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Induction of apoptosis by *Streptomyces* strain CH54-4 extract through activation of caspase-3 in human nasopharyngeal cells

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- Background** : *Streptomyces* serves as an incessant source of novel compounds with the ability to produce bioactive secondary metabolites, such as, antimicrobial, antiviral, and especially anticancer compounds. In the case of screening for new anticancer agents, a soil sample was collected from the tropical mangrove area in Chantaburi Province, Thailand which may have potential effects to induce apoptosis on nasopharyngeal cancer.
- Objectives** : To study the anticancer and apoptosis induction mechanisms of *Streptomyces* strain CH54-4 extract on human nasopharyngeal (KB) cell line.
- Methods** : *Streptomyces* strain CH54-4 was maintained in ISP2 broth. The cells and medium were extracted with methanol and ethyl acetate (1:1) and treated on KB cells. The cell viability was determined by MTT assay. The molecular apoptotic cell death was evaluated by nuclear staining with 4',6-diamidino-2-phenylindole (DAPI), DNA agarose gel electrophoresis assay and evaluated cell cycle by propidium iodide. The activation of caspase-3 was analyzed.

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Results : The IC_{50} values of strain CH54-4 extract and doxorubicin were found at 14.29 ± 1.34 and 1.04 ± 0.21 $\mu\text{g/ml}$, respectively. Cell death mechanisms have been associated with apoptotic bodies and DNA fragmentation with broad smearing bands. The nuclei displayed apoptotic nuclear condensation and fragmentation. The cell cycle analysis showed increased proportion of sub-diploid cell population. Strain CH54-4 extract induced activation of caspase-3 which was reduced by caspase-3 inhibitor.

Conclusions : The findings demonstrate that the apoptotic effects of strain CH54-4 extract were mediated through the caspase-3 pathway. This study provides good candidates for novel anticancer compounds with high potency and specificity.

Keywords : *Streptomyces* strain CH54-4, apoptosis, caspase-3, KB cells, mangrove.

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เหตุผลของการทำวิจัย : เชื้อ *Streptomyces* สร้างสารออกฤทธิ์หลายชนิด เช่น สารปฏิชีวนะ ต้านไวรัส ต้านเซลล์มะเร็ง และยังไม่ถูกค้นพบอีกมาก คณะผู้วิจัยกำลังค้นหาสารชนิดใหม่จากดินป่าชายเลน จังหวัดจันทบุรี ประเทศไทย ซึ่งน่าจะมีศักยภาพสูงต่อการตายของเซลล์มะเร็ง

วัตถุประสงค์ : ศึกษาการตายแบบอะพอโทซิสในเซลล์มะเร็งหลังโพรงจมูกของสารสกัดจาก *Streptomyces* สายพันธุ์ CH54-4

วิธีการทำวิจัย : เลี้ยงเชื้อในอาหาร ISP2 นำเชื้อร่วมกับอาหารมาสกัดด้วยเมธานอล และเอทิลแอลกอฮอล์ (1:1) นำสารสกัดบ่มกับเซลล์ และนับเซลล์มีชีวิต โดยวิธีการทดสอบ MTT assay ย้อมสีนิวเคลียสด้วย 4',6-diamidino-2-phenylindole (DAPI), แยกชิ้นดีเอ็นเอด้วยสนามไฟฟ้า ประเมินวัฏจักรของเซลล์ด้วยสาร propidium iodide และวิเคราะห์การทำงานของเอนไซม์แคสเปส-3

ผลการศึกษา : สารสกัดสายพันธุ์ CH54-4 และ doxorubicin ยับยั้งเซลล์มะเร็งหลังโพรงจมูกโดยมีค่า IC_{50} เท่ากับ 14.29 ± 1.34 และ 1.04 ± 0.21 $\mu\text{g/ml}$ ตามลำดับ เซลล์ตายสัมพันธ์กับอะพอโทติกบอดี้, การแตกของดีเอ็นเอเป็นแถบยาว, นิวเคลียสแตกหัก, วัฏจักรของเซลล์มีโครโมโซมน้อยกว่า $2n$ เพิ่มขึ้น และกระตุ้นเอนไซม์แคสเปส-3 ซึ่งถูกลดการกระตุ้นโดยสารยับยั้งแคสเปส-3

