



Research Article

Salvurmin A and Salvurmin B, Two Ursane Triterpenoids of *Salvia urmiensis* Induce Apoptosis and Cell Cycle Arrest in Human Lung Carcinoma Cells

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Abstract

Background: Ursane triterpenoids could be considered as novel multi-target therapeutic anti-cancer agents. Salvurmin A and Salvurmin B are novel cytotoxic ursane triterpenoids isolated from the aerial parts of *Salvia urmiensis*, an endemic plant species of Iran.

Methods: In this study, we assessed cytotoxicity of these compounds against two human cancer cell lines and one human normal cell line and investigated its mechanism via apoptosis and cell cycle arrest.

Results: Salvurmin A and B showed the most cytotoxic effect on A549 cells compared to other studied cancer cells. IC₅₀ values for Salvurmin A and B against A549 cells were 35.6 ± 1.5 and 19.2 ± 0.8 μ M, respectively. Based on annexin V staining, both of these compounds significantly induced apoptosis in A549 cells. Moreover, these two compounds significantly increased cell accumulation in G2/M and decreased the number of cells in G0/G1 phases in A549 cells in a dose-dependent manner.

Conclusion: Based on the results Salvurmin B can be considered as potential candidate for further studies against human lung carcinoma.

Introduction

Cancer is still the second leading cause of death in human society.¹ According to WHO (World Health Organization) report, cancer is the first or second cause of death in adults in 91 of 172 countries, and it is the third or fourth in 22 countries in 2015.² Natural products and their derivatives have been playing a fundamental role in drug discovery and also, more than 49% of FDA approved anticancer agents are natural products or their derivatives.³ Terpenoids, which are a main group of natural products, are one of the main sources for development of clinically important anti-cancer agents such as paclitaxel.⁴ On the other hand, triterpenoids could be considered as novel multi-target therapeutic anti-cancer agents, since, they affect various proteins involved in tumor development. Semi-synthetic derivatives of triterpenoids could increase their therapeutic efficacy, while their synthesis in the laboratory could be successfully developed.^{5,6} Pentacyclic triterpenoids including: the lupane, oleanane or ursane groups, act at various stages of tumor development such as inducing tumor cell differentiation and apoptosis, DNA

repair, cell cycle arrest, adjustment of different transcription and growth factors and suppressing tumor angiogenesis mechanisms.^{7,8} The anti-tumor effects of pentacyclic triterpenoids were confirmed in a series of carcinoma cells including: neuroblastoma, colon, lung, and breast.⁹⁻¹³ Furthermore, there are many studies on pentacyclic triterpenoids, such as betulinic acid as anti-cancer agents. In melanoma cells, BA (Betulinic acid) induced cell cycle arrest in the G1 phase and also decreased of cdk4 protein,^{14,15} also, it inhibited the catalytic activity of the topoisomerase I by preventing it from binding to DNA.¹⁶ In addition, BA treatment produced a decrease in cyclin D1 expression (on miRNA and protein levels) in different cell lines.¹⁷ Ursane triterpenoids are pentacyclic triterpenoids, which have shown potential as anticancer drugs. There are many studies on Ursolic acid (UA) and its natural analogues as antiproliferative agents. UA could modulate various targets in tumor development, especially in breast, colon, prostate, stomach and skin tumors.^{5,18-20} UA induced cell cycle arrest in the G1 phase and also, decreased the expression of cyclins D1, D2, and E, and their activating

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partners cdk2, cdk4, and cdk6 with concomitant induction of p21.²¹ Furthermore, UA could accelerate cell death by producing DNA fragmentation, mitochondrial disruption and modulation of key apoptotic proteins on DU145, CT26 and B16F10 cells.²² *Salvia urmiensis* belonging to the Lamiaceae family, is an endemic species growing in West Azerbaijan, a northwestern province of Iran.^{23,24} Ursane triterpenoids isolated from *Salvia* species, represented cytotoxic activities in many cases.²⁵⁻²⁷ In addition, in recent years various cytotoxic ursane triterpenoids were extracted from *S. urmiensis* and some of them showed apoptosis-inducing activity.²⁸⁻³⁰ In our recent publication, two new ursane triterpenoids (Salvurmin A and Salvurmin B) were extracted from the aerial parts of *S. urmiensis*.³¹

In the regard of antiproliferative effects of triterpenoids and specially Ursane triterpenoids as anticancer agents,^{27,28,32,33} for the first time, in this study, we have been investigated the antiproliferative properties of the Salvurmin A and B on human colon adenocarcinoma cell line (HT29), human alveolar lung carcinoma cell line (A549), and human normal breast cells (MCF-10a). Moreover, the induction of apoptosis and cell cycle analysis were conducted for both compounds in the A549 cells.

