Original Article

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Induction of programmed cell death in lung cancer cells by secondary metabolites of Nocardia carnea UTMC 863 as soil actinomycetes

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ABSTRACT

Background: Apoptosis induction is one of the effective mechanisms in cancer therapy. So far, various natural sources have been identified for inducing apoptosis in cancer cells. This study proposed identifying promising active drug pharmacophores of soil actinomycetes with the capability of apoptosis induction in A549 cells, a human alveolar adenocarcinoma cell line.

Methods: The crude extract of Nocardia carnea UTMC 863 was obtained from UTBC (University of Tehran Biocompound Collection). After 48 hours of exposure, cell viability, gene expression, and apoptosis rate were determined using MTT, quantitative real-time-PCR, and flow cytometry.

Results: The MTT assay exhibited that the effective concentrations of UTMC863 and doxorubicin (positive control) were 24 µg/ml and 1 µM, respectively. UTMC 863 with a 24 µg/ml concentration and doxorubicin could induce apoptosis in the A549 cell line. Also, apoptosis-related gene expression increased in the UTMC863 group compared to the untreated group ($p<0.01$).

Conclusions: The crude extract of Nocardia carnea UTMC 863 can induce apoptosis in A549 cells, and it may be one of the promising pharmacophores for cancer therapy.

Keywords: Apoptosis, Actinomycetes, Lung cancer, Doxorubicin

INTRODUCTION:

Lung cancer is one of the most prevalent cancers and has high mortality and morbidity rate worldwide. It has been estimated that this cancer can eventually lead to 1.5 million deaths annually [1]. There are two subgroups of lung cancer, small cell lung cancer (SCLC, 15%) and non-small cell lung cancer (NSCLC), with a prevalence of 15% and 75%, respectively. Several NSCLC subtypes with high and low grades are associated with lung cancers. Squamous cell carcinoma (30%), large cell carcinoma (9%), and adenocarcinoma (45%) have been reported as the most prevailing NSCLC subtypes. Unusual histologic variants and mixed-cell type combinations may occur in all types [2, 3]. According to recent investigations, numerous genetic and epigenetic changes are considered to participate in NSCLC, including abnormalities in tumor suppressor genes, oncogenes, and genes involved in the growth, angiogenesis, cell apoptosis, and metastasis [3-5]. Ongoing research on the molecular modifications suggests these targets can develop drug discovery processes [6, 7]. Various studies in cancer therapy show that approved cancer therapies such as chemotherapy and radiation can activate the apoptotic machinery and eradicate tumor cells [8-10]. Apoptosis or programmed cell death is a physiological process that provides an effective and non-inflammatory way to remove redundant or damaged cells from tissues, securing tissue homeostasis [11, 12]. Thus, apoptosis induction in tumor cells is one of the best specific and selective approaches for cancerous cell killing without triggering inflammatory or immune responses [11, 13]. Unfortunately, the common drugs in chemotherapy also cause damage to cells and normal tissues in the body. Therefore, new research to discover drug agents with minimal side effects is advancing rapidly. Discoveries and developments of most newly anti-cancer drugs originated in the early 20th century [14, 15]. Various studies have revealed that metabolites derived from natural sources might possess pro-apoptotic properties and great cancer prevention potential [16]. Natural products from varied sources such as plants, animals, and even microorganisms are extensively used in cancer therapy. The immense diversity, abundance, and underutilization of microorganisms' natural anti-tumor compounds have attracted many researchers to discover novel metabolites to develop pharmaceutical industries [17-19]. A Gram-positive and filamentous bacteria called actinomycetes is a prime source for new bioactive compounds [20]. They are capable of producing secondary metabolites with diverse biological activities. Comprehensive screening of terrestrial actinomycetes was started in the early 1950s and later developed into antimicrobial [amphotericin B, erythromycin, vancomycin [21], 1,4-Dihydroxy-2-(3-hydroxybutyl)-9,10-anthraquinone9,10-anthrac1,4-Dihydroxy-2-(3-hydroxybutyl)-9,10-anthraquinone 9,10-anthrac] [22], anti-cancer (daunorubicin, bleomycin, mitomycin [21], N-(2-hydroxyphenyl)-2-phenazinamine (NHP) [23, 24], Arenimycin, Antitumor [Aureolic acid [25], Elaiomycins B and C) [26] and immunosuppressive (rapamycin) drugs [21].

