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Partially Purified Pigment Extract from *Streptomyces* A 16-1 Induces Apoptosis of Human Carcinoma of Nasopharynx Cell (KB cells) via the Mitochondrial and Caspase-3 Pathway

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Abstract

The genus *Streptomycetes* are very important bacteria which produce secondary metabolites used in clinical cancer therapy and other pharmacological activities. *Streptomyces* A 16-1 was isolated from coastal soil in Chonburi, Thailand. The objective of this study is to examine the apoptotic effects and mechanisms of the partially purified pigment extract from *Streptomyces* A 16-1 against human carcinoma of nasopharynx cell (KB cells). The methanol and ethyl acetate extract from the fermentation of ISP2 broth was fractionated by using column chromatography with stationery-phase silica-gel and mobile phase chloroform: methanol mixtures with increasing polarity. Among 9 fractions (Fr), the fractions which contained red pigment were selected and the calculated IC_{50} values for Fr 5, Fr 6 and Fr 7 were 0.04 ± 0.005, 0.20 ± 0.02, and 0.55 ± 0.05 μg/ml, respectively. The molecular mechanisms of cell death were associated with DNA fragmentation, accumulation of cells in sub-G1 phase, mitochondrial membrane depolarization, and activation of caspase-3, which was significantly diminished by the caspase-3 inhibitor. These first findings demonstrate that the pro-apoptotic effects of these fractions were mediated through the mitochondrial and caspase-3 pathway.

Keywords: Apoptosis, *Streptomyces* A 16-1, DNA fragmentation, mitochondria, caspase-3

Introduction

The search for new bioactive secondary metabolites produced by microorganisms is urgently needed in order to have cancer treatment without undesirable side effects. Marine actinomycetes, filamentous gram-positive bacteria, are unlimited sources of unique bioactive secondary metabolites, which are currently used in human medicine. Actinomycetes are extensively distributed in different marine ecosystems and have evolved considerable genomic and metabolic diversities. Among actinomycetes, the genus *Streptomyces* has long been known to be a prolific producer of a wide range of bioactive metabolites, such as anti-bacterial [1-3], anti-inflammation [3], and anti-cancer [2-4]. *Streptomyces* species were assigned to a 7 color series: blue, gray, green, red, violet, white and yellow. The color of the substrate mycelium and the soluble pigment are of the greatest value in category grouping, and oftentimes show interesting bioactive activities [5].

The well-known antibiotic pigments produced from *Streptomyces* are anthracyclin glycoside, diazaindophenol, naphthoquinone, phenizaquinone, and prodigiosins [5]. Moreover, *Streptomyces* have been found to produce compounds used in anticancer medicines, such as anthracyclines (aclarubicin,

daunomycin, and doxorubicin), peptides (bleomycin and actinomycin D), aureolic acids (mithramycin), enediynes (neocarzinostatin), antimetabolites (pentostatin), carzinophilin, mitomycins, and a number of indolocarbazoles [6]. By way of this work, a new anthracene derivative, 3-hydroxy-1-keto-3-methyl-8-methoxy-1,2,3,4-tetrahydro-benz[α]anthracene, was isolated from the marine *Streptomyces* sp. W007, and this compound exhibited cytotoxicity against the human lung adenocarcinoma cell line A549 [7]. In addition, a new angucycline derivative, kiamycin, was isolated from the marine *Streptomyces* sp. M268, and this compound was found to be equally cytotoxic to leukemia HL-60 as adriamycin [8].

The crude ethyl acetate extract of *Streptomyces avidinii* strain SU4 was toxic to the Hep-2 cell line and less cytotoxic to the normal cell line, with IC_{50} values of 64.5 µg/ml and 250 µg/ml, respectively. The analysis of the crude extract showed the presence of 1,2-benzenedicarboxylic acid, bis (2-methylpropyl) ester and isooctyl phthalate, which were responsible for the anticancer activity [4]. Furthermore, marine *Streptomyces* have also produced a wide range of pigmented cytotoxic compounds. For example, streptochlorin is a yellowish crystalline molecule isolated from marine *Streptomyces* sp. (strain 04DH110). Streptochlorin induces apoptotic effects via mitochondrial membrane potential collapse and the subsequent activation of caspase-3 in human leukemic U937 cells [9] and human hepatocarcinoma Hep3B [10]. The red pigment from *Streptomyces* sp. PM4 showed cytotoxicity on cervical (HeLa), laryngeal (HEp-2), fibrosarcoma (HT1080), and breast (MCF-7) cancer cell lines [11].The natural red pigments called prodigiosins, produced by *Serratia marcescens*, showed cytotoxic activity against HeLa cells, with an IC_{50} value of 35µg/ml in 48 h [12].

The effectiveness of cancer therapy at present is limited, due to the development of resistance to multiple chemotherapeutic drugs and adverse side-effects. Cancer is an extremely complex disease, given its variety of molecular mechanisms to escape apoptosis, or "programmed cell death" [13]. To overcome these limitations, the search for natural bioactive compounds that can induce apoptosis has become a common aim in the development of anti-cancer drugs, because these compounds may offer more effective and safer therapeutic strategies for cancer treatment. Many chemotherapy drugs and natural active secondary metabolites that induce apoptosis are often found to target the death receptor or mitochondria, but both of these pathways promote the activation of caspase-3 [14]. To deal with the demand for new active secondary metabolites produced by microorganisms, researchers have been motivated to look for novel *Streptomyces* in marine ecosystems. The coastal soils in the east coast of the Gulf of Thailand are known to have prominently productive ecosystems, and *Streptomyces* A16-1 was first isolated from coastal soil in Chonburi Province, Thailand. The objective of this study is to investigate the *in vitro* anticancer and apoptosis signaling pathways induced by the partially purified pigment extract from *Streptomyces* A 16-1 in human carcinoma of nasopharyngeal cells (KB cells).