Materials and Methods

Compounds and cell lines

Salvurmin A and Salvurmin B (Figure 1), two new ursane triterpenoids, investigated for their antiproliferative effects in this study, were obtained from the dichloromethane extract of the aerial parts of *S. urmiensis*.³¹ The human cell lines: Human colon adenocarcinoma (HT29), human alveolar lung epithelial carcinoma (A549) cells and human normal breast cells (MCF-10a) were purchased from the National Cell Bank of Iran (Pasteur Institute, Tehran, Iran).

Cell culture

The cell lines: A549 and MCF-10a were cultured in RPMI 1640 media (Bio idea, Iran) supplemented with 10% fetal bovine serum (FBS; Gibco, USA) and 1% antibiotics (penicillin/streptomycin; Biosera, France). In addition, the HT-29 cell line was cultured in DMEM high glucose in the presence of 1% L-glutamine (Gibco, USA). The cell lines

were grown at 37 °C in a humidified CO₂ incubator.

Cell viability assay

The antiproliferative activities of both compounds were determined using the 3-(4, 5-dimethylthiazol-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) assay according to the previous protocols.³⁴⁻³⁷ The human cancer cell lines (A549 and HT29) and human normal breast cells (MCF-10a) were trypsinized by trypsin/EDTA 0.5% solution (Gibco, USA) and then were seeded at a density of 8×10^3 cells per well for the A549 cell line, 10×10^3 cells per well for the HT-29 cell line and 15×10^4 cells per well for the MCF-10a cell line in 100 μ l of complete culture media in 96-well microplates. After 24 h incubation at 37 °C, each cell line was treated by adding 100 μ l of media supplemented with six different concentrations (1-100 μ M) of the above-mentioned compounds and cisplatin (positive control) in triplicate manner. Moreover, the cells treated only with DMSO at concentrations equal to the test wells were used as negative control (the concentration of DMSO was less than 0.1% to avoid its cytotoxic effect). After 72 h incubation, the media was removed and replaced with fresh MTT solution at a final concentration of 0.5 mg/ml in each well. After 4 h incubation, the media was discarded and 150 μ L of dimethyl sulfoxide (DMSO; Sigma-Aldrich, UK) was added to each well and was incubated in the dark for 10 minutes to solubilize formazan crystals. The optical absorbance was measured at 490 nm by a microplate ELISA reader (Bio Tek, UK). The cytotoxic effect of the compounds on each cell line was performed in two or three independent experiments (n=3). Excel 2019 (Microsoft, USA) and Curve Expert 1.4 (Microsoft, USA) were used to delineate a plot of the percentage inhibition versus concentration and calculate IC₅₀ (demonstrating the concentration with 50% growth inhibition).^{38,39}

Apoptosis analysis

Apoptosis analysis was assessed using PE Annexin V apoptosis detection kit with 7-AAD (Biolegend, USA) based on reported protocols.^{36,40-42} Firstly, the A549 cells were cultured in 24-well plates at a density of 80×10^3 cells per 500 μ M of complete culture media. After 24 h incubation,

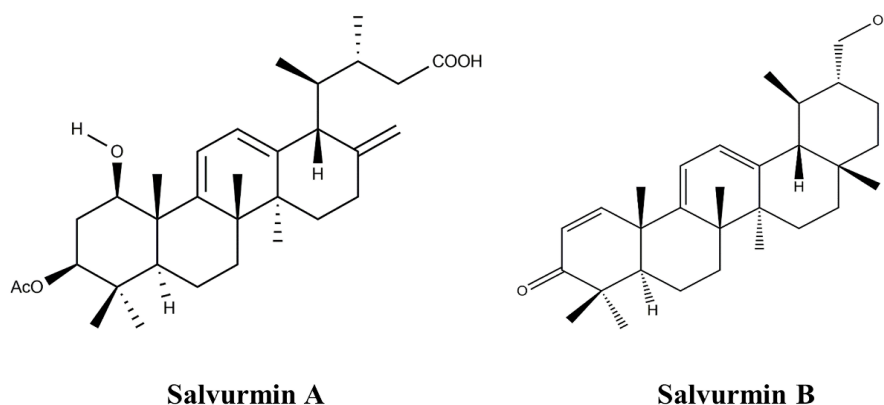


Figure 1. Structures of Salvurmin A and Salvurmin B.

the cells were treated with different concentrations of Salvurmin A (20, 40, 80 μM) and Salvurmin B (10, 20, 40 μM) and then, were incubated for 72 h. In each plate, 4 wells contained untreated cells as negative control. The cells were trypsinized and washed two times in cold phosphate-buffered saline (PBS 1X). Fifty μL annexin V-binding buffer was added to dilute the pellets and then the cells transferred to polystyrene tubes (BD Biosciences, USA). Two μL of PE-conjugated AV and 2 μL of 7-AAD were added into each tube and incubated at room temperature in the dark for a period of 15 min. Afterwards, 300 μL annexin V-binding buffer was added and mixed gently. The samples were immediately analyzed using a four-color FACSCalibur flow cytometer (BD Biosciences, USA). Finally, the data were analyzed with FlowJo software package (BD Biosciences, USA).⁴³ Apoptosis analysis was done in 3 independent experiments.

