Evaluation of the effect of Drimia Maritima and curcumin extracts in changes in the expression of Bax, Bcl-2, and P53 genes in lung cancer non-small cell

Negin Noori Sepehr
Alborz University of Medical Sciences
Fatemeh Soleimanifar (soleimanifar.abzums@gmail.com)
Alborz University of Medical Sciences
Nafiseh Khosravi Dehaghi
Alborz University of Medical Sciences
Hossein Mahboudi
Alborz University of Medical Sciences
Fereshteh zare
Alborz University of Medical Sciences
Afsaneh Tavasoli
Alborz University of Medical Sciences
Fatemeh Sameni
Shahed University
Hamed Haddad Kashani
Kashan University of Medical Sciences

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Abstract

Introduction:

Lung cancer is one of the most common malignancies in the world, with a very high mortality rate. Surgery and chemotherapy are among the first approaches to cancer treatment, which are associated with severe side effects, so more research has been done in the field of using medicinal plants with less toxicity. In the present study, we investigated the simultaneous effect of Drimia maritima plant extract (proscillaridin A), a cardiac glycoside, and turmeric plant extract (curcumin) in inducing apoptosis of non-small cell lung cancer cells.

Methods and Materials

Treatment of cancerous and non-cancerous cells with plant extracts was done by the MTT method, and the RNA of the samples was extracted using an extraction kit, and then cDNAs were synthesized using a special kit. Specific primers were designed for the sequence of P53, Bax, Bcl-2, and Gapdh genes, and the expression levels of the desired genes were checked and analyzed using the real-time PCR method. Cell cycle changes and apoptosis rates were also checked using the flow cytometry method.

Results

The results of investigating the simultaneous effect of proscillaridin and curcumin extracts on non-small cell lung cancer cells showed that we did not witness the synergistic power of the extracts together with each other, but we saw an increase in the survival of cancerous and non-cancerous cells, which, of course, in the case of non-cancerous cells were more impressive, and the two extracts seem to have neutralized each other's effect. The results indicate that the effect of each extract alone on cell lines (especially Calu-3 compared to A549) was greater.

Conclusions

Therefore, according to the research, it is possible that the use of extracts along with a suitable chemotherapy drug has a more significant effect on the life of normal or non-cancerous cells, which reduces the side effects of the drug and can increase the penetration rate of the chemotherapy drug, so in this field, more studies are needed.

Introduction

Cancer is characterized by the development of abnormal cells that divide uncontrollably, have the ability to invade and destroy normal body tissue, and often spread throughout the body. One of the most common cancers in terms of prevalence and mortality worldwide is lung cancer, with more than 2.2 million new cases of lung cancer in 2020, which is 12.5% of the total cancer statistics, so the 5-year survival rate is about 16 [1]. In Iran, the number of lung cancer cases in men and women in 2020 was approximately 10,465 (12.6%), and the number of deaths was 9,071 (10.9%) [11]. The main types of lung

cancer include small cell lung cancer (SCLC), which has a faster growth and spread rate, and non-small cell lung cancer (NSCLC), which pathologically accounts for 80% of cases. There are 3 main groups of adenocarcinoma (ADC) with a frequency of 30–40%, which is the most common form of NSCLC, squamous cell carcinoma (SCC), and large cell carcinoma (LCC). The people who are more likely to develop lung cancer are people over 50 years of age with a smoking history. The main risk factors involved in lung cancer are tobacco consumption, air pollution, being exposed to substances such as asbestos or radon at home or work, and having a family history of lung cancer [3] [4]. Common treatments for lung cancer include chemotherapy, radiation therapy, surgery, and targeted molecular treatments [3]. Currently, most of the anti-cancer treatments with all the desired efficiency are associated with many undesirable and unfortunate side effects. Inefficiency, lack of safety, drug resistance, and high costs of treatment have led to a lack of trust in these methods. For this reason, today, returning to the use of medicinal plants has received a lot of attention, and since products of plant origin are naturally They are multi-purpose, and in addition, compared to chemical agents, they are cheap and safe. Also, the active substances in herbal medicines have a biological balance due to their combination with other substances, so they are not accumulated in the body and have no side effects. There are fewer side effects, and in this sense, they are significantly superior to chemical drugs [1] and [8]. One of these medicinal plants, Drimia maritima, with the scientific name Urginea maritima, has been used since ancient times to treat various diseases, including respiratory diseases, heart defects, skin problems, and digestive disorders [1] [9]. Among the main active compounds, unique bufadienolides such as Proscillaridin A, which is a type of cardiac glycoside drug, can be used in the treatment of congestive heart failure and cardiac arrhythmia. The cardiotonic effect of cardiac glycosides is caused by their anti-Na/K pump activity; although originally prescribed for heart failure, their anticarcinogenic effect was recently discovered [9] [4]. According to studies, Proscillaridin at very low concentrations effectively induces apoptosis and potently inhibits cell survival pathways while having very little toxicity to normal cells [10]. Apoptosis (programmed cell death) is triggered through different pathways; some of these pathways occur due to the activation of caspase group enzymes. BAX and BCL2 genes are among the important genes involved in the mitochondrial pathway of apoptosis, since the balance between proliferation and apoptosis is disrupted in cancer cells, and cells need to inhibit apoptosis for their growth and proliferation, a direct relationship. There is a relationship between the expression of these genes and the cancerous process, so investigating the expression changes of these genes can be considered a therapeutic or diagnostic goal in cancer studies [11] [12].

