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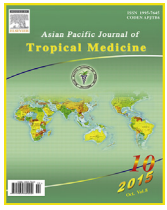

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Potential of four marine-derived fungi extracts as anti-proliferative and cell death-inducing agents in seven human cancer cell lines

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ABSTRACT

Objective: To evaluate the *in vitro* anticancer activity of crude ethyl acetate extracts of the culture of four marine-derived fungi *Aspergillus similanensis* KUFA 0013 (E1), *Neosartorya paulistensis* KUFC 7897 (E2), *Neosartorya siamensis* KUFA 0017 (E4) and *Talaromyces trachyspermus* KUFC 0021 (E3) on a panel of seven human cancer cell lines.

Methods: Effects on cell proliferation, induction of DNA damage and cell death were assessed by MTT and clonogenic assays, comet assay and nuclear condensation assay, respectively.

Results: The proliferation of HepG2, HCT116 and A375 cells decreased after incubation with the extracts E2 and E4. The anti-proliferative effect was confirmed by morphologic alterations and by clonogenic assay. Both extracts also induced cell death in HepG2 and HCT116 cells. Doxorubicin was used as a positive control and showed *in vitro* anticancer activity.

Conclusions: This study demonstrated, for the first time, that extracts of *Neosartorya paulistensis* and *Neosartorya siamensis* have selective anti-proliferative and cell death activities in HepG2, HCT16 and A375 cells. The bioactivity of these extracts suggests a potential for biotechnological applications and substantiates that both should be further considered for the elucidation of the molecular targets and signal transduction pathways involved.

1. Introduction

Cancer is one of the main causes of death worldwide. In the following decades, the number of people with cancer will continue to increase, largely due to lifestyle, nutrition and environmental conditions in developed countries [1–3]. During cancer development, cells acquire several genetic and epigenetic changes. These changes result in the progressive acquisition of biological characteristics such as sustained proliferative signaling, insensitivity to growth suppressors, evading apoptosis, increasing genomic instability, activating mobility, invasion, metastasis and angiogenesis that may thus evolve into a malignant phenotype [4].

Advances in cancer biology knowledge have allowed the development of new treatment strategies, including new anticancer drugs that may act in one or more of the hallmarks described above. In fact, compounds that reactivate cell death and/or decrease proliferative ability in cancer cells show a potential anticancer activity. However, most of the anticancer drugs currently used, such as doxorubicin, give rise to undesirable side effects, such as cardiotoxicity and tumor drug-resistance [5]. Therefore, new anticancer drugs with more efficiency and ability to mitigate side effects are in need.

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It is interesting to note that more than 50% of the drugs used in cancer treatment are from natural origin, mainly from plant sources [6]. Nonetheless, the marine environment represents about 95% of the world's biosphere and is an important source of bioactive compounds to be explored [7,8]. As terrestrial microorganisms have proved their value as sources of bioactive compounds, the focus has also turned to marine microorganisms [9]. Marine fungi have remained until recently much less studied than terrestrial fungi, nevertheless, novel metabolites have been found in marine fungi that greatly differ from those found in terrestrial counterparts [6]. The production of secondary metabolites by marine fungi can be influenced by the combination of the unique conditions of marine environment, such as variations in temperature, light, water current, salinity, and nutrient availability, all of which create a highly competitive environment and thus force marine organisms to evolve complex chemical adaptations, many of which developed under a symbiotic relationship with other species [10]. In fact, recent research has exploited these symbiotic relationships in marine ecosystems as a source for bioactive compounds. This is particularly pertinent when analyzing the case of microbe-sponge relationships, and whereas sponges are known to be notable sources for bioactive compounds, where origin has also been attributed to the sponge's microbial associates, namely fungi and bacteria [11]. Furthermore, compounds are frequently produced as a chemical manner of defense by many marine organisms, and are released into the water and thus diluted. Consequently, these compounds must be extremely efficient in order to produce their effect in spite of their dilution in the water. Hence, these metabolites seem to have interest as novel lead structures for the synthesis of new bioactive compounds [9,12,13].

In regard to biological activity, several metabolites produced by marine-derived fungi have been reported as antibacterial, antiviral, antifungal, antioxidant and anticancer agents [14–17]. Notwithstanding the increasing interest in these bioactive compounds, there is frequently an effective difficulty in extracting these compounds from nature since their source organisms are often hard to reproduce and manipulate in laboratorial conditions, limiting their availability and use. Interestingly, some marine fungi may grow efficiently under laboratory conditions, which may therefore enable the use of biotechnological tools for a massive production of the compounds of interest [18,19]. In summary, marine-derived fungi seem to be good candidates as a source of new bioactive compounds, thus making them a pivotal part of the emergent marine biotechnology applications.

Our present purpose was to assess the *in vitro* anticancer activity of crude ethyl acetate extracts of the sponge-derived fungi *Aspergillus similanensis* (*A. similanensis*) KUFA 0013 (E1), *Neosartorya paulistensis* (*N. paulistensis*) KUFC 7897 (E2) and *Talaromyces trachyspermus* (*T. trachyspermus*) KUFC 0021 (E3), and the sea fan-derived fungi *Neosartorya siamensis* (*N. siamensis*) KUFA 0017 (E4), on a panel of seven human cancer cell lines, namely, colorectal carcinoma (HT29 and HCT116), hepatocellular carcinoma (HepG2), breast adenocarcinoma (MCF-7), malignant melanoma (A375), non-small cell lung carcinoma (A549) and glioblastoma (U251) cells. The chemical composition of the ethyl extracts E1, E2 and E3 has been previously analysed by members of our research team [20–22].

