Extraction of *Micromonospora aurantiaca* from Coastal Marine Sediments Enhances Doxorubicin-Induced Apoptosis in KB Cells

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Abstract

Background. The search for substances sensitizing cancer cells to apoptosis is necessary. The potential activities of *Micromonospora* may provide novel structural diversity to be discovered. The coastal marine sediments are largely unexplored source for *Micromonospora*, however, little is known about its ability to produce anticancer activity and molecular mechanisms. The objective is to investigate the apoptosis-modulating activities of *Micromonospora aurantiaca* in human carcinoma of nasopharynx (KB cells).

Methods. Marine *Micromonospora aurantiaca* (MA) was extracted with methanol and ethyl acetate and treated on KB cells. The viable cell number was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Apoptotic cell death was assessed by nuclear staining with 4',6-diamidino-2-phenylindole (DAPI), agarose gel electrophoresis for DNA fragmentation assay and confirmed by flow cytometry using propidium iodide (PI) staining. Caspase-3 activity was analyzed using Caspase Activity Kit.

Results. The extract of MA and doxorubicin (DOX) alone inhibited KB cells with an IC_{50} of 83.46 ± 3.9 and 2.0 ± 0.1 μg/ml, respectively. The combined MA extract (50 μg/ml) and DOX (1 μg/ml) treatment produced greater cytotoxicity associated with increased chromatin condensation, DNA fragmentation and hypodiploid cells compared with treating cells with each agent alone. Apoptosis enhancement of combination treatment was accompanied by an increase in the relative activity of caspase-3 by 3.67 ± 0.31 fold, which was significantly attenuated by a caspase-3 inhibitor. The morphological evidence indicated a diminished size, rounded and easily detached cells compared with polygonal adherent cells in normal shape.

Conclusion. The induction of apoptosis by combined MA extract + DOX treatment involves the activation of effector caspases-3. Thus, the combination may provide another important advantage: MA extract would represent a promising source for discovery of interesting anticancer compounds. The elucidation of other apoptosis-related intracellular targets will be addressed in future studies.

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Actinomycetes are filamentous gram-positive bacteria belonging to the phylum Actinobateriaceae which are considered an important natural source of bioactive secondary metabolites from natural products. Because the marine environments are particularly different from terrestrial, the marine actinomycetes have developed unique pharmacological activities that would not be observed in terrestrial microorganisms.[1-3] The anticancer secondary metabolites produced by *actinomycetes* such as anthracyclines (doxorubicin), peptides (bleomycin and actinomycin D), etc., showed significant activity in medical treatment.[4,5] The secondary metabolites derived from actinomycetes display different biological activities including anti-bacterial, anti-fungal, cytotoxic, anti-oxidant, anti-inflammatory and immunosuppressive agents.[6] Actinomycetes isolated from the samples collected at different marine environments, such as the deep sea floor, marine invertebrates and coastal sediments, represent unique ecosystems that cannot be found anywhere else in the world.[7] Among the marine actinomycetes, the genus *Micromonospora* has become a focus in the search for novel secondary metabolites.

Micromonospora species are best known for synthesizing antibiotics, especially aminoglycoside (gentamicin and netamicin), enediyne, and oligosaccharide antibiotics which contribute their impact on medicinal usage.[8] Furthermore, *Micromonospora* species has been intensively investigated and isolated for anticancer antibiotics such as anthraquinones, anthracyclines, alkaloids, and macrolides.[9-12] Recently, the four new anthracyclinones were isolated from a strain of *Micromonospora* species associated with the tunicate *Eudistomavannamei*. Two compounds were cytotoxic against the HCT-8 human colon adenocarcinoma cell line, with IC_{50} values of 12.7 and 6.2 μM, respectively, while the

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other two compounds were inactive.[13] The alkaloid from *Micromonospora* secondary metabolite, diazepinomicin, has demonstrated a broad spectrum *in vitro* and *in vivo* cytotoxicity, and has been completed by EcopiaBioSciences, Inc., as an anticancer agent.[11] Streptonigrin isolated from *Micromonospora sp*. IM 2670 induced apoptosis through a p53-dependent pathway in the human neuroblastoma SH-SY5Y cells.[14] Tetrocarcin A (TCA), produced by *Micromonospora chalcea* NRRL 11289, induced apoptosis via activating the caspase-dependent cell death pathway.[15]

