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

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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 EC_{50} 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.

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.

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Until now, totally over 1 000 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[19]. 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, cembrane-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 biocenosis, 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 (Salamboo, 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/CH_2C_{12}, using C18 cartridges (Sep-pack, Supelco) and different organic solvents in the order of decreased polarity: ethanol, acetone and MeOH/CH_2C_{12} (1:1)[26,27]. Two diterpenes [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 midified 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 (10 000 IU/mL and 10 000 μg/mL, Life Technologies, Paisley, UK, Cat. No.15140-122). Cells were maintained at 37 ˚C in humidified environment containing 5% (v/v) CO_2 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 1×10^5 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 CO_2 incubator for 24 h. After incubation, supernatants were removed and cells were washed with fresh PBS; afterwards plates were centrifuged on 5 000 r/min for 10 min. Supernatants were discarded and 200 μL of trypsin-EDTA (BioWest, Nuaille, France, Cat. No. L0930-100) was added to each well. Cells

were then incubated for 15 min at 37 °C, centrifuged at 5000 r/min for 10 min and supernatants eliminated. Finally, trypsinized cells and supernatant with non-adherent cells of each well were mixed, and 3 μL/well of Annexin-V-FITC (Immuno Tools, Friesoythe, Germany, Cat. No. 31490013) and PI (Miltenyl Biotec Inc., Auburn, USA, Cat. No. 130-093-233) were added and plates were incubated for 15 min in the dark prior to cytometry analysis. Annexin-V-FITC is a fluorescent agent which binds to phosphatidylserine on cell membrane[29]. PI is a fluorescent intercalating agent which binds to DNA; it stains cells in late apoptosis or necrosis with damaged cell membranes[30]. Combination of both Annexin-V-FITC (green fluorescence) and PI (red fluorescence) enables classification and characterization of live, pre-apoptotic, late-apoptotic and necrotic cells[31]. Plates were analyzed on a Guava EasyCyteTM micro-capillary flow cytometer (Millipore, Merck, Darmstadt, Germany) using InCyte® software package.

Gates were drawn around the appropriate cell populations using a forward scatter (FSC) versus side scatter (SSC) acquisition dot plot to exclude debris. Tests were performed in duplicates.

2.4.2. Determination of EC_{50} concentrations

Percentages of pre-apoptotic and late-apoptotic cells were computed for each concentration of C1–C5 and presented on a concentration–response graph. EC_{50} value, which represents the concentration of compound inducing apoptosis in 50% of all cells after 24 h incubation, was determined using symmetric four parameter logistic equation (EasyCyte software, Merck Millipore).

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, with media exchanged on the Day 4. Spheroids, derived from cancer stem cells are compact globes with central zones clearly distinguished within, whose growing is characterized by regular increase in diameters[33]. Thus, we have followed diameter as change parameter for non-treated and treated spheroids and that has been assessed on a Celigo® imaging cytometer (Nexcelom, San Mateo, CA, USA). Growth rates of treated and non-treated spheroids were computed for each day during ten-day incubation by dividing the diameter on the Day n with the diameter on the Day 0.

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).

Concentration response curves and EC_{50} values for apoptotic activity of C1–C5 were displayed in Figure 3. C1 exhibited the highest apoptotic activity with EC_{50} value of 13 μg/mL. An interesting apoptotic activity was also observed with C2 and C3, whose EC_{50} values were 49 and 30 μg/mL, respectively. Apoptotic potencies of C4 and C5 were similar with EC_{50} values of 66 and 65 μg/mL, respectively.

3.2. Anticancer activity on 3D cell model

Compounds C1–C5 were further tested for their anticancer activity in a 3D multicellular spheroids model at different concentrations (20, 50, 100 and 200 μg/mL).

