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Original Article

In Vitro Comparative Study on Antineoplastic Effects of Pinoresinol and Lariciresinol on Healthy Cells and Breast Cancer-Derived Human Cells

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Abstract

Background: Herbal medicines are the preferred anticancer agents due to their lower cytotoxic effects on healthy cells. Plant lignans play an important role in treating various diseases, especially cancer. The present study aimed to evaluate the effect of podophyllotoxin, pinoresinol, and lariciresinol on cellular toxicity and inducing apoptosis in fibroblasts, HEK-293, and SkBr3 cell lines.

Methods: An *in vitro* study was conducted from 2017 to 2019 at the Faculty of Biological Sciences, Tarbiat Modares University (Tehran, Iran). The cell lines were treated for 24 and 48 hours with different concentrations of lignans. Cell viability and apoptosis were examined using MTT and flow cytometry, respectively. Expression levels of cell cycle and apoptosis regulator genes were determined using quantitative real-time polymerase chain reaction. Data were analyzed using a two-way analysis of variance followed by Tukey's HSD test. $P<0.05$ was considered statistically significant.

Results: Podophyllotoxin significantly increased apoptosis in fibroblast cells compared to pinoresinol and lariciresinol ($P<0.001$). The percentage of cell viability of fibroblast cells treated for 48 hours with pinoresinol, lariciresinol, and podophyllotoxin was reduced by 49%, 47%, and 36%, respectively. Treatment with pinoresinol and lariciresinol significantly overexpressed pro-apoptotic genes and underexpressed anti-apoptotic genes in SkBr3 cells ($P<0.001$). SkBr3 cells treated with lariciresinol significantly reduced gene expression ($P<0.001$).

Conclusion: Pinoresinol and lariciresinol can potentially be used as new therapeutic agents for the treatment of breast cancer.

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Keywords ● Lignans ● Natural products ● Breast neoplasms ● Cell viability ● Apoptosis

What's Known

- Podophyllotoxin exhibits significant antineoplastic effects on various types of cancer cell lines. However, it has cytotoxic effects on healthy cells.
- Lariciresinol and pinoresinol are precursors of podophyllotoxin in the lignin biosynthesis pathway and are used to assess cytotoxic and apoptotic effects on healthy and cancer cells.

What's New

- Compared to podophyllotoxin, lariciresinol, and pinoresinol showed lower cytotoxic effects on healthy cells and induced apoptosis in SkBr3 cell lines through intrinsic and extrinsic pathways.
- Lariciresinol and pinoresinol have a potential therapeutic value in the development of apoptosis-inducing agents to treat breast cancer.

Introduction

Cancer is predicted to dramatically reduce life expectancy in the 21st century, with malignant neoplasms in the female breast being one of the leading causes of cancer death.[1-3] Herbal medicines are the preferred anticancer agents due to their lower cytotoxic effects on healthy cells.[4-6] Recently, several studies have

investigated the antioxidant, anti-inflammatory, and anticancer effects of lignans.[7-9] Lignans are a large group of plant polyphenols that have anticancer properties and are capable of inducing apoptosis in cancer cells. Due to their structural similarity to estrogens, lignans, also known as phytoestrogens, are converted by gut bacteria into enterolignans.[10] Enterolignans can inhibit estrogen-dependent cancers, such as breast cancer.[11] In addition, enterolignans can reduce the risk of developing prostate and colon cancer.[12] Podophyllotoxin (PTOX) is an important lignan that induces apoptosis in cancer cells. However, due to its high cytotoxic effect on healthy cells, it is not an effective natural anticancer agent.[13, 14] Therefore, it is important to find other natural compounds that induce cancer cell death, but with minimum cytotoxic effect on healthy cells.

Pinoresinol (PINO) and lariciresinol (LARI) are known as precursors of PTOX in the lignin biosynthesis pathway,[15] which may have cytotoxic effects on breast cancer. Among plant lignans, PINO has the strongest anti-inflammatory properties in human intestinal cells.[16, 17] Furthermore, some studies reported that LARI induces apoptosis in HepG2 cells and secoisolariciresinol (a natural dietary lignan of flaxseeds) and has anti-inflammatory effects in mice with breast cancer.[9, 18, 19]

Given the above, the present study aimed to evaluate lignans that induce apoptosis in cancer cells while having low cytotoxic effects on healthy cells. The cytotoxic effects of LARI, PINO, and PTOX were evaluated on the $HER2^+$ SkBr3 breast cancer cell line, normal fibroblast cell line, and nonmalignant HEK-293 immortalized cell line.