Cell cycle analysis

The cell cycle analysis was performed on A549 cell line using flow cytometry according to the published protocols.³⁶ A549 cells (80×10^3 cells per 500 μM of complete media) were seeded in a 24-well plate. The cells were treated with Salvurmin A (20, 40, 80 μM) and Salvurmin B (10, 20, 40 μM) and incubated for a period of 72 h. After harvesting the cells by mild trypsinization and washing in cold phosphate-buffered saline (PBS 1X), the cells were fixed in cold 70% ethanol at 4 $^{\circ}\text{C}$ for a week. Afterwards, the cells were washed with PBS 1X two times and centrifuged at $400 \times g$, 4 $^{\circ}\text{C}$. Then, 50 μL ribonuclease A (100 $\mu\text{g}/\text{mL}$, Sigma-Aldrich, Germany) was added to remove RNA. At the final step, 200 μL propidium iodide (PI; 50 $\mu\text{g}/\text{mL}$ solution, Sigma-Aldrich, Germany) was added to stain DNA.⁴² The stained cells were then analyzed by a four-color FACSCalibur flow cytometer (BD Biosciences, USA) and FlowJo software. Cell cycle analysis was done in 3 independent experiments.

Statistical analysis

SPSS software package V.16 (SPSS Inc, USA) was used to perform statistical analyses. One-way ANOVA followed by post-hoc Tukey HSD (Honestly Significant Difference) test were used to determine statistical differences between groups. P-value less than 0.05 was considered statistically significant.^{44,45} GraphPad Prism 8 software package (Inc; San Diego CA, USA, 2003) was also used for drawing the statistical graphs.

Results

Cytotoxic activity studies

In our previous report, the antiproliferative effects of Salvurmin A and Salvurmin B were assessed against human breast adenocarcinoma cells (MCF-7)³¹. In the first step of this study, we assessed the antiproliferative effects of the above compounds against human normal breast cells (MCF-10a) to assess the cytotoxicity of the compounds on normal cells and determine the selectivity index between

cancerous and normal cells. Both compounds showed a significant decrease in cytotoxic effect on the normal breast cells (MCF-10a) with an IC_{50} of $117.2 \pm 8.1 \mu\text{M}$ for Salvurmin A and $129.9 \pm 9.9 \mu\text{M}$ for Salvurmin B (Table 1) in comparison to the breast carcinoma cells, indicating their relative selectivity in growth inhibitory effect between tumorigenic and non-tumorigenic cell lines. The selectivity index for Salvurmin A, Salvurmin B and Cisplatin on breast cancerous and normal cells were 2.16, 3.23 and 1.65, respectively. Salvurmin B showed a better selectivity index than Salvurmin A and Cisplatin. In addition, Salvurmin B showed lower cytotoxic effect on normal cells (MCF-10a) compared to Salvurmin A.

In the latter step, we assessed the cytotoxic effects of Salvurmin A and B on two human cancer cell lines: human lung carcinoma cells (A549) and human colon adenocarcinoma cells (HT-29), as summarized in Table 2. These two carcinoma cell lines commonly utilize for evaluating antiproliferative effects of triterpenoids.⁴⁶⁻⁴⁹ Three days (72 hours) after treatment, the compounds, induced growth inhibitory effect on the cell lines compared with cisplatin and paclitaxel as the standard drugs. Among carcinoma cell lines in this study (A-549 and HT-29) and in our previous report (MCF-7 and SW1116), the best cytotoxic effects were observed on A549 cells for both compounds. Salvurmin B with an IC_{50} of $19.2 \pm 0.8 \mu\text{M}$ was more potent compared to Salvurmin A ($\text{IC}_{50} = 35.6 \pm 1.5 \mu\text{M}$) on A549 cells. Similarly, in HT-29 cells, Salvurmin B with an IC_{50} of $35.2 \pm 2.9 \mu\text{M}$ showed more cytotoxic activity compared to Salvurmin A ($\text{IC}_{50} = 38.9 \pm 3.4 \mu\text{M}$).