Materials and methods

Sampling and isolation

Streptomyces A16-1, which produces a red pigment, was isolated from tropical coastal soil from Chonburi Province, Thailand on starch casein agar. The soil sample was pretreated at 55 °C for 30 min before being spread on the agar medium and incubated at 32 °C for a week. The colony was purified on the same medium after appearing on the isolation plate. The isolated *Streptomyces* A16-1 was kept at room temperature (25 °C) for further studies, and then preserved in 20 % glycerol at –40 °C in the Culture Collection of the Institute of Marine Science, Burapha University.

Culture characteristics and morphological study

Culture characteristics such as growth patterns, aerial mycelium, substrate mycelium and pigments were observed on actinomycete isolation agar (AIA), yeast extract-malt extract agar (ISP2), oatmeal agar (ISP3), inorganic salt starch agar (ISP4), glycerol asparagines agar (ISP5), and starch casein agar (SCA), after being cultured for 7 days at 30 °C. The morphological characteristics of *Streptomyces* A16-1 were examined by light microscopy and scanning electron microscopy (SEM) (Leo-1450VP). Aerial mycelium, substrate mycelium, and spore chain types were observed coarsely under a light microscope. Spore morphology and other ultra-structures were examined under SEM. Samples were fixed with a 2.5

% glutaraldehyde buffer solution, dehydrated through an acetone series, and finally critical point dried. Before observation, samples were gold coated.

Molecular identification

Chromosomal DNA of *Streptomyces* A16-1 was prepared by DNA Trap Kit (Cat No 100-10009, BIOTEC, Thailand). PCR amplification of 16S rRNA gene was performed by Hot Star Taq Master Mix Kit protocol (Qiagen, Germany) in a final volume of 50 µl containing 10 µl of 10x PCR buffer, 25 mM $MgCl_2$, 2 µl dNTP (10 mM), 2 µl 9F (5'-GACTTTGATCCTGGCTCAG-3'), 2 µl 1510R (5'-GGCTACCTTGTTACGA-3'), and distilled water. Direct sequencing of the purified PCR product was carried out with an autosequencer (ABI PRISM model 377). The resultant 16 rRNA gene sequences were aligned and compared with data base sequences in GenBank using BLAST tool (http://eztaxon-e.ezbiocloud.net/ezt_identify).

The ability of *Streptomyces* A16-1 to utilize different sole carbon sources (L-arabinose, D-fructose, Meso-inositol, D-mannitol, D-raffinose, L-rhamnose, D-sucrose, and D-xylose) was examined in ISP9 medium (Basal mineral salt agar medium), as described by Shirling and Gottlieb [15]. Results were observed after 14 days incubation at 30 °C, and growth of the isolate was described as positive.

Extraction and partial purification of crude bioactive compounds

Streptomyces A16-1 was cultured in ISP2 broth, and reciprocally shaken at 100 rpm, at 30 °C for 10 days. Cells and medium were separated by centrifugation before extraction with methanol and ethyl acetate, respectively. The crude product was first separated by thin layer chromatography on a silica gel plate (Merck) to investigate roughly how many components were mixed. Partial purification of the crude product (150 mg) was continued through silica gel (70 - 230 mesh) column (1.8×30 cm) chromatography, eluted by chloroform: methanol mixtures with increasing polarity at ratios of 100:0, 95:5, 90:10, 80:20, 70:30, 50:50, and 0:100, respectively. Fractions of 50 ml each were collected, and solvents were then concentrated by evaporation. Fractions which contained a red pigment were selected to be investigated for cytotoxicity and apoptosis against KB cells.

Primary fresh cultures of peripheral blood mononuclear cells (PBMCs)

Human PBMCs were obtained from healthy individuals. The study was approved by the Ethical Committee of Burapha University, according to document number 85/2556, and informed consent was obtained from all subjects. Immediately after venipuncture into heparinized tubes, PBMCs were separated from whole blood using a Ficoll-isopaque gradient (1.077) that separates layers of blood. The buffy coat under the layer of plasma was aspirated, and the cells were washed 3 times in PBS [16]. PBMCs were cultured at 37 °C in RPMI 1640 medium, supplemented with 10 % (v/v) fetal bovine serum (FBS), 100 µg/ml streptomycin, and 100 units/ml penicillin, under an atmosphere of 5 % CO_2.

Cell culture and maintenance

KB cells were obtained from the National Center Institute (Bangkok, Thailand). They were grown in RPMI 1640 containing 10 % (v/v) heat-inactivated fetal bovine serum, 100 µg/ml streptomycin, and 100 units/ml penicillin. KB cells were cultured and incubated in a humidified atmosphere with 5 % CO_2, 95 % air at 37 °C. Cells at the exponential stage were used for all experiments and subcultured every 2 - 3 days. All experiments were done within < 20 passages.