Non-treated spheroids recorded a linear growth rate, with a maximal increase of 2.30 ± 0.32 fold at Day 10. Taxol induced growth regression of spheroids, and steadily downhill growth rate curves were observed with different concentrations (0.01, 0.02, 0.05 and 0.1 μg/mL); the growth rate, at Day 10, was about 1.19 ± 0.11 at 0.1 μg/mL (Figure 4). Spheroids treated with the two diterpenes C1 and C2 showed concentration-dependent changes in diameter (Figure 4). At the concentration of 200 μg/mL, they induced reduction in spheroid size after second day of incubation; growth rates were about

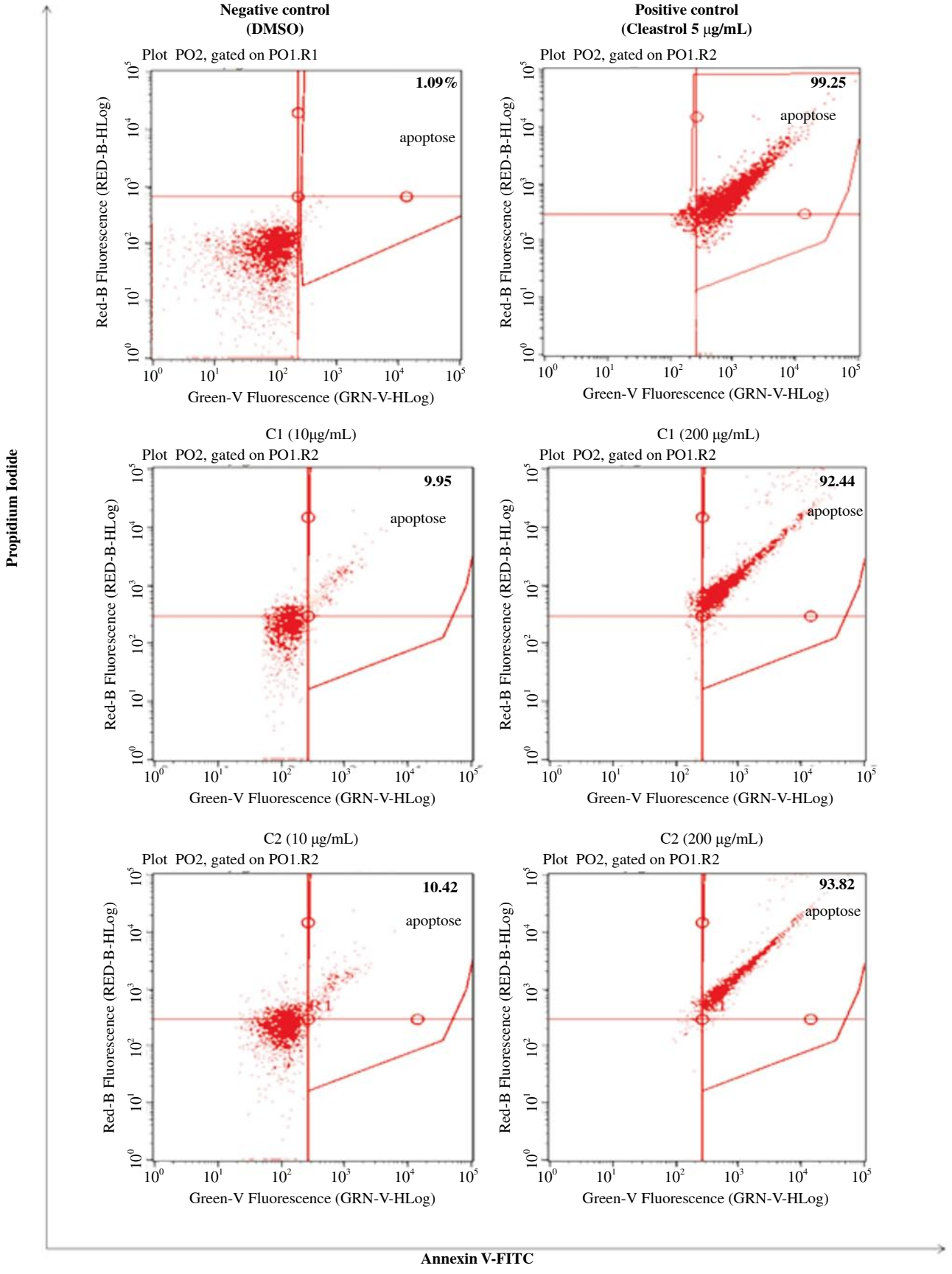

Figure 1. Flow cytometric analysis of apoptotic cell death using Annexin-V-FITC/PI staining of MCF-7 cells treated with DMSO (negative control) or celastrol (5 µg/mL) (positive control) or the two diterpenes C1 and C2 (10–200 µg/mL).