Among the plants that have been of great interest due to the presence of natural and safe active ingredients is the turmeric plant, with the scientific name Curcuma longa. Turmeric has been used for thousands of years in the treatment of various diseases such as colds, fevers, skin diseases, and liver diseases, and its use is not toxic even in high doses. Recently, this substance has been considered an effective combination for the treatment of cancer [15] [16]. Turmeric itself consists of three main compounds: demethoxycurcumin, curcumin, and bisdemethoxycurcumin. Among these components, curcumin, the effective ingredient of the rhizome of the turmeric plant, is beneficial in preventing the growth of cancer cells. This compound has anti-inflammatory, anti-viral, anti-bacterial, anti-fungal, and

anti-cancer properties [16]. Many in vitro and in vivo studies have shown that curcumin can inhibit cancer in three stages: progression, angiogenesis, and tumor growth. The anticancer activities of proscillaridin and curcumin are through their effects on various biological pathways involved in mutagenesis, oncogene expression, cell cycle regulation, apoptosis, tumors, and cell metastasis [18]. The purpose of this research is to simultaneously investigate the effectiveness of proscillaridin and curcumin in inducing apoptosis and cell cycle changes in NSCLC cells.

Methods and Materials

Plant Extraction(Maceration)

At first, we ground 1 kg of turmeric and also chopped 1 kg of Drimia maritima plant, immersed each in 2 liters of 80% ethanol, and kept them in the same condition for 48 hours. After 48 hours, the liquid was poured into a rotary evaporator to concentrate. Ethanol was poured again for the second and third times, and the liquid was concentrated after 48 hours each time. Finally, the concentrated extract was kept at room temperature for a few days to completely remove the remaining water.

Cell culture

The human non-small cell lung cancer cell lines (A549, Calu-3) and human diploid lung fibroblast cell line (MRC5) were obtained from the Pasteur Institute of Iran (Tehran, Iran). Our cell lines were cultured in DMEM/F-12 medium containing 10% fetal bovine serum (Gibco, USA) and 1% penicillin-streptomycin under 5% CO2 at 37°C. The cultural media were replaced every other day.

Cell cytotoxicity assay

The inhibitory effect of proscillaridin A and curcumin on cell proliferation was determined using the MTT assay. A549, Calu-3, and MRC5 cells were seeded as 8 *103 cells per well in 96-well microtiter plates. The culture media were replaced after 48 h, and various concentrations of Proscillaridin A (100, 125, 150, and 175 nmol/L) were added to treat cells for 12 h and 24 h. Concentrations of curcumin (125, 250, 375, and 500 µg/L) were added to treat cells for 24 h, 48 h, and 72 h.The combination of proscillaridin and curcumin concentrations((125,100) (250,100)(375,10)(500,100)(125,125)(250,125)(375,125)(500,125) (125,150)(250,150)(375,150) (500,150) (125,175)(250,175)(375,175)(500,175)) were also added for 24 hours. Controls were maintained to determine the control cell survival (CS) and the percentage of live cells after culture. After incubation, the medium was removed and 10 µL of MTT reagent was added to each plate, followed by incubation for 3 h at 37°C. The yellowish MTT is reduced to dark-colored formazan by viable cells only. After the removal of the medium, 90 µL of DMSO was added and incubated at 37°C for 10 min. The color developed was quantified with an ELISA plate reader (570 nm).

RNA extraction and cDNA preparation

The mRNA expression of p53, bcl-2, and Bax was quantitatively estimated in cancerous and normal cells treated with curcumin at a dose of 500µg for A549 and MRC5 and a dose of 375µg for Calu-3, also

treated with proscillaridin at a dose of 175 nM for A549 and 150 nM for MRC5 and Calu-3. The combination of proscillaridin and curcumin concentrations for A549 was 500,175, for Calu-3 it was 375,150, and for MRC5 it was 500,150. This experiment used the RNA extraction kit (Sinacolon, Tehran, Iran) to extract total RNA from the normal and cancerous treated cell lines, and the integrity was examined with nanodrops. According to the manufacturer's instructions, total RNA was reverse transcribed to cDNA by using the cDNA Synthesis Kit (Roje, Tehran, Iran).