สรุป : สารสกัดสายพันธุ์ CH54-4 เหนี่ยวนำให้เซลล์ตายแบบอะพอโทซิสผ่านทางการกระตุ้นเอนไซม์แคสเปส-3 การศึกษาครั้งนี้ทำให้มีทางเลือกที่ดีในการค้นหาสารที่ยับยั้งเซลล์มะเร็งชนิดใหม่ ซึ่งมีประสิทธิภาพและความจำเพาะสูง

คำสำคัญ : เชื้อ *Streptomyces* สายพันธุ์ CH54-4, อะพอโทซิส, แคสเปส-3, เซลล์มะเร็งหลังโพรงจมูก, ป่าชายเลน.

Cancer is a major cause of death worldwide and chemotherapy results in therapeutic failure by multiple-drug resistance. Research indicates that cancer cells develop a complex signaling cascade mechanism to escape apoptosis.⁽¹⁾ Therefore, the apoptotic pathways may possibly be an interesting target of cancer therapy. Although the anticancer drugs in clinical use have been shown to induce apoptosis, they are still challenged by causing normal cells to die and reversing cancer cells to survive.⁽²⁾ Several drugs have been designed to be activators of caspases such as caspase-based drug therapy, caspase-based gene therapy which had selectively induced apoptosis in cancer cells. At the execution phase of apoptosis, caspase-3 is an important mediator and indispensable for chromatin condensation and DNA fragmentation associated with the typical morphological changes in apoptosis.^(3, 4) Ongoing researches should focus on therapeutic strategies from natural sources that can induce cancer cell apoptosis by targeting multiple pathways with minimized side effects.

The screening of microbial natural products has been underexplored and it is an important route to the discovery of novel bioactive products from less-known and/or new bacterial taxa.⁽⁵⁾ The genus *Streptomyces* is Gram-positive, filamentous bacteria that are abundant in the marine environment. It is suggested that marine microorganisms have unique characteristics from those of their terrestrial counterparts because of their cellular adaptation to salt content. Marine *Streptomyces* have been proved to be efficient producers for drug discovery that have shown a range of biological activities such as antibacterial, antifungal, anticancer, anti-inflammatory

agents or other pharmaceutical compounds.⁽⁶⁾ The chemotherapeutic agents produced by *Streptomyces* species have been approved to be used in human including anthracyclines (aclarubicin, daunomycin and doxorubicin), glycopeptides (bleomycin and actinomycin D), aureolic acids (mithramycin) and others.⁽⁷⁾

Mangrove-derived *Streptomyces* has the potentiality of producing bioactive anticancer secondary metabolites of great interest in medicinal research.⁽⁸⁾ However, they need to be confirmed and developed. In our previous study, *Streptomyces* strain CH54-4 was isolated from mangrove sediment deposits in Chantaburi Province, Thailand. Red pigment extract of this strain exhibited a broad spectrum of antimicrobial activities against Gram-positive, Gram-negative bacteria, yeast and fungi. Additionally, this strain showed strong cytotoxicity against breast cancer cells with the IC_{50} value of 2.91 μ /ml.⁽⁹⁾ Consequently, the objective of this study was to investigate the cytotoxicity and apoptotic mechanism of the extract from *Streptomyces* CH54-4 on human nasopharyngeal epidermoid carcinoma (KB cells).

Materials and Methods

Preparation of Streptomyces strain CH54-4 extract

Streptomyces strain CH54-4, which is aerial and substrate mycelia in red color, was isolated from mangrove soil in Chantaburi Province, Thailand. Starch casein was used as isolation media after the soil was pretreated at 100°C for 1 h. Crude extract in red color was prepared by culturing the strain CH54-4 in 1 liter of ISP2 broth medium for 7 days. The active metabolites in the cells and the medium were extracted

with methanol and ethyl acetate (1:1) and the solvent was evaporated with rotary vacuum evaporator (Eyela A-10005).