The potential activities of *Micromonospora* may provide novel structural diversity to be discovered. The search for the substances sensitizing cancer cells to apoptosis induction by chemotherapeutic agents is urgently required in the current strategy and hope to improve therapeutic properties by using combinatorial biosynthesis approaches. The coastal sediments from the gulf of Thailand are a largely unexplored source for *Micromonospora* with the potential to produce biologically active secondary metabolites, however, little is known about its ability to produce anticancer activity and the molecular mechanisms by inducing apoptosis. Nasopharyngeal carcinoma is known as the most common cause of death and chemotherapy treatment produces many systemic side effects. The human carcinoma of the nasopharynx (KB cells) was used by the National Cancer Institute (NCI) for some of the earliest *in vitro* anticancer drug-screening research. The aim of the study was to investigate the apoptosis-modulating activities of *Micromonospora aurantiaca* (MA) extraction in human carcinoma of nasopharynx (KB cells).

Materials and Methods

Chemicals

The following chemicals were purchased from the following suppliers: propidium iodide (PI), 4'-6-diamidino-2-phenylindole (DAPI) and SYBER Gold from Invitrogen (Paisley, UK); dimethyl sulfoxide (DMSO) and [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] (MTT) from Sigma Chemical (St Louis, MO, USA); cell culture media or materials were purchased from Gibco BRL (Gaithersburg, MD, USA) and InVitromex (Grevenbroich, Germany).

Isolation and identification of marine *Micromonospora*

The coastal sediment samples were collected at 1.3 meter in depth, in Na-Klua sub-district of Chonburi province, which is located on the east coast of the Gulf of Thailand. Sample soil was diluted, inoculated and isolated on Mineral Agar Guase 1 in 50 percents sea water adding with nystatin (50 mg/l) and novobiocin (20 mg/l). After incubation at 30 ˚C for 14 days, all colonies with different pigmentation and morphology were chosen for isolation. *Micromonospora* strain (MAG 9-7) appeared on Mineral Agar Guase 1 in orange-black colonies after 10-day incubation at 30°C of 100 µl aliquot inoculation on petri dish. After purification, the pure colonies were preserved in 20% glycerol at -80°C for further studies. Standard morphological property and chemotaxonomic analysis were proved, including molecularly identification by 16S rRNA gene sequencing.[16,17] Analysis of the DNA sequence revealed genomic features characteristic of *Micromonospora aurantiaca* (Accession no CP002162).

Crude Extract Preparation

Micromonospora aurantiaca was cultured in 1 L ISP2 broth medium and 105 rpm reciprocal shacked at 30°C for 10 days before extraction. The culture cells were separated from medium by centrifugation. Cells were extracted with methanol and ethyl acetate and medium was extracted with ethyl acetate. The solvent was evaporated off with vacuum evaporator and dry crude *Micromonospora aurantiaca* extract (MA extract) was kept in vial at -20°C.

Cell culture and viability assay

KB cell line, human carcinoma of the nasopharynx, was obtained from National Cancer Institute of Thailand. KB cells were cultured in RPMI 1640 medium supplemented with 10% inactivated fetal bovine serum, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin. Cell cultures were maintained in a humidified atmosphere of 95% air and 5% CO_2, 37°C.

Measurement of cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. This assay was based on cleavage of the tetrazolium salt by mitochondrial dehydrogenase of viable cells to formazan dye.[18] At least three separate experiments for each sample were used to determine the cell viability. In brief, cells in exponential growth phase were incubated with absolute ethanol (EtOH) (0.21%), MA extract (0-300 µg/ml), doxorubicin (DOX) (0-10 µg/ml) and MA extract + DOX for 48 h incubation. The MTT-formazan was measured at 570 nm with a microplate reader (Cecil Bioquest 2000 Series). Under these conditions, EtOH (0.21%) was not toxic and cell survival in vehicle control was assumed 100%. The percentage of cell viability was calculated relative to the vehicle control according to the following equation:

$$\% \text{ Cell viability} = \frac{\text{absorbance at 570 nm of treated cells}}{\text{absorbance at 570 nm of vehicle control cells}} \times 100$$

IC_{50} is the MA extract concentration under which 50% inhibition of cell proliferation occurred.