2. Materials and methods

2.1. Chemicals

Doxorubicin, DMEM, MEM, RPMI-1640, sodium pyruvate, sodium bicarbonate, N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid), penicillin/streptomycin, trypsin solution, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) and 4,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Fetal bovine serum was purchased from Biochrom KG (Berlin, Germany). All other reagents and chemicals used were of analytical grade.

2.2. Fungal material

A. similanensis KUFA 0013 was isolated from the marine sponge *Rhabdermia* sp., as described by Prompanya *et al* [22], *N. paulistensis* KUFC 7897 was isolated from the marine sponge *Chondrilla australiensis*, as previously reported [20], and *T. trachyspermus* KUFC 0021 was isolated from the marine sponge *Clathria reianwardii*, as reported by Kumla *et al* [21].

N. siamensis KUFA 0017 was isolated from sea fan (*Rumphella* sp.), collected from the coral reef at Similan island, Phang Nga province, Southern Thailand, in April 2010. Briefly, the sea fan tissue was cut into a piece of 0.5 cm × 0.5 cm, placed on the malt extract agar (MEA) with 70% sea water and incubated for 28 °C for 7 d. The fungus was identified by one of us (T. Dethoup) by morphological features, including the characteristic of ascospores and colonies, and by sequence analysis of the β-tubulin gene [23]. The pure cultures were deposited as KUFA0017 at Kasetsart University Fungal Collection, Department of Plant Pathology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand.

2.3. Preparation of crude ethyl acetate extracts from marine-derived fungi

A. similanensis KUFA 0013, *N. paulistensis* KUFC 7897 and *T. trachyspermus* KUFA 0021 were cultured as described by Prompanya *et al* [22], Gomes *et al* [20] and Kumla *et al* [23], respectively. Briefly, the fungi were cultured for one or two weeks in Petri dishes with malt extract agar. Erlenmeyer flasks, containing rice and water, were autoclaved at 121 °C for 15 min and then inoculated with mycelia plugs of the fungi and incubated at 28 °C for 30 d, after which the moldy rice was macerated in ethyl acetate and filtered. The two layers were separated, and the ethyl acetate solution was concentrated at a reduced pressure to yield 97.51 and 102.00 g of the crude ethyl acetate extracts, respectively [21,22].

N. siamensis KUFA 0017 was cultured for one week in Petri dishes with 25 mL of potato dextrose agar per dish. Thirty-five Erlenmeyer flasks containing 200 g of rice and 100 mL of water were autoclaved at 121 °C for 15 min, and then inoculated with 10 mycelium plugs of the fungus. The culture was incubated at 28 °C for 30 d. To each flask with the mouldy rice was added 500 mL of ethyl acetate and the content was left to macerate for 7 d. The content of the flasks was filtered by filter paper and the filtrate was evaporated under reduced pressure to give 1000 mL of the solution and then anhydrous sodium sulphate was added and filtered. The ethyl acetate solution was evaporated under

reduced pressure to give 5 g of dark brown viscous mass of a crude ethyl acetate extract.

2.4. Cell culture

Seven human cancer cell lines were used to assess anti-proliferative activity. HT29 and HCT116 (colorectal carcinoma) were kindly provided by Prof. Carmen Jerónimo from CI-IPO, Porto. HepG2 cells were kindly provided by Prof. Rosário Martins from ESTSP and CIIMAR, Porto. MCF-7, A375, A549 and U251 cells were obtained from European Collection of Cell Cultures (ECACC). Human cancer cell lines were maintained as monolayer cultures in DMEM (HT29, A375 and A549), MEM (HepG2, MCF-7 and U251) and RPMI (HCT116), supplemented with 10% fetal bovine serum and 1% of antibiotic solution (100 U/mL penicillin and 100 μg/mL streptomycin), 0.1 mmol/L sodium pyruvate and 10 mmol/L N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) under an atmosphere of 5% CO_2 at 37 °C. Cells were trypsinized when nearly confluent.

Stock solutions of Dox and crude ethyl acetate extracts of the marine fungi were prepared in dimethyl sulphoxide (DMSO) and aliquots kept at −20 °C. The final concentration of DMSO in the medium was <0.5% (v/v). The controls received only DMSO. All the cells were incubated with the extracts and Dox dissolved in RPMI just before use, in order to maintain the same conditions for all cell lines.