Evaluating the apoptotic effects of Salvurmin A and Salvurmin B on A549 cell line

In order to investigate the antiproliferative effects of Salvurmin A and Salvurmin B, cells were treated with three different concentration of the compounds (20, 40, and 80 μM for Salvurmin A and 10, 20, and 40 μM for Salvurmin B) for 72 hours and then stained with PE-annexin V (AV)/7-amino actinomycin D (7-AAD) apoptosis detection kit using flow cytometry method. In this method, PE-AV in the presence of calcium binds to phosphatidylserine which then migrates to the outer plasma membrane during cell apoptotic process. 7-AAD is a DNA specific membrane-impermeable dye that detects live and dead cells. Annexin V and 7-AAD were used to distinguish between intact ($\text{AV}_{\text{neg}}/\text{7-AAD}_{\text{neg}}$), apoptotic (early phase apoptotic cells ($\text{AV}_{\text{pos}}/\text{7-AAD}_{\text{neg}}$) and late phase apoptotic cells ($\text{AV}_{\text{pos}}/\text{7-AAD}_{\text{pos}}$)), and necrotic cells ($\text{AV}_{\text{neg}}/\text{7-AAD}_{\text{pos}}$). After 72

Table 1. IC_{50} values of Salvurmin A, Salvurmin B and Cisplatin on MCF-10a cells. The values represented the mean \pm standard deviation (SD), based on three independent experiments. Cisplatin was used as positive control.

Compounds	MCF-10a
Salvurmin A	$117.2 \pm 8.1 \mu\text{M}$
Salvurmin B	$129.9 \pm 9.9 \mu\text{M}$
Cisplatin	$30.6 \pm 3.5 \mu\text{M}$

Table 2. IC₅₀ values of Salvurmin A, Salvurmin B, Cisplatin and paclitaxel on A-549 and HT-29 cell lines. The values represented the mean \pm standard deviation (SD), based on three or two independent experiments. Cisplatin and paclitaxel were used as positive control.

Compounds	(IC ₅₀ \pm SD) μ M	
	A549	HT29
Salvurmin A	35.6 \pm 1.5 μ M	38.9 \pm 3.4 μ M
Salvurmin B	19.2 \pm 0.8 μ M	35.2 \pm 2.9 μ M
Cisplatin	23.6 \pm 1.8 μ M	26.2 \pm 0.3 μ M
Paclitaxel (nM)	7.28 \pm 0.9	1.2 \pm 0.7

hours of treatment, the concentrations of 40 μ M (71.9 \pm 7.8) and 80 μ M (86.6 \pm 4.6) of Salvurmin A significantly induced apoptosis in A549 cells ($p < 0.0001$) compared to the negative control (1.5 \pm 0.26 %) as illustrated in Figure

2. Moreover, Salvurmin B showed a substantial rise in the cell apoptosis by increasing its concentrations (for 10 μ M: 15.0 \pm 1.9, $p = 0.03$, for 20 μ M: 79.0 \pm 4.7 %, $p < 0.0001$ and for 40 μ M: 86.2 \pm 4.5, $p < 0.0001$) (Table 3). Based on the results, the apoptotic effects of Salvurmin A and Salvurmin B on the A549 cells are dose dependent. Figure 3 illustrated apoptotic effects in A549 cells by various concentrations of Salvurmin A and Salvurmin B. Untreated cells were mostly viable as they were negative for both Annexin V and 7-AAD. ANOVA with Tukey's post-hoc tests were applied to analyze the data.

The potential effect of Salvurmin A and Salvurmin B on the A549 cells' cell cycle

In order to investigate the effects of Salvurmin A and Salvurmin B on cell cycle phases in A549 cell line, a flow