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.

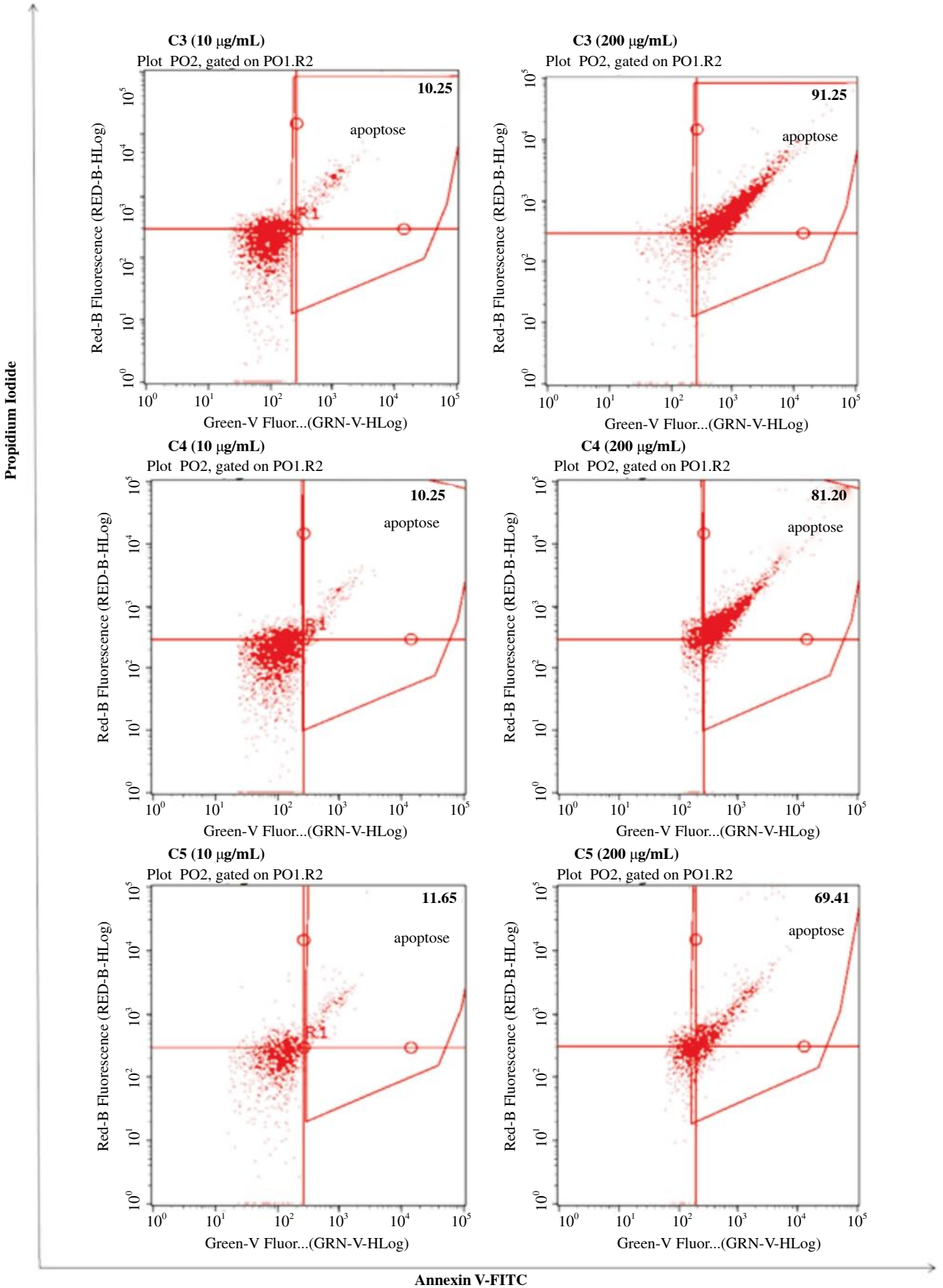

Figure 2. Flow cytometric analysis of apoptotic cell death using Annexin-V-FITC/PI staining of MCF-7 cells treated by steroid C3 or C4 or C5 (10–200 μg/mL).

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.