Real-time PCR

The expression of p53, bcl-2, and Bax genes was determined using the real-time PCR method, and SYBR Green q PCR Master mix (Yekta Tajhiz Azma, Teh, Iran) was used for quantitative PCR reactions in a Rotorgene machine. The primer PCR sequences were: Bcl-2 forward 5'-CATCAGGAAGGCTAGAGTTAC-3' and reverse 5'- CAGACATTCGGAGACCACAC-3'; Bax forward 5'-GATGCGTCCACCAAGAAG-3' and reverse 5'-AGTTGAAGTTGCCGTCAG-3'; P53 forward 5'-GCCGCAGTCAGATCCTA-3' and reverse 5'-CTGGGAGCTTCATCTGGA-3'; Gapdh forward 5'-CTCTTGCTACTCTGCTCTG-3' and reverse 5'-GCCTGCCTGGTGATAATC-3'.

Flowcytometry

Flow cytometry was performed using the Annexin V-FITC/PI double staining kit. Briefly, A549, Calu-3, and MRC5 cells were cultured in 6-well plates overnight. The cells were then treated with the combination of proscillaridin A and curcumin for 24 hours (A549 (500,175), Calu-3 (375–150), and MRC5 (500,150). Following treatments, adherent cells were collected and washed with PBS. The cell pellets were resuspended in 0.500 mL of binding buffer. Finally, 5 µL annexin V-FITC and 10 µL PI were added into a cell suspension, and samples were incubated in the dark for 15 min according to kit instructions. After filtration, the cell samples were analyzed by flow cytometry for the evaluation of apoptosis and cell cycle changes.

Statistical analysis

Using SPSS 26.0 software, comparisons between groups were made using a one-way ANOVA. All results are presented as the mean ± standard error. $P < 0.05$ was considered to indicate a statistically significant difference.

Result

Proscillaridin A exerted a cytostatic effect on A549 and calu-3. The cell cytotoxicity assay was used to analyze the anti-proliferation effect of proscillaridin. According to the obtained results, proscillaridin at a concentration of 175 nanomolars after 24 hours has destroyed about 65% of cancer cells (Figs. 1(A-C)).

Curcumin exerted a cytostatic effect on A549 and calu-3. To investigate whether curcumin could increase the number of apoptotic cells in both the A549 and Calu-3 cell lines (Figs. 2(A-C)). Curcumin at a concentration of 136 µM has destroyed about 55% of cancer cells within 24 hours. Curcumin and proscillaridin showed less toxicity than the effects of each one alone.

Examining the combined effect of two extracts on the Calu-3 and A549 cell lines compared to the control group showed a decrease in cell death compared to the effect of each extract alone (Figs. 7–9). With the effect of each extract of proscillaridin and curcumin on lung fibroblast cells (MRC5), we have seen 57% and 29% cell survival, respectively, but the simultaneous examination of the extracts, fortunately, led to the survival of more non-cancerous cells up to 150% (Figs. 3(A-C).

The expression of the Bcl-2 gene as a known anti-apoptotic gene in A549 and Calu-3 cells compared to the control group showed that the combination of two extracts increased the expression of the Bcl2 gene in A549 cells (Fig. 4-C). Also, the effect of each extract alone did not significantly change the increase or decrease of this gene. The combination of two extracts also increased Bax gene expression in A549 and MRC5 cells as a known pro-apoptotic gene (Fig. 4-A).

Also, in examining the expression of the P53 gene as a tumor suppressor gene, the effect of curcumin alone has shown an increase in expression in A549 cells (Fig. 4-B).

The simultaneous effects of the extracts did not show any significant changes in cell apoptosis.

Cell apoptosis was detected using Annexin V/PI staining, and the findings show no significant increase or decrease, which was in line with the results of our other tests (Figs. 5 (A-C)).

