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ORIGINAL ARTICLE

Marine-derived Fungi Extracts Enhance the Cytotoxic Activity of Doxorubicin in Nonsmall Cell Lung Cancer Cells A459

Bruno Castro-Carvalho[1,2*], Alice A. Ramos[1,2*], Maria Prata-Sena[1,2], Fernanda Malhão[1,2], Márcia Moreira[1], Daniela Gargiulo[1,3], Tida Dethoup[4], Suradet Buttachon[1,5], Anake Kijjoa[1,5], Eduardo Rocha[1,2]

[1]Interdisciplinary Center for Marine and Environmental Research, University of Porto, 4450-208 Matosinhos, Departments of [2]Microscopy and [5]Chemistry, Institute of Biomedical Sciences Abel Salazar, University of Porto, 4050-313 Porto, Portugal, [3]University Center of Belo Horizonte, University of Minas Gerais, Belo Horizonte, Brazil, [4]Department of Plant Pathology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand

*Joint first authors

ABSTRACT

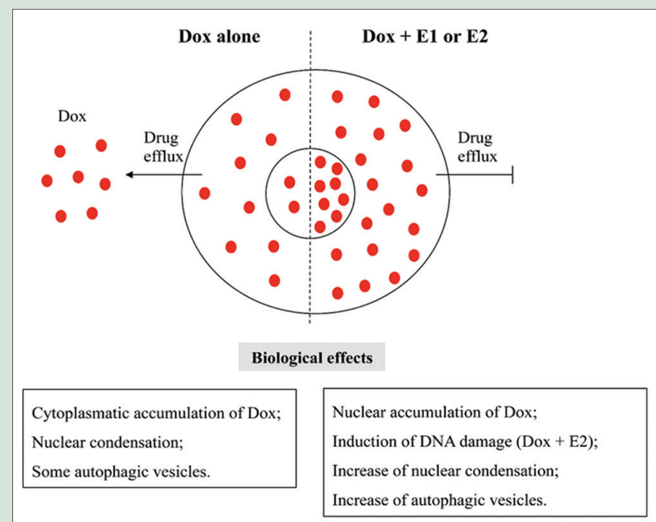
Background: Drug resistance is a major concern in the current chemotherapeutic approaches and the combination with natural compounds may enhance the cytotoxic effects of the anticancer drugs. Therefore, this study evaluated the cytotoxicity of crude ethyl extracts of six marine-derived fungi – *Neosartorya tsunodae* KUFC 9213 (E1), *Neosartorya laciniosa* KUFC 7896 (E2), *Neosartorya fischeri* KUFC 6344 (E3), *Aspergillus similanensis* KUFA 0013 (E4), *Neosartorya paulistensis* KUFC 7894 (E5), and *Talaromyces trachyspermum* KUFC 0021 (E6) – when combined with doxorubicin (Dox), in seven human cancer cell lines. **Materials and Methods:** The antiproliferative activity was primarily assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. **Results:** Two extracts, E1 and E2, demonstrated a significant enhancement of Dox's cytotoxicity in nonsmall cell lung cancer A549 cells. Accumulation of Dox in the nuclei increased when A549 cells were treated in combination with extracts E1 and E2, with induction of cell death observed by the nuclear condensation assay. The combination of E2 with Dox increased the DNA damage as detected by the comet assay. Ultrastructural observations by transmission electron microscopy suggest an autophagic cell death due to an increase of autophagic vesicles, namely with the combination of Dox with E1 and E2. **Conclusion:** These findings led to the conclusion that the fungal extracts E1 and E2 potentiate the anticancer action of Dox, through nuclear accumulation of Dox with induction of cell death mainly by cytotoxic autophagy.

Key words: Autophagy, cell death, drug combination, marine-derived fungi extracts, *Neosartorya* sp, nonsmall cell lung cancer

SUMMARY

- Fungal extracts increase the cytotoxic activity of doxorubicin (Dox) in lung cancer cells
- Nuclear accumulation of Dox, DNA damage, and cell death as a mechanism of action
- Fungal extracts may potentiate the anticancer activity of conventional drugs.

Abbreviations Used: A375: Human malignant melanoma cell line, A549: Human non small lung cancer cell line, DAPI: 4,6-Diamidino-2-phenylindole, DMEM: Dulbecco's Modified Eagle Medium, DMSO: Dimethylsulfoxide, Dox: Doxorubicin, DSBs: DNA double-strand breaks, E1: *Neosartorya tsunodae* KUFC 9213, E2: *Neosartorya laciniosa* KUFC 7896, E3: *Neosartorya fischeri* KUFC 6344, E4: *Aspergillus similanensis* KUFA 0013, E5: *Neosartorya paulistensis* KUFC 7894, E6: *Talaromyces trachyspermum* KUFC 0021, FBS: Fetal bovine serum, HCT116: Human colorectal carcinoma cell line, HEPES: (N-[2-hydroxyethyl] piperazine-N'-[2-ethane-sulfonic acid]), HepG2: Human hepatocellular carcinoma cell line, HT29: Human Caucasian colon adenocarcinoma Grade II cell line, IC_{50}: Concentration of the extract or Dox that inhibits cell viability by 50%, LRP: Lung resistance-related protein, MCF7: Human breast adenocarcinoma cell line, MEM: Minimum Essential Medium Eagle, MRPs: Multidrug resistance-associated proteins, MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, PBS: Phosphate-buffered saline, NSCLC: Nonsmall cell lung cancer, P-gp: P-glycoprotein, ROS: Reactive oxygen species, RPMI: Roswell Park Memorial Institute Medium, TEM: Transmission electron microscopy, U251: Human glioblastoma astrocytoma cell line.