Materials and Methods

An *in vitro* study was conducted from 2017 to 2021 at the Faculty of Biological Sciences, Tarbiat Modares University (Tehran, Iran). The study was approved by the Ethics Committee of Tarbiat Modares University (code: IR.BHN.REC.1400.025).

Chemical Compounds

PINO and LARI were purchased from Oy Arbonova Ab (Turku, Finland). As described in a previous study,[15] PTOX was purified from hairy root cultures of the *Linum album*. To prepare initial stock solutions at a concentration of 100 mmol, each compound was dissolved in pure methanol (Merck, Germany) and kept at -20 °C. The stock solutions were made in HPLC-grade methanol with a final alcohol concentration of 1% v/v.

Cell Culture and Maintenance

HEK-293, SkBr3, and fibroblast cell lines were purchased from Pasteur Institute (Tehran, Iran). Fibroblast and SkBr3 cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented medium. The HEK-293 cell line was cultured in DMEM/F12 with 10% fetal bovine serum (FBS), 100 units of penicillin, and 100 µg of streptomycin. The cell cultures were then incubated at 37 °C and 5% CO_2. All cell culture media were purchased from Gibco Invitrogen (Carlsbad, CA, USA).

Cytotoxicity Assessment

The inhibitory concentration (IC_{50}) of all compounds was evaluated using MTT colorimetric assay kit (Roche, Mannheim, Germany). Using a 96-well plate, 6×10^3 of fibroblast, 1×10^4 of HEK-293, and 18×10^3 of SkBr3 cells per well were cultured. The inhibitory concentration of each compound was determined in two steps. First, PINO and LARI concentrations of 0, 50, 100, 200, 400, 600, 800, and 1000 µM, and PTOX concentrations of 0, 0.1, 0.2, 0.4, 0.6, 0.8, 1, and 1.2 µM were used. Then, based on these concentrations, a smaller range was selected to determine their exact inhibitory concentration. The final IC_{50} was evaluated with PINO and LARI concentrations of 0, 400, 425, 450, 475, 500, 525, 550, 575, and 600 µM and PTOX concentrations of 0, 0.025, 0.05, 0.75, 0.1, 0.125, 0.15, 0.175, and 0.2 µM. MTT assay was performed after 24 h and 48 h of treatment, and optical density at 570 nm was measured using an enzyme-linked immunosorbent assay (ELISA) microplate reader (Randox Toxicology, Crumlin, UK). Tests were performed in triplicate. Methanol alcohol 1% v/v and PTOX were used as negative and positive controls, respectively.

Apoptosis Analysis

The cells were treated with 0.175 µM, 575 µM, and 500 µM of PTXO, PINO, and LARI, respectively. After 24 hours and 48 hours of treatment, cells were harvested and stained using an annexin V-FITC/PI kit, and apoptosis was examined according to the manufacturer's protocol (BioLegend, CA, USA). The cells were washed with phosphate-buffered saline (Gibco, Invitrogen, Carlsbad, CA, USA) and dissolved in 500 µl of 1×binding buffer containing 5 µl annexin and 10 µl PI. Then, it was incubated in the dark at room temperature for 15 min. Flow cytometry (BD FACSCalibur, BD Bioscience, CA, USA) was then used to assess the extent of annexin binding and PI staining.

Real-time Polymerase Chain Reaction (PCR)

The total RNA of treated cell lines was extracted using RNXTM-PLUS solution according to the manufacturer's protocol and then treated with DNase I (CinnaGen, Iran). A reverse transcription reaction for cDNA synthesis was performed using 3 µg of total RNA according to the manufacturer's protocol (Fermentas, USA). Real-time PCR was performed using ABI PRISM® 7500 system (Applied Biosystems, CA, USA). Samples without cDNA were used as negative control, and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an/the internal control (housekeeping gene). Based on published sequences obtained from GenBank®, primers were designed using OLIGO™ software version 7.0 (National Biosciences Inc., Plymouth, MN, USA). All primers listed in table 1 were purchased from Pishgam Co., (Tehran, Iran). Each test was performed in triplicate, and fold-change in gene expression was calculated using the $2^{-\Delta\Delta CT}$ method.