Discussion

Lung cancer is one of the most common malignancies in the world, with a very high mortality rate. Surgery and chemotherapy are among the first approaches to cancer treatment, which are accompanied by severe side effects, so more research has been done in the field of using medicinal plants with less toxicity [1]. In the present study, we investigated the simultaneous effect of Drimia maritima plant extract (proscillaridin A), a cardiac glycoside, and turmeric plant extract (curcumin) in inducing apoptosis of non-small cell lung cancer cells. By performing cell culture and MTT assays on the A549 cancer strain, we investigated the toxicity of the extracts separately and simultaneously. According to the obtained results, proscillaridin at a concentration of 175 nanomolars after 24 hours has destroyed about 65% of cancer cells, while in the study of Li et al. [21] at a dose of 50 nanomolars (this difference in concentration was probably due to the purity percentage of the extract), 65% of A549 cells underwent apoptosis. Curcumin at a concentration of 136 μM has destroyed about 55% of cancer cells within 24 hours. In the study by Wang et al. [22], curcumin at a dose of 80 μM was able to destroy 55% of A549 cells in the same period of time. In the present study, by examining the simultaneous effect of two extracts of proscillaridin and curcumin at an effective dose of 175–136 in a period of 24 hours, contrary to expectations, we saw a decrease in the death of A549 cells and, in fact, a decrease in the cytotoxicity effect compared to the effect of each extract. Separately. The results of the MTT test on the Calu-3 cancer strain showed that proscillaridin caused the death of 87% of cancer cells, and curcumin caused the death of 70% of the cells within 24 hours. This shows that the lethality of the extracts in the Calu-3 strain was higher than A549. In order to not find similar research on the effect of these two extracts on the Calu-3 strain, a study on the effect of proscillaridin and curcumin on the same strain (H1975) was selected for comparison. In the

study by Li et al. [21] of the effect of proscillaridin on the H1975 strain at a dose of 50 nM, 65% of the cells underwent apoptosis. Also, Lee et al. [24] with the effect of curcumin on this strain dramatically witnessed the death of 90% of cells at a dose of 20 µM. Examining the combined effect of two extracts on the Calu-3 line has shown a decrease in cell death compared to the effect of each extract alone. In the study by Lee et al. [23] on non-small lung cancer cells, curcumin interestingly neutralized the toxic effect of the chemotherapy drug (Paclitaxel) and reduced its side effects. With the effect of each extract of proscillaridin and curcumin on lung fibroblast cells (MRC5), we have seen 57% and 29% cell survival, respectively, but the simultaneous examination of the extracts, fortunately, led to the survival of more non-cancerous cells up to 150%. The expression of the Bcl-2 gene as a known anti-apoptotic gene in A549 and Calu-3 cells compared to the control group showed that the combination of two extracts increased the expression of the Bcl2 gene in A549 cells. Also, the effect of each extract alone did not significantly change the increase or decrease of this gene. According to the research conducted by Maryam et al. [10] and Salehi et al. [25], respectively, proscillaridin and curcumin individually decreased the expression of the Bcl-2 gene, which in the present study, by examining the simultaneous effect of the extracts, was not found. The combination of two extracts also increased Bax gene expression in A549 and MRC5 cells, as Bax is a known pro-apoptotic gene. In other studies, such as the study by Gabr et al. [26] and Li et al. [21], we have seen the effect of curcumin and proscillaridin alone, respectively, on increasing Bax gene expression [9, 15]. Also, in examining the expression of the P53 gene as a tumor suppressor gene, the effect of curcumin alone has shown an increase in expression in A549 cells. Tang et al. [27] also noted in a review study the effect of curcumin on non-small cell lung cancer cells, increasing the expression of the P53 gene.

There are very few studies on the effect of proscillaridin extract on non-small cell lung cancer, which requires more research in this area. The comparison of early and delayed apoptosis and necrosis in each cell line with the simultaneous treatment of extracts by flow cytometry also shows no significant increase or decrease, which was in line with the results of our other tests.

Conclusion

The results of the simultaneous effect of proscillaridin and curcumin extracts on non-small cells of lung cancer showed that we did not see the synergy of extracts together, but we saw an increase in the life of cancer and non-cancer cells. Of course, it has been more impressive in the case of non-cancer cells, and it seems that two extracts have neutralized each other's effects. The results indicate that the effect of each extract alone on cellular categories (especially Calu-3 and A549) was greater. Therefore, the use of extracts, along with an appropriate chemotherapy drug, may have a greater impact on the life of normal or non-cancer cells that reduces the effects of the drug and increases the penetration rate of chemotherapy, so in this regard, further studies are needed.

Declarations

Acknowledgments

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Availability of data and materials

All the authors confirm the availability of data and materials.

Authors' Contributions

FS and NNS conducted the research, analyzed and interpreted the data, and contributed to writing the manuscript. FS, FS, NKD and HM conducted part of the research, analyzed and helped interpreting the data. FS, HHK, FZ and AT provided research material, discussed the project, analyzed and interpreted the data. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

All the authors confirm that the manuscript represents their honest work and agree to consent to its publication.

Ethics approval and consent to participate

Not applicable.

