Anticancer activity of diterpenes and steroids from *Eunicella singularis* against two- and three-dimensional breast cancer cell models

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1. Introduction

Cancer is one of the most lethal diseases that threaten human life; according to the World Health Organization, it is responsible for approximately 13% of all deaths each year [1] and its incidence is increasing with changing lifestyle, nutrition, and global warming. Breast cancer causes significant morbidity and mortality among women worldwide making it a major public health concern and one of the major challenges in the scientific community [2]. However, despite substantial research efforts, cancer therapy is still limited to classical radiotherapy and chemotherapy. Furthermore, current breast cancer treatments are also limited due to drug resistance and toxicity of drugs which indiscriminately kill both cancerous and normal cells [3-5]. Thus it is important to develop effective new anticancer compounds from natural sources with potent pro-apoptotic activity and weak side effects for breast cancer treatment and prevention.

The marine environment has become increasingly recognized as an excellent source of structurally diverse bioactive compounds [6,7]. Soft corals and gorgonians of Octocorallia have recently attracted a great deal of attention from scientists in the fields of chemistry and pharmacology as new marine sources of novel bioactive natural products with a wide range of pharmacological activities and health promoting properties including antimicrobial [8-11], antifouling [12], antiproliferative [13], cytotoxic [14] and anti-inflammatory [15] effects.

**ARTICLE INFO**

**ABSTRACT**

**Objective:** To investigate the anticancer activity of two diterpenes [palmonine F (C1) and palmonine D (C2)] and three steroids [cholesta-5,22-dien-3β-ol (C3), stigmasterol (C4) and 5α-cholest-5-en-3β-ol (C5)], isolated from the Mediterranean gorgonian *Eunicella singularis*, against MCF-7 breast cancer cell line.

**Methods:** This study was performed on standard monolayer two-dimensional (2D) model to evaluate apoptosis by means of AnnexinV-FITC/PI flow cytometry and on three-dimensional (3D) spheroid model using Celigo imaging cytometer for spheroids size analysis.

**Results:** Results indicated that both diterpenes and steroids exhibited an important apoptotic activity in a concentration-dependent manner with EC₅₀ values of 13, 49, 30, 66 and 65 μg/mL for C1, C2, C3, C4 and C5, respectively. Treatment of MCF-7 3D cell model with C1–C5 induced growth regression of spheroids in a concentration-dependent manner similar to the clinical anti-breast cancer drug Taxol; over ten days of incubation, growth rates were < 1.5 at Day 10 with all tested compounds at 200 μg/mL.

**Conclusions:** The present study indicates that the two diterpenes C1 and C2 and the three steroids C3, C4 and C5, isolated from *Eunicella singularis*, might be used as anti-breast cancer candidate drugs for further development.
Until now, totally over 1000 new natural products have been obtained from approximately 1% of the worldwide gorgonian corals. The Mediterranean Sea represents one of the rich centers of coral biodiversity with an important distribution and abundance of gorgonian of the genus *Eunicella*.[16]

In relation to anticancer properties, gorgonian of the genus *Eunicella* has been demonstrated to contain a wide variety of bioactive natural compounds such as steroids and diterpenes, some of which are believed to have a cytotoxic activity.[17,18]

Steroids are one of the most natural products isolated from gorgonians[9]. These metabolites exhibited a large variety of biological activities, especially cytotoxicity against a wide variety of tumor cell lines including human breast carcinoma cells MCF-7[20]. Besides, diterpenes represent about 65% of all the reported metabolites from gorgonians. The representative structures of diterpenes by carbon skeleton class from gorgonians included briarane-type, emembrane-type, eunicellan-type, xenicane-type, pseudopterosin-type, dilophol-type, etc.[21]. These kinds of molecules were reported to have diverse biological and pharmacological activities, including cytotoxic effects. In *in vitro* bioassays, these secondary metabolites exhibited different levels of growth inhibitory activity against different tumor cell lines.[18]

Therefore, in continuation of our study for the phytochemical and pharmacological investigations of bioactive secondary metabolites from gorgonian[22,23], we attempted to investigate the anticancer activity of two diterpenes and three steroids, previously isolated from the white gorgonian *Eunicella singularis* (Cnidaria: Octocorallia, Esper 1791) (*E. singularis*) with characteristic of coralligenous bioenocosis, and one of the most abundant species in the Mediterranean area, against human breast cancer cells MCF-7. The study was performed on standard monolayer two dimensional (2D) cell culture model and on three dimensional (3D) spheroid model which serves as a screening platform known to provide more reliable and meaningful therapeutic readouts compared to 2D assays[24,25].