Statistical Analysis

The tests were performed in triplicate, with each test containing 18 samples per treatment (three cell lines, two treatment durations), i.e., a total of 54 samples for three experiments. Statistical analysis was performed using GraphPad Prism 7 (San Diego, CA, USA). Data obtained from flow cytometry were analyzed using FlowJo™ software (Tree Star Inc., Ashland, OR, USA). Two-way analysis of variance (ANOVA), followed by Tukey's HSD (or Tukey's multiple comparisons) test was used to assess significant differences between the groups. Qualitative variables for each cell line were the type and duration of treatments. $P<0.05$ was considered statistically significant.

Results

Effects of Compounds on Cell Proliferation

Cytotoxic effects of PTOX, PINO, and LARI were examined at different concentrations of SkBr3, HEK-293, and fibroblast cell lines. The results showed a significant decrease in cell viability in a time- and dose-dependent manner. The IC_{50} values of compounds for different cell lines are shown in table 2.

After 48 hours of treatment with PINO at 575 µM concentration, cell viability for SkBr3, fibroblast, and HEK-293 cells was 50%, 49%, and 49% respectively. Furthermore, cell viability for these cells after 48 hours of treatment with LARI at 500 µM concentration was 50%, 47%, and 49%, respectively. However, after 48 hours treatment with PTOX at 0.175 μM concentration, cell viability for SkBr3, HEK-293, and fibroblast cells was reduced by up to 50%, 39%, and 36%, respectively (figure 1). Therefore, it can be concluded that PTOX had a significantly higher cytotoxic effect on fibroblast and HEK-293 cell lines than PINO and LARI.

Apoptosis assay was performed using annexin V-FITC/PI staining and flow cytometry.

Table 1: The sequence of primers used for the real-time polymerase chain reaction

Name	Forward Primers	Reverse Primers
BAX	5'-GGAGCTGCAGAGGATGATTGCC-3'	5'-TCCCGCCACAAAGATGGTCACG-3'
BID	5'-AGTCACACGCCGTCCTTGCTCC-3'	5'-TGTGCAGATTCATGTGTGGATG -3'
BCL-2	5'-GATACTGAGTAAATCCATGCAC-3'	5'-AGTGTTGCAGAATATCAGCCAC -3'
FAS	5'-TAGTTGTGCTTTGCTTAGGGTTC-3'	5'-AAGTTCTTGCTCTGAGTTCACC -3'
CASP3	5'-ATGCATACTCCACAGCACCTGG-3'	5'-GTGAGCATGGAAACAATACATGG -3'
CASP8	5'-CCTGTTGAGACTGATTCAGAGG-3'	5'-TGAAAGTAGGCTGAGGCATCTG -3'
CASP9	5'-CAGTCCCTCCTGCTTAGGGTCG-3'	5'-CCTCCACTGTTCAGCACTTGTCG -3'
TP53	5'-GGACGGAACAGCTTTGAGGTGC-3'	5'-TATGGCGGGAGGTAGACTGACC -3'
CDKN1A (P21)	5'-CAGGGGACAGCAGAGGAAGAC-3'	5'-GGGCGGCCAGGGTATGTAC -3'
GAPDH*	5'-CCAGCCGAGCCACATCGCTC-3'	5'-ATGAGCCCCAGCCTTCTCCAT -3'

*Housekeeping gene

Table 2: The half-maximal inhibitory concentration values (μM) of fibroblast, HEK-293, and SkBr3 cell lines after 24- and 48-hour treatment with pinoresinol, lariciresinol, and podophyllotoxin

Treatment duration	Cell lines	Treatments		
		Pinoresinol	Lariciresinol	Podophyllotoxin
24-hour	Fibroblast	650	475	0.175
	HEK-293	600	550	0.150
	SkBr3	675	600	0.300
48-hour	Fibroblast	550	400	0.075
	HEK-293	550	475	0.075
	SkBr3	575	500	0.175