2.5. MTT reduction assay

To evaluate the effects of the crude ethyl acetate extracts on cell viability/proliferation, cells were plated in 96-multiwell culture plates at a density of 0.8×10^4 – 1×10^4 cells/well. Twenty-four hours after plating, the medium was discarded and fresh medium containing the extracts of the marine-derived fungi at different concentrations (0.1–500.0 μg/mL) and Dox at (0.001–10.000 μmol/L) as positive control, as well as 0.5% DMSO as negative control was added. After 48 h incubation with the extracts, MTT was added at a final concentration of 0.5 mg/mL and incubated for 2 h. Then, the medium was removed, and the formazan crystals were dissolved in a DMSO:ethanol solution (1:1) (v/v). Absorbance was measured at 570 nm in a microplate reader (Multiskan EX, Labsystems, USA). The MTT colorimetric assay is based on mitochondrial conversion of tetrazolium salt (MTT) into formazan crystals, and thus alterations in the number of viable cells can be detected by measuring formazan crystals optical density [24,25]. IC_{50} corresponds to the concentration of the extract or Dox that decreases the number of viable cells by 50%. In this case, the absorbance in the control at 48 h corresponds to 100% viability. The IC_{50} values were determined using GraphPad Prism v5.0 software (GraphPad Software, La Jolla, CA, USA). To evaluate the effect on cell proliferation, the absorbance at the beginning of incubation (t = 0 h) was subtracted from all the experimental conditions used, including the control (0.5% DMSO) at 48 h. Therefore, negative values can be interpreted as direct and short-term cytotoxic effect of the extracts that may, in turn, indicate necrotic cell death [26]. Positive values (between 0 and 100%) can be interpreted as inhibition of cell proliferation. This variant of the MTT assay allows a rapid and simple discrimination between inhibition of cell proliferation and cell death [26]. The results are expressed as the percentage of cell viability/proliferation relative to control (untreated cells) of at least six independent experiments; each one was carried out in duplicate. For the following assays, only the fungal extracts that presented an IC_{50} equal to or less than the arbitrary cut-off threshold of 200 μg/mL and without direct cytotoxic effects were used.

2.6. Clonogenic assay

To study the effect of the extracts of the marine-derived fungi on the proliferation of clonogenic cells, cells were plated (1×10^5 cells/well) in 24-multiwell culture plates and treated with extracts or Dox (IC_{50}). After a 48 h treatment, survival cells were trypsinized, counted and plated in 6-multiwell culture plates at 200 cells/well with fresh medium. After 10 d of culture, colonies were fixed, stained with crystal violet (5 g/mL) for 30 min and washed with water. Colonies containing more than 50 individual cells were counted using a stereomicroscope (Leica, ZOOM 2000). Plating efficiencies (PE) were calculated from the ratio between the number of colonies counted and the number of cells plated. The surviving fraction (SF) was calculated using the formula: $SF = PE$ of treated cells/PE of control × 100 [27–29]. Results are expressed as the surviving fraction relative to control (cells treated with 0.5% DMSO).

2.7. Comet assay

To investigate the genotoxic effect of the tested fungal extracts, cells were plated (1×10^5 cells/well) in 24-multiwell culture plates. Twenty-four hours after plating, cells were incubated with the extracts of the marine-derived fungi (IC_{50}) for 4, 24 and 48 h and DNA damage (strand breaks and alkali-labile sites) was assessed by the alkaline version of the single cell gel electrophoresis (comet) assay [30,31]. In short, after treatment, cells were trypsinized, washed and centrifuged, and the pellet suspended in low melting point agarose and about 2×10^4 cells/gel were placed on a slide pre-coated with 1% normal melting point agarose. Slides were placed in lysis solution (2.5 mol/L NaCl, 100 mmol/L Na_2EDTA, 10 mmol/L Tris base, pH 10 plus 1% Triton X-100) for 1 h at 4 °C, and then placed in a horizontal electrophoresis chamber with a buffered solution (300 mmol/L NaOH, 1 mmol/L Na_2EDTA, pH > 13), for 40 min at 4 °C, for the DNA to unwind. The electrophoresis was run at 21 V for 20 min and the slides were washed twice with PBS and dried at room temperature. For the analysis of the comet images, the slides were stained with DAPI solution (1 μg/mL) and visualized in a fluorescence microscope (Olympus IX71). Images were registered and studied with the image analysis software CometScore® (CometScore, TriTek Corp.), for quantifying the percentage of tail intensity. At least 100 randomly selected cells were analyzed per sample.

2.8. Nuclear condensation assay

To evaluate the ability of the extracts of the marine-derived fungi to induce cell death, cells were plated (1×10^5 cells/well) in 24-multiwell culture plates. After cell adhesion, cells were treated with the extracts or Dox (IC_{50}) for 48 h and nuclear condensation was assessed by observation in the mentioned fluorescence microscope. Briefly, both adherent and non-adherent cells were collected, washed, centrifuged, fixed with 4% (w/v) paraformaldehyde in PBS for 20 min at 37 °C, and

then attached into a polylysine-treated slide using a Cytospin Cytocentrifuge (Thermo Scientific, USA). Once dried, slides were incubated with DAPI (1 μg/mL) for nuclei staining. The percentage of cells with condensed nuclei was calculated from the ratio between cells with nuclear condensation and total number of cells (nuclei staining with DAPI). More than 300 cells were counted per sample.

2.9. Statistical analysis

Results are expressed as mean ± SD from at least three independent experiments. The GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA, USA) was used for inferential statistics. Eventual significant differences ($P < 0.05$) were evaluated by one-way ANOVA, followed by the post-hoc Newman–Keuls multiple comparison test or Dunnett's test, as appropriate. In specific cases, Student's *t*-test was used as described. Data from the MTT assay were analysed for normal distribution using the D'Agostino-Pearson omnibus test. For the remaining data, normal distribution was not tested due to low 'n', and was assumed to occur, which is consistent with the normal behavior of cell culture responses as observed in similar published studies.