Nuclear staining with DAPI

KB cells were placed in the six-well plate attached with cover slide. Following various treatments, the

cells were washed with phosphate-buffered saline (PBS) and fixed with 2.5% glutaraldehyde. RNaseA (10 µg/ml) treatment was performed in the dark for 30 min at room temperature. The fixed cells were washed with PBS, and stained with 4,6-diamidino-2-phenylindole (DAPI) (5 µg/ml) for 10 min at room temperature. After unbound dye removal, cells were mounted on a glass slide with mounting solution (PBS:glycerol, 1:9), the edges sealed with nail polish and then cells visualized with fluorescence micro - scope (Olympus BX51) at 100x magnification. For each treatment group, approximately 500 different nuclei were counted in random microscopic fields. Data were expressed as percentage of nuclei in different phases.[19] At least three separate experiments for each sample were performed.

Agarose gel electrophoresis for DNA fragmentation assay

The GF-1 Tissue DNA Extraction Kit (Vivantis) was used according to the manufacturer's instructions. After treatment, floating and adherent cells were washed with PBS and then lysed with digestion buffer containing proteinase K (400 µg/ml) at 60°C. RNase A (10 µg/ml) was added and incubated for 10 min at 37°C. Genomic DNA was extracted with ice-cold absolute ethanol. High-purity genomic DNA is eluted in low salt buffers and has an absorbance 260/280 ratio between 1.7 and 1.9, making it ready to use. Equal amounts of DNA samples (300 ng) were mixed with SYBER Gold (0.1 mg/ml, 1 µl) and loading buffer and then loaded onto pre-solidified 1.5% agarose.[19] The agarose gels were run at 125 V for 30 min in TBE buffer. Gels were observed and photographed under transilluminator (Clare Chemical Research).

Flow cytometry analysis for measurement of sub-G1 phase

Each group of cells were harvested and washed once with cold PBS, fixed in 4% paraformaldehyde (cold) for 15 min and stored at 4°C. Prior to analysis, the cells were washed twice again with PBS, suspended in 1 ml of a cold propidium iodide (PI) solution containing RNase A (10 µg/ml), PI (50 µg/ml), EDTA disodium (0.1 mM), Triton X-100 (0.1%) (v/v) and further incubated on ice in the dark until analysis. Flow cytometry analysis was carried out using a flow cytometer (FACS Calibur, Becton Dickinson, USA). Data were analyzed by using CellQuest software (BD Biosciences, USA) which was used to determine the cellular DNA content based on the presence of PI-labeled cells.[20]

Measurement of Caspase-3 Activity

Caspase-3 activity was measured by a colorimetric assay kit (Clontech, Texas, USA) according to the manufacturer's instructions. Briefly, cell lysates from 4×10^6 cells in each group were prepared using the lysis buffer (50 µl) and then incubated on ice for 30 min. After centrifugation at 14,000 g, 4°C for 5 min, the supernatant (50 µl) was collected and mixed with 50 µl 2x reaction buffer and 5 µl caspase-3 substrate (DEVD-pNA).The samples were incubation at 37°C in the dark for 4 h, and were read on a spectrophotometer at a wavelength of 405 nm, which represented the intracellular activity of caspase-3. Data were expressed as fold increase on the control level.[21]

Data processing and statistical analysis

Data were expressed as mean ± standard error of the mean (S.E.M) from independent 3-4 experiments and analyzed with the software Microcal™ Origin 6. Statistical comparisons were performed using Student's *t*-test. A P value < 0.05 was considered statistically significant.