Correspondence:
Prof. Eduardo Rocha,
Department of Microscopy, Institute of Biomedical Sciences Abel Salazar, University of Porto, Rua De Jorge Viterbo Ferreira No. 228, 4050-313 Porto, Portugal.
E-mail: erocha@icbas.up.pt
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INTRODUCTION

Cancer is a complex disease where multiple factors such as genetic, epigenetic, environment, and lifestyle strongly interact during the carcinogenic process.[1] The main strategies for cancer treatment include chemotherapy, radiotherapy, and surgery, and in some cases, combined strategies showed the best results. However, attending the high heterogeneity of cancer cells, it is very difficult to predict the cells that will respond to the treatment, and treatment resistance appears as a major problem for patients.[2]

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Doxorubicin (Dox) is an anthracycline used in the treatment of several types of cancers. Dox is known to act by interacting with the enzyme topoisomerase IIα, which leads to the accumulation of DNA breaks and ultimately to cell death.[3] However, Dox resistance is a serious problem and several mechanisms such as reduction of drug uptake, activation of drug detoxification, increased drug efflux and DNA repair capacity, and deflecting apoptotic pathway[4,5] have been proposed. P-glycoprotein (P-gp), multidrug resistance-associated proteins, and lung resistance-related protein (LRP) are some of the best-known proteins involved in drug resistance in several cancers, namely in the chemoresistance of nonsmall cell lung cancer (NSCLC), where drugs (e.g., Dox) are actively pumped from the cell to the outer membrane.[6,7] A current approach to overcome this problem is the use of multidrug combinations in an attempt to implement multitarget therapy as an alternative treatment by affecting diverse cellular mechanisms implicated in cell resistance which can result in cell death.[8] Apoptosis induction is one of the most successful approaches to kill cancer cells, and is portrayed by hallmarks such as cell shrinkage, membrane blebbing, nuclear condensation, and fragmentation into apoptotic bodies.[9] However, in some cases, cancer cells showed intrinsic or acquired resistance to apoptotic pathways.[10] In this context, autophagy has been suggested as a promising anticancer mechanism.[11] Autophagy is a multistep process characterized by initiation, elongation, and maturation of autophagosomes and fusion with lysosomes allowing cellular self-digestion. Nowadays, a dual role of autophagy, i.e., cytoprotective and cytotoxic effects, in the carcinogenic process, has been proposed, depending on the stage of cancer development.[12,13]

Bio-prospection of marine-derived products is currently one of the main interests of pharmaceutical research, and several bioactive attributes, such as antibacterial, antidiabetic, antifungal, anti-inflammatory, anti-protozoal, anti-tuberculosis, and antiviral activities,[14] have been found in these compounds. Considering the anticancer potential of marine natural products, their combination with conventional anticancer drugs may constitute a strategy to overcome cancer drug resistance and mitigate some of the hazardous side effects associated with chemotherapy; for instance, by decreasing the administered dose of the commonly used drugs.[15]

The aim of our study was to assess whether the combination of the ethyl acetate crude extracts of six marine-derived fungi, namely *Neosartorya tsunodae* KUFC 9213 (E1), *Neosartorya laciniosa* KUFC 7896 (E2), *Neosartorya fischeri* KUFC 6344 (E3), *Aspergillus similanensis* KUFA 0013 (E4), *Neosartorya paulistensis* KUFC 7894 (E5), and *Talaromyces trachsypermus* KUFC 0021 (E6), with Dox could enhance the *in vitro* cytotoxic activity of Dox on a panel of seven human cancer cell lines (HT29, HCT116, A375, A549, MCF7, U251, and HepG2 cells). The effects of the combination of the extracts and Dox were also evaluated for cell morphology, induction of DNA damage, nuclear condensation, and Dox accumulation (in the case of A549 cells).

MATERIALS AND METHODS

Chemicals

Dimethyl sulfoxide (DMSO) was purchased from AMRESCO LLC (Solon, SO, USA). Fetal bovine serum (FBS) was purchased from Biochrom AG (Berlin, Germany). Dox, Roswell Park Memorial Institute (RPMI) 1640, Dulbecco's Modified Eagle Medium (DMEM), Minimum Essential Medium (MEM), trypsin solution, penicillin/streptomycin solution, 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), sodium pyruvate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and 4,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Glutaraldehyde was purchased from Merck (Darmstadt, Germany) and osmium tetroxide at TAAB (Aldermaston, UK). All other reagents and chemicals used were of analytical grade.