Cytotoxic assay

Cell proliferation upon treatment with the test compounds was measured by MTT (1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan) assay according to the manufacturer's instructions (Sigma Chemical Co., USA). KB cells and PBMCs were seeded at 5×10^4 cells/well into a 96-well plate. Test compounds were dissolved in absolute ethanol (EtOH) and were serial diluted at appropriate concentrations with a cultivation medium. After incubation for 24 h, cells were treated with different concentrations (0 - 8 µg/ml) of test compounds and doxorubicin (positive control) for 48 h incubation.

The negative control was 0.04 % EtOH in a cell culture medium that was expected to cause, or not interfere with, normal cell growth. After incubation for 48 h, MTT solution (0.5 mg/ml) was added to each well, followed by further incubation for 4 h at 37 °C. The products of MTT cleavage, insoluble formazan crystals, were dissolved in dimethyl sulfoxide (80 µl/well), and then the optical density was read with an ELISA reader (Cecil Bioquest 2000 Series) at 570 nm. The relative cell viability (%) related to control was calculated using the following equation;

$$\text{Percentage of cell viability} = \frac{\text{OD (570 nm) of treated cells}}{\text{OD (570 nm) of control cells}} \times 100 \quad (1)$$

The IC_{50} value was calculated as the concentration of the test compound which inhibited 50 % of cell growth compared with the untreated control cells [17]. Data were presented as the mean ± SEM of 3 independent experiments.

Agarose gel electrophoresis for DNA fragmentation assay

After incubation with IC_{50} concentrations of test compounds for 48 h, the KB cells were collected and the genomic DNA was extracted via the GF-1 Tissue DNA Extraction Kit (Vivantis, Poland) according to the manufacturer's instructions. Briefly, KB cells were lysed in a lysis buffer containing proteinase K (400 µg/ml) for 10 min at 60 °C, and subsequently with RNase A (10 µg/ml) for 10 min at 37 °C. Genomic DNA was extracted and precipitated in ice-cold absolute ethanol. DNA concentration was determined spectrophotometrically by measuring the absorbance at 260 nm (A260). High-purity genomic DNA had an A260/A280 ratio of 1.8 - 2.0. Thereafter, the same amount of DNA (80 ng/band) was mixed with the loading dye, containing SYBER Gold (Invitrogen, USA), and then loaded onto pre-solidified 1 % agarose. DNA samples were electrophoresed at 125 V for 30 min in TBE buffer [17]. Gels from 3 independent experiments were photographed under a UV transilluminator (Clare Chemical Research).

Nuclear staining with DAPI for nuclear morphology

KB cells were grown in an 8-well slide chamber at a density of 5×10^4 cells/well, and then incubated with IC_{50} concentrations of test compounds for 48 h. After the incubation, KB cells were washed twice with PBS and fixed with 4 % paraformaldehyde for 10 min at room temperature. Next, the fixed cells were washed in PBS and stained with 5 µg/ml DAPI (4,6-diamidino-2-phenylindole) (Invitrogen, USA) for 10 min in the dark. After removing unbound dye, the samples were prepared with a mounting solution (1:9; PBS: glycerol), and the nuclear morphology of cells were examined using fluorescence microscopy (BX51TR, Olympus, Tokyo, Japan) at 100 × magnification with a DAPI filter. At least 5 fields of view were randomly selected for image capture. Approximately 200 different nuclei were counted for each treatment condition [17]. The data were expressed as percentages of apoptotic nuclei from at least 3 independent experiments.

Cell cycle analysis by flow cytometry

KB cells were grown in 6-well plates at a density of 1×10^5 cells/well, and then incubated with IC_{50} concentrations of test compounds for 48 h. Both floating and adherent cells were harvested and fixed in 4 % cold paraformaldehyde at room temperature for 15 min. Cells were washed twice with ice-cold PBS and stained with the fluorescent probe solution containing 0.1 % Triton X-100, 0.1 mM EDTA disodium, 10 µg/ml RNase A, and 10 µg/ml propidium iodide (PI) (Invitrogen, USA) for 30 min at room temperature. Approximately 10,000 cells from each sample were then analyzed for PI-DNA complex and cell cycle phase using flow cytometry (Becton Dickinson, BD LSR II) with an excitation at 488 nm. DNA histograms were determined by BD FACSDiva Software. Cells in the sub-G1 phase were compared with that of the control [17]. The flow cytometry analysis was executed 3 times.

Mitochondria membrane potential (ΔΨm) assay

KB cells were treated with IC_{50} concentrations of test compounds for 48 h. At the end of the treatment period, KB cells were detached by trypsinization, followed by centrifugation (400 × g for 5 minutes at room temperature) and resuspension in PBS (0.5 ml). The membrane-permeable lipophilic cationic probe JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide) was utilized following the manufacturer's protocol (Invitrogen, UK). KB cells were incubated with 5 µg/mL of JC-1 at 37 °C for 15 min in a humidified 5 % CO_2 incubator, and then washed twice with PBS. Approximately 10,000 cells from each sample were analyzed by flow cytometry (Becton Dickinson, BD LSR II) with an excitation at 488 nm. The emission peaks of JC-1 monomer and JC-1 aggregate forms could be detected separately in FL1 and FL2 channels, respectively. The populations between JC-1 aggregate/JC-1 monomers were gated, and the percentages of cells were calculated using the BD FACSDiva Software [9]. The flow cytometry analysis was executed 3 times.