Results

Combined MA extract and DOX treatment inhibits viability in KB cells

KB cells were exposed to 0-300 µg/ml of MA extract for 48 h. The MA extract treatment significantly inhibited the cell viability in a concentration-dependent manner as determined using MTT assay. It was found that absolute ethanol (0.21%) was not toxic for cells. At 200 µg/ml of MA extract, the viability of KB cells was reduced to less than 15%. The IC_{50} values of MA extract and DOX alone were 83.46 ± 3.9 and 2.0 ± 0.1 µg/ml, respectively (Figure 1). Direct observation by inverted microscopy demonstrated that KB cells treated with MA extract or DOX exhibited diminished size, rounded and detached from the monolayer, with condensed cytoplasm accompanied by transforming cell into a cluster of membrane-bound bodies when compared with cuboidal and polygonal adherent cells in normal shape. In the combined treatment, the MA extract (0-100 µg/ml) + DOX (1 µg/ml) produced significantly greater cytotoxicity compared with that observed by treating cells with each agent individually (Figure 2). Overall, the IC_{50} value for combined treatment was 8.8 ± 1.2 µg/ml and it was approximately 10 folds lower than treating cells with MA extract alone.

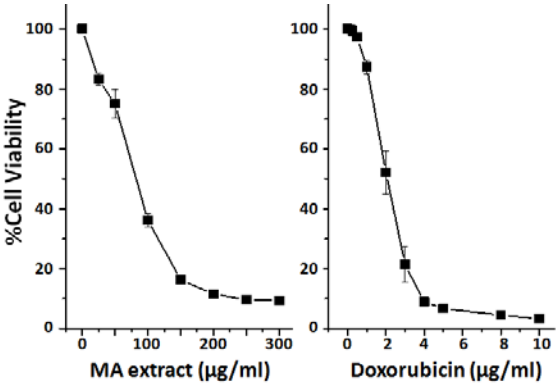

Figure 1 Effects of MA extract and doxorubicin on viability of KB cells. Cells were treated with MA extract (0-300 µg/ml) or doxorubicin (0-10 µg/ml) for 48 h. Viable cell number was measured with MTT assay. Control cells were treated with 0.21% EtOH. Data were expressed as mean ± SEM (n = 4).

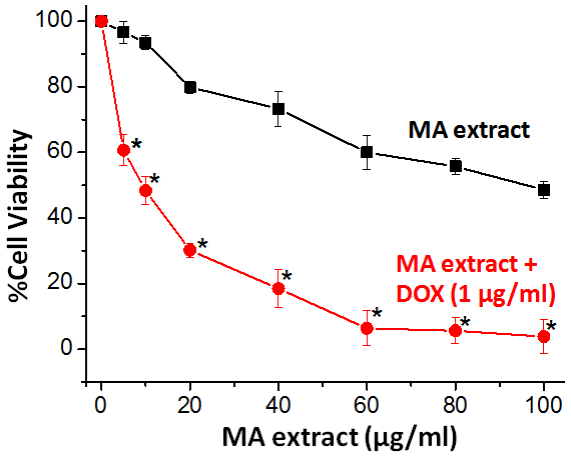

Figure 2 Effects of MA extract and doxorubicin in combination on viability of KB cells. Cells were treated with MA extract (0-100 μg/ml) alone (■) or MA extract (0-100 μg/ml) + doxorubicin (1 μg/ml) (●) for 48 h. Viable cell number was measured with MTT assay. The first data points in each line were control cells treated with 0.21% EtOH. Data were expressed as mean ± SEM (n = 4). The significance was determined by Student's *t*-test (*$P < 0.05$ *vs* MA extract).