2. Materials and methods:

2.1. Strain supply and extract preparation

*Nocardia carnea UTMC 863 was supplied from UTMC (University of Tehran Microorganisms Collection). Seeding medium (ISP2 medium) consisted of (g0 l-1): glucose, 4; yeast extract, 4; malt extract, 10; and pH value was adjusted at 7.4, whereas the fermentation medium consisted of (g l-1): soybean meal, 30; starch, 20; CaCO3, 10; MgSO4.7H02O, 1; and pH value was adjusted to 7.1 ± 0.1. All the media were sterilized at 121ºC for 20 min. Spore suspension of Nocardia carnea UTMC 863 (10 % v/v) was injected into each 100 mL flask containing a 9 mL seeding medium and was shaken at 28ºC with 220 rpm for 36 h. The seeding substance (10% v/v) was transferred to 250 mL flasks containing a 20 mL fermentation medium. The inoculated flasks were incubated at 28 ºC at 220 rpm for 7 to 11 days. After extracting metabolites with ethyl acetate and drying, crude extracts were prepared and stored at -20 ºC.

2.2. Lung cancer cell culture

A human lung adenocarcinoma cell line, A549, was used for this study. The cells were obtained from the Iranian Biological Resource Center and cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM;

Gibco-BRL) supplemented with 10% fetal bovine serum (Gibco-BRL) and 100 U/ml penicillin and 100 mg/ml streptomycin (GIBCO BRL). Cells were kept in a humidified atmosphere of 95% air with 5% CO2 at 37 ºC.

2.3. Investigating the cytotoxicity of actinomycete metabolite

The MTT assay evaluated cell viability based on the MTT reduction to a purple formazan mediated by viable cells' mitochondrial dehydrogenases [26]. A549 cells were seeded into 96-well culture plates and cultured for 24 h under 5% CO2 at 37°C. The cells were exposed to Nocardia carnea UTMC 863 metabolite at different concentrations (3-24 µg/mL) and 48 h. After treatment, 10µl MTT was added to the plate well and incubated for three hours at 5% CO2 and 37°C. The medium was subsequently removed, and 100 µl DMSO was added to the plate well to dissolve the formed formazan crystals. Finally, the soluble formazan product was evaluated by an ELISA reader [28]. Doxorubicin was employed as a positive control in this study, and all the procedures were performed for doxorubicin.

2.4. A549 Cells treatment with secondary metabolites of Actinomycete

Nocardia carnea UTMC 863 metabolite was dissolved in DMSO at a 4000 µg/mL concentration and was stored in a dark-colored bottle at -20 °C as a stock solution. The stock was diluted to the required concentration immediately before using growth media. The cells were exposed to actinomycete metabolite at a 24 µg/mL concentration for 48 h. Cells grown in media containing an equivalent amount of DMSO without actinomycete metabolites were considered control. A549 cells were seeded into 48-well plates at a density of 4 × 104 cells per well in 150 µL of media. After 24 h, cells were treated with actinomycete metabolite diluted in complete DMEM and incubated at 37°C for 48 h.

2.5. Apoptosis detection in treated cells

2.5.1. Quantitative real-time polymerase chain reaction [Q-RT-PCR]

Total cellular RNA was extracted using RNX-Plus (Low Copy RNA Isolation)-reagent (CinnaGen) for Q-RT-PCR analysis. The cDNA was synthesized with M-MuLV reverse transcriptase (RT) and random hexamer as the primer, according to the manufacturer's instructions [Vivantis]. The GAPDH housekeeping gene was used as a control. Furthermore, apoptosis-related gene expression, including BCL2 Associated X (Bax), p21, caspase-7 (Casp-7), p53, and retinoblastoma (Rb), was studied using Q-RT-PCR. PCR amplification was performed using SYBR Premix Ex Taq TM Master mix (TAKARA) with a two-step procedure of an initial denaturation at 95°C for 2 minutes, followed by cycles circulating 5 seconds of denaturation at 95°C and 30 seconds of annealing/extension at 60°C. The total number of cycles was 40. Further details and specific primers are illustrated in Table 1.