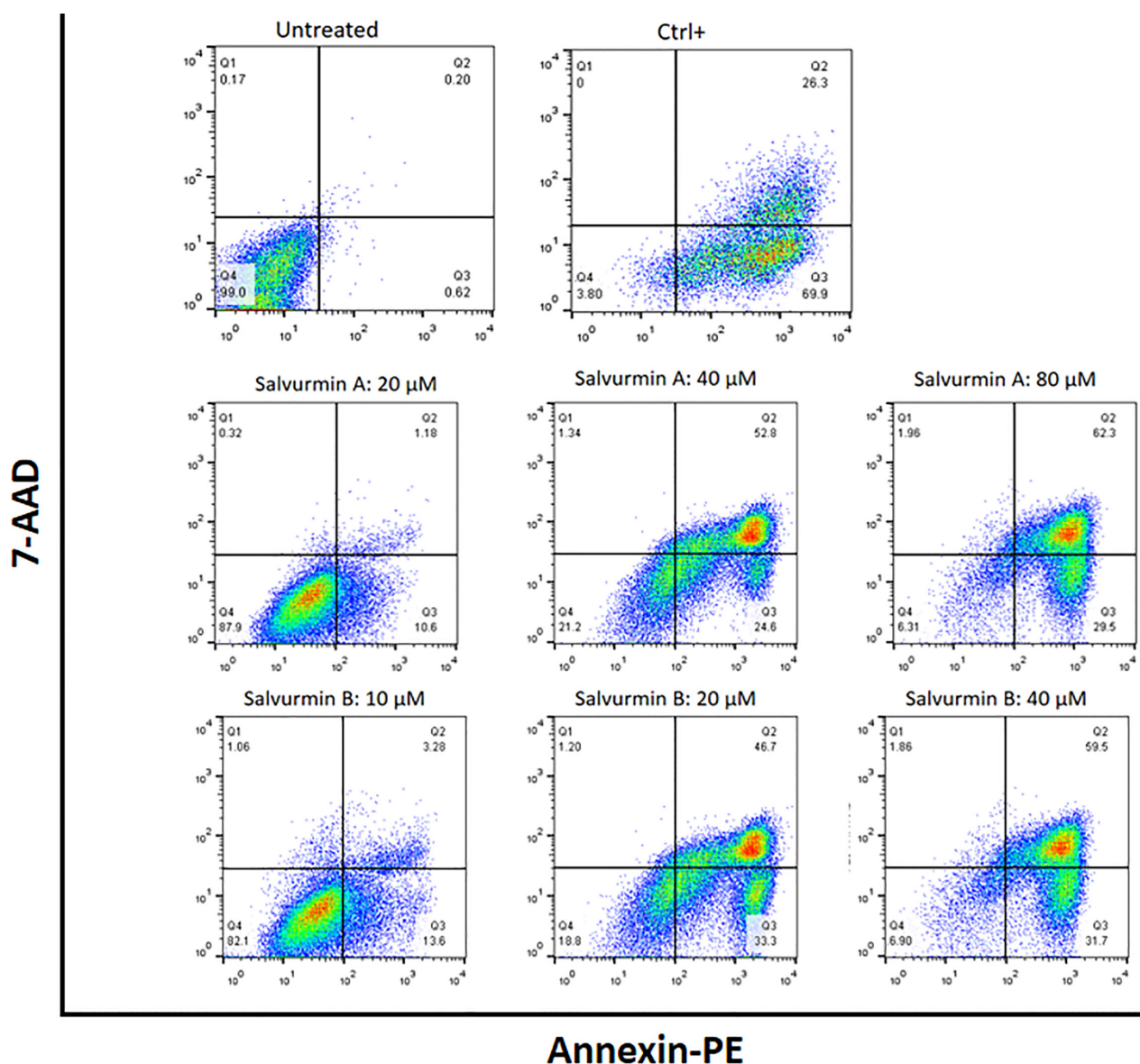


Figure 2. Flow cytometry dot plots of A549 cells after 72 hours of treatment with various concentrations of Salvurmin A and Salvurmin B. Untreated cells were mostly viable as they were negative for both Annexin V and 7-AAD and positive control (Ctrl+) cells were heated at 56 $^{\circ}$ C.