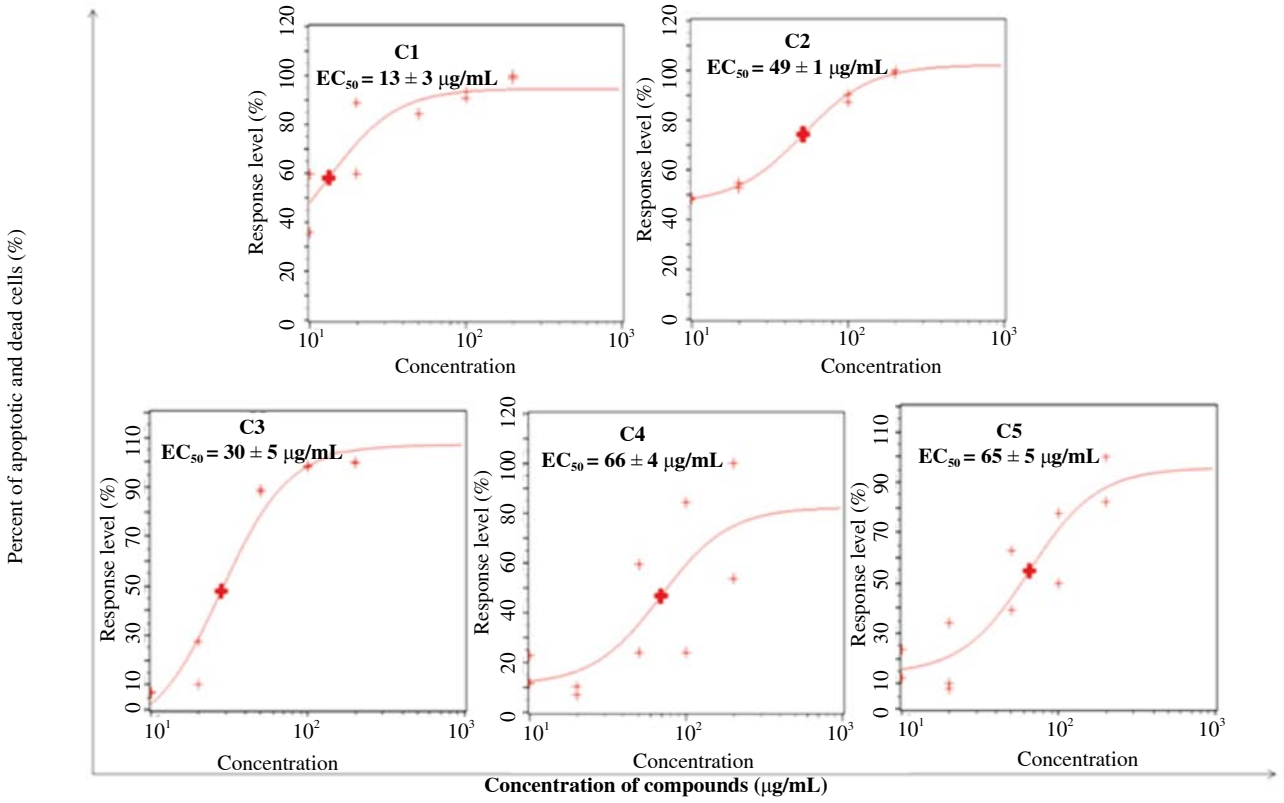

Figure 3. Dose response curves and EC_{50} values for apoptotic activity of C1–C5 after 24 h incubation in MCF-7 cells (n = 2 independent experiments run in triplicates, 2 000 events analyzed).