Caspase-3 activation assay

After treatment with IC_{50} concentrations of test compounds, caspase-3 activities in cell lysates were assayed using a colorimetric assay kit (Clontech, Texas, USA) based on the manufacturer's protocol. Briefly, KB cells in each group were suspended in 50 µl of the lysis buffer, kept on ice for 30 min, and then centrifuged (14,000 × g, 5 min at 4 °C). The supernatants containing equal amounts of protein (50 µl) were mixed with 2× reaction buffer (50 µl) and caspase-3 substrate (DEVD-pNA) (5 µl). The caspase-3 inhibitor (DEVD-fmk) was used as a negative control. The samples were incubated at 37 °C in the dark for 4 h. Then, the concentration of chromophore, p-nitroanilide (pNA) cleaved from the substrate, was read on a spectrophotometer at 405 nm, which represented the intra-cellular activity of caspase-3 [17]. Data were expressed as fold increase in protease activity compared with the control level from 3 independent experiments.

Data processing and statistical analysis

The data were presented as the mean ± standard error of the mean (SEM) in at least 3 independent experiments and analyzed with the software Microcal TM Origin 6. Statistical comparisons between control and treated groups were performed using a student t-test. A P-value < 0.05 was considered to be statistically significant.

Results and discussion

Morphology and molecular studies

The culture characteristic study revealed that *Streptomyces* A16-1 exhibited good growth on the ISP 2 and ISP 3 media, moderate growth on the AIA and SCA media, and poor growth on the ISP 4 and ISP 5 media. This strain produced diffusible pigment on all of the media tested. The reddest pigment was produced on ISP2 medium (**Table 1**). In this study, *Streptomyces* A16-1 was subjected to standard morphological and biochemical tests. Data from the morphological study revealed that *Streptomyces* A16-1 produced red pigment and a whitish-gray spore mass with some characteristics typical of the genus *Streptomyces*. Scanning electron micrograph observation showed long flexuous (rectiflexibiles) spore chains, which contained more than 20 spores per chain. Spores were spherical in shape, exhibited a spiny surface, and were 0.6 - 0.9 µm in diameter. No spirals, hooks, verticils, or sporangia were observed (**Figure 1**).

Table 1 Culture characteristics of *Streptomyces* A16-1 on different media.

Medium	Growth	Aerial mycelium	Substrate mycelium
AIA	Moderate	Pale-purple	Purple
ISP2	Good	Red	Dark red
ISP3	Good	Whitish-pink	Pink
ISP4	Poor	Pale pink	Pink
ISP5	Poor	Pale pink	Pink
SCA	Moderate	Pinkish-orange	Pinkish-orange

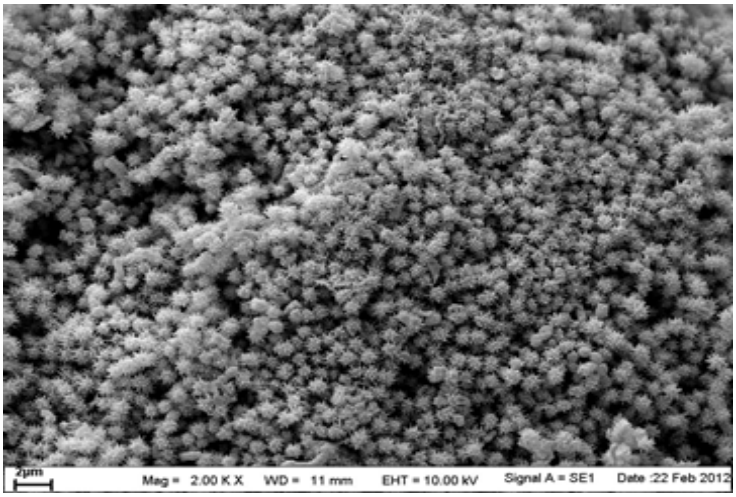

Figure 1 Scanning electron micrograph of *Streptomyces* A16-1 grown on ISP2 medium for 7 days at 30 °C, showing rectiflexibile spore chains. The spores exhibited a spiny surface and were spherical in shape. Scale bar is 2 µm.

Molecular identification based on the nucleotide sequence of 16S rRNA gene revealed that *Streptomyces* A16-1 was 99.1 % homologous and closest to *Streptomyces indiaensis*. *Streptomyces* A16-1 utilized L-arabinose, Meso-inositol, D-mannitol, D-raffinose, D-sucrose, and D-xylose as sole carbon sources, but not D-fructose or L-rhamnose. Utilization of compounds as sole sources of carbon by *Streptomyces* A16-1 and comparison of some other morphological properties showed that *Streptomyces* A16-1 was much different from *S. indiaensis*, although both the strains showed the closest match on the basis of 16S rRNA gene base sequences (**Table 2**). The results of biochemical and morphological characteristics, including the 16S rRNA gene sequence of *Streptomyces* A16-1, strongly indicated that it was not *S. indiaensis*, and could possibly have been a new species. Therefore, the phylogenetic classification of *Streptomyces* A16-1 has to be determined by additional analysis in the future.

Table 2 Some phenotypic properties of *Streptomyces* A16-1 compared with those of related species [5].

