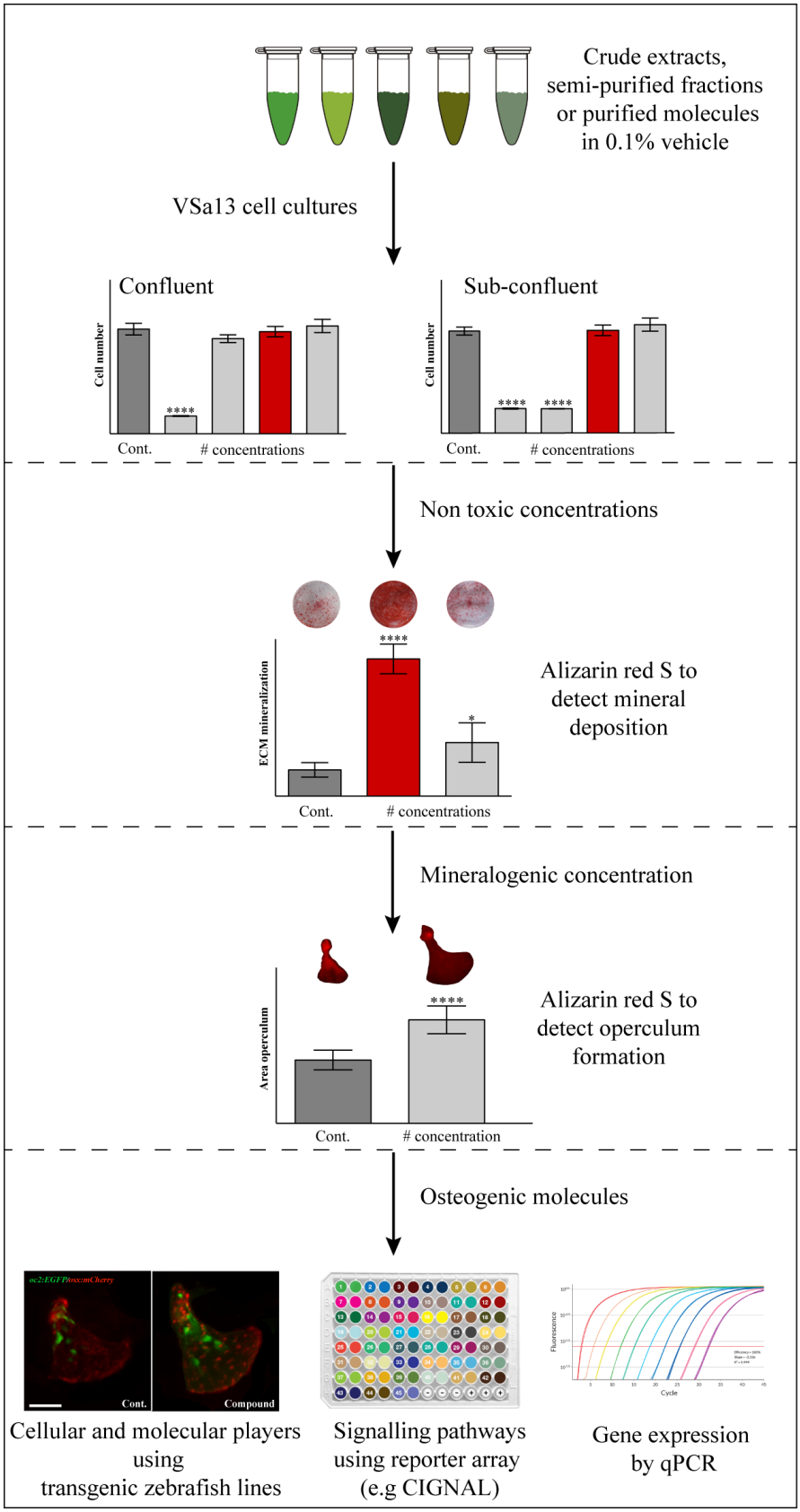


**Figure 6.1.** Pipeline to assess the mineralogenic/osteogenic potential of extracts/compounds from natural resources.

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