Fungal material

Neosartorya tsunodae KUFC 9213 (E1) was isolated from a marine sponge, *Aka coralliphaga*, *Neosartorya laciniosa* KUFC 7896 (E2) was isolated from a diseased coral (ulcerative white spot in *Porites lutea*), and *Neosartorya fischeri* KUFC 6344 (E3) was isolated from coastal forest soil, as described by Ramos *et al*.[16] *Aspergillus similanensis* KUFA 0013 (E4) was isolated from a marine sponge, *Rhabdermia* sp., *Neosartorya paulistensis* KUFC 7894 (E5) was isolated from a marine sponge, *Chondrilla australiensis*, and *Talaromyces trachyspermum* KUFC 0021 (E6) was isolated from a marine sponge, *Clathria reianwardii*, as reported by Ramos *et al*.[17]

Cultivation and preparation of the crude extracts

N. tsunodae, *N. laciniosa*, *N. fischeri*, *A. similanensis, N. paulistensis, and T. trachyspermus* were cultured, and the crude ethyl extracts were prepared according to our previous report.[16,17] Briefly, the fungi were cultured for 1 or 2 weeks in Petri dishes with potato dextrose agar (strain 6344) or malt extract agar (strains 9213, 7896, 0013, 7894, and 0021). Autoclaved Erlenmeyer flasks, containing water and rice, were inoculated with mycelial plugs of the fungi and incubated for 30 days at 28°C. The moldy rice was macerated in ethyl acetate, filtered, and the two layers were separated, and the ethyl acetate solution was concentrated under a reduced pressure to obtain crude ethyl acetate extracts.

Cell culture

A549, A375, U251, and MCF7 were purchased from the European Collection of Cell Cultures. HT29 and HCT116 cell lines were provided by Prof. Carmen Jerónimo from IPO, Porto. HepG2 cell line was provided by Prof. Rosário Martins from ESTSP and CIIMAR, Porto. Human cancer cells were cultured in their corresponding media, including DMEM, RPMI, and MEM, supplemented with 1% antibiotic solution (100 U/ml penicillin and 100 μg/ml streptomycin), 10 mM HEPES, 0.1 mM sodium pyruvate, and 10% FBS.

3-(4,5-Dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide colorimetric assay

The screening of cytotoxic activity of the fungal extracts in combination with Dox was performed by the MTT colorimetric assay, as previously described.[18] Briefly, the seven cell lines were cultured in 96-multiwell culture plates (0.1×10^6 cells/ml) at 37°C and 5% CO_2. The fungal extracts at 100 μg/ml, alone or combined with Dox (concentration that inhibits cell viability in 50% [IC_{50}] of each cell line), were added to cells and left to incubate for 48 h. Cell viability was calculated according to the following equation: cell viability (%) = $(OD_{sample}/OD_{control}) \times 100$, where OD is the optical density. For the following assays, only combinations that decreased cell viability were used, and in these cases, two concentrations (100 and 200 μg/ml) of extracts were tested to evaluate the dose effect.

Comet assay

The alkaline version of the single-cell gel electrophoresis assay was performed according to the previous report.[19] A549 cells were cultured in 24-multiwell culture plates (0.1×10^6 cells/ml) at 37°C and 5% CO_2. The fungal extracts (100 and 200 μg/ml) and Dox (0.54 μM), either alone or combined, were added to cells and left to incubate for 24 and 48 h. Samples were stained with DAPI (1 μg/ml) and observed under a fluorescence microscope (Olympus IX71, Tokyo, Japan). Images were

collected, and at least 100 randomly selected cells were scored with image analysis software CometScore® version 1.5 (TriTek Corporation, Sumerduck, VA, USA) to quantify a percentage of tail intensity.

Nuclear condensation assay

A nuclear condensation assay was performed according to a previous description.[20] A549 cells were cultured in 24-multiwell culture plates (0.1×10^6 cells/ml) at 37°C and 5% CO_2. The fungal extracts (100 and 200 μg/ml) and Dox (0.54 μM), either alone or combined, were added to cells and left to incubate for 48 h. Cells were attached to polylysine-treated slides using a Cytospin™ Cytocentrifuge (Thermo Scientific, Waltham, MA, USA) by centrifugation at 500 rpm for 5 min and nuclei were stained with DAPI (1 μg/ml). At least 300 cells were counted per sample under a fluorescence microscope (Olympus IX71, Tokyo, Japan). The percentage of cells with condensed nuclei was calculated according to the ratio between cells presenting nuclear condensation and the total number of cells.

Electron microscopy

A549 cells were cultured in 12-multiwell culture plates (0.1×10^6 cells/ml) at 37°C and 5% CO_2 for 24 h. The fungal extracts (200 μg/ml) and Dox (0.54 μM), either alone or combined, were added to cells and left to incubate for 48 h. After the exposure period, cells were collected and processed for transmission electron microscopy (TEM) according to the protocol described by Madureira *et al.*[21] Ninety-nm-thick ultrathin sections were obtained with a diamond knife (Diatome, Biel, Switzerland), in an ultramicrotome Reichert Supernova (Leica, Heidelberg, Germany) and contrasted with uranyl acetate and lead citrate. Sections were observed with an electron microscope JEOL 100CXII (Jeol, Tokyo Japan) and photographed with a digital camera (Gatan, West Coast, USA).