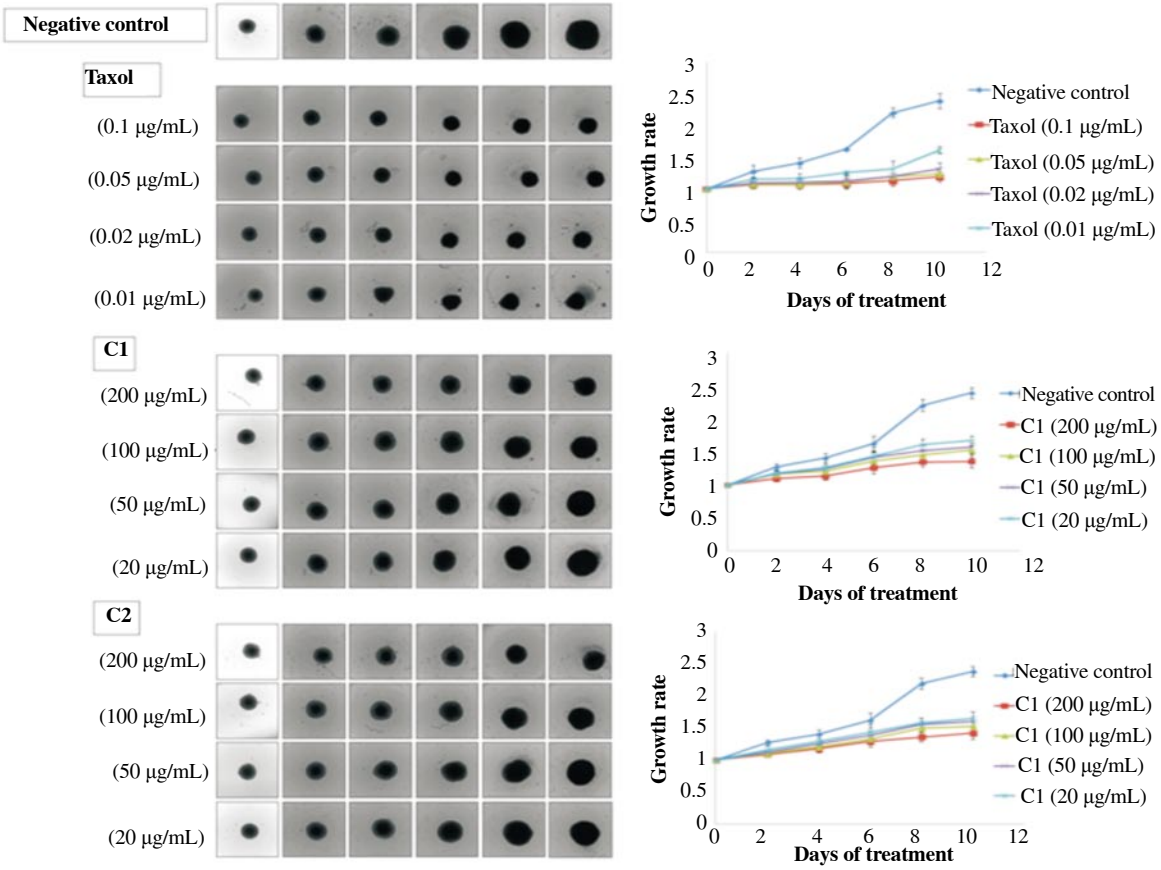

Figure 4. MCF-7 spheroid size reduction induced when treated with Taxol [0.01, 0.02, 0.05 and 0.1 µg/mL)] or C1 or C2 (20, 50, 100 and 200 µg/mL) over 10 days. Images were acquired every other day, starting from Day 0 (Celigo image cytometer) (A); Growth rate changes when MCF-7 spheroids were treated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