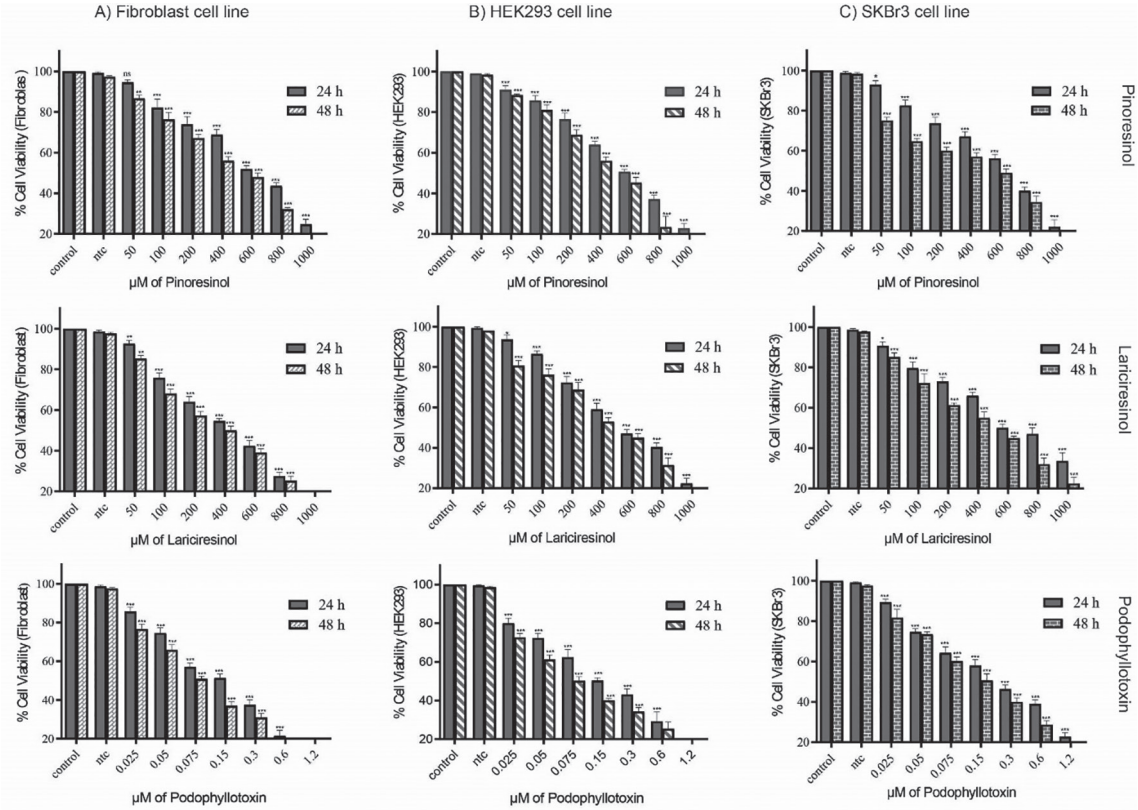

Figure 1: The cytotoxicity effects of lignan compounds in terms of different treatment durations and concentrations. Fibroblast (A), HEK-293 (B), and SkBr3 (C) cell lines were treated with different concentrations of pinoresinol, lariciresinol, and podophyllotoxin for 24 and 48 hours. One group of each cell line was treated with alcohol methanol and used as negative control (final methanol concentration: 1% v/v). Error bars represent mean±SD for n≥3 (*P<0.05, **P<0.01, ***P<0.001 compared to the control group).

Cell lines were treated with PTXO, PINO, and LARI at concentrations of 0.175 µM, 575 µM, and 500 µM, respectively (figure 2). The results showed that the treatments caused a significant increase in apoptosis in a time-dependent manner compared to the control cells. PINO increased apoptosis in fibroblast, HEK-293, and SKBR3 cells by 5.2-, 6.6-, and 9-fold after 24 hours of treatment and 10.5-, 9-, and 11-fold after 48 hours of treatment, respectively. Whereas LARI increased apoptosis by 7.4-, 6.7-, and 10.7-fold after 24 hours of treatment and 20.2-,7.4-, and 12.7-fold after 48 hours of treatment, respectively. Therefore, compared to control cells, PINO and LARI induced higher levels of apoptosis in cancer cells than in healthy cells after 24 hours of treatment. In addition, in comparison with PINO, 48 h of treatment with LARI induced higher apoptosis in all cell lines (figure 3). On the other hand, PTOX induced higher levels of apoptosis in fibroblast cells than HEK-239 and SKBR3 cells. The minimum and maximum levels of induced apoptosis in fibroblast cells were achieved after 24 hours and 48 hours of treatment with PINO and PTOX, respectively. Compared to PTOX, LARI induced higher levels of apoptosis in cancer cells and less in healthy cells after 48 hours of treatment. The majority of cell death caused by all compounds was due to apoptosis rather than necrosis (figures 3B and 3C).