2. Materials and methods

2.1. Sample collection and extraction

*E. singularis* was collected from the Northwestern coast of Tunisia in June 2010 at a depth between 20 and 30 m and identified by the National Institute of Marine Sciences and Technologies (Salambo, Tunisia). A voucher specimen was deposited under the number 1132. After collection, samples were washed with sea water and distilled water to remove salt, epiphytes and other particles. Gorgonians were then air dried in the shade at (30 ± 2) °C for 1–2 weeks and powdered using electric mixer grinder. A total of 600 g of powdered samples were finely packed in small bags (5 × 10 cm) of Whatman No. 1 filter paper (15 g of powder in each bag); all bags were sealed and soaked in 2 L of methanol-dichloromethane (1:1, v/v) for 48 h with frequent stirring, then, filtered. A ratio of 1:2 (w/v) for solvent to the dry mass of the coral material was used for the extraction. This process was repeated 3 times on the residue. The crude extract was evaporated using rotary evaporator (Buchi, B-480) at low temperature (<40 °C).

2.2. Purification and isolation of natural compounds

Crude extract was fractionated into three semi-purified fractions F-EtOH, F-Ac and F-MeOH/CH2Cl2, using C18 cartridges (Sep-pak, Supelco) and different organic solvents in the order of decreased polarity: ethanol, acetone and MeOH/CH2Cl2 (1:1)[26,27]. Two diterpnes [palmonine F (C1) and palmonine D (C2)] and three steroids [cholesta-5,22-dien-3β-ol (C3), stigmasterol (24-ethylcholesta-5,22-dien-3β-ol) (C4) and 5α-cholest-5-en-3β-ol (C5)] were purified from F-EtOH fraction as described previously[22,23].

2.3. MCF-7 cell culture

Human mammary adenocarcinoma (MCF-7, ATCC® HTB-22) cell line was cultured in Dulbecco’s modified eagle medium high glucose (Dominique Dutscher, 67172 Brumath cedex, France, Cat. No. L0102-500), supplemented with heat-inactivated 10% fetal bovine plasma (Life Technologies, Paisley, UK, Cat. No. 10270-106) and 1% penicillin-streptomycin (10000 IU/mL and 10000 μg/mL, Life Technologies, Paisley, UK, Cat. No.15140-122). Cells were maintained at 37 °C in humidified environment containing 5% (v/v) CO2 during the growth phase and treatment with compounds.

All compounds C1–C5 were first dissolved in dimethyl sulfoxide (DMSO) to reach a concentration of 200 μg/mL. Appropriate concentrations applied on the cells have been prepared with dilution in culture media immediately before each assay, so that the highest final DMSO concentration never exceeded 0.5% (v/v) to avoid side effects such as cell toxicity or induction of differentiation.

2.4. Apoptosis

2.4.1. Annexin-V/propidium iodide (PI) double staining assay

Cells were seeded into 96-well plates (BD Falcon, Cat. No. 353072) at a density of 1 × 10⁵ cells/mL of media. Increasing concentrations of compounds (10, 20, 50, 100 and 200 μg/mL) in culture medium were prepared prior to each assay. To each well containing 100 μL of cells in culture medium, 100 μL of each concentration of C1–C5 was added. Non-treated cells and cells treated with 0.5% DMSO were used as negative controls. Celastrol (5 μg/mL) (Enzo Life Sciences, Farmingdale, US), a natural pentacyclic triterpenoid, was used as positive control[28]. The 96-well plates were then incubated in CO2 incubator for 24 h. After incubation, supernatants were removed and cells were washed with fresh PBS; afterwards plates were centrifuged on 5000 r/min for 10 min. Supernatants were discarded and 200 μL of trypsin-EDTA (BioWest, Nuaillé, France, Cat. No. L0930-100) was added to each well. Cells
2.5. Growth inhibition of 3D tumor spheroid using Celigo Imaging Cytometry

3D MCF-7 mammospheres were seeded in 96-well plates (Corning, Sigma-Aldrich, St. Louis, MO, USA, Cat. No. 4515). Tumors were left to grow for additional four days; afterwards C1–C5 were added in concentrations of 20, 50, 100 and 200 μg/mL. Taxol, a plant alkaloid and a cancer chemotherapeutic drug[32], was used as positive control at doses 0.01, 0.02, 0.05 and 0.1 μg/mL, in order to study the growth evolution of spheroids as a function of dose and time. Non-treated spheroids were used as negative control. Evaluation has been maintained during a ten days incubation period, and C1 and C2 exhibited an apoptotic activity in a concentration-dependent manner; they showed a high induction of cell death at the concentration of 200 μg/mL. About 92% and 93% of cells were dead by apoptosis, respectively with C1 and C2 at 200 μg/mL after 24 h incubation (Figure 1). Also, C3, C4 and C5 induced apoptosis in a concentration-dependent manner. At 10 μg/mL, these steroids compounds caused cell death in less than 15% of MCF-7 cells, while at 200 μg/mL, more than 70% of cells were dead by apoptosis after 24 h incubation (Figure 2).

