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Original Article

Soil *Actinomycetes*-derived Secondary Metabolites-Induced Apoptosis in Human Lung Cancer Cells

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Background and Aims: Natural compounds derived from animal, plant, and microbial sources participate in treating various types of cancers, including lung cancer. This survey attempted to explore the anticancer activity of two novel metabolites extracted from soil-derived *actinomycetes* in the human lung cancer A549 cells.

Materials and Methods: The crude extracts of UTMC 638 and UTMC 877 secondary metabolites were obtained from the University of Tehran Microorganisms Collection (UTMC). When doxorubicin was applied as a positive control, cell viability, apoptosis detection, and mRNA expression were assessed by MTT assay, flow cytometry, and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) technique.

Results: The results of the MTT assay showed that UTMC 638, UTMC 877, and doxorubicin reduce A549 cell viability in a concentration-dependent manner. Cell treatment with UTMC 877, UTMC 638, and doxorubicin could promote apoptosis in the A549 cell line. However, the effect of UTMC 638 on apoptotic induction was more than doxorubicin or UTMC 877. The q-RT-PCR results highlighted that the gene expression associated with apoptosis was augmented in the treated group compared to the untreated group.

Conclusion: Our findings provide evidence that the crude extract of UTMC 676 could promote apoptosis in A549 cells and can be a very promising source for designing a potent antitumor agent against lung cancer cells.

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Introduction

Globally, lung cancer continues to be the major cause of cancer-related deaths among women and men. In 2018, the estimated number of new lung cancer cases and death were 11.6% and 18.4%, respectively [1]. This cancer is the second leading cause of cancer death among Iranian men and the third leading cause of cancer death among Iranian women [2]. Substantially, lung cancer incidence is tightly affected by cigarette smoking patterns; nearly 85% of lung cancer cases are caused by carcinogens in tobacco smoke [3]. Molecular heterogeneity is responsible for the critical problem in diagnosing and treating patients with lung cancer. Based on decades of study and research, lung cancer can be considered a multi-stage event, and the accumulation of genetic and epigenetic modifications leads to DNA damage and the transformation of normal lung epithelial cells into cancerous cells [4-6]. Non-small cell lung carcinoma (NSCLC) and small cell lung carcinoma (SCLC) are two main types of lung cancer in histological and clinical features. NSCLC accounts for about 85% of all types of lung cancer and divides into the subtypes of adenocarcinoma, squamous cell carcinoma, and large cell carcinoma [7]. Various treatment strategies have been used to treat this cancer, which target various events involved in cancer, such as angiogenesis and apoptosis. [8, 9]. Therefore, using treatment agents that can induce apoptosis is a very good idea in cancer therapy. Treatments for lung cancer include surgery, chemotherapy, radiotherapy, targeted therapy, or combination therapies. On the other hand, it is necessary to discover new chemotherapy drugs due to drug resistance [10, 11].

Natural compounds derived from animal, plant, and microbial sources have become popular in various sciences and industries, especially in drug production [12-14]. Microorganisms are the source of many important drugs, such as antibiotics, antitumor compounds, antiviral compounds, and antiparasitic agents [15-18]. A*ctinomycete* strains are valuable and produce many natural bioactive products with antitumor properties [19]. It should be noted that the use of secondary metabolites of *actinomycetes* in various fields of pharmaceutical science and drug discovery, including the production of antitumor compounds, is promising and has attracted the attention of researchers [9, 20, 21]. Various studies on *actinomycete* strains have been performed in Iran, some of which aimed to identify *actinomycetes* that produce biologically active compounds. In 2014, Sarrami et al. proved the cytotoxic effects of some soil *actinomycete* strains stored at the University of Tehran. Microorganisms Collection (UTMC) against human lung cancer A549 cells [22]. However, isolated secondary metabolites of *actinomycetes* have not been studied to treat lung cancer by introducing apoptosis. Therefore, this study intended to evaluate the ability of *actinomycete* strains to induce apoptosis in A549 cells by measuring the quantitative expression of genes involved in apoptosis induction.