Doxorubicin accumulation assay

A549 cells were cultured in glass coverslips in 12-multiwell culture plates (0.1×10^6 cells/ml) at 37°C and 5% CO_2 for 24 h. The fungal extracts (100 and 200 μg/ml) and Dox (0.54 μM), either alone or combined, were added to cells and left to incubate for 48 h. Cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde (w/v) for 15 min, and then washed with PBS. Coverslips were placed onto the slides and the nuclei were stained with DAPI (1 μg/ml) for 10 min. Samples were imaged with a fluorescence microscope (Olympus IX71, Tokyo, Japan).

Statistical analysis

The statistical analyses were performed with GraphPad Prism v6.0 software (GraphPad Software, La Jolla, CA, USA). Results were expressed as mean ± standard deviation from at least three independent experiments. Outlier detection was performed using a ROUT test (Q = 10%), as included in the cited software. Data were analyzed for homogeneity of variances and normal distribution using Bartlett's test and Kolmogorov–Smirnov test, respectively. The one-way ANOVA was used to assess significant differences ($P \leq 0.05$) between different conditions, followed by the *post hoc* Newman–Keuls procedure for multiple comparisons.

RESULTS

Effects on cell viability: 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide colorimetric assay

Combinations of the fungal extracts E1 and E2 with Dox were found to induce a statistically significant decrease in cell viability when compared with the sole use of Dox in A549 cells [Figure 1]. The positive control, Dox (0.54 μM), exhibited a decrease of 40% in cell viability in relation to the negative control (0.5% DMSO). Extracts E1 and E2 alone, at 100 μg/ml, did not exhibit a statistically significant decrease in cell viability when compared to the negative control. The combinatory regimens of the extract E1 + Dox (100/0.54) (from this point on, the notation, e.g., E1 + Dox (100/0.54) refers to extract E1 at 100 μg/ml and Dox at 0.54 μM in A549 cells) and the extract E2 + Dox (100/0.54) revealed a significant cell viability inhibition of 26%, in both cases, when compared with the positive control. The remaining extracts did not present any significant decrease in cell viability when combined with Dox, in any of the cell lines tested [Supplementary Figure 1]. The assays that followed the MTT were made solely on the extracts E1 and E2 in the lung cancer A549 cell line, considering that only the combination of these two extracts with Dox, in this particular cell line, demonstrated an enhancement of Dox's cytotoxic effect.

Effects on DNA damage: Comet assay

The potential induction of DNA damage, in the form of strand breaks and alkali labile sites, caused by the combination of the fungal extracts and Dox, was assessed by comet assay after a 24 h and 48 h exposure. No genotoxicity was detected at 24 h of exposure to treatment (data not shown). In contrast, DNA damage was observed at 48 h as shown in Figure 2. In the tested conditions, Dox alone did not induce significant DNA damage in A549 cells. Furthermore, none of the treatments with E1 presented a significant increase of DNA damage relative to the use of Dox alone [Figure 2a]. Figure 2b shows that E2 alone, at 200 μg/ml, significantly increases DNA damage by 6%, relative to the negative control. The combination E2 + Dox (200/0.54) induced an increase of 9% in comparison with Dox alone. However, E2 + Dox (100/0.54) triggered only a slightly but not significant trend (around 4%) of DNA damage relative to the respective positive control (Dox).

Effects on cell death: Nuclear condensation assay

As shown in Figure 3, the positive control for this experiment, Dox at 0.54 μM, induced a significant increase in the percentage of cells with nuclear condensation by 8%, when compared with the negative control.

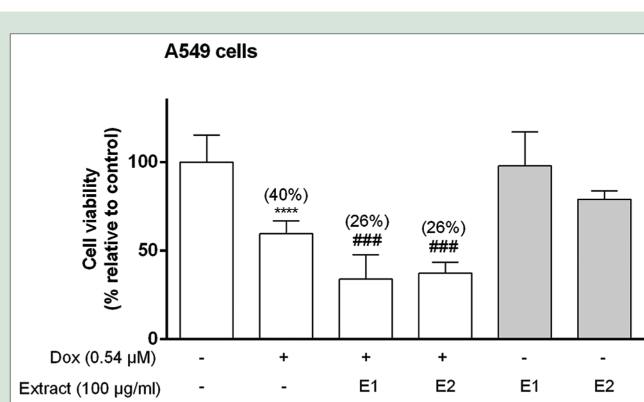

Figure 1: Effect of extracts E1 and E2 at 100 μg/ml alone and in combination with doxorubicin at 0.54 μM on cell viability of the A549 lung cancer cell line after 48 h of incubation. Percentages in brackets refer to the relative decrease in cell viability in relation to the negative control (medium with 0.5% dimethyl sulfoxide) or positive control (doxorubicin at 0.54 μM). Results are the mean ± standard deviation of at least four independent experiments. Significant differences (***$P \leq 0.001$) when compared with the negative control and (###$P \leq 0.001$) with the positive control (doxorubicin alone) were determined by one-way ANOVA followed by the *post hoc* Newman–Keuls multiple comparison test