Concentration response curves and EC50 values for apoptotic activity of C1–C5 were displayed in Figure 3. C1 exhibited the highest apoptotic activity with EC50 value of 13 μg/mL. An interesting apoptotic activity was also observed with C2 and C3, whose EC50 values were 49 and 30 μg/mL, respectively. Apoptotic potencies of C4 and C5 were similar with EC50 values of 66 and 65 μg/mL, respectively.

2.6. Statistical analysis

Experiments were repeated at least three times and results were expressed as mean ± SEM. Statistical differences were determined using the One-way ANOVA followed by the post-hoc Bonferroni test using GraphPad Prism software (Prism version 5.04 for Windows, GraphPad Software, CA, USA).

3. Results

3.1. Apoptotic activity

Investigation on anticancer activity of compounds (C1–C5) was initiated by evaluation of their ability to induce apoptosis in a classical MCF-7 2D monolayer culture over 24 h of incubation. MCF-7 apoptosis was quantified by microcapillary flow cytometry using Annexin V-FITC/PI assay. Celastrol showed total inductions (99%) of cell death by apoptosis, whereas negative control showed only 1% of dead cells. The two diterpenes C1 and C2 exhibited an apoptotic activity in a concentration-dependent manner; they showed a high induction of cell death at the concentration of 200 μg/mL. About 92% and 93% of cells were dead by apoptosis, respectively with C1 and C2 at 200 μg/mL after 24 h incubation (Figure 1). Also, C3, C4 and C5 induced apoptosis in a concentration-dependent manner. At 10 μg/mL, these steroids compounds caused cell death in less than 15% of MCF-7 cells, while at 200 μg/mL, more than 70% of cells were dead by apoptosis after 24 h incubation (Figure 2).
Figure 1. Flow cytometric analysis of apoptotic cell death using Annexin-V-FITC/PI staining of MCF-7 cells treated with DMSO (negative control) or celastrol (5 μg/mL) (positive control) or the two diterpenes C1 and C2 (10–200 μg/mL).

Lower left quadrant [Annexin V (-)/ PI (-)] represents live cells, lower right quadrant [Annexin V (+)/ PI (-)] represents early apoptotic cells, upper right quadrant [Annexin V (+)/ PI (+)] represents late apoptotic cells and upper left quadrant [Annexin V (-)/ PI (+)] represents cells dead by necrosis but without apoptosis induction.
Figure 2. Flow cytometric analysis of apoptotic cell death using Annexin-V-FITC/PI staining of MCF-7 cells treated by steroid C3 or C4 or C5 (10–200 μg/mL).

Lower left quadrant [Annexin V (-)/ PI (-)] represents live cells, lower right quadrant [Annexin V (+)/ PI (-)] represents early apoptotic cells, upper right quadrant [Annexin V (+)/ PI (+)] represents late apoptotic cells and upper left quadrant [Annexin V (-)/ PI (+)] represents cells dead by necrosis but without apoptosis induction.
Day 0   Day 2   Day 4   Day 6   Day 8   Day 10

Figure 4. MCF-7 spheroid size reduction induced when treated with Taxol (0.01, 0.02, 0.05 and 0.1 μg/mL) or C1 or C2 (20, 50, 100 and 200 μg/mL) over 10 days. Images were acquired every other day, starting from Day 0 (Celigo image cytometer) (A); Growth rate changes when MCF-7 spheroids were treated with either Taxol, or C1 or C2 (B).

Growth rates were determined by means of spheroid diameter size estimated by Celigo software, then computed for every other day up to 10 days of incubation (diameter on Day n divided by diameter at Day 0). Results are presented as the mean ± SD of two replicates of 2 independent experiments.

Figure 3. Dose response curves and EC₅₀ values for apoptotic activity of C1–C5 after 24 h incubation in MCF-7 cells (n = 2 independent experiments run in triplicates, 2000 events analyzed).