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Materials and Methods

Cell culture

The human lung carcinoma cell line, A549, used in this study was obtained from the Iranian National Center for Genetic and Biologic Resources. A549 cells were plated in Dulbecco's Modified Eagle's medium (DMEM) (Gibco, USA) containing antibiotic (mixed 100 U/mL penicillin and 100 µmol/L streptomycins) (Gibco, USA) and 10% fetal bovine serum (FBS) (Gibco, USA) and maintained in a humidified incubator at 37 °C.

Microbial extract preparation

The secondary metabolites of UTMC 638 and UTMC 877 were obtained from the UTMC. The concentration of stock solutions prepared from each secondary metabolite was 4000 g/mL dissolved in dimethyl sulfoxide (DMSO) solution (Sigma, USA) [22]. The stock solutions were diluted with a culture medium to prepare the desired concentrations.

3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay

After treatment with UTMC 638, UTMC 877, and doxorubicin (Sigma, USA), the viability of A549 cells was evaluated by MTT assay (Sigma, USA). Briefly, 10^4 cells/well were plated in 96-well plates and exposed to different concentrations of UTMC 638 (0.8, 1.6, 3.2, and 6.4 µg/ml), UTMC 877 (6, 12, 24, and 48 µg/ml), and doxorubicin (0.125, 0.25, 0.5, and 1 µM) for 48 hours. In this study, doxorubicin was applied as a positive control. After the exposure, the medium was discarded from each well and replaced with a new medium containing MTT solution (1 mg/ml). After exposure to MTT, cells were incubated for 3 hours at 37 ˚C until a purple color of formazan product was visible [23]. The resulting formazan product was dissolved with DMSO, and the absorbance was detected at 570 nm using a microplate reader (BioTek, H1M).

Apoptosis detection via flow cytometry

The apoptosis rate of A549 cells was evaluated by annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) staining according to the manufacturer protocol (BD Biosciences, Clontech, USA). Shortly after treatment with UTMC 638, UTMC 877, and doxorubicin for 48 hours, A549 cells were trypsinized, rinsed, and then suspended in the Annexin-V binding buffer. Next, Annexin V-FITC and PI were added and incubated for 15 min at room temperature in the dark condition [24]. Finally, the rate of apoptotic cells was assessed by a flow cytometer using FlowJo software version 10 (FlowJo LLC, Ashland, OR, USA).

Real-Time polymerase chain reaction (PCR) Analysis

A real-time PCR technique was executed to determine the mRNA levels of the *p53*, *Bax*, *p21*, *retinoblastoma*, and *caspase 7* genes. A549 cells were seeded in 6-well plates and exposed to UTMC 638, UTMC 877, and doxorubicin for 48 hours, and then total RNA isolation was performed using a total RNA isolation kit (CinnaGen, Iran) according to the manufacturer's instructions. One µg of total RNA was converted into the first-strand cDNA by First Strand cDNA Synthesis Kit (TAKARA, Japan).

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Table 1. The specific sequences of selected primers used in this study

Gene product	Primer sequences	Product size(bp)	References
GAPDH	Sense 5′- CCTCAAGATCATCAGCAATG-3′ Antisense 5′- CATCACGCCACAGTTTCC-3′	90	[26]
Bax	Sense 5′-CAAACTGGTGCTCAAGGC-3′ Antisense 5′-CACAAAGATGGTCACGGTC-3′	178	[27]
Caspase-7	Sense 5′-CACGGTTCCAGGCTATTAC-3′ Antisense 5′-GGCAACTCTGTCATTCACC-3′	139	[27]
p21	Sense 5′-CCAGCATGACAGATTTCTACC -3′ Antisense 5′- AGACACACAAACTGAGACTAAGG-3′	150	[28]
P53	Sense 5′-GGAGTATTTGGATGACAGAAAC-3′ Antisense 5′-GATTACCACTGGAGTCTTC-3′	181	[29]
Retinoblastoma	Sense 5′-AATCATTCGGGACTTCTG-3′ Antisense 5′-ACTTCCATCTGCTTCATC-3′	154	[29]

Q-RT-PCR was conducted by RotorGene 6000 Q real-time analyzer (Corbett, Qiagen), and results were displayed by RotorGene 6000 Q real-time analyzer (Corbett, Qiagen). The sequences of the specific primers for *p53*, *Bax*, *retinoblastoma*, *p21*, *Caspase7*, and *Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH)*, were given in Table 1. GAPDH was applied as an internal housekeeping control, and the selected mRNA expression was normalized to the GAPDH gene [25]. The present investigation results from a research project of the Department of Microbial Biotechnology University of Tehran through the following approved code: 1398/17.