Table 1. Primer sequences used in Real-time PCR

Gene product	Primer sequences	Product size(bp)	Tm(0C)
GAPDH	Sense 5′- CCTCAAGATCATCAGCAATG-3′ Antisense 5′- CATCACGCCACAGTTTCC-3′	90	56 56
Bax	Sense 5′-CAAACTGGTGCTCAAGGC-3′ Antisense 5′-CACAAAGATGGTCACGGTC-3′	178	56.1 57.3
Caspase-7	Sense 5′-CACGGTTCCAGGCTATTAC-3′ Antisense 5′-GGCAACTCTGTCATTCACC-3′	139	57 57
p21	Sense 5′-CCAGCATGACAGATTTCTACC -3′ Antisense 5′-AGACACACAAACTGAGACTAAGG-3′	150	59 61
P53	Sense 5′-GGAGTATTTGGATGACAGAAAC-3′ Antisense 5′-GATTACCACTGGAGTCTTC-3′	181	58 55
Rb	Sense 5′-AATCATTCGGGACTTCTG-3′ Antisense 5′-ACTTCCATCTGCTTCATC-3′	154	52 52

2.5.2. Apoptosis assay

Detection of apoptotic A549 cells was performed using Annexin V-FITC (fluorescein isothiocyanate) / propidium iodide (PI). Briefly, A549 cells were cultured in a 48-well plate, the medium was removed after 24 hours, and cells were treated with the Streptomyces UTMC 863. After 48 hours, both adherent and floating cells were harvested and centrifuged. PBS washing was subsequently executed, and cells were suspended in 100 µl 1x binding buffer. Afterward, cells were incubated with 5µl Annexin V-FITC and 5µl PI for 15 min in the dark at room temperature. In the final stage, samples were analyzed using flow cytometry.

2.6. Statistical analysis

Real-time PCR reactions were monitored in Rotor-gene Q real-time analyzer (Corbett), and statistical analyses were followed with Rest software. Statistical studies related to MTT and flow cytometric analyses were proceeded using IBM SPSS software, version 19. A one-way ANOVA analysis of variance was used in this study, and $p \leq 0.05$ was considered statistically significant.

3. Results:

*In this study, a secondary metabolite of Nocardia carnea UTMC 863 was used as a treatment for examining apoptosis induction on A549 cell lines.

3.1. Metabolites' effects on cell viability

For the cytotoxicity studies, an MTT assay was performed. The MTT assay result indicated that the crude extract of Nocardia carnea UTMC 863 reduces cell growth and viability. Inhibitory Concentration (IC) of cell viability demonstrated that the extract with 24 µg/ml concentration inhibits 50% of A549 cell viability (Figure 1) [29].

3.2. Induction of apoptosis

3.2.1. Q-RT-PCR

3.2.2. Flow cytometric evaluation of apoptosis induction

Table 2. Effects of treatment on apoptosis of A549 cell line

Flow cytometry (%)				
Group	**Early apoptosis**	**Late apoptosis**	**Necrosis**	**alive**
Control	**0.178±0.007**	**2.905±0.417**	**21.5±3.111**	**75.4±2.687**
treated with crude extract of Streptomyces UTMC 863	**0.01±0.01**	**77.3±0.141***	**17.7±0.848**	**4.98±1.08***
Treated with Doxorubicin	**1.755±0.2***	**38.35±8.697***	**37.45±8.838**	**22.45±0.07***
Values are reported at the mean±S.D. *indicated that p≤0.05 toward the un-stimulated group.				

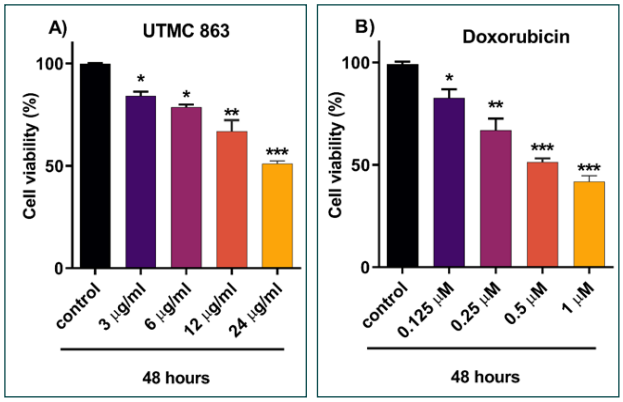

Figure.1. The effects of A(secondary metabolites of Nocardia carnea UTMC 863 and B) Doxorubicin on A549 cells viability. Doxorubicin is considered a positive control.