Cell line

The human nasopharyngeal epidermoid carcinoma cell line or KB cells were obtained from the National Center Institute (Bangkok, Thailand).

Viability assay by MTT assay

MTT assay was performed as previously described.⁽¹⁰⁾ KB cells were treated with strain CH54-4 extract (0 - 100 μ /mL) for 48 hours. Thereafter, 10 μ L of MTT (5 mg/mL) solution was added to each well for 4 h. The resulting formazan crystals produced by the mitochondrial dehydrogenase enzymes were dissolved in DMSO and measured at 570 nm in a microplate reader (Cecil Bioquest 2,000 Series). Data were expressed as relative cell viability (%) against the control cells (100% of viability). The half maximal inhibitory concentration (IC_{50}) value was done through dose dependent curve which was calculated by the following formula:

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

DNA fragmentation by agarose gel electrophoresis

At the end of incubation period, KB cells were collected and the GF-1 tissue DNA extraction kit was used for DNA purification. DNA was stored at -20°C and has a A_{260}/A_{280} ratio between 1.8 and 2.0. DNA samples were separated by electrophoresis on a 1.5% (w/v) agarose gel containing SYBER Gold (1 μ /ml) and visualized under a UV transilluminator (Clare Chemical Research).

Nuclear morphological analysis by 4',6-diamidino-2-phenylindole (DAPI) staining

KB cells were treated at IC_{20} , IC_{50} , & IC_{80} concentrations of strain CH54-4 extract for 48 h. The monolayer of cells was fixed with 4 % para for maldehyde and stained with 5 μ /mL 4',6-diamidino-2-phenylindole (DAPI) for 30 min. The unbound dye was then removed and 20 μ L of mounting solution (PBS: glycerol, 1:9) was added. The morphological changes of apoptotic nuclei were observed using fluorescence microscopy (BX51TR, Olympus, Tokyo, Japan) at 100 \times magnification with a DAPI filter. Approximately, 200 different nuclei were counted under the microscope with at least 5 fields of view for each treatment condition.⁽¹⁰⁾ Percentage of apoptotic nuclei was calculated as follows:

$$\% \text{ apoptotic nuclei} = \left(\frac{\text{amount of apoptotic nuclei}}{\text{amount of all nuclei}} \right) \times 100$$

DNA cell cycle analysis by propidium iodide (PI) staining

KB cells were treated with IC_{50} , & IC_{80} concentrations of strain CH54-4 extract for 48 h. Both floating and adherent cells were collected, washed with PBS, and fixed in 4% cold paraformaldehyde for 15 min. Fixed cells were treated with the PI solution containing 0.1% Triton X -100, 0.1 mM EDTA disodium, 10 μ /ml RNase A, and 10 μ /ml PI for 30 min. The cell cycle distribution of 10,000 cells per sample was then determined using flow cytometry (Becton Dickinson, BD LSR II). The fluorescence activity of PI-stained DNA was analyzed by BD FACSDiva software. Apoptotic cells with hypodiploid DNA content in the sub-G1 phase were compared with that of the control.⁽¹⁰⁾

Caspase-3 activity assay

After incubation with IC_{50} concentrations of strain CH54-4 extract for 48 h, caspase-3 activities in cell lysates were assayed using a colorimetric assay kit. The pellets were lysed and then centrifuged at 14,000 g for 5 min at 4°C. The supernatant fraction containing 50 mg of protein were incubated with 2 × reaction buffer (50 µl) and caspase-3 substrate (DEVD-pNA) (5 µl) for 4 h at 37°C in the dark. The caspase-3 inhibitor (DEVD-fmk) was used as a negative control. Then, the accumulation of pNA cleaved from the substrate was measured using a spectrophotometer at 405 nm which represented the intra-cellular activity of caspase-3. ⁽¹⁰⁾

Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM) of three independent experiments and performed with Microcal TM Origin 6 software. The statistical differences between control and treatment were analyzed by unpaired student *t*-test. *P*-value less than 0.05 were accepted as statistically significant.