Statistical analysis

All data were analyzed by GraphPad Prism software version 8 and presented as mean ± standard deviation (SD). Statistical differences were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. $P < 0.05$ was considered a statistically significant difference.

Results

The effect of UTMC 638 and UTMC 877 secondary metabolites on A549 cell viability

As presented in Figure 1, data from the MTT assay showed that the crude extracts of UTMC 638, UTMC 877, and doxorubicin metabolites could reduce the viability of A549 cells in a dose-dependent manner. UTMC 877, UTMC 638, and doxorubicin at 24 µg/ml, 6.4 µg/ml, and 1 µM could decrease approximately 50% of A549 cell viability. These effective concentrations were used for subsequent analyses.

The effect of UTMC 638 and UTMC 877 secondary metabolites on morphological changes of A549 cells

As demonstrated in Figure 2, the lung cancer cells exposed to UTMC 638 and UTMC 877, as well as doxorubicin, morphologically exhibited cell death compared with the lung cancer cells exposed to DMSO (as a negative control).

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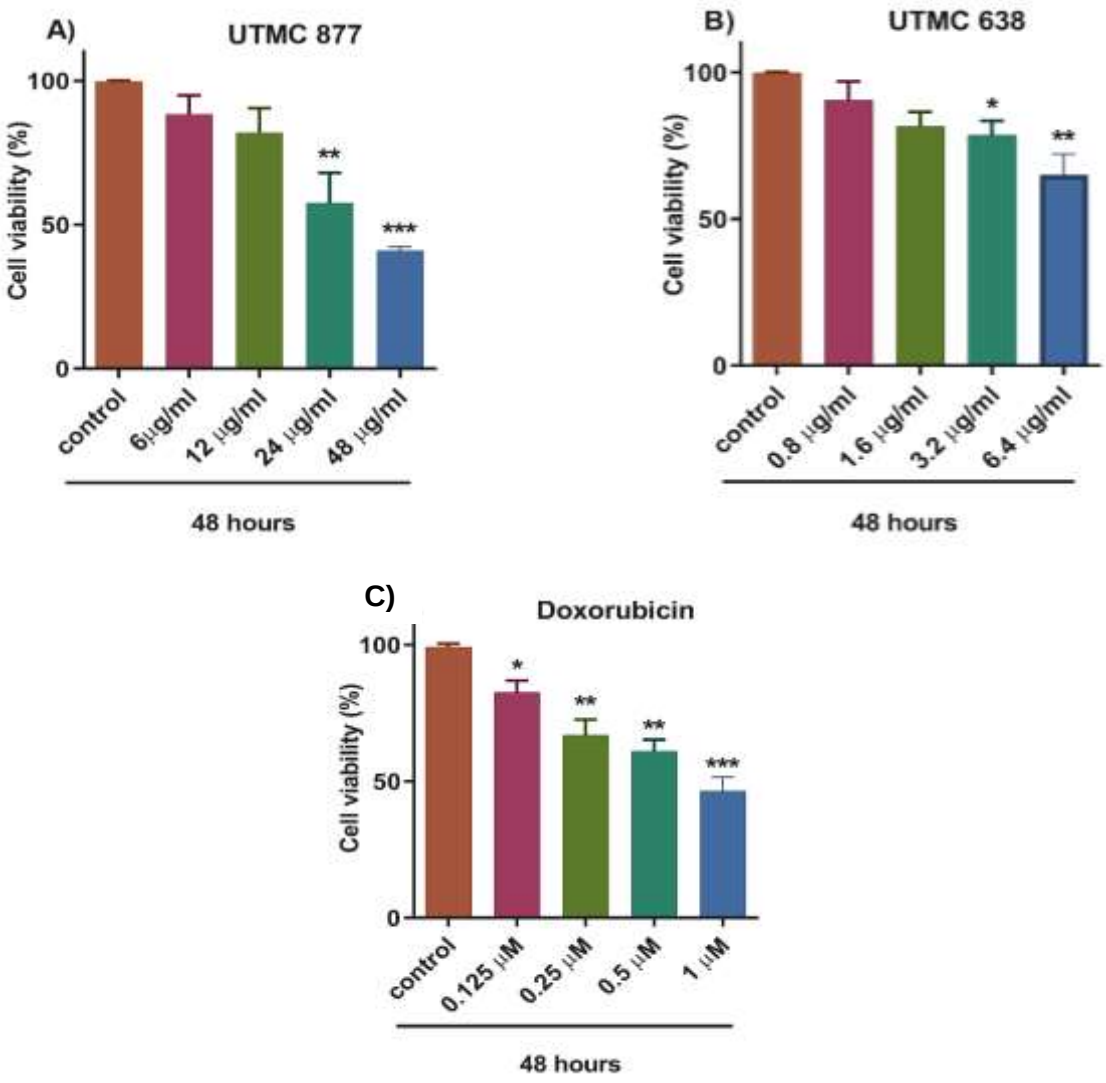