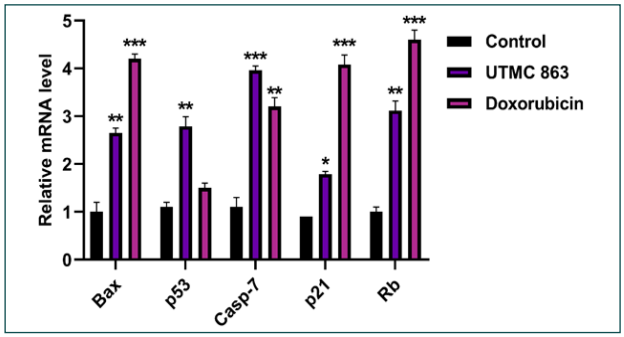

Figure.2. Comparative expression of Bax, Rb, p21, p53, and Casp7 genes related to the control group. These data confirmed that the crude extract of Nocardia carnea UTMC 863 and doxorubicin as a positive control augmented apoptotic associated markers' expression related to the untreated group. * and ** show significant data with $p<0.05$ and $p<0.01$ compared to the untreated group.

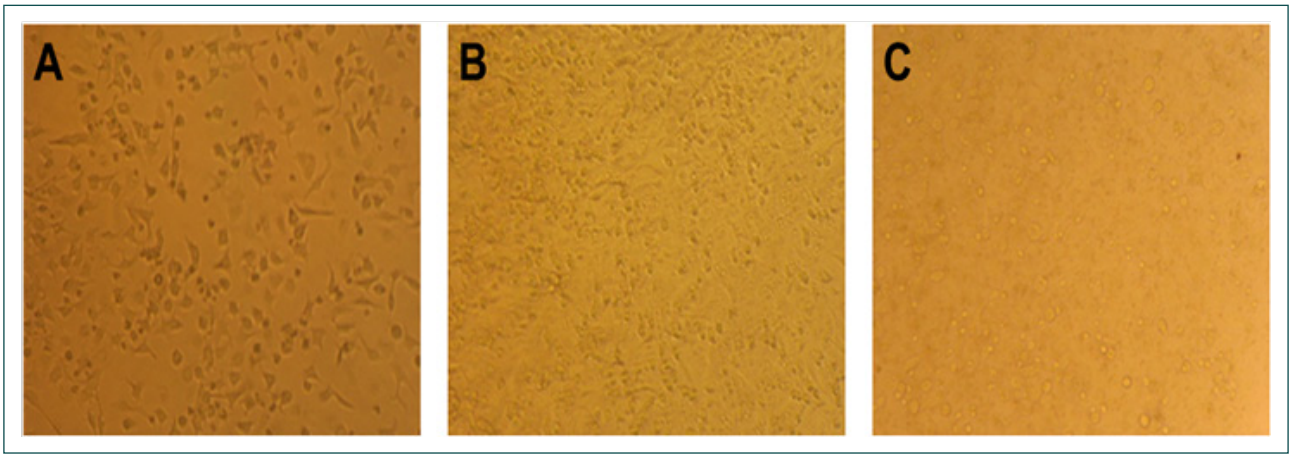

Figure.3. Morphological changes in A549 cells treated with A) DMSO as negative control; B) Doxorubicin as the positive control; and C) The crude extract of Nocardia carnea UTMC 863 (× 10 magnification)

We found that the crude extract of Nocardia carnea UTMC 863 with a 24mg/ml concentration and doxorubicin could induce apoptosis in the A549 cell line. Apoptosis was evaluated by staining with Annexin V-FITC/Pi (Table 2, Figure 4). Table 2 elucidates that apoptotic cell percentage in microbial extract treatment was higher than those exposed to doxorubicin.

4. Discussion:

The data of this manuscript showed that the crude extract of Nocardia carnea UTMC 863 and doxorubicin increased apoptotic gene expression. However, this extract at a 24 μg/ml concentration is a promising target and has a more apoptotic effect on A549 cells than doxorubicin.