Expression of Apoptosis-associated Genes in Cell Lines

Evaluation of fibroblast, HEK-293, and SkBr3 cell lines showed that PINO, LARI, and PTOX significantly overexpressed pro-apoptotic *BAX* and *FAS* genes, as well as the CDKN1A inhibitory gene. In contrast, the expression of anti-apoptotic *BCL-2* genes was decreased in all treated cell lines compared to untreated cells. However, the fold-change of apoptotic genes in fibroblast cells treated with PTOX was more significant than those treated with PINO and LARI (figure 4). In comparison with PINO, LARI caused higher overexpression of apoptotic genes in HEK-293 and SKBR3 cells. Moreover, LARI significantly overexpressed pro-apoptotic genes and underexpressed anti-apoptotic genes in SkBr3 compared to fibroblast cells. The full extent of *BCL-2* underexpression was observed in SkBr3 cells after 48 hours of treatment with LARI. The highest overexpression was found in the *FAS* gene (a death receptor in the extrinsic apoptotic pathway), followed by the *CASP8* gene (a key caspase in this pathway) of the fibroblast and SkBr3 cell lines (figures 4E and 4G).

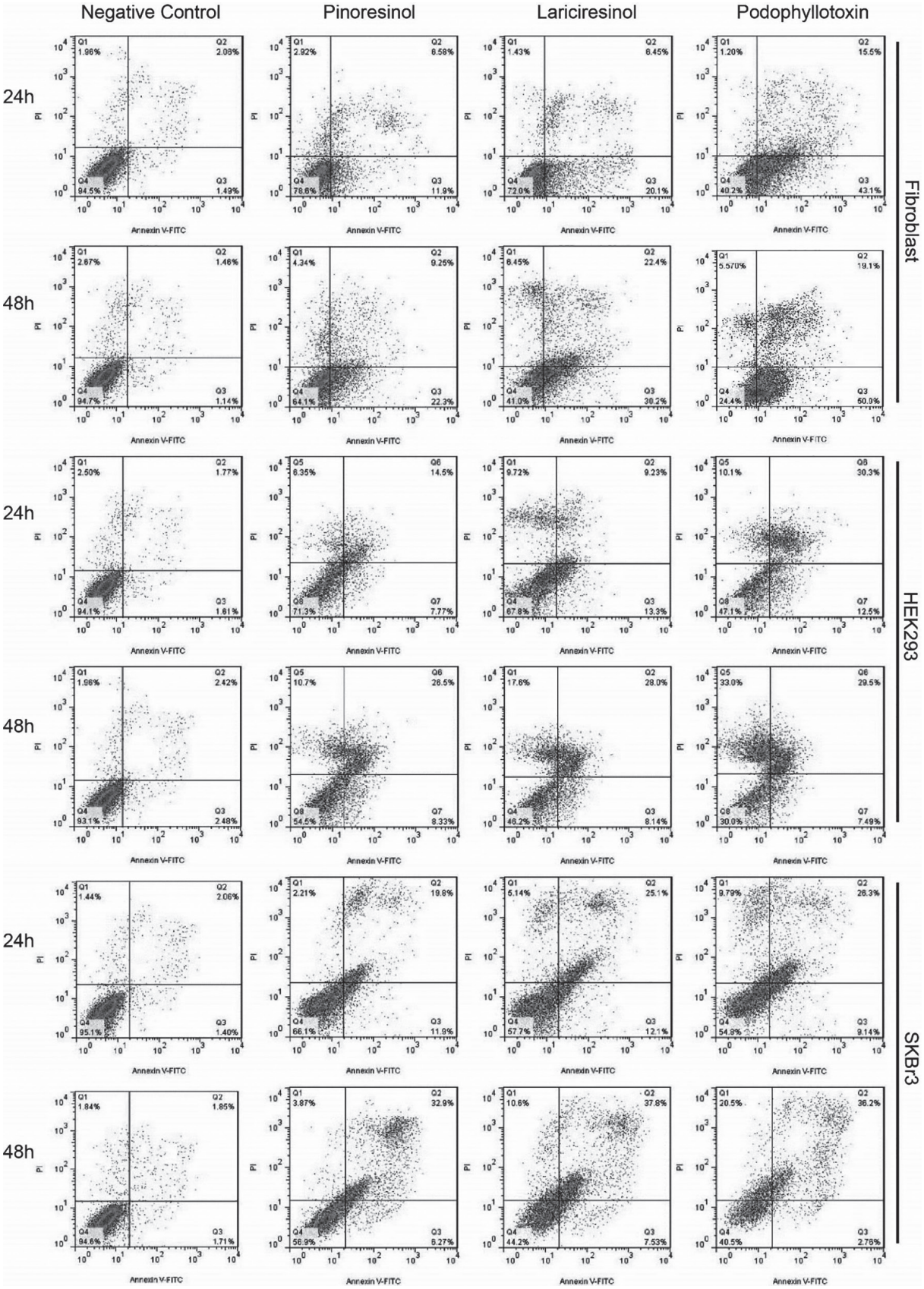