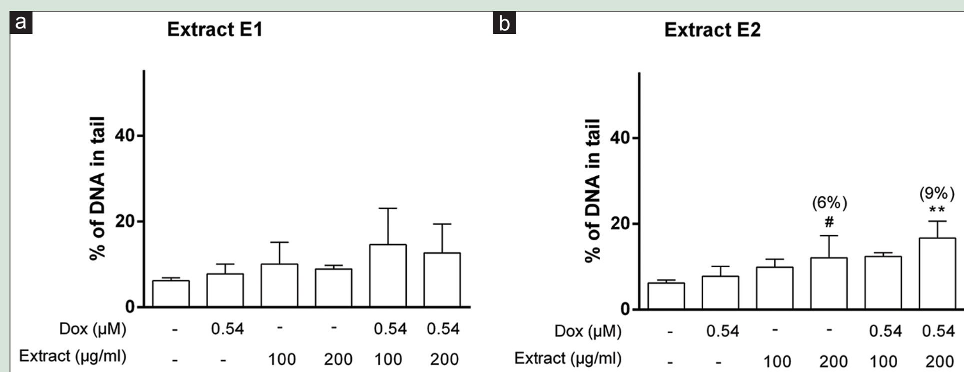

Figure 2: Effect of extracts E1 (a) and E2 (b) at 100 µg/ml and 200 µg/ml alone and in combination with doxorubicin at 0.54 µM on the induction of DNA damage after 48 h in A549 cell line assessed by comet assay. Percentages in brackets refer to the increase of DNA damage in relation to the negative control (medium with 0.5% DMSO) or positive control (doxorubicin alone). Results are the mean ± standard deviation of at least three independent experiments. Significant differences (#$P \leq 0.05$) when compared with the negative control and (**$P \leq 0.01$) when compared with doxorubicin alone were determined by a one-way ANOVA followed by the *post hoc* Newman–Keuls multiple comparison test

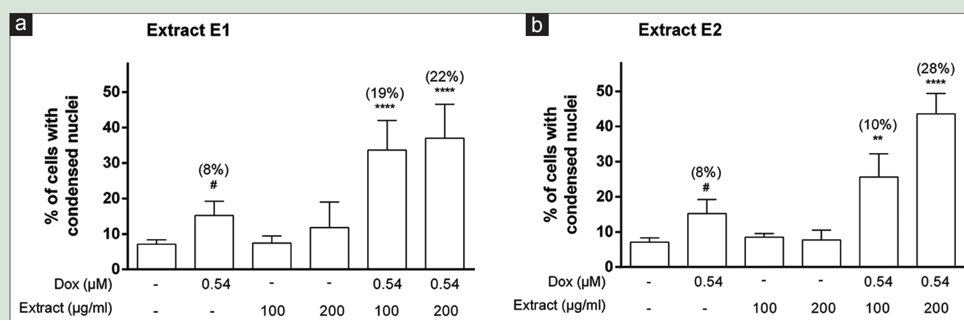

Figure 3: Effect of extracts E1 (a) and E2 (b) at 100 µg/ml and 200 µg/ml alone and in combination with doxorubicin at 0.54 µM on the induction of nuclear condensation in A549 cell line after 48 h of incubation. Percentages in brackets refer to the increase in cells with condensed nuclei in relation to the negative control (medium with 0.5% DMSO) and positive control (doxorubicin alone), respectively. Results are the mean ± standard deviation of at least three independent experiments. Significant differences (#$P \leq 0.05$) when compared with the negative control and (**$P \leq 0.01$ and ****$P \leq 0.0001$) when compared with the positive control were determined by a one-way ANOVA followed by the *post hoc* Newman–Keuls multiple comparison test

Combinations of Dox with the extract E1: E1 + Dox (100/0.54) and E1 + Dox (200/0.54) exhibited an increase of 19% and 22%, respectively, when compared with Dox alone [Figure 3a]. In addition, the extract E2 combined with Dox increased the number of cells with nuclear condensation. The combination of E2 + Dox (100/0.54) exhibited an increase of 10%, and the combination of E2 + Dox (200/0.54) caused an increase of 28% relative to Dox alone [Figure 3b]. None of the extracts alone, E1 and E2, at 100 or 200 µg/ml, induced increases in the number of cells with nuclear condensation when compared with the negative control.

Effects on cell morphology: Electron microscopy

Cell morphology evaluation by TEM revealed negative control cells with poor organelle content, essentially constituted by few mitochondria and cisternae of rough endoplasmic reticulum [Figure 4a]. Some lipid droplets and dense bodies, probably corresponding to lysosomes in various stages, were also observed. Dox-exposed cells [Figure 4b] showed a few autophagic vesicles/compartments (Av), exhibiting a morphology that is compatible with autolysosomes. Cells exposed to E1 [Figure 4c] were quite similar to the control cells, while E2-exposed cells revealed an increased load of Av [Figure 4d]. Both combinations of extracts with Dox resulted in a great increase in the number of autophagic vesicles (Avs) [Figure 4e and f]. In addition, even under a qualitative observation, this increase was obviously accompanied by a greater number of lipid droplets. In all instances, the Av displayed a range of morphological aspects [Figure 5], from mild-to-strong electron dense in content, some of them still presenting organelle debris, particularly from mitochondria. It was also quite common to find collated Avs that looked as if they were merging with each other.