Results

Strain CH54-4 grew well on ISP2 medium and morphological observation of the 15-day-old showed convex colonies and spore chains around the colony edge which were well developed. After removal of solvent, the percent yield of crude extracts was 15%. The viability of KB cells decreased by almost 80% after exposure to 26 ± 1.93 µg/ml of CH54-4 extract for 48 h. In addition, 0.42% ethyl alcohol (EtOH) had no effect on the cell number. The IC_{50} values of strain CH54-4 extract and doxorubicin (Dox) were at

14.29 ± 1.34 and 1.04 ± 0.21 µg/ml, respectively (Figure 1). Under phase-contrast light microscopy, the strain CH54-4 extract-treated KB cells became shrunken with the appearance of small vesicle bodies (apoptotic bodies), low cell confluence, and easily detached from the monolayer. Similar events were also observed when the KB cells were treated with 1 µg/mL of Dox. Moreover, untreated or EtOH-treated cells were attached to the culture plates with greater than 90% confluence with polygonal adherent cells in normal shape. With the assessment of cytotoxicity using brine shrimps (*Artemiasalina*), the 30 µg/ml of strain CH54-4 extract exhibited significant non-cytotoxic activities. The total mortality was 100% in the highest concentration (>100 µg/mL) of strain CH54-4 extract (Data not shown).

The DNA molecules isolated from control or vehicle control cells showed clear bands of intact DNA. Because DNA fragmentation rate of the movement differs based on its length, DNA molecules isolated from KB cells treated with strain CH54-4 extract and Dox showed a significant degree of DNA degraded with broad smearing bands. DNA fragmentation pattern was apparent progress in a dose dependent manner (Figure 2). Morphological alterations of DAPI-stained nuclei were further studied via fluorescence microscopy (Figure 3). In the untreated or EtOH treated KB cells, the stained nuclei were rounded and homogeneously stained with DAPI that was estimated nearly 100% of normal cells. Strain CH54-4 extract induced alteration in nuclear morphology characterized by chromatin condensation and nuclear fragmentation, proportionally to the concentration. KB cells treated with strain CH54-4 extract (6.19, 14.29, 26.66 µg/ml) and Dox (1.04 µg/ml) exhibited

19.8 ± 8.9, 44.2 ± 3.3, 70.1 ± 5.6 and 69.8 ± 4.9% of apoptotic cells, respectively. In agreement with the loss of metabolic activity, KB cells became membrane

blebbing, round shaped as well as irregularities in cell contour and size.

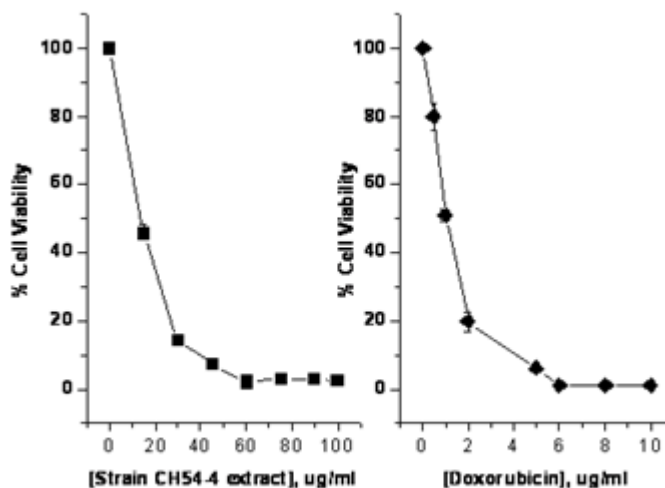


Figure 1. Dose-effect of the strain CH54-4 extract and doxorubicin on percentage of cell viability in KB cells using MTT assay. Data were expressed as mean ± SEM of three independent experiments.