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Figures

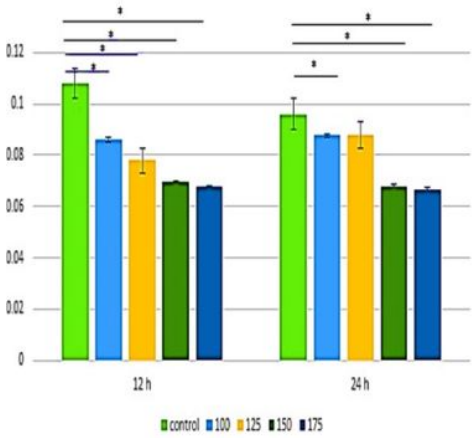

Figure 1-A: Proscillaridin A effect on MRC5

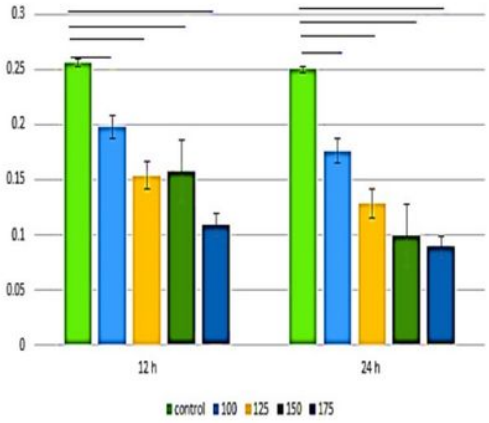

Figure 1-B: Proscillaridin A effect on A549

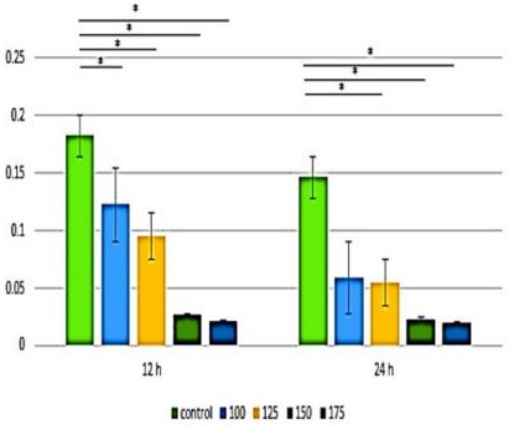

Figure 1-C: Proscillaridin A effect on Calu-3

Figure 1

See image above for figure legend.

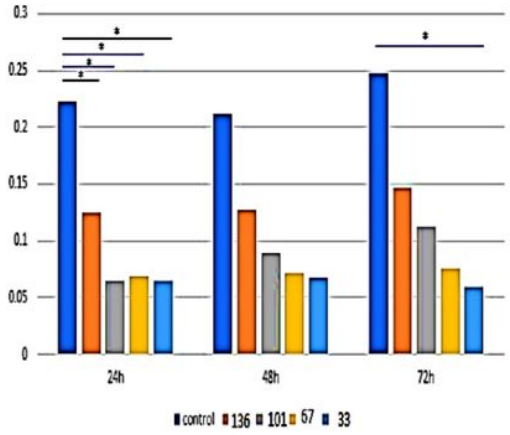

Figure 2-A: Curcumin effect on MRC5

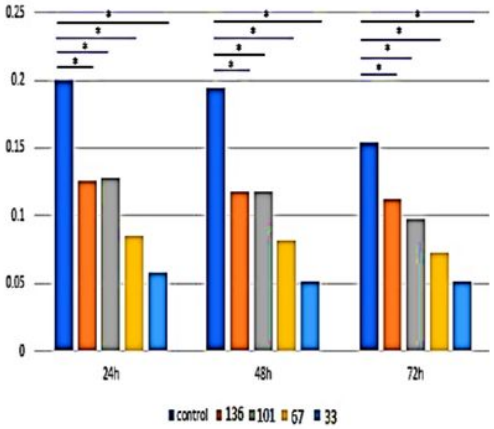

Figure 2-B: Curcumin effect on Calu-3

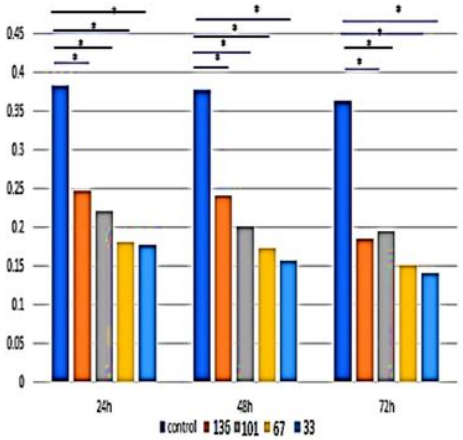

Figure 2-C: Curcumin effect on A549

Figure 2

See image above for figure legend.