Characteristics	***S.* A16-1**	***S. echinoruber***	***S. indiaensis***	***S. massasporeus***
Aerial spore mass	Gray	Gray	Gray	-
Spore surface	Spiny	Smooth	Smooth	-
Reverse substrate	Red	Rose-brown	Red	-
Spore chain	Long/flexuous	Retinaculiaperti	Spirales	Spirales
Diffusible pigment	Red	Red	Red	Red
Carbon utilization (1% w/v)				
L-arabinose	w/+	+	+	NA
D-fructose	w/ -	+	+	NA
Meso-inositol	+	-	+	+
D-mannitol	w/+	+	+	+
D-raffinose	w/+	-	-	+
L-rhamnose	-	-	+	+
D-sucrose	+	+	+	+
D-xylose	w/+	+	-	NA

Note: w = weak; + = positive; - = negative; NA = not applicable

It appeared that the crude extract from both the cells and culture supernatant of *Streptomyces* A16-1 exhibited almost similar patterns of compounds on the chromatogram, so they were combined for purification. Large scale fermentation was carried out to obtain a sufficient quantity of crude extract for the partial purification of the bioactive compound, and the % yield of the crude extract was 0.3900 g/l in the ISP2 medium. Partial purification of the crude product on a silica gel column gave individual fractions and, based on TLC, similar profiles were combined and gave 9 fractions. The extracts which were partial purification combined to Fr 5, Fr 6, and Fr 7 were obtained from eluted solvents, chloroform: methanol, 95:5; 90:10, and 70:30, respectively. However, the production of any anticancer substance is highly dependent on the fermentation conditions; therefore, it is very essential to develop protocols in the scaling-up process to enhance growth and pigment production [11].

Cytotoxic activity and DNA fragmentation

In preliminary studies, KB cells were treated with different concentrations of test compounds for 24 and 48 h, and significant cytotoxic effect at 48 h was observed. The longer the incubation time, the lower the concentration required to destroy or induce apoptosis in KB cells. The MTT assay results demonstrated that Fr 5, Fr 6, and Fr 7 exhibited cytotoxic activity *in vitro* against the KB cell line, with IC_{50} values of 0.04 ± 0.005, 0.20 ± 0.02, and 0.55 ± 0.05µg/ml, respectively. KB cell growth was inhibited in a dose-dependent manner, with an almost complete inhibition at 4 µg/ml in all test compounds (**Figure 2a**). However, Fr 5 exhibited the lowest IC_{50} value, which might be due to the strong synergistic effect of the active compounds in the fraction. These IC_{50} values showed potent cytotoxic effects, as the criteria established by the American National Cancer Institute (NCI) for cytotoxic activity of chemical agents and natural products against animal tumors was IC_{50} < 30 µg/mL [18]. In addition, the IC_{50} values of Fr 5, Fr 6 and Fr 7 were even lower than that of DOX (1.35±0.23 µg/ml), which has been well-documented in clinical studies since NCI approval in 1974 [19]. However, the effectiveness of DOX is sometimes limited by the generation of drug resistance, as well as by cardiotoxicity [20]. Conversely, the same concentrations of the test compounds produced little cytotoxic effect on PBMCs obtained from healthy donors. The viability of PBMCs remained above 80 % at a concentration of 4 µg/ml (data not shown); nevertheless, the obvious cytotoxic effect of DOX was observed.

It is significant that these partially purified pigment fractions contain bioactive ingredients that are specific to KB cancer cells. These results are in agreement with the previous statement that the crude ethyl acetate extracts of *Streptomyces avidinii* strain SU4 exhibited an IC_{50}value of 64.5 µg/ml against the

Hep-2 cell line, and were less cytotoxic to normal cell lines [4]. The flavonoid type isolated from *Streptomyces* sp. (ERINLG-4) showed cytotoxic activity, with an IC_{50} value of 82 µM against the A549 lung cancer cell line, but it showed no toxicity up to 2000 µg/ml in Vero cells (normal cells) [21]. The F3-2-5, which was isolated from a *Streptomyces* sp., had an anti-proliferative effect on various human cancer cells, but not on normal lymphocytes or normal fibroblasts [22]. The above results suggested that the compounds extracted from *Streptomyces* exhibited high cytotoxic activity against various cancer cell lines and less cytotoxicity in normal cell lines.

Similarly, a red pigment isolated from *Streptomyces* sp. PM4 showed potential anticancer activity against cervical (HeLa), laryngeal (HEp-2), fibrosarcoma (HT1080), and breast (MCF-7) cancer cell lines, with IC_{50} values of 18.5, 15.3, 9.6, and 8.5 µg/ml, respectively [11]. Two compounds, 8-O-methyltetrangulol and naphthomycin, isolated from the ethyl acetate extract of *Streptomyces* sp. nov. WH26 via silica gel column chromatography and HPLC, demonstrated potent cytotoxic activity against several human tumor cell lines, including A549, HeLa, BEL-7402, and HT-29 [23]. The ethyl acetate extracts of *Streptomyces* galbus ERINLG-127 was subjected to activity-guided fractionation by column chromatography over silica gel. The compound showed prominent cytotoxic activity *in vitro* against the A549 lung adenocarcinoma cancer cell line, with an IC_{50} value of 60 µg/ml [2]. In these results, the highest cytotoxic activity recorded in Fr 5 may be due to the strong nonpolar nature of the eluted compounds. In various fractions, the difference in activity might be due to the presence of different components that can exert synergistic effects of compounds within themselves. The cytotoxic activities were found in descending order from non-polar to polar, so the type of solvent serves as a guideline for the isolation of active components responsible for their activities.