3. Results

3.1. Effect of extracts of marine-derived fungi on cell viability and proliferation in cancer cells by MTT

In order to test the effects of the extracts of four marine-derived fungi on cell viability and proliferation, seven human cancer cell lines (HT29, HCT116, HepG2, MCF-7, A375, A549 and U251) were used. The assay was performed on exponentially growing cells, as previously determined (data not shown). Each cell line was incubated for 48 h with the extracts at different concentrations (0.1–500.0 μg/mL) and then cell viability and proliferation were assessed by MTT assay. Doxorubicin was tested in parallel over a concentration range (0.001–10.000 μmol/L), corresponding to (5.8×10^{-4}–5.8 μg/mL), as a positive control. The IC_{50} (concentration of the extract or Dox that reduces the number of viable cells by 50%) for each cell line was calculated from the dose–response curves. The values of the IC_{50} are summarized in Table 1. The extracts E2 and E4 significantly decreased the number of viable cells in HepG2, HCT116 and A375 cells. Both extracts showed similar IC_{50} values for HepG2 cells. Regarding HCT116 and A375 cells, the extract E4 showed a lower IC_{50} value (124 and 150 μg/mL, respectively) when compared to the extract E2 (165 and 184 μg/mL). The extracts E1 and E3 had an IC_{50} higher than the cut-off level of 200 μg/mL in all tested cell lines, and therefore they were not used in the following experiments. Dox decreased cell viability in all tested cell lines and the related IC_{50} values ranged

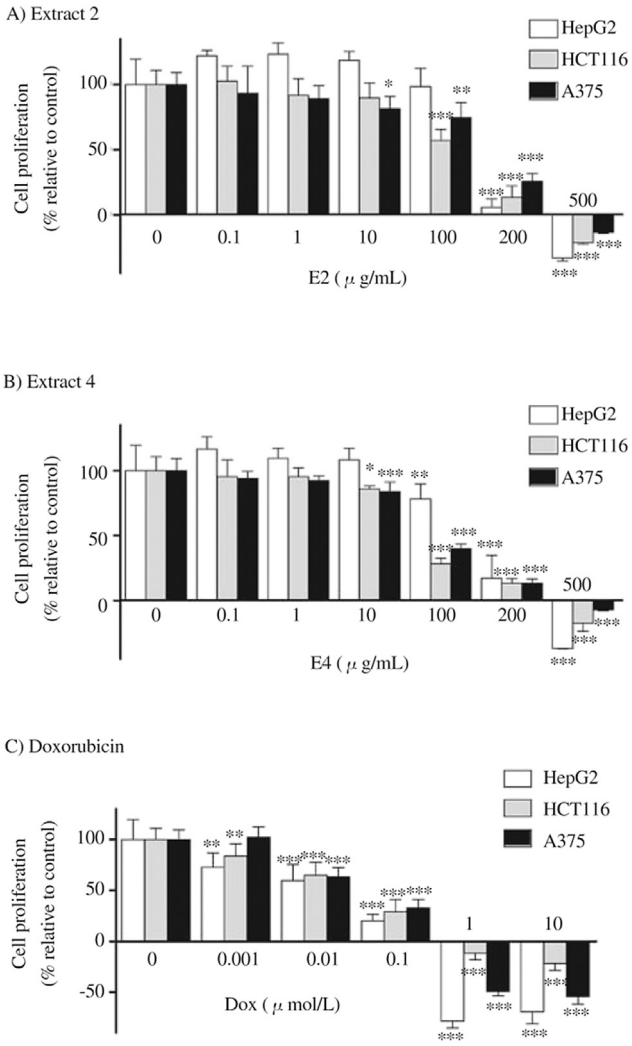

Figure 1. Dose-response effects of extracts E2 (A), E4 (B) and doxorubicin (C) on cell viability/proliferation in HepG2, HCT116 and A375 cells after 48 h, evaluated by MTT assay. Results are expressed as mean ± SD of at least six independent experiments, each made in duplicate. Significant differences ($^{*}P < 0.05$; $^{**}P < 0.01$ and $^{***}P < 0.001$) between groups per situation of exposure were tested by one-way ANOVA, followed by the post-hoc Newman–Keuls multiple comparison test.

Table 1
Determination of IC_{50} values[a] (concentration that inhibits the number of viable cells in 50%) of four extracts (μg/mL) in seven cells lines, with doxorubicin as a positive control.

Cancer cell lines	Doxorubicin (μmol/L)		KUFA 0013 (E1) IC_{50}	KUFC 7897 (E2)		KUFC 0021 (E3) IC_{50}	KUFA 0017 (E4)	
	IC_{50}	95% CI		IC_{50}	95% CI		IC_{50}	95% CI
HepG2	0.11	(0.07–0.17)	>200	198	(179–208)	>200	197	(182–213)
HT29	0.87	(0.54–1.39)	>200	>200		>200	>200	
HCT116	0.13	(0.09–0.19)	>200	165	(136–201)	>200	124	(81–180)
U251	1.55	(0.70–2.50)	>200	>200		>200	>200	
A549	0.54	(0.30–0.94)	>200	>200		>200	>200	
A375	0.12	(0.09–0.16)	>200	184	(160–209)	>200	150	(131–173)
MCF7	0.37	(0.27–0.50)	>200	>200		>200	>200	

[a] IC_{50} values are the mean of at least six independent experiments, each in duplicate.