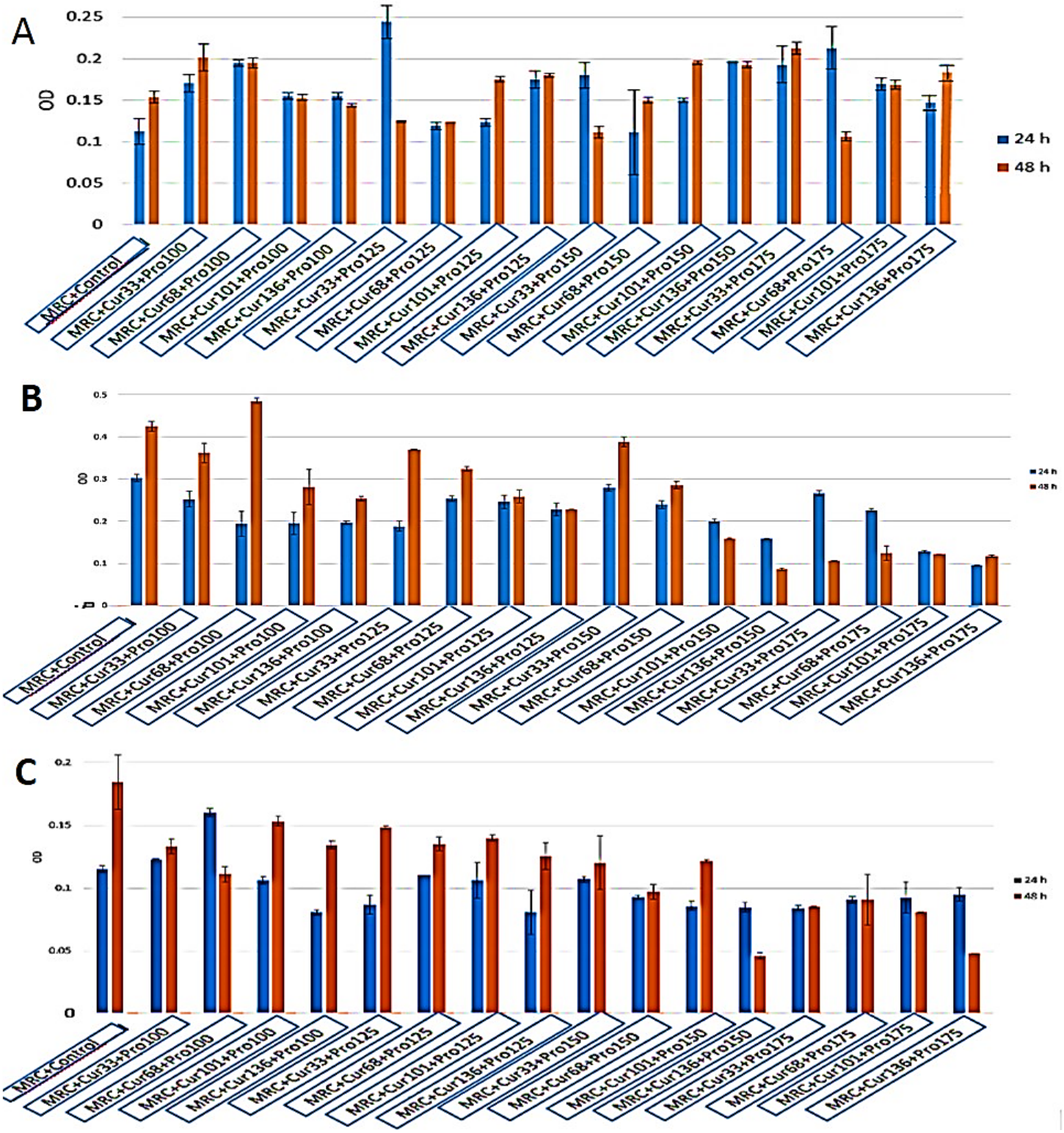

Figure 3

(A-C): The simultaneous effect of proscillaridin and curcumin on the cell viability of MRC5, A549 and Calu-3.