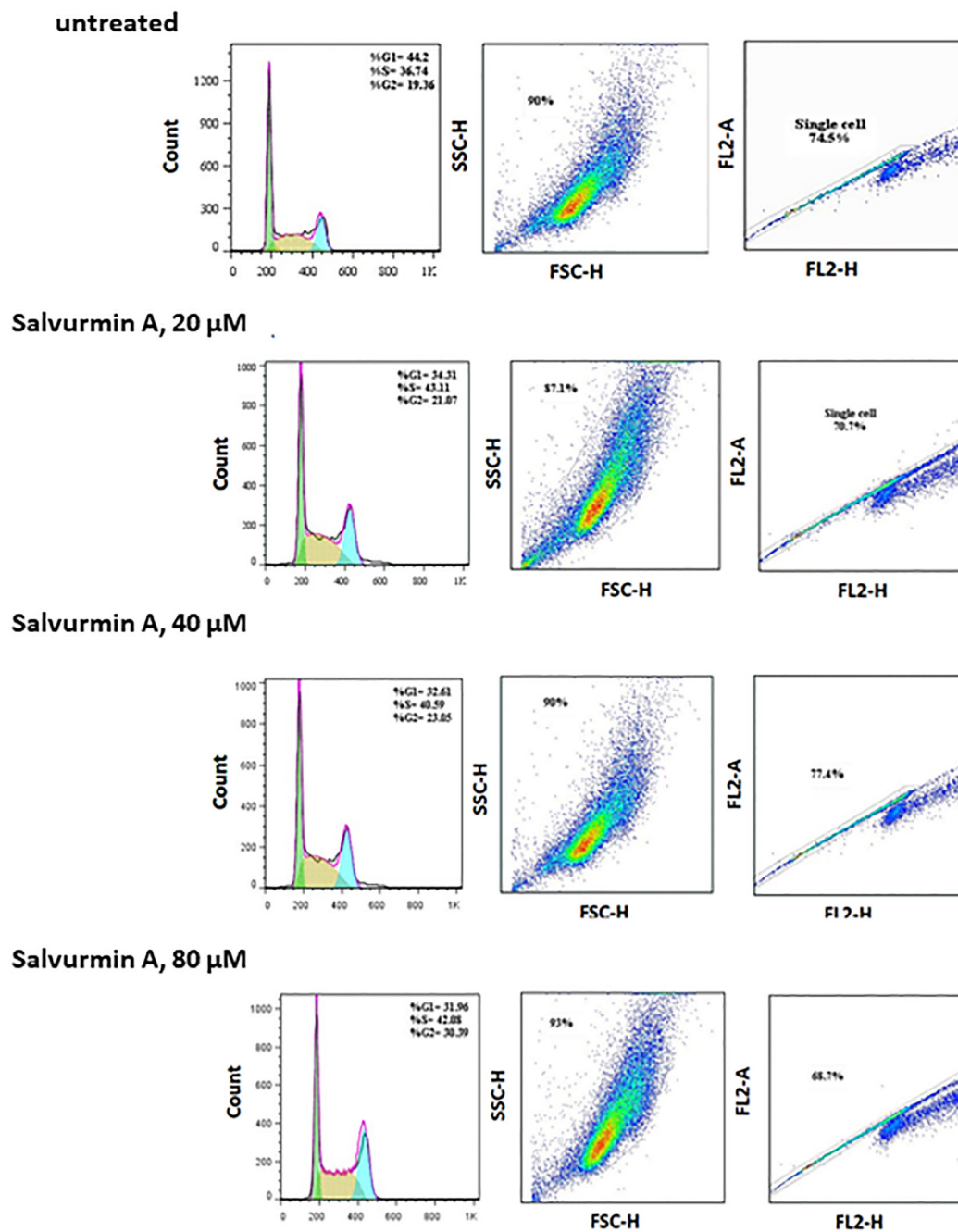


Figure 3. Flow cytometry cell cycle histograms of A549 cells treated with 20 μM, 40 μM and 80 μM of Salvurmin A for 72 hours.

cytometry-based method was performed. Briefly, the A549 cells were treated with 20 μM, 40 μM and 80 μM of Salvurmin A, and 10 μM, 20 μM and 40 μM of Salvurmin B for 72 hours and then stained with PI. We observed a rise in the proportion of G2/M cells by increasing the concentrations of the compounds in all treatments. After 72 hours treatment with Salvurmin A, the number of G2/M cells was significantly higher than the control ($17.1 \pm 2.5\%$) and was increased from $25.3 \pm 3.9\%$ at 20 μM to $34.1 \pm 4.1\%$ at 80 μM ($p < .0001$). Similarly, treatment of A549 cells for 72 h with Salvurmin B increased the number of G2/M cells from $12.1 \pm 2.6\%$ at 10 μM to $33.4 \pm 2.4\%$ at 40 μM compared to untreated cells (15.9 ± 4.6). In

addition, the number of cells in G0/G1 phase decreased in almost all concentrations of the compounds (Figures 3 and 4, and Table 4). The comparison of these results with the results of the annexin V/7-AAD test, showed that both compounds were able to inhibit the cell cycle by inducing apoptosis in A549 cells.

Discussion

Ursane triterpenoids are pentacyclic triterpenoids, which have shown desirable potential as anticancer drugs.^{7,8} Salvurmin A and B are novel cytotoxic ursane triterpenoids, which demonstrated antiproliferative effects against human carcinoma cells (MCF-7 and SW1116).³¹

Table 3. Percentage of apoptotic cells upon the effect of various concentrations of Salvurmin A and Salvurmin B on the A549 cells. Data represents the mean ± standard deviation (SD) based on three independent experiments.