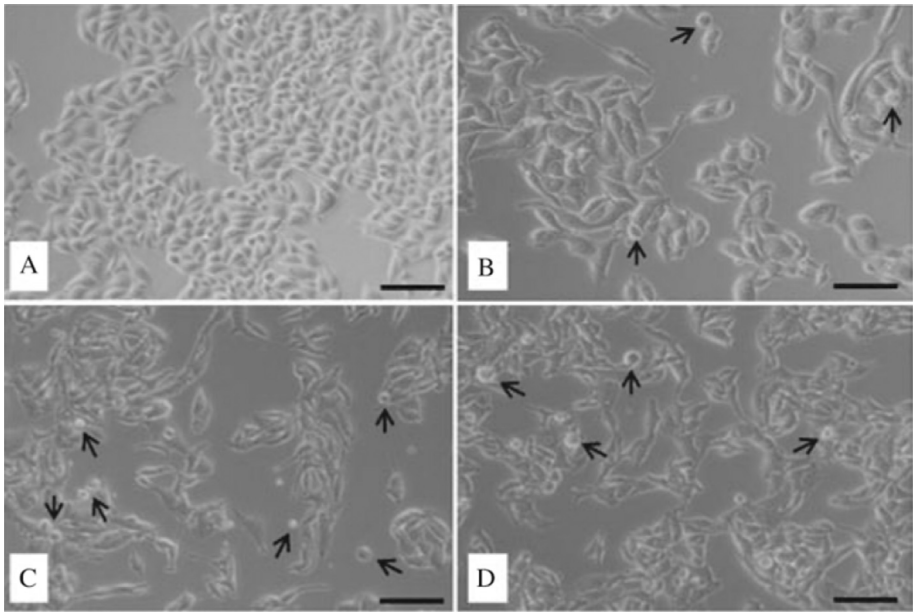

Figure 2. Morphology of HepG2 cells under phase contrast, after 48 h incubation with A) 0.5% of DMSO, B) Dox, C) extract E2, and D) extract E4, all three at IC_{50}. Situation A) represents control cells, with normal morphology. In B), C) and D) arrows indicate rounded and detaching cells. Scale bar = 10 μm.

from 0.11 to 1.55 μmol/L (corresponding to 0.06–0.9 μg/mL) (Table 1).

In relation of cell proliferation, it was observed that all cell lines, when treated only with 0.5% DMSO (negative control), grew significantly between the beginning (t = 0 h) and the end of the incubation (t = 48 h) (data not shown). The results showed that the extracts E2 and E4 decreased cell proliferation in a concentration-dependent manner in HepG2, HCT116 and A375 cells (Figure 1). The anti-proliferative effect of both extracts was higher in HCT116 and A375 cells. The extract E2, at 100 μg/mL, significantly decreased proliferation of HCT116 and A375 cells by 43% and 25%, respectively relative to control (Figure 1A). At the same concentration, the extract E4 significantly decreased proliferation of HCT116 and A375 cells by 72% and 60%, respectively (Figure 1B). In the MTT assay, the extract E4 was more active in the cells lines tested. For concentrations higher than 200 μg/mL, both extracts showed a direct cytotoxic effect in HepG2, HCT116 and A375 cells. Dox decreased cell proliferation in a concentration-dependent manner in all cell lines tested. At 1 and 10 μmol/L, Dox exhibited a direct cytotoxic effect in HepG2, HCT116 and A375 cells (Figure 1C).

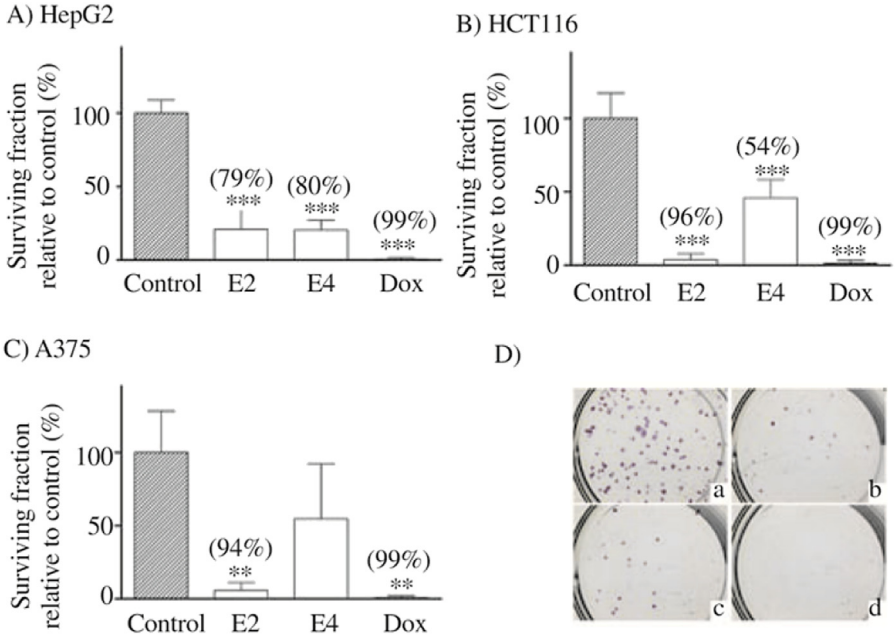

Figure 3. Clonogenic survival of HepG2 (A), HCT116 (B) and A375 (C) cells after pre-treatment with extracts E2, E4 and Dox (all three at IC_{50}) for 48 h, followed by 10 d in fresh medium. Results are expressed as mean ± SD of at least three independent experiments. Significant differences ($^{*}P < 0.05$; $^{**}P < 0.01$ and $^{***}P < 0.001$) when compared with control cells were evaluated by one-way ANOVA, followed by the post-hoc Dunnett's test. D) Representative images of clonogenic assay in HepG2 cells after pre-treatment with a) 0.5% of DMSO, b) extracts E2, c) extract E4 and d) Dox at IC_{50}, showing a decrease of the number of colonies formed relative to control (a).