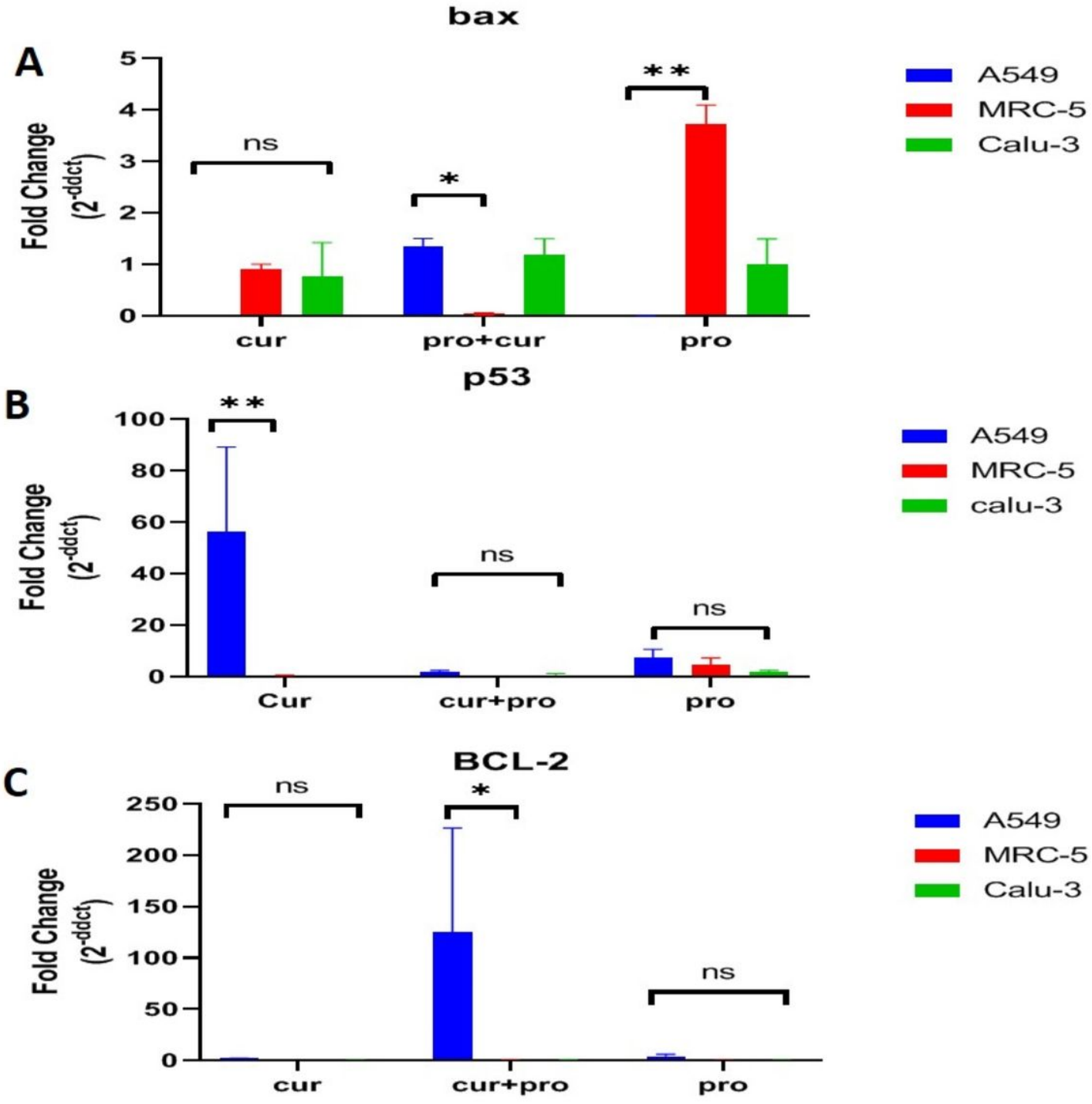

Figure 4

A: Comparison of Bax gene expression in A549 and Calu-3 cell lines treated with curcumin (cur) and proscillaridin (pro) and curcumin-proscillaridin combination (cur+pro) compared to the control group of MRC-5 cell line in RT-PCR reaction: The curcumin-proscillaridin combination led to a significant increase

in bax gene expression in the A549 cell line compared to the control group of the MRC-5 cell line. Also, proscillaridin alone led to a decrease in bax gene expression in the A549 cell line compared to the control group of the MRC-5 cell line. * and ** indicate statistically significant differences between the groups compared to the control group, respectively (P<0.05) and (P<0.01).

B: Comparison of p53 gene expression in A549 and Calu-3 cell lines treated with curcumin (cur) and proscillaridin (pro) and the combination of proscillaridin-curcumin (cur+pro) compared to the control group of the MRC-5 cell line in the RT-PCR reaction: Curcumin led to a significant increase in p53 gene expression in the A549 cell line compared to the control group of the MRC-5 cell line. ** indicates a statistically significant difference between the groups compared to the control group (P<0.01).