To understand the underlying mechanism of cytotoxicity, a molecular method used to analyze the ability of genomic DNA to move through a gel based on their size was investigated, using agarose gel electrophoresis. When KB cells were treated with Fr 5, Fr 6, Fr 7, and DOX (0.04, 0.20, 0.55, 1.35 µg/mL, 48 h), the smaller DNA migrated faster than the bigger DNA, indicating DNA fragmentation and signs of apoptosis. In the control group, the DNA band did not diffuse when subjected to an electric field, which indicated no evidence of DNA fragmentation. During programmed cell death, or apoptosis, fragmentation of chromatin into units of single or multiple nucleosomes is specific, and the nucleosomal DNA ladder was clearly visible in agarose gels [13]. As shown in **Figure 2b**, the DNA laddering was diffused and interposed with smear, suggesting that some late apoptotic or necrotic cells caused random nucleosomal DNA fragmentation [24]. Cells in culture have no phagocytic cells to destroy apoptotic cells, so DNA fragmentation due to both apoptosis and necrosis was considered in this study.

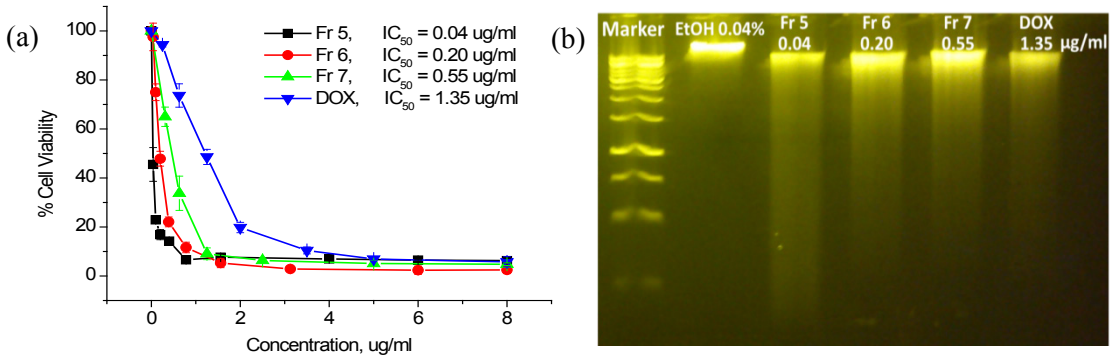

Figure 2 (a) Cytotoxic effects of Fr 5, Fr 6, Fr 7, and DOX on viability of KB cells with MTT assay. Data were expressed as mean ± SEM of n = 3. (b) A photograph of the SYBER Gold-stained DNA and electrophoresed agarose gel. Similar results were obtained in 3 different experiments.

Nuclei stained with DAPI were UV-excited for observation of nuclear morphology under fluorescence microscopy. The nuclei of untreated KB cells showed round morphology of intact nuclei, whereas the treatment with Fr 5, Fr 6, Fr 7, and DOX (0.04, 0.20, 0.55, 1.35 µg/ml, 48 h) displayed typical features of apoptosis, such as chromatin condensation and nuclear fragmentation into granular particles or apoptotic bodies (**Figure 4a; DAPI**). In the 0.04 % EtOH with absence of test compounds, the quantitative estimation of normal cells was 100 %, whereas the apoptotic nuclei in cells treated with Fr 5, Fr 6, Fr 7, and DOX were 43.75 ± 1.14, 38.51 ± 1.20, 36.65 ± 1.82, and 42.21 ± 1.20 %, respectively. Consistent with the results of genomic DNA electrophoresis, the nuclear changes seemed to be due to upregulation of Caspase-Activated DNase (CAD), which cleaves DNA into oligonucleosomal fragments and changes chromatin structure [25]. The observation by microscopy demonstrated that KB cells treated with Fr 5, Fr 6, Fr 7, and DOX showed many morphological alterations, including cell shrinkage, rounding, poor adherence, and cytoplasmic blebs (**Figure 4a; Bright field**).

To analyze cellular DNA content, DNA was labeled with a nucleic acid dye,, PI, that binds to DNA by intercalating between the base pairs of DNA in a solution containing RNase A. KB cells were treated with test samples at a concentration of IC_{50}. As shown in **Figure 4b**, the number of KB cells in the sub-G1 phase (hypodiploid cells) was markedly increased, suggesting the appearance of apoptotic DNA. The percentages of sub-G1 cells were 1.1, 17.5, 19.6, 20.3, and 23.2 % after 48 h of incubation with EtOH, Fr 5, Fr 6, Fr 7, and DOX, respectively. Sub-G1 cell populations after treating KB cells with test samples were significantly different from that of the EtOH-treated control ($P < 0.01$). The representative histogram was presented from 3 individual experiments. These results indicated that KB cells were partially blocked at the G1 phase of the cell cycle in accordance with the results from apoptotic DNA fragmentation analysis. Similarly, doxorubicin is well known to arrest cell growth by increasing the levels of cell cycle inhibitors p53 and p21 and reducing the levels of cell cycle accelerators CDK (cyclin dependent kinase) [26].