Fig. 1. The secondary metabolites' effects of A) UTMC 877, B) UTMC 638, and C) doxorubicin on A549 cell viability. Doxorubicin is considered a positive control. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ were statistically significant.

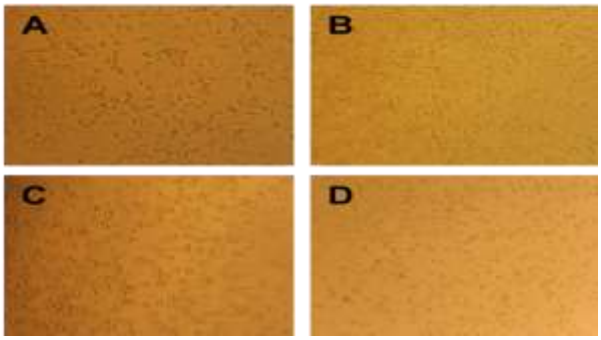

Fig. 2. Morphological modification (×10 magnification) of lung cancer A549 cells were incubated with A) DMSO, B) doxorubicin (positive control), C) the crude extract of UTMC 877 metabolite, and D) the crude extract of UTMC 638 metabolite.

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The effect of UTMC 638 and UTMC 877 secondary metabolites on the apoptosis rate of A549 cells

Data from the flow cytometry indicated that UTMC 877, UTMC 638, and doxorubicin could promote apoptosis in the human lung cancer A549 cell line (Table 2, Figure 3). As depicted in Table 2, the necrotic and late apoptotic cell percentages in the A549 cells exposed to UTMC 877 were 47.3% and 17.7%, respectively, and in the cancer cells exposed to UTMC638 were 13.35% and 74.4%, respectively. However, after cell treatment with doxorubicin, the percentage of apoptotic and necrotic cells was about 38.35% and 37.45%, respectively. Furthermore, the late apoptotic rate in the A549 cells treated with UTMC 877 was notably lower than those exposed to doxorubicin or UTMC 638. However, the percentage of necrotic cells in UTMC 638-treated cells was lower than those treated with doxorubicin or UTMC 877 (Table 2).

The effect of UTMC 638 and UTMC 877 secondary metabolites on apoptosis-related gene expression

The quantitative analysis of *retinoblastoma*, *Bax*, *p21*, *Caspase7* and *p53* genes in cancer cells exposed to UTMC 877, UTMC 638, and doxorubicin was presented in Figure 4. The q-RT-PCR results showed that the expression of *retinoblastoma*, *Bax*, *p21*, *Caspase7*, and *p53* genes were increased in the treated cells compared with control cells ($p < 0.05$, $p < 0.01$, $p < 0.001$).