C: Comparison of BCL-2 gene expression in A549 and Calu-3 cell lines treated with curcumin (cur) and proscillaridin (pro) and curcumin-proscillaridin combination (cur+pro) compared to the control group of MRC-5 cell line in RT PCR reaction: curcumin-proscillaridin combination led to a significant increase in BCL-2 gene expression in A549 cell line compared to the control group of MRC-5 cell line. * indicates a statistically significant difference between the groups compared to the control group (P<0.05).

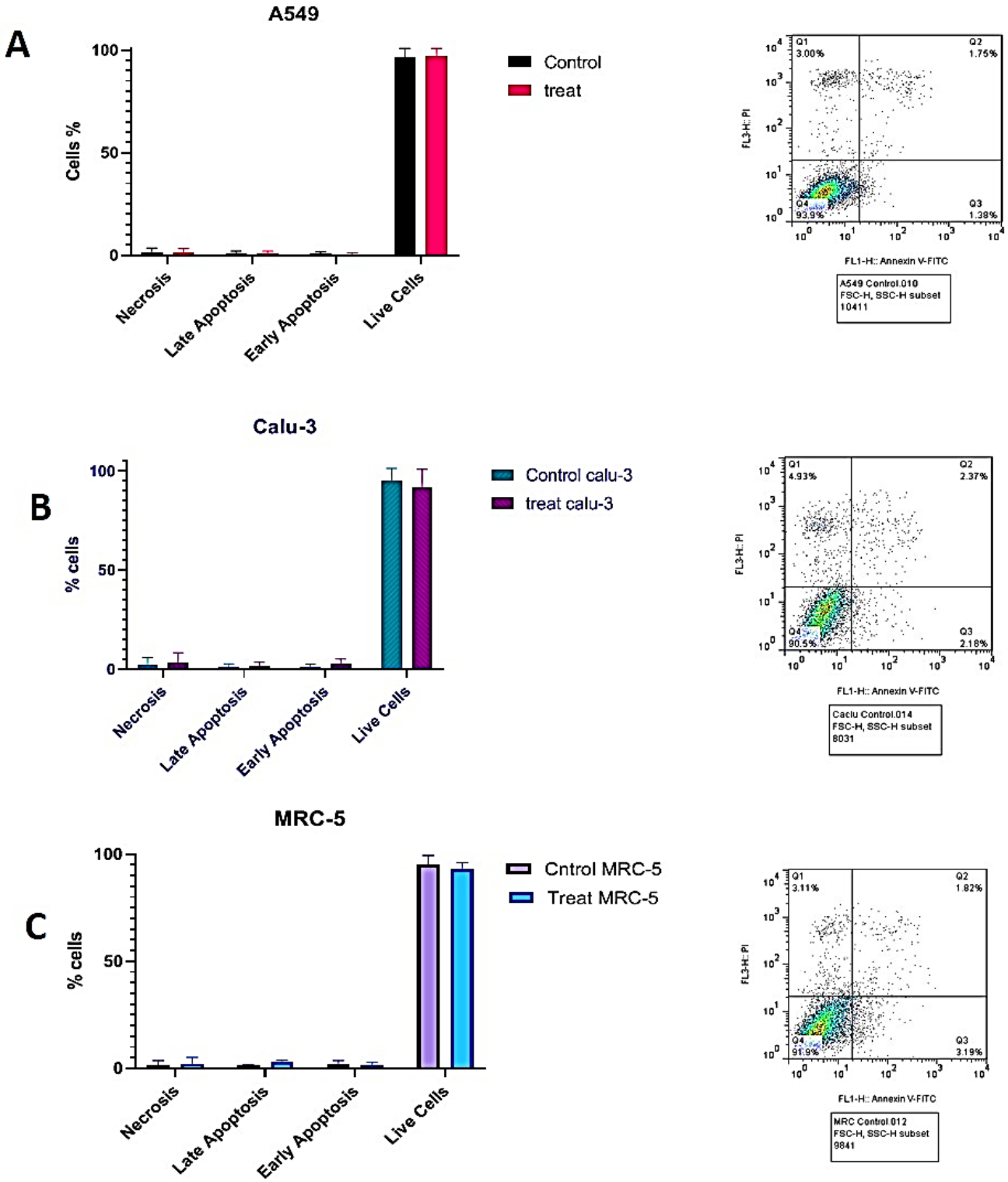

Figure 5

(A-C): Comparison of early and delayed apoptosis and necrosis in the A549, Calu-3 and MRC-5 cell line treated with curcumin (136) and proscillaridin (175) within 24 hours compared to the untreated A549, Calu-3 and MRC-5 cell line (control group) The graph shows no significant increase or decrease in the rate of apoptosis or necrosis. The data was analyzed by the T-test method.