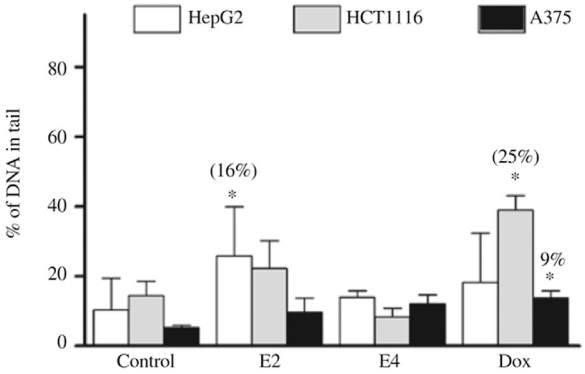

Figure 4. Effect of extracts E2, E4 and Dox (all three at IC_{50}) on DNA damage (strand breaks and alkali-labile sites) after 48 h in HepG2, HCT116 and A375 cells, assessed by comet assay.
Values are mean ± SD of at least three independent experiments. Significant differences ($^{*}P < 0.05$) when compared with control cells were judged by one-way ANOVA, followed by the post-hoc Dunnett's test. Additionally, a Student's *t*-test was selectively used to access significant differences in HepG2 (E2) and A375 (Dox) in relation to the respective control.

To select the extracts and cell lines for use in the following experiments, two aspects were considered: 1) extracts should inhibit cell proliferation without significant direct cytotoxic effects; and 2) the IC_{50} value of the extract should be lower than 200 μg/mL. Having met these two criteria, the extracts E2 and E4 were selected, and the anti-proliferative and pro-cell death effects were evaluated in HepG2, HCT116 and A375 cells.

The anti-proliferative effect of the extracts E2, E4 and Dox in HepG2, HCT116 and A375 was also confirmed by a decrease of cell density and also by structural alterations, such as rounded and detached cells as observed in a phase contrast microscope (Figure 2).

3.2. Effect of extracts of marine-derived fungi on reproductive viability by clonogenic assay

To evaluate the long-term anti-proliferative effects of the extracts, cells were pre-treated for 48 h with the extracts (IC_{50}) and then survival cells were allowed to grow for 10 d in fresh medium without extracts. Results from the clonogenic survival assay showed that the extract E2 significantly decreased the proliferative ability of a single cell to form a viable colony in HepG2, HCT116 and A375 cells (Figure 3). The extract E4 significantly reduced the number of colonies in HepG2 and HCT116 cells when compared with control (cells pre-treated with 0.5% DMSO) (Figure 3A and B). Meanwhile, no significant results were observed in A375 cells (Figure 3C). Cells pre-treated with Dox did not show an ability to proliferate and form colonies.

3.3. Evaluation of the effect of the extracts of the marine-derived fungi on DNA damage by the comet assay

To assess the genotoxic effect of the extracts, cells were incubated with extracts at the IC_{50} concentration for 4, 24 and 48 h, and DNA damage (strand breaks and alkali-labile sites) was assessed by the comet assay. Results are expressed as the percentage of DNA in tail. At 4 and 24 h of incubation, none of the extracts at the tested concentrations induced DNA damage in HepG2, HCT116 or A375 cells (data not shown). However, after incubation for 48 h, the extract E2 significantly increased by 16% the extent of DNA damage in the HepG2 cells (see white bars, Figure 4), when compared with control cells (0.5% DMSO). No effect was observed in HCT116 (grey bars) and A375 (black bars) cells when treated with the extract E2. Moreover, the extract E4 did not induce DNA damage detectable by the comet assay at any of the tested experimental conditions. However, at 48 h, Dox evidenced a significant increase of DNA damage in HCT116 (by 25%) and A375 (by 9%) cells, when compared to the control (Figure 4).

3.4. Effects of extracts of marine-derived fungi on cell death by nuclear condensation assay

Since the anti-proliferative effects of the extracts could be due to cell death, we evaluated nuclear condensation after 48 h cell incubation with the extracts (at IC_{50}). As shown in Figure 5, the extract E2 significantly induced nuclear condensation in