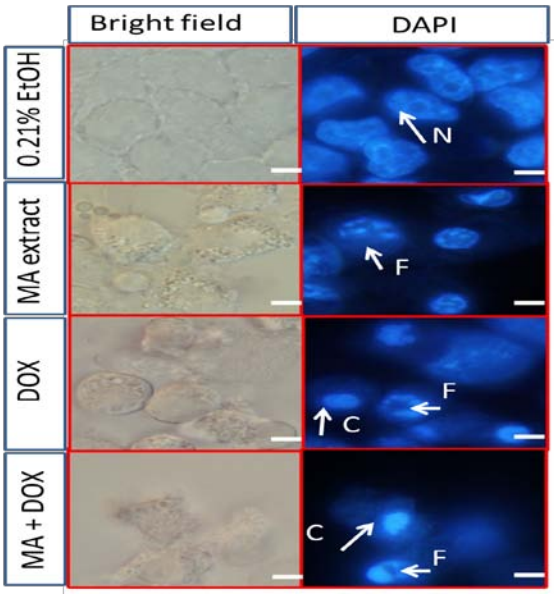

Figure 3 Correlation of surface morphologies with nuclear features. KB cells were treated with 0.21% EtOH, MA extract (50 μg/ml), doxorubicin (1 μg/ml) and MA extract (50 μg/ml) + doxorubicin (1 μg/ml) for 48 h, and then stained with DAPI. Cells were detected by bright field and blue filter (DAPI) at the same view point. N, normal nuclei; F, nuclear fragmentation; C, chromatin condensation. Data were expressed as mean ± SEM (n = 3). Scale bar is 10 μM.

Combined MA extract and DOX treatment enhances apoptosis in KB cells

To determine the mechanism of the cytotoxic effect of the combined treatment, KB cells with various treatments were carried out by using fluorescent microscopy, agarose gel electrophoresis, and flow cytometry analysis. Nucleic acids were stained with DAPI and observed under a fluorescence microscope. In the control group, the nuclei were homogeneously stained and the quantitative estimation of normal cells was 100%. The apoptotic nuclei showed fragmented in nuclei with chromatin condensation. When the KB cells were treated with MA extract (50 μg/ml) and DOX (1 μg/ml), the apoptotic nuclei were 24.27 ± 0.35 and 29.68 ± 3.49%, respectively. The sensitization effect of the combination treatment was significantly evident in the increasing apoptotic cell as 74.64 ± 6.19% (Figure 3).

As for the DNA purity, the ratio of wavelengths at 260/280 nm of most samples are in the 1.9 range, suggesting little protein contamination in a DNA sample. Agarose gel electrophoresis indicated that treatment with MA extract and DOX alone induced some fragmented DNA. DNA fragmentation induced by combined MA extract + DOX treatment was progressive accumulation of nucleosomal fragmentation and the intensity of smear-like DNA degradation was more prominent compared to solitary treatment (Figure 4). The flow cytometry analysis was used to determine the magnitude of apoptosis. KB cells treated with MA extract, DOX and MA extract + DOX were stained with propidium iodide. In normal cell cycle, the percentage of hypodiploid cells was 2.76% that revealed very little in sub-G1 phase. After treatment with MA extract or DOX alone, the hypodiploid cells in sub-G1 phase were increased to 13.13 and 10.51%, respectively. On the other hand,

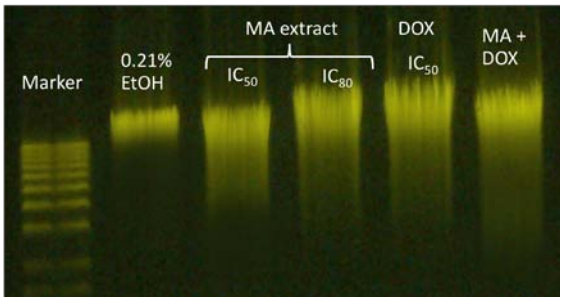

Figure 4 A photograph of the SYBER Gold-stained agarose gel. KB cells were treated with 0.21% EtOH, MA extract (IC_{50} and IC_{80}), doxorubicin (IC_{50}) and MA extract (50 μg/ml) + doxorubicin (1 μg/ml) for 48 h, and then DNA was extracted and electrophoresed. Similar results were obtained in three different experiments.

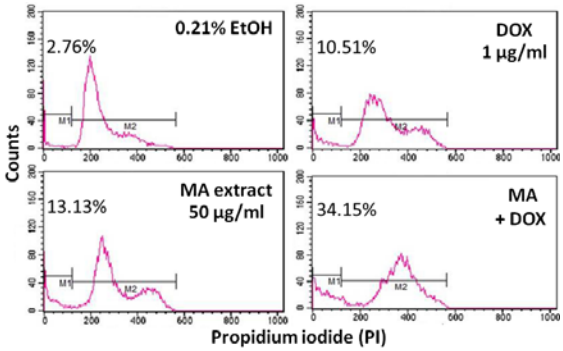

Figure 5 Histograms of flow cytometric analysis. KB cells were treated with 0.21% EtOH, MA extract (50 μg/ml), doxorubicin (1 μg/ml) and MA extract (50 μg/ml) + doxorubicin (1 μg/ml) for 48 h, and then stained with PI. M1 was the percentage of cells in sub-G1 phase. Similar results were obtained in three different experiments.