Effects on doxorubicin accumulation: Fluorescence microscopy

Accumulation of Dox in A549 cells was studied by fluorescence microscopy after 48 h of treatment [Figure 6]. No fluorescence was observed in cells treated with E1 and E2 alone. In cells exposed to Dox alone, the drug remained mainly in the cytoplasm as low fluorescence was seen in the nucleus. However, when in combination with extracts E1 and E2, either at 100 or 200 µg/ml, Dox remained in the cytoplasm but the fluorescence in the nucleus increased, thus confirming that the mixtures induced changes in the intracellular drug distribution with an accumulation in the nucleus.

DISCUSSION

Dox has been used in the treatment of several types of cancer. However, drug resistance is one of the main factors that limits its efficacy. Reduction of intracellular accumulation of drug, an increase of DNA

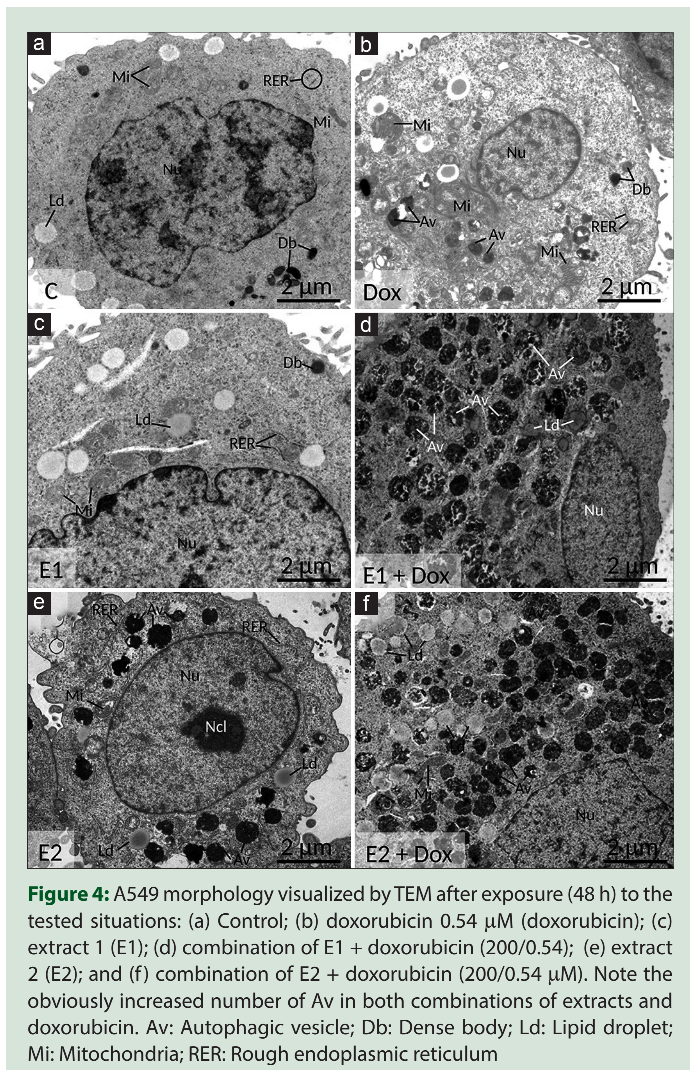

Figure 4: A549 morphology visualized by TEM after exposure (48 h) to the tested situations: (a) Control; (b) doxorubicin 0.54 μM (doxorubicin); (c) extract 1 (E1); (d) combination of E1 + doxorubicin (200/0.54); (e) extract 2 (E2); and (f) combination of E2 + doxorubicin (200/0.54 μM). Note the obviously increased number of Av in both combinations of extracts and doxorubicin. Av: Autophagic vesicle; Db: Dense body; Ld: Lipid droplet; Mi: Mitochondria; RER: Rough endoplasmic reticulum