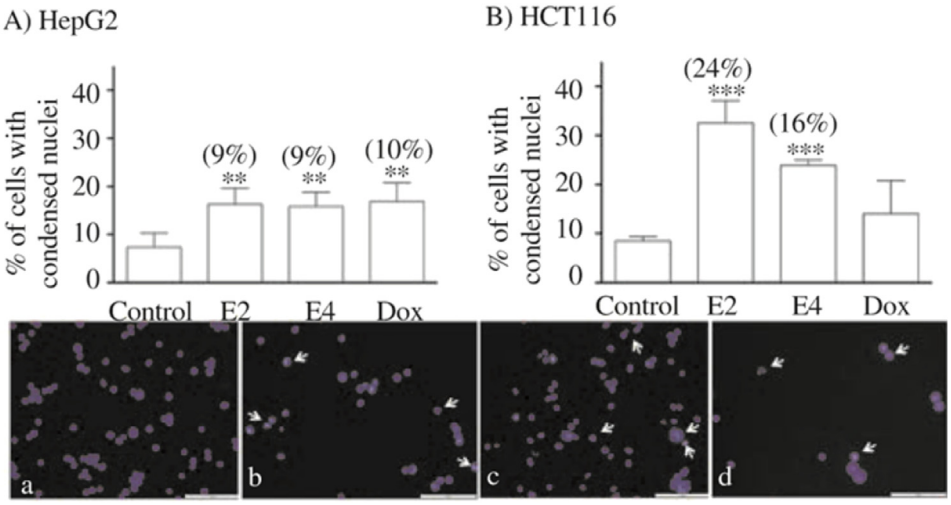

Figure 5. Effect of extracts E2, E4 and Dox (all three at IC_{50}) on the induction of nuclear chromatin condensation in HepG2 (A) and HCT116 cells (B) by the nuclear condensation assay after 48 h of incubation.
Values are mean ± SD of at least three independent experiments. Significant differences ($^{**}P < 0.01$ and $^{***}P < 0.001$) when compared with control cells were determined by one-way ANOVA, followed by the post-hoc Dunnett's test. Representative images of nuclear condensation in HCT116 cells after exposure to a) 0.5% of DMSO, b) extracts E2, c) extract E4 and d) Dox at IC_{50}. Scale bar = 100 μm. Nuclear condensation (arrow).

HepG2 (Figure 5A) and HCT116 cells (Figure 5B). The number of cells with condensed nuclei was increased by 9% in HepG2 and 24% in HCT116 cells. The extract E4 also induced an increase of nuclear condensation by 9% in HepG2 and 16% in HCT116 cells. Dox induced nuclear condensation also by 10% in HepG2 and by 8% in A375 cells. However, none of the tested extracts induced an increase of cells with nuclear condensation in A375 cells (data not shown). The HCT116 cells were the most sensitive to cell death induction by the extracts, with the extract E2 being the most effective against this cancer cell line.

4. Discussion

Marine-derived fungi biosynthesize a vast range of secondary metabolites with complex and unique structures [32]. However, very little is known about the biological activity of the extracts and compounds of these fungi. In this study, we investigated the *in vitro* anticancer activity of the crude ethyl acetate extracts obtained from four marine-derived fungi, *A. similanensis* (E1), *N. paulistensis* (E2), *T. trachyspermus* (E3) and *N. siamensis* (E4), on seven human cancer cell lines.

To the best of our knowledge, this is the first report on the *in vitro* anticancer activity of the marine-derived fungi *N. paulistensis* KUFC 7897 (E2) and *N. siamensis* KUFA 0017 (E4). In this study we showed that extracts E2 and E4 decrease the number of viable cells, with an IC_{50} lower than 200 μg/mL in HCT116, A375 and HepG2 cells. Both extracts decreased cell proliferation in a dose-dependent manner and exhibited a direct cytotoxic effect in HepG2, HCT116 and A375 cells at concentrations greater than 200 μg/mL. Some authors refer that intense insults (exposure to high concentrations) are able to induce uncontrolled cell death (necrosis) [33]. The extract E3 did not exhibit significant anticancer bioactivity (IC_{50} > 200 μg/mL) in the tested panel of cell lines. Several compounds of the ethyl acetate extract of *T. trachyspermus* KUFA 0021 (E3) have been isolated by Kumla *et al* [21], namely, 3-acetyl ergosterol 5,8-endoperoxide, ergosta-4,6,8(14),22-tetraen-3-one, spiculisporic acid E, glaucanic and glauconic acid. The latter three were tested for cytotoxic activity against breast MCF-7, lung NCI–H460 and melanoma A375-C5 cancer cell lines, however, did not present significant bioactivity [21], much alike to our crude ethyl acetate extract. Similarly, the assessment of anticancer bioactivity of the crude extract of *A. similanensis* (E1) did not result in an observation of meaningful cytotoxic activity (IC_{50} > 200 μg/mL) against our panel of cell lines. The chemical composition of *A. similanensis* KUFA 0013 (E1) ethyl acetate extract was described by Prompanya *et al* [22], and resulted in the isolation of five compounds, chevalone E, pyripyropene S, 5-hyroxy-8-methyl-2*H*, 6*H*-pyrano[3,4-g] chromen-2,6-dione and 6,8-dihydroxy-3,7-dimethylisocoumarin. After the initial screening of antiproliferative activity, the extracts E2 and E4 that showed an IC_{50} lower than 200 μg/mL were selected for the following studies.

The reduction of the number of viable cells could be due to an increase of cell death and/or decreased cell proliferation. In our study, the suggestive induction of cell death was first noticed through the observation of morphological alterations (cell shrinkage, membrane blebbing, rounded and detached cells) in a phase contrast microscope and further confirmed by a nuclear condensation assay. Moreover, to discern whether the antiproliferative activity prevailed over time, *i.e.*, a cytostatic effect, a clonogenic cell survival assay was performed to assess the extracts' ability of arresting indefinite proliferation of cells by reducing reproductive viability [29].

In this perspective, the *in vitro* anticancer effect of the extract E4 in HepG2 and HCT116 cells could be due to both an increase of cell death and a decrease of long-term cell proliferation. In A375 cells, the extract E4 decreased the number of viable cells without an induction of cell death and presented a slight decrease of clonogenic potential. In this case, the extract E4 seems to inhibit only cell proliferation in short term, however, in long term the alterations induced by the extract seem to be reverted or repaired in A375 malignant melanoma cells. The lack of long-term inhibition may be explained by the observations of other studies, which have shown that malignant melanoma is resistant to chemotherapy based on DNA damage induction, besides the normal function of p53, and may be related to the up-regulation of some DNA repair genes [34,35].