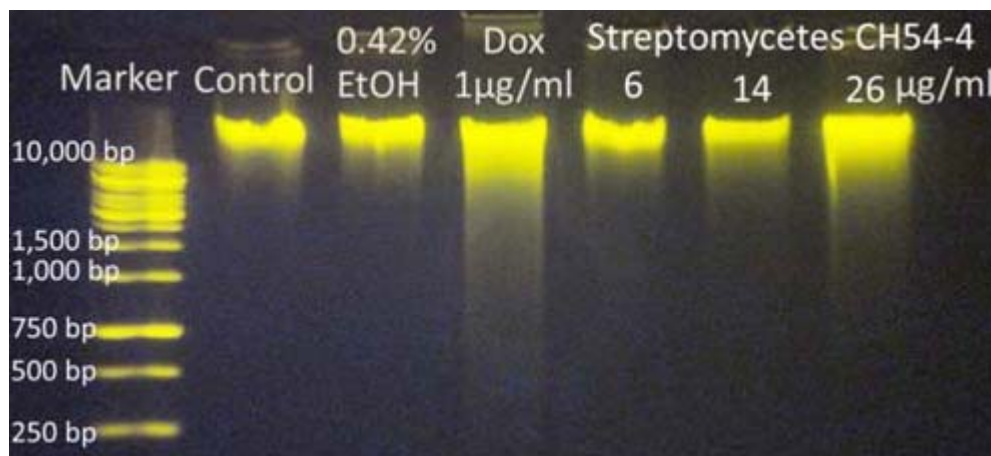


Figure 2. Electrophoresis of DNA extraction. KB cells were either untreated (control & 0.42% EtOH) or treated with doxorubicin (1 µg/ml) or strain CH54-4 extract (6, 14, 26 µg/ml) for 48 h. DNA was extracted and purified genomic DNA was electrophoresed on 1.5% agarose gel, stained with SYBER Gold and visualized under ultraviolet. A photograph was representative of three independent experiments.