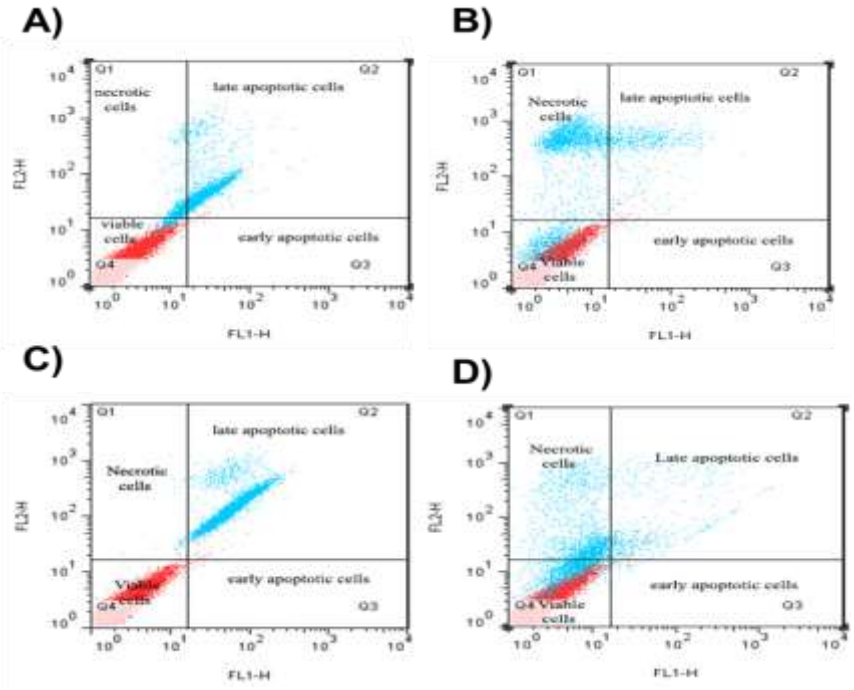

Fig. 3. The effect of A) DMSO, B) doxorubicin (1 µM), C) UTMC 638 secondary metabolites (6.4 µg/ml), and D) UTMC 877 secondary metabolites (24 µg/ml) on the apoptosis rate of A549 cells. Doxorubicin is considered a positive control.

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Table 2. The impact of UTMC 877, UTMC 638, and doxorubicin on the apoptosis rate of A549 cells

Group	Flow cytometry (%)			
	Early apoptosis	Late apoptosis	Necrosis	Alive
Control	0.178 ± 0.007	2.905 ± 0.417	21.5 ± 3.111	75.4 ± 2.687
UTMC 877	1.076 ± 0.585	17.70 ± 6.646	47.3 ± 3.67	34.25 ± 3.606
UTMC 638	1	74.4 ± 8.83*	13.35 ± 0.919	11.185 ± 6.809*
Doxorubicin	1.755 ± 0.2*	38.35 ± 8.697*	37.45 ± 8.838	22.45 ± 0.07*

All results are indicated at mean ± SD. *P < 0.05 was regarded as statistically significant.

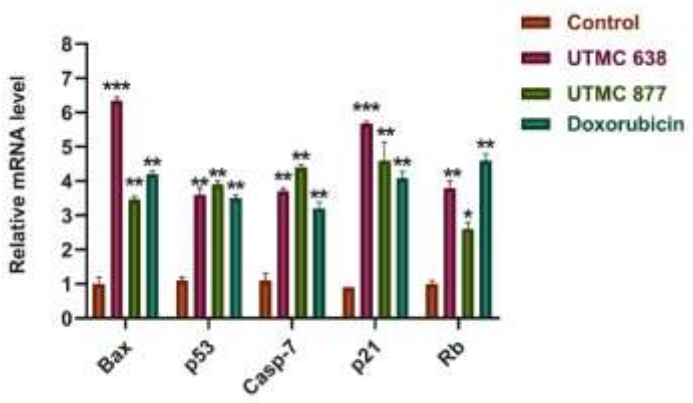

Fig. 4. Relative mRNA expression of *Bax*, *p53*, *Casp7*, *p21*, and *retinoblastoma* genes in A549 cells exposed to DMSO (control), the crude extract of UTMC 638 (6.4 µg/ml), the crude extract of UTMC 877 (24 µg/ml), and doxorubicin (1 µM). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.01$ were statistically significant.

Figure 4 shows that the mRNA expression of *Bax*, *Casp7*, and *p21* genes was higher in the cells exposed to UTMC 638 than in the A549 cells treated with doxorubicin. The *retinoblastoma* gene expression in UTMC 638-treated cells was lower than in doxorubicin-treated cells (Figure 4). On the other hand, the mRNA expression of *Casp7* and *p21* genes was higher in the UTMC 877-treated cells than in doxorubicin-treated cells; however, the expression of the *Bax* and *retinoblastoma* genes in UTMC 877-treated cells was lower than doxorubicin-treated cells.