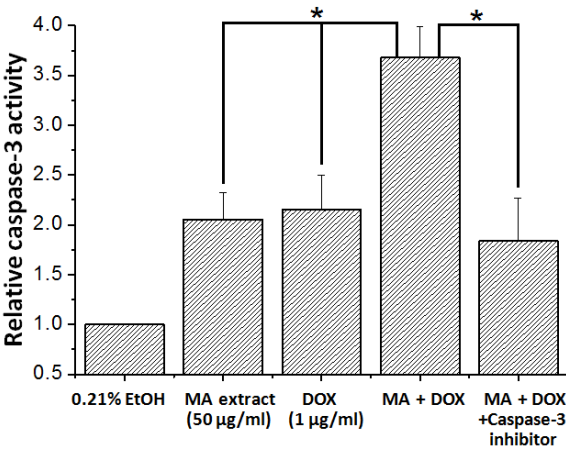

Figure 6 Relative caspases-3 activities in KB cells were measured by spectrophotometry. The control group (0.21% EtOH) was set as 1, and the values of other groups were standardized against it. Data were expressed as mean ± SEM (n = 3); *P < 0.05.

treatment with a combination of MA extract + DOX resulted in obvious increase in hypodiploid cells in sub-G1 phase to 34.15% (Figure 5).

Combined MA extract and DOX treatment enhances caspase-3 activity

To further evaluate the apoptotic pathway, the activities of caspase-3 were detected by colorimetric analysis. After treatment with MA extract and DOX alone, the relative activity of caspase-3 increased by 2.05 ± 0.07 and 2.15 ± 0.04 fold, respectively, when compared with the control. The relative activity of caspase-3 in combined treatment increased significantly by 3.67 ± 0.31 folds which was statistically different (P < 0.05) compared with non-combined treatment. z-DEVD-fmk (caspase-3 inhibitor) prevented the MA extract-induced caspase-3 activation (Figure 6). For inhibited apoptosis samples, z-DEVD-fmk was added to the cells at a final concentration of 20 µM. Co-treatment of cells with MA extract and different doses of z-DEVD-fmk (10, 20 and 50 µM) strongly inhibited the MA extract-induced apoptosis. The lower doses of the inhibitor showed almost similar effect.

Discussion

The marine microorganisms, particularly *Micromonospora*, have been proven to be a rich source for the discovery of novel secondary metabolites. Although collecting soil and marine sediments are relatively inexpensive, their pharmacological potential has not yet to be fully explored. With growing and intense interest, *Micromonospora* is being investigated for the discovery of new bioactive compounds that have no terrestrial equivalents.[2-4,7] During cancer progression, cancer cells are conferred with a capacity to proliferate independently and produce their own growth factors, which can make growth factor receptors more responsive to stimulation. Thus, the anti-proliferative effect of natural products produced by microorganisms on cancer cells is one of the mechanistic ways in chemoprevention and chemotherapy.

In the present study, *Micromonospora aurantiaca* was collected from coastal sediments on the east coast of the Gulf of Thailand and extracted with methanol and ethyl acetate to be dry crude extract. The MA extract showed a moderate cytotoxicity (IC_{50} = 83.46 ± 3.9 µg/ml) against KB cells. These results did not meet the criterion set by the National Cancer Institute (NCI) for the crude extract in that the acceptable IC_{50} value is lower than 30 µg/ml.[22] Further, the MA extract is about 42-fold less toxic than DOX. However, MA extract in combination with DOX caused 10-fold enhanced cell death at concentrations that each agent alone is poorly effective. Furthermore, the enhanced cytotoxicity of the combined treatment may result from augmentation of DOX-induced apoptosis by MA extract.