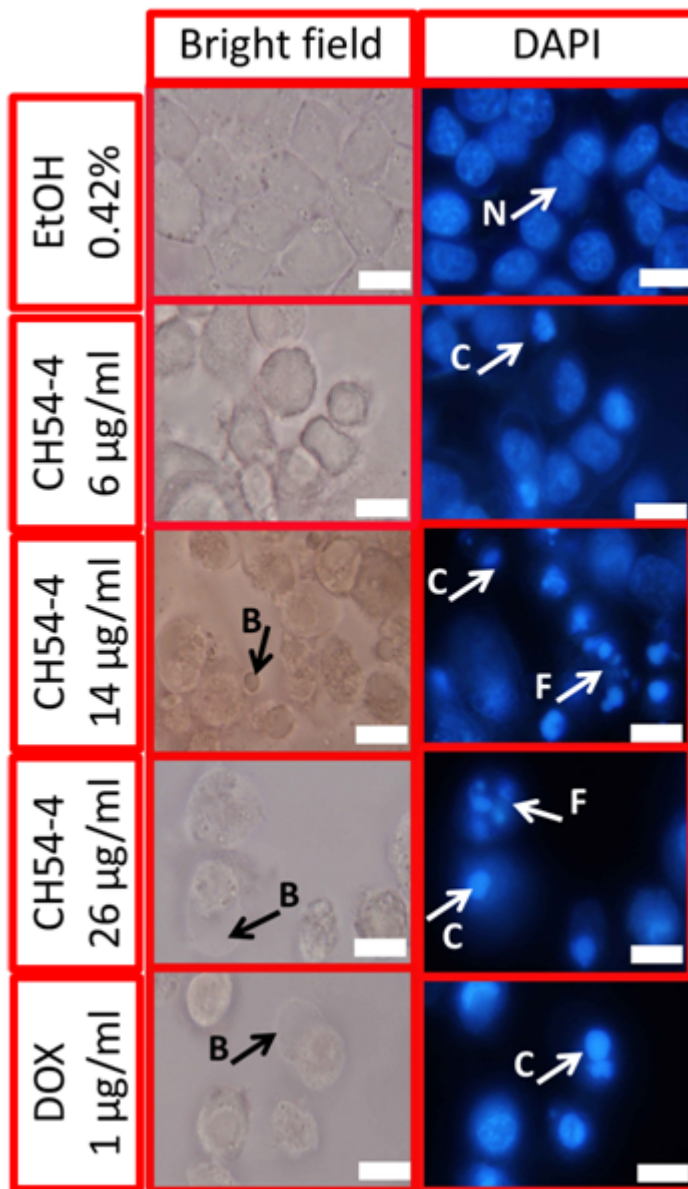


Figure 3. Parallel of surface morphology with nuclear features. KB cells were treated with ethyl alcohol (EtOH) (0.42%) or strain CH54-4 extract (6, 14, 26 µg/ml) or doxorubicin (1 µg/ml) for 48 h. Morphological features of KB cells were stained with DAPI and detected by a bright field and fluorescence microscope at the same view point. Similar results were observed in triplicate experiments. B: represents cytoplasmic blebs; C: chromatin condensation; F: nuclear fragmentation; and N: normal nuclei. Scale bar is 10 µM.

By using flow cytometric analysis of DNA content, strain CH54-4 extract induced cell cycle arrest and increased sub-G1 apoptotic cell population along with the increase in dose as compared to that of the control. After treatment with strain CH54-4 extract (14.29, 26.66 µg/ml) and Dox (1.04 µg/ml), the

percentage of hypodiploid cells (DNA < 2n) increased significantly to 25.65 ± 2.2, 35.28 ± 3.44 and 28.84 ± 2.47%, respectively; while only 1.21 ± 0.03% of the controlled cells had DNA content in the sub-G1 phase (Figure 4). The activity of caspase-3 was increased in cells treated with strain CH54-4 and Dox resulting in

DNA damage. In the presence of strain CH54-4 extract (14.29 $\mu\text{g/ml}$) and Dox (1.04 $\mu\text{g/ml}$), the relative caspase-3 activity was significantly increased to 2.7 ± 0.14 ($P < 0.05$) and 3.0 ± 0.15 ($P < 0.05$) as

compared with the untreated control. On the other hand, the caspase-3 inhibitor, DEVD-fmk, decreased the activity of caspase-3 to 1.5 ± 0.09 ($P < 0.05$) as compared with only strain CH54-4 extract (Figure 5).

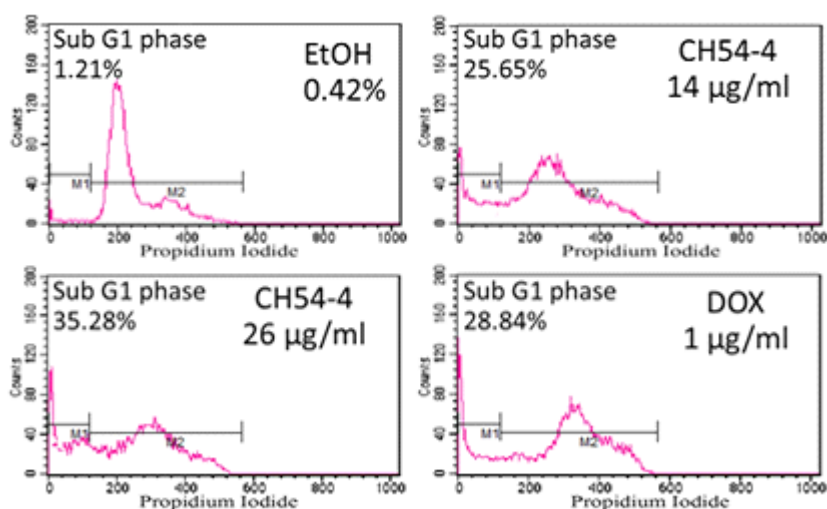


Figure 4. Cell cycle histogram of the DNA content. KB cells were exposed to EtOH (0.42%) or strain CH54-4 extract (14, 26 $\mu\text{g/ml}$) or doxorubicin (1 $\mu\text{g/ml}$) for 48 h. The floating and adherent cells were collected and fixed in 4% paraformaldehyde. Fixed cells were stained with PI and their DNA content was analyzed by flow cytometry using the BD FACSDiva software. Similar results were observed in three independent experiments.