The analysis of the ethyl extract of *N. siamensis* KUFA 0017 (E4) composition is still not available. However, Buttachon *et al* have reported the isolation of several compounds from a terrestrial strain of *N. siamensis* KUFC 6349, namely, sartorymensin, tryptoquivaline, tryptoquivalines F, H, L and O, fiscalins A and C, *epi*-fiscalin A and C, *epi*-neofiscalin A, neofiscalin A and 2,4-dihydroxy-3- methylacetophenone [36]. In the same study, selected compounds were screened for the *in vitro* cytotoxic activity in a panel of cancer cell lines, and the results showed that sartorymensin, tryptoquivaline O and F showed an IC_{50} range between 72 and 91 μmol/L in breast MCF-7 and glioblastoma U373 cells. Additionally, Sondgam *et al* showed that tryptoquivaline L, *epi*-fiscalin A and C, isolated from *Xylaria humosa*, have cytotoxic effects in MCF-7 cells with an IC_{50} range between 21 and 33.6 μg/mL [37]. If the nature of the compounds of the marine-derived strain is similar to that of the mentioned terrestrial counterpart, it is possible to hypothesize that some of the referred compounds may be responsible for the anticancer activity of the extract E4. However, further studies should be ensued to confirm this hypothesis.

The *in vitro* anticancer activity of the extract E2 in HepG2 and HCT116 cells was mainly due to the induction of cell death and decrease of long-term proliferation of survival cells. In HepG2 cells, the induction of cell death could be related with the ability of the extract E2 to induce DNA damage. This characteristic is common to several chemotherapeutic drugs, which reveal an anticancer activity mainly due to their ability to induce DNA damage; if such DNA damage is not properly repaired, its accumulation ultimately ensues in cell death [38]. In A375 cells, the extract E2 decreased long-term cell proliferation (diminished colony formation) without effects on induction of cell death, at the tested conditions. Decrease of clonogenic survival may be related with the induction of senescence and mitotic catastrophe [39,40]. The analysis of the chemical composition of the ethyl extract of *N. paulistensis* KUFC 7896 (E2) has been described by Gomes *et al* [20], which led to the isolation of several compounds, namely, sartorypyrone C, tryptoquivalines H, L, F, 4(3H)-quinazolinone and 3′-(4-oxoquinazolin-3-yl) spiro [1H-indole-3,5′]-2,2′-dione. As referred previously, tryptoquivaline L exhibited cytotoxic activity against MCF-7 cells [37], while tryptoquivaline F exhibited cytotoxic activity against MCF-7 and U373 cells [36]. It is thus possible to suggest that tryptoquivalines L and F, among other compounds, may be in some measure responsible for the anticancer activity of the extract E2.

Doxorubicin is an anticancer drug commonly used in the chemotherapy and its cytotoxic effect has been related to free radical formation, inhibition of DNA topoisomerase II and nucleotide intercalation, resulting in cell cycle arrest and apoptosis [41]. In our study, the *in vitro* anticancer activity of Dox, used as a positive control, seems to be related to a decrease in long-term cell proliferation and induction of cell death. However, in accordance with other studies, in HCT116 cells Dox reduces the number of viable cells without an induction of apoptosis [42]. Induction of cell death by Dox seems to depend on factors such as dose, exposure time and cell line used. Even so, facing our aims, Dox's exact mechanisms do not jeopardize it as control.

Genetic characteristics of cells, namely p53 status, influence its susceptibility to chemotherapy [43]. Some studies have associated, in part, the Dox resistant phenotype with p53 status [44,45], and this can in fact be observed in our results regarding HT29 and U251 cells, which are p53 mutant, presenting higher IC_{50} values for Dox. Our results indicate that the extracts E2 and E4 only present an effect in the p53 mutant cells (HCT116, HepG2 and A375), which suggests that the *in vitro* anticancer activity of the extracts could be p53 dependent. In spite of this, it must be considered that the response to the extract varies according to cell line, which varies in multiple genetic factors, it is probable that other molecular targets are involved, and thus it is pressing to clarify the molecular mechanisms involved in the activity of the extracts E2 and E4.

Our results reinforce the interest in pursuing further studies on the anticancer bioactivity of extracts and compounds originating from marine fungi from the *Neosartorya* genus. Some marine fungi of the *Neosartorya* genus are known to produce several compounds with the *in vitro* anticancer activity, for example, Liang *et al* exemplified five compounds (four gliotoxin analogues and one diketopiperazine) isolated from *Neosartorya pseudofischeri* 2014F27-1 which presented cytotoxic activity against colon cancer HCT116, RKO and kidney HEK 293 cell lines [46]. Tan *et al* isolated several compounds from *Neosartorya fischeri* 1008F, resulting in two compounds, AGI-B4 and 3,4-dihydroxybenzoic acid, with cytotoxic activity against gastric SGC-7901 and hepatic BEL-7404 cancer cell lines [47].

In summary, our study shows for the first time that the crude ethyl acetate extracts obtained from the marine-derived fungi *N. paulistensis* and *N. siamensis* have *in vitro* anticancer activities, by causing a decrease in cell proliferation and/or induction of cell death in hepatocellular carcinoma, colon carcinoma and melanoma cells. The inherent molecular targets and related signal transduction pathways should be researched in the future.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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