The induction of apoptosis in cancer cells is one of the advantageous strategies for anticancer drug development and many studies were performed for screening of apoptosis-inducing compounds from marine actinomycetes.[1,4,5,7] The search of novel substances with considerable potential for chemosensitization was ideally suited for enhancing the cytotoxic activity of chemotherapeutics. In the present study, the MA extract in combination with DOX at each sub-cytotoxic concentration exhibits the morphological and biochemical changes that characterize apoptosis *in vitro*. The combined treatment significantly increased apoptosis as shown by loss of cell viability (Figure 2), chromatin condensation (Figure 3), DNA fragmentation (Figure 4) sub-G1 phase accumulation (Figure 5) and relative caspase-3 activity (Figure 6) as compared with single treatment. Because no phagocytic cells were presented *in vitro*, the apoptotic cells eventually reached a late stage similar to necrosis. At any time point, the cell asynchrony would demonstrate a mixture of apoptosis events together with necrosis or late apoptosis. Smearing in agarose gels electrophoresis in late apoptotic cells can be caused by the enzyme that function improperly and characterized by random DNA fragmentation. Based on these results, the mechanisms of such enhancement seem to be related to caspase-3 dependent cell death pathway in KB cell line.

In the finding of others studies, *Micromonospora* has been intensively investigated and also produced several active secondary metabolites, such as anthraquinones,[23] anthracyclines,[13] alkaloids,[24] and macrolides[12]. The macrolide, a spiroketal lactone structure produced by *Micromonospora* sp, showed cytotoxic activity against mouse leukemia P-388 and human lung non-small cell A-549, colon adenocarcinoma HT-29 and melanoma MEL-28 cell lines.[25] The aminoquinone antibiotic streptonigrin and its novel derivative from an actinomycete strain,

Micromonospora sp. IM 2670 induced apoptosis through a p53-dependent pathway in human neuroblastoma SH-SY5Y cells as the model system.[14] Four new anthracyclinones were isolated from a strain of *Micromonospora*. Two compounds were cytotoxic against the HCT-8 human colon adenocarcinoma cell line, with IC_{50} values of 12.7 and 6.2 μM, respectively, while the other two compounds were inactive.[13] Additionally, doxorubicin belonging to anthracyclines caused DNA damage as a result of topoisomerase II poisoning that inhibits cancer cells mainly by apoptosis.[26]

Thus, the use of combinations may provide a matter of great interest for improving the anticancer activity of DOX because it allows lower and safer doses of each compound. Natural compounds are ideally suited for this application because they allow a variety of large combinations to be used safely. The different components in the MA extract may have synergistic activities and may offer greater therapeutic or preventive activity when used in combination. A well-designed combination of compounds has attracted special attention for further studies. Taken together with this present study, the elucidation of other apoptosis-related intracellular targets will also be addressed in future studies. However, cancer is a multifactorial disease; it demands multimodal therapeutic approaches. Recent studies indicated that marine microorganisms are economically and biotechnologically attractive in that many are not general cytotoxic agents but rather display a broad spectrum of biological activities including anti-microbial, anti-fungal, anti-inflammatory and immunosuppressive agents.[6] So, the combinations or synergistic interactions provide one other important advantage. In this study, the active compounds in MA extract actually are cytotoxic agents but they may present in a low amount. Further studies to identify the chemical structures are necessary. MA extract would represent a promising source for its potential for the discovery of interesting anticancer compounds.

Conclusion

In summary, this study demonstrates that the methanol and ethyl acetate extraction from marine *Micromonospora aurantiaca* significantly enhanced human carcinoma of the nasopharynx (KB cell) to apoptosis induced by DNA topoisomerase II poisoning (doxorubicin). This apoptotic response was associated with caspase-3 activation. Thus, the combination may provide a more effective treatment for nasopharyngeal carcinoma. The MA extract would represent a promising source the discovery of interesting anticancer compounds. A well-designed combination of compounds and the apoptosis-related others intracellular targets will be addressed in future studies.

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Conflict of interest

None to declare.

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