Cancer is a heterogeneous disease identified as the growth and invasion of a cell population, which interferes with normal physiological functions. Lung cancer is one of the leading causes of death in developing and developing countries [30, 31]. Therefore, it is necessary to create treatment strategies for this cancer. Natural products have been a rewarding source for treating specific diseases [32]. These biological compounds play a crucial role in developing new drugs as they possess enormous diversity serving as scaffolds for drug discovery. Biological compounds can be obtained from various sources such as plants, animals, and microorganisms. The most notable bacteria with diverse biological compounds and many biological activities belong to the actinobacteria phylum. Due to this fact, these biological systems have been screened for compounds possessing anti-cancer potential. Some of these microbial metabolites, including doxorubicin, pentostatin, streptozotocin, anthracyclines, and bleomycin, are being used to treat cancers, and these compounds are approved by the Food and Drug Administration (FDA) [33]. Numerous conventional chemotherapies, such as doxorubicin and vincristine lead to cell apoptosis and cytotoxicity [34, 35].

On the other hand, due to drug resistance in chemotherapy and the side effects of chemotherapy drugs, it is necessary to identify new anti-cancer compounds. In the recent decade, the use of various natural compounds with apoptosis-inducing properties has become popular. In line with these studies, secondary metabolites of various microorganisms and their derivative compounds have been used [36, 37]. A study by Gao et al. on marine actinomycetes of the BM-17 strain indicates the toxicity of the bacterium extract on cancer cells [23]. Since 1950, many soil actinomycetes have been studied and screened for antibiotic, anti-cancer, and anti-tumor agents [38].

This study investigated the effect of the extracted metabolite from one of the soil actinomycetes in the collection of microorganisms at the University of Tehran to induce apoptosis in lung cancer cells. Since 85% of lung cancers are related to NSCLC type, one of the most important NSCLC adenocarcinoma, a lung adenocarcinoma cell line, A549, was used. The present study verified the cyto-