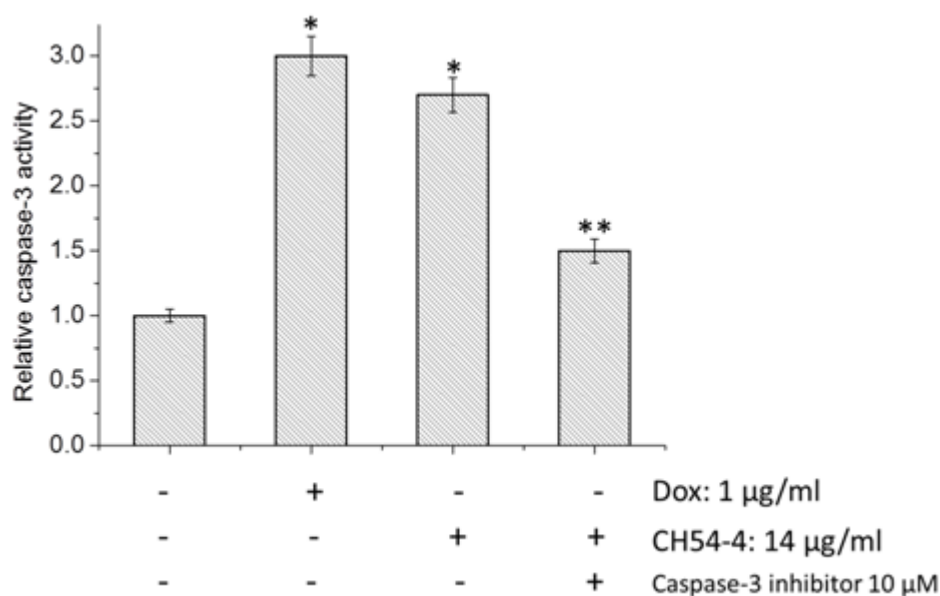


Figure 5. Relative caspase-3 activity was measured by the cleavage of the substrate (DEVD-pNA). The pNA was quantified by using a colorimetric assay. KB cells were treated with ethyl alcohol (EtOH) (0.42%) or doxorubicin (1 $\mu\text{g/ml}$) or strain CH54-4 extract (14 $\mu\text{g/ml}$) or strain CH54-4 extract + caspase-3 inhibitor for 48 h. The control group (0.42 % EtOH) was set as 1; the values of treatment groups were standardized against it. Each bar represents mean \pm SEM of $n = 3$ samples, * $P < 0.05$ vs. control group, ** $P < 0.05$ vs. non caspase-3 inhibitor group.

Discussion

In previous study, the phylogenetic analysis of a 16S rRNA gene sequence data showed that strain CH54-4 was closely related to *Streptomyces thermocarboxydus*. It might possibly be a new species. Strain CH54-4 produced red carotenoid pigment on ISP2 medium and the aerial spore mass color was whitish-pink.⁽⁹⁾ During a screening program, the ethyl acetate crude extract from strain CH54-4 displayed strong anticancer activity against HeLa cancer cell line and showed good antimicrobial activity in bioautography assay (Data not shown). Strain 54-4 crude extract had so many red components of substances isolated by thin-layer chromatography (TLC) using solvent mixture chloroform: methanol at the ratio 9:1 (Data not shown). HPLC analysis of partial purification products in red showed many spectrum peaks of substances and some of them may resemble prodigiosin analogues (Data not shown).