repair, and apoptosis dysregulation are important mechanisms which are involved in multidrug resistance.[22,23] The combination of Dox with natural compounds can be a promising therapeutic strategy.[15,24,25] In this study, the effect of six crude ethyl extracts derived from *Neosartorya tsunodae* KUFC 9213 (E1), *Neosartorya laciniosa* KUFC 7896 (E2), *Neosartorya fischeri* KUFC 6344 (E3), *Aspergillus similanensis* KUFA 0013 (E4), *Neosartorya paulistensis* KUFC 7894 (E5), and *Talaromyces trachyspermum* KUFC 0021 (E6) in combination with Dox on cell viability was assessed by a panel of seven cancer cell lines (HepG2, HCT116, HT29, A549, A375, MCF-7, and U251). Our exploratory screening showed that noncytotoxic concentrations of extracts E1 and E2 significantly enhance the cytotoxic effect of Dox in NSCLC cells. However, the other extracts did not potentiate the cytotoxic activity of Dox in the panel of cell line tested. Regarding drug, combination is important to characterize the type of drug interaction and several methods have been developed. Chou-Talalay method is widely used allowing the determination of the combination index (CI) that provides a quantitative definition of synergistic, additive, and antagonistic effects.[26,27] However, our extracts did not evoke cytotoxic effects singly which according with the Chou-Talalay method does not allow CI determination. According to the literature, if a drug A has no effect and drug B has an effect and the combination of drug A and B has an effect greater than the drug B alone, this could be defined as a potentiation or enhancement.[26-28] Therefore, extracts E1 and E2 potentiate Dox's cytotoxicity in A549 cells.

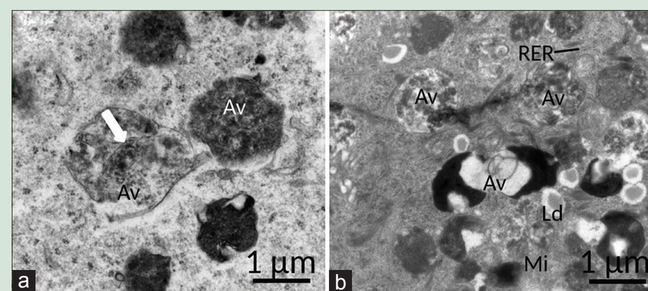

Figure 5: Autophagic vesicles as seen in transmission electron microscopy. (a) Av (presumptive autolysosomes) in different stages of maturation, with a more lucent one at left, still displaying a heterogeneous content resulting from organellar debris, and with an electron denser one at the right. (b) At the image center, two Avs are merging with each other. Avs: Autophagic vesicles; Ld: Lipid droplet; Mi: Mitochondria

Several mechanisms of action have been proposed for the cytotoxic effect of Dox, including: (1) DNA intercalation that prevents DNA replication; (2) inhibition of topoisomerase II resulting in DNA double-strand breaks; and (3) generation of reactive oxygen species (ROS) with induction of DNA damage.[29,30] The level, type, and persistence of DNA damage influence the cellular response to DNA damage. While low levels of DNA damage induce cell cycle arrest and definitive DNA repair, such reparation under high levels of DNA damage may not be enough to solve the problem and the induction of apoptosis may occur.[31,32] We recently demonstrated that Dox can decrease cell viability in several cell models, and in some of them, induction of DNA damage and cell death occur.[16] However, one of the most resistant cell lines with higher IC_{50} was A549 cell.[17] In this study, the results show that Dox at 0.54 µM decreased cell viability of A549 cells with induction of cell death being accomplished with nuclear condensation but without induction of strand breaks detected by comet assay. This reinforced the issue of drug resistance in NSCLC, where several mechanisms such as drug transport, DNA repair, and apoptosis invasion have been described.[33,34] The extracts E1 and E2, at noncytotoxic concentrations, when combined with Dox greatly decreased A549 cell viability, increasing the number of cells with condensed nuclei in comparison to Dox alone, and in a dose-dependent way. In the case of the combination of the extract E2 with Dox, induction of cell death was accomplished with an increase of DNA damage. Accumulation of DNA damage may appear as a result of a decrease of antioxidant defenses and/or impaired DNA repair mechanisms.[35,36] Similar to our results, enhancement of the cytotoxic effect of Dox against breast cancer cell lines has been also reported for other ethanolic extracts, from *Citrus aurantifolia* lime peels (25) and *Ficus septica* Burm. f. (*Moraceae*).[37]

Low sensitivity of NSCLC cells has been related with drug efflux due to upregulation of efflux pumps and impaired intracellular accumulation of drug.[23,38] In our study, marked cytoplasmic confinement of Dox was observed when A549 cells were treated solely with the drug, while nuclear localization increased in combination with extracts E1 and E2. Our results corroborate other reports suggesting that some natural compounds can enhance the cytotoxic activity of Dox by avoiding drug efflux and changing its intracellular localization. Some authors refer that inhibitors of P-gp (such as verapamil among others) promote an intracellular redistribution of Dox by reducing the drug in the cytoplasm while accumulating it in the nucleus. The removal of P-gp inhibitors reverses the intracellular localization of Dox back into cytoplasmic vesicles.[39,40] Neferine (found in lotus seeds – *Nelumbo nucifera*) was found to enhance the apoptotic effect of Dox in A549 cells with an increase of intracellular accumulation of Dox with an increase of ROS.[41]