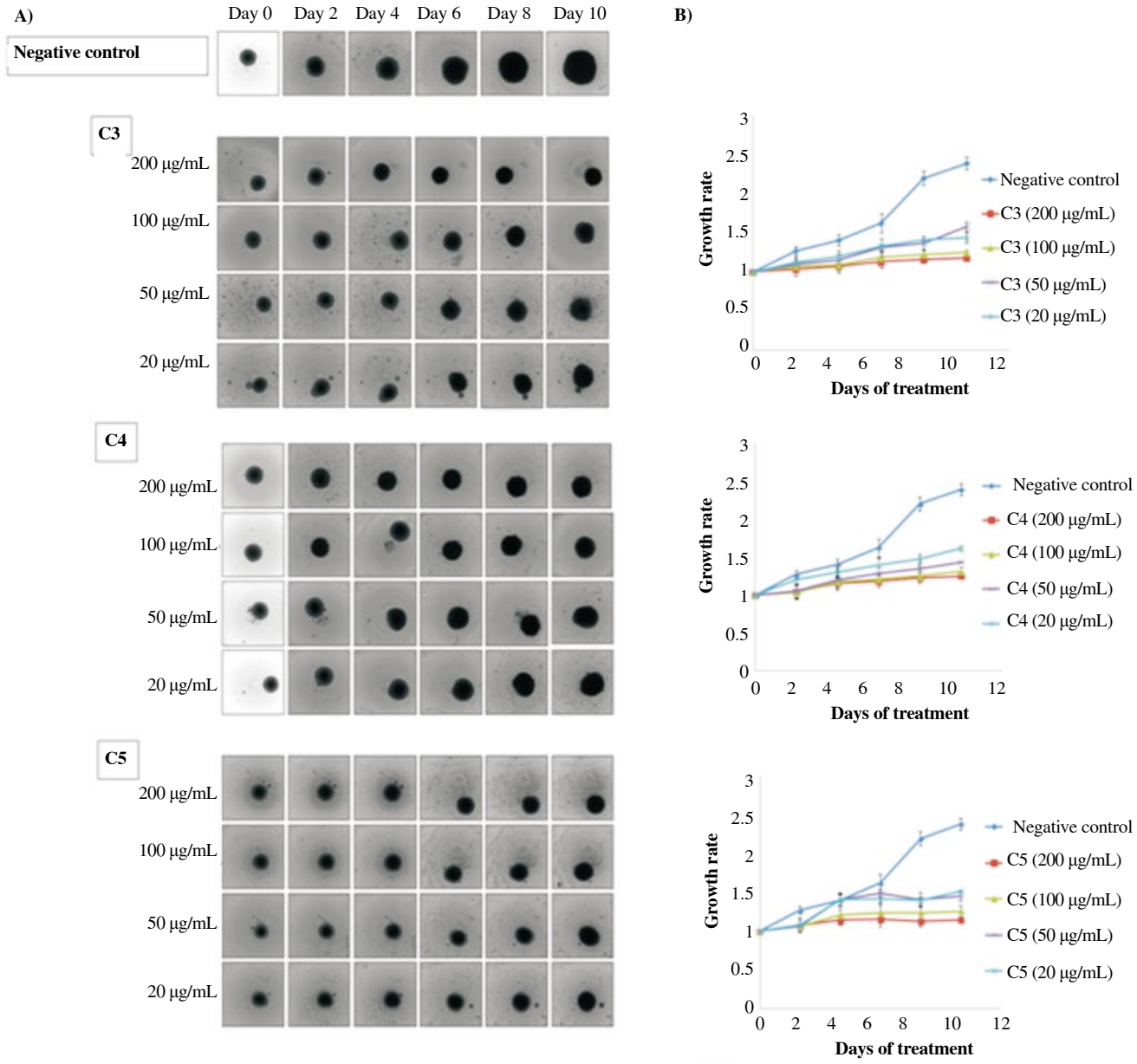

Figure 5. MCF-7 spheroid size reduction induced when treated with C3 or C4 or C5 at 20, 50, 100 and 200 µg/mL over 10 days. Images were acquired every other day, starting from Day 0 (Celigo image cytometer) (A); Growth rate changes when MCF-7 spheroids were treated with either C3 or C4 or C5 (B).

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.

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-

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 diterpenes plamonine F and palmonine 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 eunicellan-type diterpenes have been isolated from gorgonian *Acalycigorgia*, *Muricella*, *Briareum*, *Erythropodium* and *Eunicella*[47]. Ortega *et al*.[48] showed that palmonines 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 promising anticancer activity[49-51]. Byju *et al*.[52] reported the anticancer activity of four steroids compounds [cholesta-5,22-diene-3β-ol, ergosta-5-22-dien-3-β-ol, 26,26-Dimethyl-5,24(28)-ergostadien-3β-ol and β-sitostero] isolated from the soft coral *Subergorgia reticulata*. It has been demonstrated that these steroids induced apoptosis and presented potent chemopreventive and chemotherapeutic activities. Besides, Ali *et al*.[53] evaluated the anticancer effect of 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 for anticancer drug development[54]. Apoptosis implicates multiple signaling pathways and a balance of anti- and pro-apoptotic proteins. Up regulation of antiapoptotic proteins, down regulation of proapoptotic proteins, and decreased expression of caspases may lead to insufficient apoptosis leading to cancer. Induction of 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 isolated from the Mediterranean soft coral *E. singularis* may be related to their prominent apoptotic activity.

Based on the findings reported here, we can conclude that the two diterpenes palmonine F and palmonine 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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