Percent of apoptotic and dead cells (%)
1.35 ± 0.21 and 1.39 ± 0.08. C3–C5 exhibited also an important inhibition of spheroids growth in a concentration-dependent manner. The growth rates were 1.17 ± 0.02, 1.25 ± 0.05 and 1.15 ± 0.02 at 200 μg/mL, respectively after 10 days of incubation, similar of that of positive control at 100 μmol/L (Figure 5).

4. Discussion

The inability to treat, high costs and severe side effects caused by chemotherapy and radiotherapy are the major problems for cancer patients and their physicians[34]. Thus, recently, the search of bioactive natural products exhibiting anticancer effect has received a great attention. Actually, more than half of the anticancer drugs are derived from natural organisms[35-37].

Marine natural compounds are a rich source of complex chemicals, having an excellent therapeutic potential, especially anticancer proprieties. Therefore, over the past few decades, scientists made significant efforts to isolate new marine-derived natural products[38-41]. Until now, among marine sources, invertebrates have been the mainstream source in marine-derived drug discovery, contributing approximately to 65% of the marine natural compounds, serving as promising sources of new anticancer agents in preclinical development[42]. The objective of this study was to determine the anticancer effect of two diterpenes (palmonine F and palmonine D) and three steroids (cholesta-5,22-dien-3β-ol, stigmasterol and 5α-cholest-5-en-3β-ol), isolated from the Mediterranean gorgonian _E. singularis_, on the human breast cancer MCF7 cell line. Anticancer activity was investigated first, on standard monolayer 2D cell culture model to evaluate apoptosis by means of AnnexinV-FITC/PI on flow cytometer. However, owing to the highly artificial environment, 2D monolayer cells are unable to mimic the pathophysiology of _in vivo_ tumor and to reproduce the real complexity and 3D structure found in the human body such as cell-cell communication and cell-

Figure 5. MCF-7 spheroid size reduction induced when treated with C3 or C4 or C5 at 20, 50, 100 and 200 μg/mL over 10 days. Images were acquired every other day, starting from Day 0 (Celigo image cytometer) (A); Growth rate changes when MCF-7 spheroids were treated with either C3 or C4 or C5 (B).

Growth rates were determined by means of spheroid diameter size estimated by Celigo software, then computed for every other day up to 10 days of incubation (diameter on Day n divided by diameter at Day 0). Results are presented as the mean ± SD of two replicates of 2 independent experiments.
extracellular matrix. Thus, we have then, evaluated the activity in 3D multicellular spheroids model which closely reflects the in vivo tumor characteristics, by taking into consideration different parameters like drug concentration, molecular weight, solubility, kinetics, charge, oxygenation, metabolism, and sequestration,[43], leading to better prediction power as a useful and effective technique for investigating anticancer activity of drugs.[44-46].

Results showed that the two diterpene plamalone F and palmonline D exhibited prominent apoptotic activity and induced growth regression of spheroids over ten days of incubation. Few reports have focused on the cytotoxicity of diterpenes, despite more than 40 eunicellian-type diterpenes have been isolated from gorgonian Acalystgorgia, Muricella, Briareum, Erythropodium and Eunicella.[47] Ortega et al.[48] showed that palmonlines isolated from the gorgonian Eunicella verrucosa presented cytotoxicity to human cancer cells (A549 lung carcinoma, HT29 colon carcinoma, and MEL28 melanoma).

On the other hand, investigated steroids (cholesta-5,22-dien-3β-ol, stigmasterol and 5α-cholest-5-en-3β-ol) induced apoptosis and growth regression of spheroids in a dose-dependent manner. Many studies showed that steroids isolated from soft corals showed chemotherapeutic activities. Besides, Ali et al.[54] showed that stigmasterol and demonstrated that this steroid exhibited a potent anticancer effect and a chemopreventive activity. The ability to induce apoptosis of cancer cells is a useful strategy to treat many types of cancers especially breast cancer.[55-57]. Thus apoptosis is one of the active strategies to arrest proliferation of cancer cells. Many chemical agents, such as tamoxifen, exert their anticancer effects by inducing apoptosis and they have been used to treat many types of cancers especially breast cancer.[55-57]. Thus the anticancer activity observed with all the investigated compounds related to their prominent apoptotic activity.

Based on the findings reported here, we can conclude that the two diterpene plamalone F and palmonline D and the three steroids cholesta-5, 22-dien-3β-ol, stigmasterol and 5α-cholest-5-en-3β-ol from the gorgonian E. singularis present a potent apoptotic activity on MCF-7 cancer cells and furthermore prevent spheroid growth even after ten days of incubation. Yet, further work is required to determine mechanisms involved in such a mighty anticancer effect before proving their use as potent new anticancer drugs.

Conflict of interest statement
We declare that we have no conflict of interest.

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