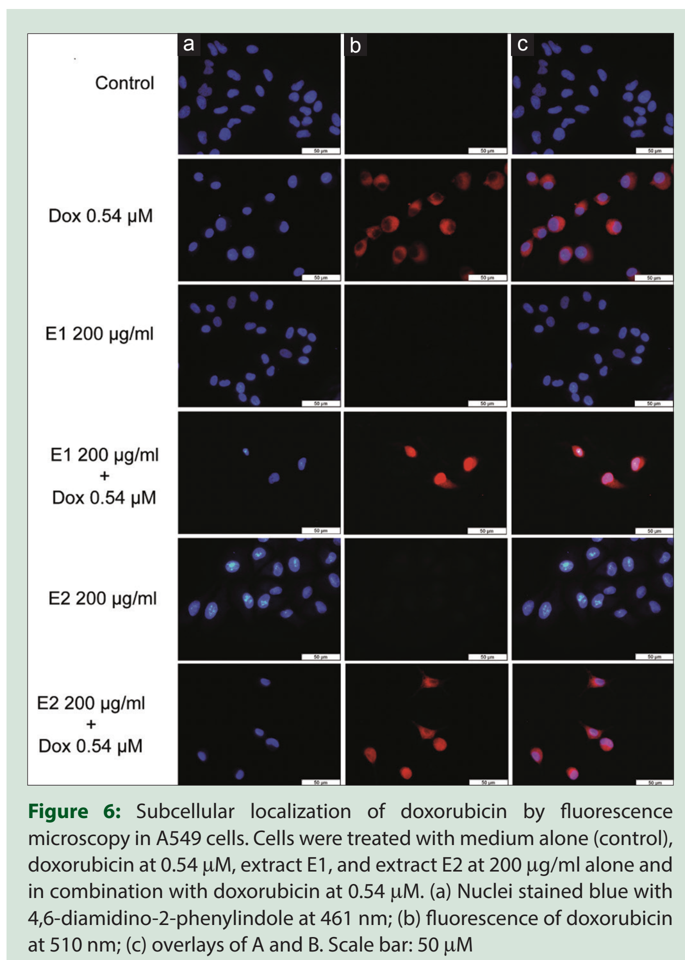

Figure 6: Subcellular localization of doxorubicin by fluorescence microscopy in A549 cells. Cells were treated with medium alone (control), doxorubicin at 0.54 μM, extract E1, and extract E2 at 200 μg/ml alone and in combination with doxorubicin at 0.54 μM. (a) Nuclei stained blue with 4,6-diamidino-2-phenylindole at 461 nm; (b) fluorescence of doxorubicin at 510 nm; (c) overlays of A and B. Scale bar: 50 μM

A propos, it was shown that A549 cells are more resistant than MCF7 cells to the Dox treatment via its accumulation in intracytoplasmic vesicles, connected with an increase of LRP expression.[6] Accordingly, other evidence suggests that LRP is involved in the subcellular distribution of Dox.[42,43]

In our study, enhancement of the cytotoxic effect of Dox-induced by extracts E1 and E2 seems to be due to the accumulation of Dox inside the cells with an increase of cell death. The exact mechanism of cell death is not clear yet, but our ultrastructural observations suggest the occurrence of cytotoxic autophagy due to the increase of Avs – or autophagic compartments as recommended elsewhere[44] – visualized herein by TEM when Dox was combined with extracts E1 and E2. TEM is considered a sensitive technique to reliably detect the presence of Avs.[45,46] Apoptosis and autophagic cell death could be both involved in cell death induced by extracts when combined with Dox. However, the connection between apoptosis and autophagy is a controversial topic that requires additional studies.[47] In accordance with our results, other natural compounds can induce cell death by different mechanisms. For instance, oleifolioside B, a cycloartane-type triterpene glycoside isolated from *Dendropanax morbifera* Leveille, was found to induce cell death by activation of apoptosis and autophagy in A549 cells.[48] Tai *et al*. showed that aqueous extract of *Solanum nigrum* leaves induced cell death in colorectal cancer cell lines with induction of autophagy.[49] Curcumin, isolated from turmeric (*Curcumalonga longa* L.), enhanced the cytotoxicity of Dox in human hepatoma cells through induction of apoptosis and autophagy.[50] Voacamine, a bisindole alkaloid isolated from the plant *Peschiera fuchsiaefolia*, was found to increase the cytotoxic effect of Dox in multidrug-resistant human osteosarcoma cells (U-2 OS-R). Voacamine is also a substrate of P-gp and acts as a competitive antagonist of Dox.[51] The induction of apoptosis was minimal, and ultrastructural alterations indicated the activation of autophagic cell death as the main process of cell death.[51] Induction of autophagic cell death has been described as a possible anticancer mechanism to be explored in apoptotic-resistant lung cells.[52] However, the autophagy induced by Dox may trigger a cytoprotective effect in (at least Hep3B) cancer cells, acting with self-protecting behavior.[53]

CONCLUSION

Our results show that noncytotoxic concentrations of ethyl extracts of *N. tsunodae* KUFC 9213 and *N. laciniosa* KUFC 7896, when in combination with Dox, enhance its cytotoxicity against the lung cancer cell line A549. The potentiation effect is (at least partially) due to the nuclear accumulation of Dox, resulting in the induction of cell death, possibly by an autophagic process. Future work should focus on the characterization of drug–extracts interactions and the underlying mechanisms, and on which compounds from the extracts (isolated/mixed) may display potentiation.

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Conflicts of interest

There are no conflicts of interest.

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