Figure 2: The percentage of apoptosis and necrosis as determined by flow cytometry using annexin V and PI staining. Fibroblast, HEK-293, and SkBr3 cell lines were treated with 0.175 µM of podophyllotoxin, 575 µM of pinoresinol, and 500 µM of lariciresinol for 24 and 48 hours. One group of each cell line was treated with alcohol methanol and used as negative control (final methanol concentration: 1% v/v).

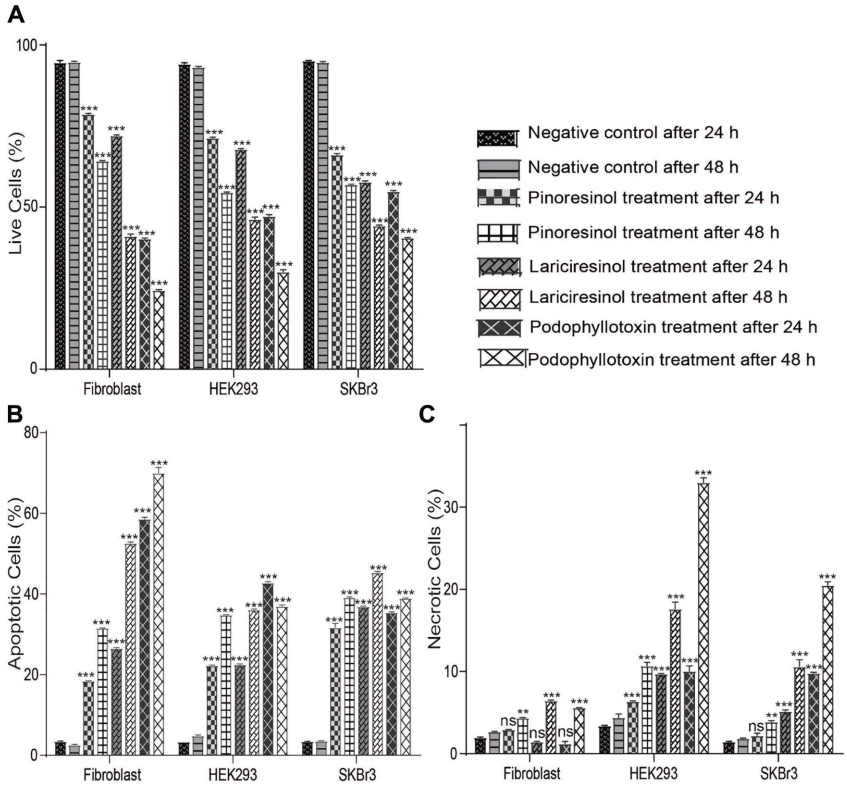

Figure 3: The percentage of viability (a), apoptosis (b), and necrosis (c) of cells treated with lignans. Fibroblast, HEK-293, and SkBr3 cell lines were treated with 0.175 µM of podophyllotoxin, 575 µM of pinoresinol, and 500 µM of lariciresinol for 24 and 48 hours. One group of each cell line was treated with alcohol methanol and used as negative control (final methanol concentration: 1% v/v). Values are presented as means±SD from two independent experiments. Significant differences with respect to the negative control group are indicated by *P<0.05, **P<0.01, and ***P<0.001. ns: Not statistically significant

However, the expression of these genes in HEK-293 was less than the key genes (*BAX*, *CASP9*, and *TP53*) in intrinsic apoptotic pathways (figures 4A, 4F, and 4H).

Discussion

Cytotoxicity assessment showed that PINO and LARI reduced the growth and proliferation of all cell lines in a time- and dose-dependent manner. Most importantly, these compounds had lower cytotoxic effects on healthy cells than PTOX.