	Salvurmin A (Mean ± SD %)			Salvurmin B (Mean ± SD %)			Positive Control	Negative Control
	80 μM	40 μM	20 μM	40 μM	20 μM	10 μM		
Apoptotic cells	86.6 ± 4.6	71.9 ± 7.8	7.6 ± 3.7	86.2 ± 4.5	79.0 ± 4.7	15.0 ± 1.9	77.7 ± 16.1	1.5 ± 0.6
Early apoptosis	26.8 ± 10.3	22.5 ± 3.4	6.3 ± 3.8	29.4 ± 2.5	33.4 ± 2.2	12.4 ± 1.6	49.7 ± 17.5	0.9 ± 0.2
Late apoptosis	59.8 ± 9.3	49.4 ± 4.4	1.3 ± 0.3	56.8 ± 2.4	45.6 ± 2.6	2.6 ± 0.6	28.0 ± 1.7	0.6 ± 0.4
Necrotic cells	9.5 ± 1.1	1.3 ± 0.3	0.3 ± 0.2	1.6 ± 0.4	1.2 ± 0.3	1.0 ± 0.4	7.4 ± 6.5	0.7 ± 0.5

Table 4. Effects of the various concentrations of Salvurmin A and Salvurmin B on cell cycle progression of cancerous cell line A549. Data represents the mean ± standard deviation (SD) based on three independent experiments.

Groups	Concentration (μM)	G0/G1 (%)	P value	S (%)	P value	G2/M (%)	P value
Untreated		48.1 ± 3.3		34.2±2.7		17.1 ± 2.5	
Salvurmin A	20	33.7 ± 3.1	0.001	38.0 ± 5.2	0.7	25.3 ± 3.9	0.05
	40	32.6 ± 3.1	0.001	41.5 ± 1.1	0.2	25.8 ± 2.2	0.05
	80	31.2 ± 1.0	0.0001	34.8 ± 3.9	1.0	34.1 ± 4.1	0.001
Untreated		69.9 ± 1.7		14.2 ± 4.2		15.9 ± 4.6	
Salvurmin B	10	67.7 ± 0.7	0.8	19.8 ± 1.7	0.3	12.1 ± 2.6	0.5
	20	60.7 ± 5.0	0.03	18.8 ± 3.3	0.5	17.3 ± 2.7	0.9
	40	52.2 ± 3.1	0.001	22.6 ± 3.6	0.05	33.4 ± 2.4	0.001