KB cell line, human cancer-derived, is the most frequently utilized *in vitro* models in cancer research for the study of the biological mechanisms involved in cancer and for anticancer drug-screening work.⁽¹¹⁾ It is noteworthy that the ethyl acetate solvent extraction of strain CH54-4 was considered to have highly cytotoxic activity against KB cells. In this study, the IC_{50} value was conducted in accordance with the National Cancer Institute (NCI, USA) criteria. It is recommended that IC_{50} values $\leq 20 \mu\text{g/mL}$ should be considered significant for crude extracts.⁽¹²⁾ The ethyl acetate and EtOH have low toxicity solvent; thus, the effect of KB cells cannot be considered. Due to the fact that strain CH54-4 extract usually occurs as a combination of various types of polar and non-polar bioactive compounds, especially red carotenoid

pigment of secondary metabolite strain CH54-4.⁽⁹⁾ Further investigation should be performed to isolate these bioactive compounds to obtain pure compounds and then to trail on more cancer cell lines.

The results agree with previous reports that the ethyl acetate crude extract of *Streptomyces avidinii* strain SU4 exhibited cytotoxic and less cytotoxic against Hep-2 cell line and VERO normal cell line with IC_{50} values of $64.5 \mu\text{g/ml}$ and $250 \mu\text{g/ml}$, respectively, with a view to assess their therapeutic potentials.⁽¹³⁾ The ethyl acetate crude extract of *Streptomyces parvulus* VITJS was found with IC_{50} value of $500 \mu\text{g/ mL}$ on HepG2 cell line, and it was also reported to have antifungal, antibacterial activities. These results could be chance of finding new bioactive compound in the crude extract.⁽¹⁴⁾ The most typical characteristics of apoptosis observed in the loss of normal shape followed by breaking up of the nucleus into fragmentation. Although the characteristic of apoptosis was DNA fragmentation into oligonucleosomal ladders, the late apoptosis or necrosis showed complete digestions of DNA into multiples sizes of nucleosomal fragments.⁽⁴⁾ The apoptotic cells were phagocytosed and destroyed *in vivo*; while no presence of phagocytic cells within a single culture could suggest that apoptosis takes place concurrently with necrosis or late apoptosis.⁽¹⁵⁾

Apoptotic signal transductions, which ultimately cause DNA fragmentation, are largely mediated by caspases. Caspases, a family of cysteinyl aspartate-specific protease, mediate apoptosis by cleavage a variety of important cellular proteins such as inhibitor of caspase-activated DNase (ICAD) and nuclear enzyme poly (ADP-ribose) polymerase (PARP) leading to DNA damage. Among

caspase family, caspase-3 is the final executioner enzyme associated with programmed cell death.^(1,2,4) The results showed good correlation by which the strain CH54-4 extract induced anti-cancer and apoptotic effects through the activation of caspase-3 in KB cells. It has been previously reported that strain CH54-4 extract contained red carotenoid pigment.⁽⁹⁾ The mechanisms underlying the apoptosis of carotenoids may involve different pathways including activation of caspase-3, release of cytochrome c from mitochondria and dysregulation of Bcl-2 family proteins.⁽¹⁶⁾

The mangrove ecosystem is part of the unique ecological places between the terrestrial and marine environments which are full of unexplored microbial diversity, and it could be a source of novel pharmaceutical compounds. Different studies should be focused on novel bioactive secondary metabolites from mangrove ecosystem for effective drug development because of its unique and rich in biological diversity.⁽⁸⁾ In recent years, *Streptomyces* from mangrove ecosystem were reported to be a producer of novel compounds with unique structure and potential medicinal use. From mangrove sediments collected in Malaysia, the pyrrolopyrazine, β -carboline and dicarboxylic acid ester were isolated from *Streptomyces* sp. MUM256, so were the deferoxamine and pyrrolizidines from *Streptomyces pluripotens* MUSC 137. These compounds were proved to exhibit antioxidant and cytotoxic activities against several cancer cell lines and could be potential sources for the development of anti-oxidative and chemopreventive agents.^(17, 18) The same research group continued to reveal the novel species named *Streptomyces malaysiense* sp. nov. which exhibited

strong anti-oxidant and cytotoxic activities together with pharmaceutical value.⁽¹⁸⁾ *Streptomyces* is the most prolific genus found in the microbial world and the bioactive constituents have been proved as a potential anticancer candidate. These results provide adequate rationale for further studies on the active metabolites and further evaluation for cancer chemotherapeutic potential.

Acknowledgments

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