Regardless of the type and etiology of cancer, all cancer cells show similar characteristics, including uncontrolled cell growth, angiogenesis, and evading apoptosis and necrosis.[20] The most common mechanisms of programmed cell death are apoptosis and necrosis. Since apoptosis occurs within each cell, unlike necrosis, it does not lead to inflammation of surrounding cells, making it an ideal mechanism for anticancer drug development.[21] Extrinsic pathway triggers cell apoptosis through extracellular signals by binding external ligands to death receptors in plasma membranes. The occupation of *FAS* receptors by their specific ligand causes trimerization and leads to the assembly of death-inducing signaling complexes. In this complex, pro-*CASP8* is activated and, in turn, leads to the activation of executive caspases.[22]

The results of flow cytometry confirmed that treatment with PINO, LARI, and PTOX induced apoptosis in all cell lines in a time-dependent manner. Compared to PTOX, treatment with PINO and LARI induced higher levels of apoptosis in the SkBr3 cell line, but left more live cells in the fibroblast cell line. Moreover, it was found that PTOX had higher cytotoxic and apoptotic effects on healthy cells in a time- and dose-dependent manner. These findings were consistent with previous studies reporting the anticancer effects of PINO and LARI through the induction of apoptosis in cancer cells.[23-25]

BCL-2 protein inhibits apoptosis in the mitochondrial pathway.[26, 27] Our results showed that treatment with LARI, PINO, and PTOX overexpressed *TP53*, *CDKN1A*, and *BAX* while reducing anti-apoptotic *BCL-2* gene expression. Moreover, treatment with PTOX significantly altered the expression of apoptotic genes in fibroblast and HEK-293 cell lines. The results also showed that *FAS* genes had the highest increase in expression. In fibroblast and SkBr3 cell lines, it was observed that the expression of *FAS* and *CASP8* genes was lower in HEK-293 than in the key genes (*TP53*, *CASP9*, and *BAX*)