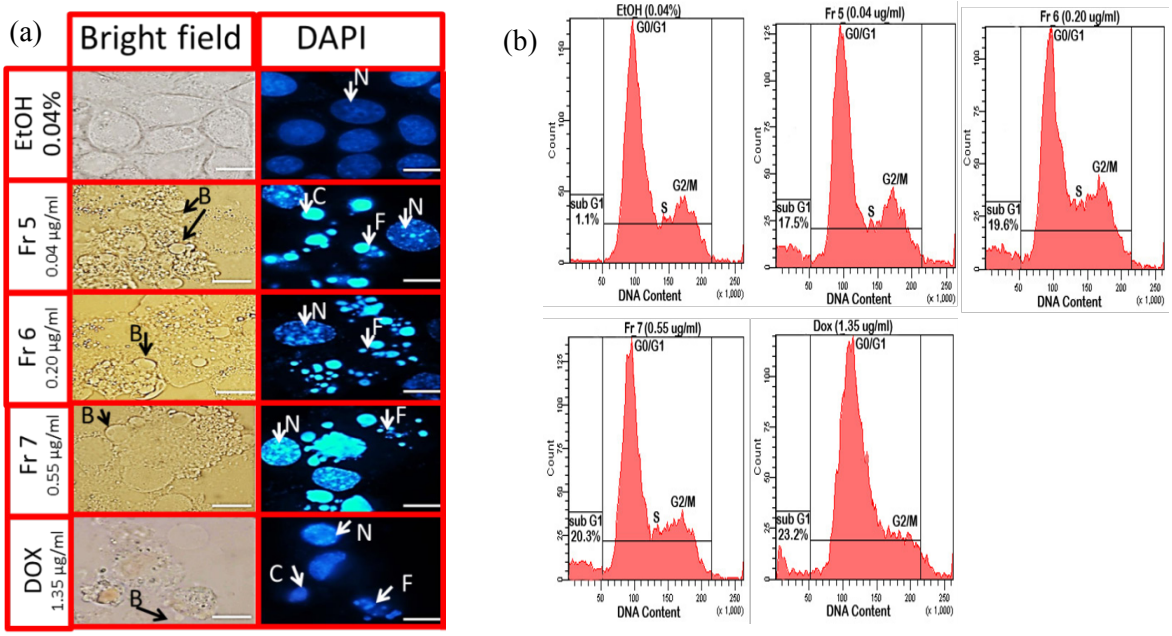

Figure 4 (a) Correlation of surface morphology with nuclear features. KB cells were stained with DAPI and detected by a bright field and blue filter (DAPI) at the same view point. B: cytoplasmic blebs, C: chromatin condensation, F: nuclear fragmentation, and N: normal nuclei. Scale bar is 10 µM. (b) Cell cycle phase distribution. KB cells were stained with PI and quantified by flow cytometry using the BD FACSDiva software.

Induction of apoptosis

To define the induction of apoptosis via mitochondrial pathways, the change in mitochondrial membrane potential (Δψm) was examined using JC-1 staining of whole cells. In live cells, JC-1 exhibits potential-dependent accumulation in mitochondria, forming J-aggregates. These aggregates can be detected within the red fluorescence spectrum (~590 nm), in contrast with the green fluorescence (~529 nm) emitted by JC-1 monomers. An increase in green fluorescence indicates depolarization of Δψm. As shown in **Figure 5a**, upon treatment with Fr 5, Fr 6, Fr 7, and DOX, the JC-1 fluorescence profile in KB cells shifted from red to green. The percentage of cells with green fluorescence following treatment with Fr 5, Fr 6, Fr 7, and DOX was 17.4, 20.4, 21.5, and 12.6 %, respectively compared to control cells (1.9 %). The representative histogram was presented from 3 individual experiments. The data for untreated and treated samples showed high statistical significance ($p < 0.001$) in all samples, suggesting that Fr 5, Fr 6, Fr 7, and DOX induced the loss of ΔΨm in KB cells.

During apoptosis, caspase-3 is the final pathway of apoptosis, and functions as an executioner caspase, which ultimately activates the endonuclease CAD in the process of apoptotic DNA fragmentation and cleaves various substrates, including cytoskeletal protein [14]. To determine the enzymatic activities of caspase-3 during apoptosis, the specific caspase-3 substrate (DEVD-pNA), and caspase-3 inhibitor (DEVD-fmk) were applied by colorimetric analysis. As shown in **Figure 5b**, the KB cells treated with Fr 5, Fr 6, Fr 7, and DOX significantly increased the relative activity of caspase-3 by 5.22 ± 0.2, 4.22 ± 0.15, 4.53 ± 0.4, and 4.58 ± 0.17 folds, respectively, when compared with the control cells. The caspase-3 inhibitor significantly prevented the caspase-3 activation induced by Fr 5, Fr 6, and Fr 7 ($p < 0.05$). These data indicated that apoptotic death was induced by Fr5, Fr 6, and Fr 7 in KB cells associated with the activation of caspase-3.