Discussion

In the present study, an NSCLC cell line, A549, was chosen as a cell model to investigate cell death and apoptosis induction in lung cancer cells. In the present study, we, for the first time, provided evidence using crude extracts UTMC 877 and UTMC 638 that two secondary metabolites of soil *actinomycetes* induce apoptosis in A549 cells. Our data indicated a decrease in the necrosis rate and an increase in the cell apoptosis rate after A549 cell incubation with UTMC 638 but not UTMC 877

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secondary metabolites. The morphological examination also aligned with the results obtained from apoptosis in A549 cells.

The results of the present study were in line with the findings of previous studies on actinomycetes. According to previous information, *actinomycetes* were regarded as the major producers of new natural secondary metabolites with great pharmaceutical potential against chemotherapeutic-resistant tumors. *Actinomycetes* are an important resource of new anticancer metabolites, including metabolites displaying the capability to promote apoptosis [30, 31]. Elmallah et al. highlighted that marine *actinomycetes*-derived secondary metabolites could induce the downregulation of survivin and XIAP, leading to sensitization of MDA-MB-231 and HCT116 cells to cell death [32]. In another study, Zhou et al. reported that a natural product of marine *actinomycete*, Ilamycin E, induces apoptosis in triple-negative breast cancer cell lines HCC1937 and MDA-MB-468 [33]. Farnaes et al. found that napyradiomycin, as a derivative isolated by a marine-derived *Actinomycete*, induces cytotoxicity and apoptosis in the colon adenocarcinoma cell line HCT-116 [34].

Balachandran et al. proved that soil-obtained filamentous bacterium *Streptomyces sp* triggers apoptosis in A549 cells through p53, cytochrome c release, and caspase-dependent pathway [35]. Jeong et al. illustrated that *Streptomyces sp*. SY-103 metabolites induce apoptosis in human leukemia cells through activation of caspase-3 and inactivation of Akt [36]. Lin et al. revealed that marine *actinomycetes*-derived Actinomycin V alleviated the growth of A549 cells by inducing cell cycle arrest and apoptosis via the upregulation of *p53*, *p21*, and *Bax* genes. Therefore, this metabolite suppresses the growth of A549 cells by inducing *p53* expression and promoting p53-dependent cellular events, including cell cycle arrest and apoptosis [37, 38]. In the present work, we also assessed the effect of UTMC 877 and UTMC 638 secondary metabolites on the expression of apoptosis-related genes. The gene expression analysis showed that UTMC 638 and UTMC 877, in line with apoptosis induction, effectively increase the expression of genes associated with cell apoptosis.

P53 is one of the classic tumor suppressor genes, which is frequently dysregulated in a large type of cancer cells. In normal cells, p53 contributes to transcriptional activation of downstream targets, including p21 and the pro-apoptotic proteins of BAX, PUMA, and NOXA [39]. The p21 protein is considered a universal inhibitor of cell cycle progression and exerts its functions on the cell cycle by inhibiting cyclin/CDK complexes and inactivating pRB [40]. The *retinoblastoma* and *p53* genes are classified as tumor suppressors, preventing tumor formation and progression. This tumor suppressor protein regulates the cell cycle and several other biological functions. *Retinoblastoma* forms a transcriptional repression complex with the E2F transcription factor and inhibits the progression from G1 to S phase of the cell cycle by regulating E2F target genes [41]. Our findings collectively emerged that both UTMC 638 and UTMC 877 induced *Bax*, *p21*, *p53*, *retinoblastoma*, and *Caspase7*

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mRNA expression. Consequently, apoptosis is raised, resulting in growth inhibition in A549 cells.

This study includes some limitations. Due to financial issues, the expression of the studied genes was executed only at the mRNA level, and their protein expression was not examined. Also, studying other genes involved in the intrinsic and extrinsic apoptosis pathways was impossible.

Conclusion

Our findings potentiate the idea that soil-derived secondary metabolites are probably introduced as novel pharmacological substances whose development may result in therapeutic pattern changes in oncology. The finding of current research highlighted that these metabolites could induce apoptosis in the human lung cancer cell line. However, these results may begin more extensive in vitro and in vivo studies.

Conflict of Interests

The authors declare that there is no conflict of interest associated with this work.

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