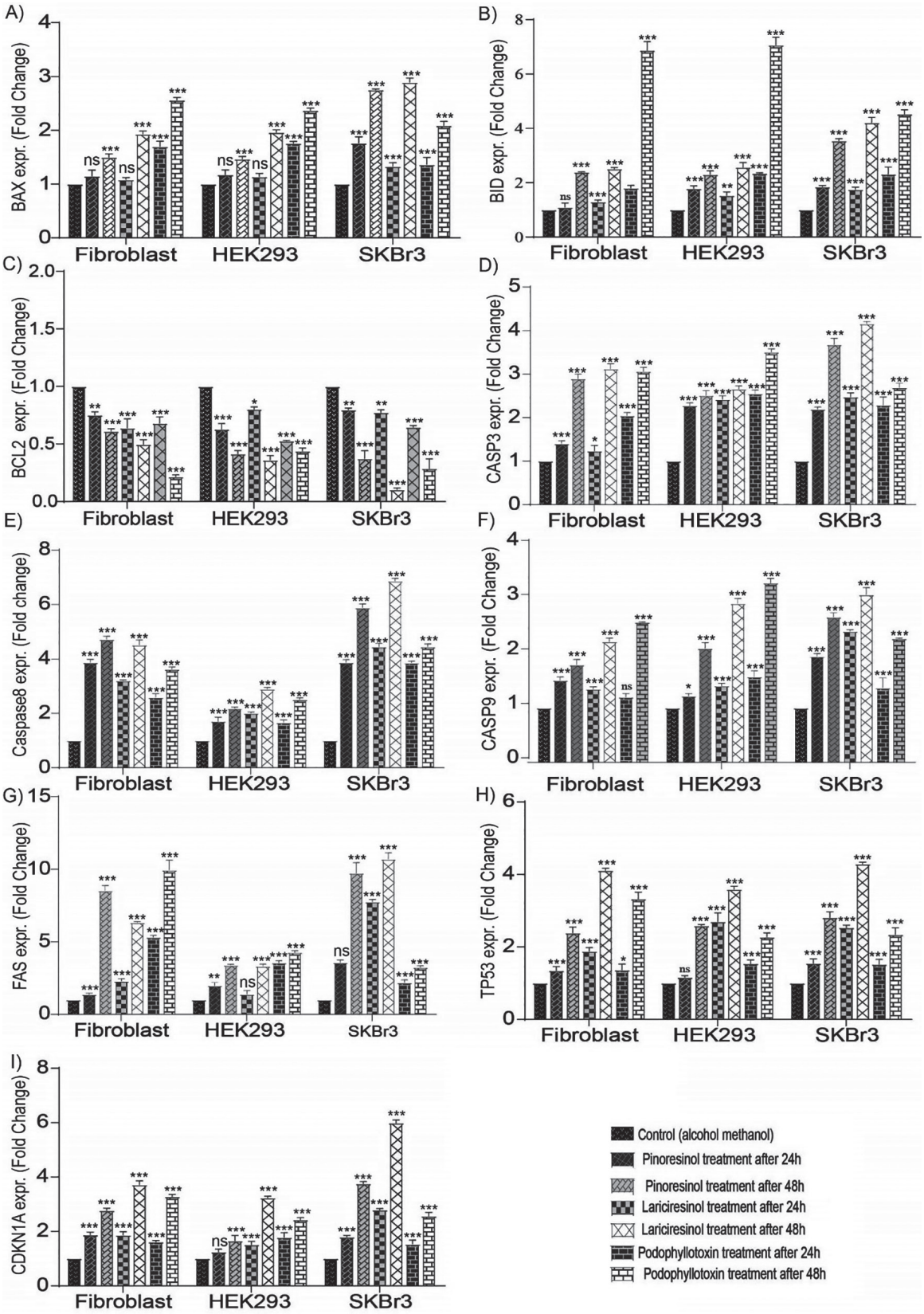

Figure 4: The effect of podophyllotoxin, pinoresinol, and lariciresinol on the expression of genes involved in apoptosis and cell cycle. Fibroblast, HEK-293, and SkBr3 cell lines were treated for 24 and 48 hours with pinoresinol, lariciresinol, and podophyllotoxin at a final concentration of 575, 500, and 0.175 µM, respectively. One group of each cell line was treated with alcohol methanol and used as negative control (final methanol concentration: 1% v/v). Fold change was calculated using the $2^{-\Delta\Delta CT}$ method. Each value is the mean±SD of the three experiments. Significant differences with respect to the negative control are indicated by *P<0.05, **P<0.01, and ***P<0.001. ns: Not statistically significant

involved in the intrinsic apoptosis pathway. Moreover, the highest expression of *CASP3* occurred in the SkBr3 cell line after 48 hours treatment with LARI. Previous studies reported that extrinsic and intrinsic pathway cascades were not completely separated. *CASP8* can directly cause apoptosis by activating *CASP3* or indirectly by degrading *BID* and triggering the mitochondrial pathway. In most cells, small amounts of initiator caspases, inadequate to induce apoptosis, are activated in the extrinsic pathway. In these cells, the extrinsic pathway activates the intrinsic apoptotic pathway through *CASP8* activation and the degradation of *tBID*, thus releasing apoptotic substances from the mitochondria and causing cell death.[28-30] Therefore, the overexpression of *BID* (as an intermediate) could explain the effect of PINO and LARI on both intrinsic and extrinsic pathways and the subsequent induction of apoptosis. It is also reported that induction of apoptosis can occur via *TP53*-dependent or independent pathways.[31, 32] This indicates that *TP53* gene expression is affected by the treatment of cells with LARI and PINO. Interestingly, we found that this effect was greater in SkBr3 than in other cell lines.

As the main strengths of the study, for the first time, we investigated the cytotoxic effects and mechanism of apoptosis induced by PINO and LARI compared to PTOX. The data suggest that LARI and PINO are less cytotoxic to healthy cells and are likely to have fewer side effects. Moreover, compared to PINO and PTOX, LARI had the highest apoptotic effect on breast cancer cells. As a limitation of the study, given the tests were performed *in vitro*, it is recommended to perform *in vivo* studies to determine the molecular mechanism of these compounds in inducing apoptosis. However, developing synthetic derivatives of PINO and LARI for clinical studies will be challenging due to limited access to lignan as plant secondary metabolites.

Conclusion

Treatment with PINO and LARI induced apoptosis in SkBr3 cells by promoting the overexpression of pro-apoptotic genes and downregulation of anti-apoptotic genes. Although these compounds induced apoptosis in SkBr3 cells through the extrinsic pathway, both intrinsic and extrinsic pathways may be involved. Further studies are required to understand the exact mechanism of the activity of these compounds. PINO and LARI and their derivatives might have potential therapeutic value in the treatment of breast cancer.

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Authors' Contribution

M.S. performed the experiments. All authors contributed to the study design, data analysis and interpretation, and critical review of the manuscript. They have read and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflict of Interest: None declared.

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