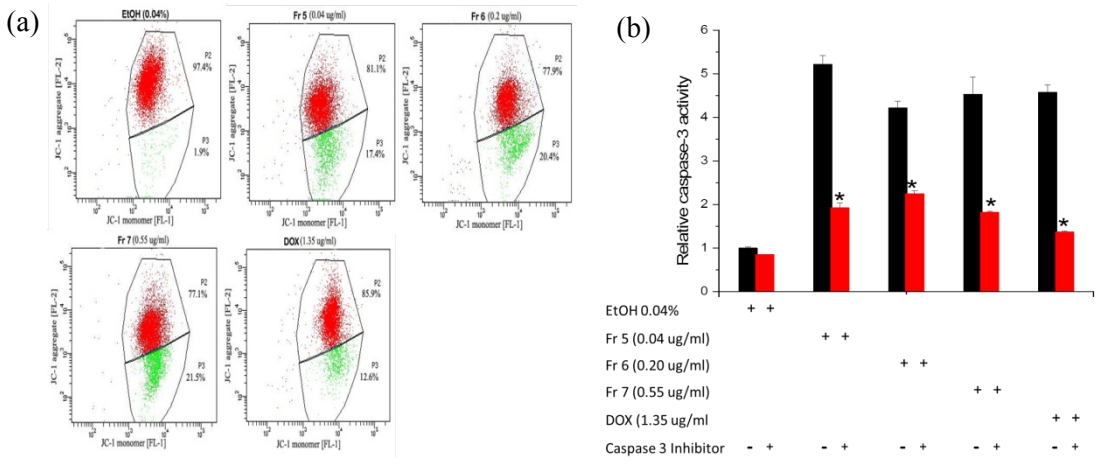

Figure 5 (a) Mitochondrial membrane potential. KB cells were stained with JC-1, followed by FACS analysis. P2 and P3 indicate the percentage of cells that emitted red and green fluorescence. Representative data from 3 individual experiments with similar results is shown. (b) Relative caspase-3 activities were measured by spectrophotometry. The control group (0.04 % EtOH) was set as 1, and the values of other groups were standardized against it. Data were expressed as mean ± SEM of n = 3 samples, *$p < 0.05$ vs. non caspase-3 inhibitor group.

The caspase-3activity is involved in apoptotic cell death by doxorubicin in human gastric cancer cells [26]. These results with KB cell apoptosis are closely related to or dependent on the loss of mitochondrial membrane potential (ΔΨm), although the role of apoptosis-related proteins and levels of p53, p21 and CDKs -related cell cycle regulators were not investigated. Recent studies have demonstrated

that a novel benzyldihydroxyoctenone derivative (F3-2-5), isolated from the culture supernatant of *Streptomyces* sp., KACC91015, inhibited growth and induced apoptosis via a p53-dependent pathway in human cervical adenocarcinoma HeLa cells. Moreover, it caused G1 phase arrest, along with increased levels of p53 and p21WAF1/CIP1, while reducing the expression of cyclin dependent kinase-4 and -6, and cyclin D1 and E [22]. The phenethyl acetate, a secondary metabolite isolated from a marine bacterium, *Streptomyces griseus*, exhibited strong anticancer activity against human promyelocytic leukemia HL-60 cells, and induced apoptosis through DNA fragmentation, DNA accumulation in G0/G1 phase, caspase-3 activation, and cleaved poly-ADP-ribose polymerase (PARP). Therefore, it may be a potential anticancer drug candidate for leukemia [27].

The bioactive molecules produced by the genus *Streptomyces*, which may have therapeutic properties, have been discovered. For example, migrastatin, a secondary metabolite from *Streptomyces*, has potent cytotoxicity on hepatoma HepG2 cells with induction of p53 and activation of caspase-3 [28]. The bioactive secondary metabolite from *Streptomyces* sp., kosinostatin, showed significant antimicrobial activity against *Candida albicans*, *Micrococcus luteus*, and *Saccharomyces cervicea*, and induced apoptosis against the mammalian carcinoma cell line (MCF-7) by expressing the p53 protein. The results acquired from this study demonstrate that kosinostatin will become a promising novel source for new anticancer drugs [29]. The results of many studies have suggested that the induction of apoptotic cell death is an important mechanism in the elimination of cancer cells, and the marine *Streptomyces* are a novel and rich source of bioactive compounds, owing to their potential pharmacological activities. According to HPLC analysis of fractions of red pigment samples from *Streptomyces* A16-1, this pigment is different from prodigiosin family pigments, and it may be an unknown pigment that is likely involved in the cytotoxicity and apoptosis (data not shown). This may be explained if the fractions were a mixture of analogues of the pigment which have the same chromophore but underwent various modifications. Taken together, the preliminary data by bioautographic assay showed that the partially purified pigment fractions of the extract from *Streptomyces* A16-1 could inhibit many methicillin resistant *Staphylococcus aureus* and MRSA P45 (hospital isolates) (data not shown). In the present study, the induction of apoptosis was conducted *in vitro* on mammalian cells, which provided useful information concerning critical cellular targets. Further investigation, including identification and purification of the active compounds, will need to be followed by *in vivo* evaluation.

Conclusions

Streptomyces A16-1, a possible new species of the genus *Streptomyces*, produced secondary metabolites, which included red pigments. The methanol and ethyl acetate extract was subjected to fractionation by column chromatography over silica gel according to polarity. The partial purification of Fr 5, Fr 6, and Fr 7 has strong cytotoxic and apoptotic effects, as shown by loss of cell viability, chromatin condensation, DNA fragmentation, and sub-G1 phase accumulation, which can be evaluated in a quantitative and qualitative method by MTT assay, DAPI staining, agarose gel electrophoresis, and flow cytometry analysis, respectively. Fraction 5 showed the best results. These apoptotic responses were associated with morphological changes, mitochondrial transmembrane depolarization, caspase-3 activation, and DNA fragmentation. These results are expected to further contribute to the understanding of the multiple mechanisms of apoptosis in nasopharyngeal cancer.

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