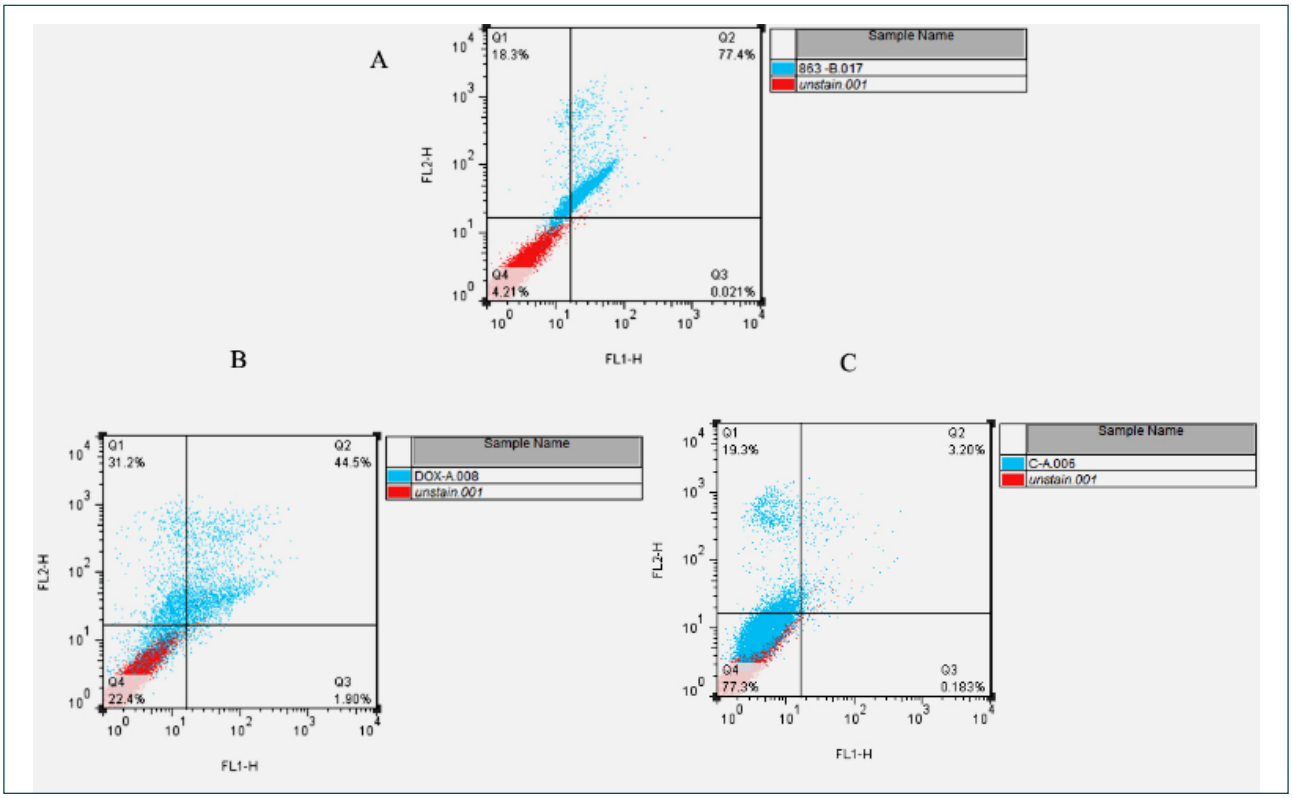

Figure.4. Dot-plot Flow cytometry of A549 treated cells stained with Annexin V-PI. A) 24 µg/ml of secondary metabolites of Nocardia carnea UTMC 863. B) 1µM doxorubicin as a positive control. C) DMEM supplemented with DMSO as a negative control.

toxic effect of Nocardia carnea UTMC 863 by MTT assay. Results indicated that this metabolite is lethal at a 24 µg/ml concentration, killing 50% of cells. [39]. Rajan et al. reported that actinomycete metabolites had 68% cytotoxic activity [40]. Induction of apoptosis in A549 cells treated with this metabolite is more effective than doxorubicin. Late apoptosis and necrosis in cells treated with UTMC 863 were 77.3% and 17.7%, respectively, while cells treated with doxorubicin were 38.35% and 37.45%, respectively. Therefore, UTMC 863 has a better effect than doxorubicin at a 24 µg /ml concentration.

High mutations in the p53 gene have been reported in various cancers, including lung, colon, and breast cancers [42]. P53, as a tumor suppressor gene, can induce p21 gene expression downstream, and then activated p21 can activate RB through suppressing cyclin E and CDK2. RB also acts as a tumor suppressor by binding to E2F, blocking the cell cycle progression from G1 to S phases, thereby causing cell cycle arrest [43-45]. There are observations that the p53 gene directly affects mitochondrial function in apoptotic processes. P53 induces the expression of pro-apoptotic genes such as Bax. On the other hand, it reduces the amount of anti-apoptotic gene Bcl2, thereby inducing apoptosis through the mitochondrial pathway [internal pathway]. Bax leads to the permeability of the mitochondrial membrane and the translocation of cytochrome c from the mitochondria to the cytosol. Then, cytochrome c, by affecting the apoptotic protease activating factor (Apaf-1), leads to apoptosome formation and downstream caspase cascade activity, increasing the expression of activating caspases such as Casp 7 [46-48]. In 2014, Rambabu et al. showed the apoptotic effect of kosinostatin, a purified compound from Streptomyces sp., in the MCF-7 carcinoma cell line. This compound induces p53 expression, one of the most important regulators of apoptosis [49]. In another study by C. Balachandran et al., the effect of flavonoids extracted from Streptomyces sp. was examined on A549 cells. In this study, increased expression of

p53, caspase-3, and Bcl2 genes after treatment of A549 cells with flavonoids indicates the induction of apoptosis in these cells [50]. In line with previous studies, A549 cells were treated with bacterial metabolites in our study. The main genes of the internal apoptotic pathway and some genes involved in developing lung cancer were investigated. Real-time PCR analysis proved that Nocardia carnea UTMC 863 increased Bax, P53, Rb, p21, and Casp7 gene expression in the A549 cell line. Doxorubicin and crude extract of Nocardia carnea UTMC 863 verified increasing apoptotic gene expression. Therefore, all results confirm the efficacy of the UTMC 863 strain metabolite.

However, this study has some limitations. Based on the available findings, studying other essential genes involved in apoptosis, especially the extrinsic pathway of apoptosis, is also necessary. Due to financial constraints, genes involved in apoptosis have been evaluated at the mRNA level, and their expression at the protein level has not been studied.

Conclusion:

In conclusion, Nocardia carnea UTMC 863 could be an ideal candidate for apoptosis induction in lung cancer cells. Apoptotic induction is a distinguished process for cancer treatment; therefore, this extract can be considered an anti-cancer agent. Considering the use of crude bacterial extracts in this study, purifying the extracted metabolites and finding the effective component in the first step is necessary. It should also be noted that the specificity of anti-cancer drugs is one of the most challenging topics in treating various cancers. Therefore, it is suggested that the apoptosis-inducing compound, in addition to cancer cells, be tested on non-cancerous cells such as fibroblasts in future studies.

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Conflict of interests :

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