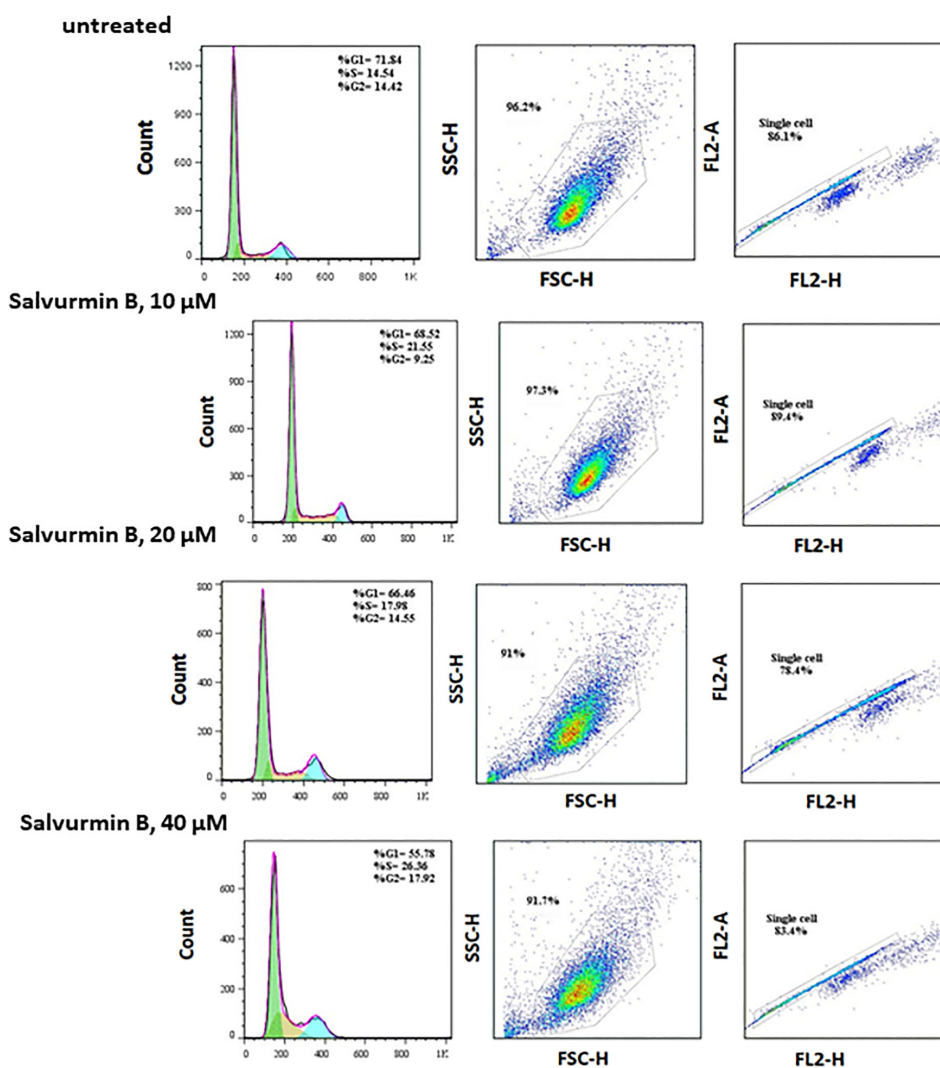


Figure 4. Flow cytometry cell cycle histograms of A549 cells treated with 10 μM, 20 μM and 40 μM of Salvurmin B for 72 hours.

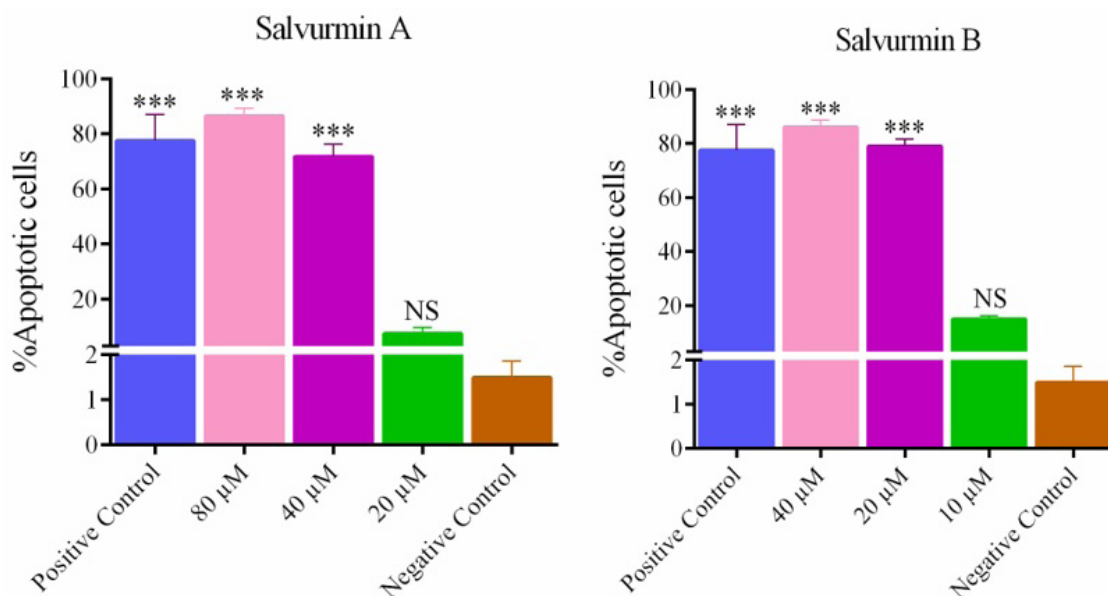


Figure 5. Apoptotic effects in A549 cells by various concentrations of Salvrurin A and Salvrurin B. Data are expressed as mean \pm SEM of three independent experiments performed in triplicate. * $p < 0.01$, *** $p < 0.0001$ and ns= non-significant compared to negative control.

In this study, the antiproliferative effects of Salvrurin A and B assessed against human carcinoma cells (A-549 and HT-29) and normal cells (MCF-10a). These cell lines commonly utilize for evaluating antiproliferative effects of triterpenoids.⁴⁶⁻⁴⁹ The results showed that both compounds have cytotoxic effects on above cell lines. Salvrurin B showed better cytotoxic effect among all carcinoma cells in current study (A-549 and HT-29) and in our previous report (MCF-7 and SW1116). In addition, Salvrurin A and B demonstrated lower cytotoxic effect on normal cells (MCF-10a) compared to carcinoma cells, indicating their relative selectivity in growth inhibitory effect between tumorigenic and non-tumorigenic cells. The best cytotoxic effects were observed on A549 cells for both compounds. Based on literature ursane triterpenoids isolated from *Salvia* species demonstrated remarkable antiproliferative effects against various human carcinoma cells.^{27,28,30,33} For instance, corosolic acid (CA) isolated from the roots of

S. syriaca showed cytotoxicity against A549 human lung epithelial cancer cells with an IC_{50} of 12 $\mu\text{g/mL}$.⁵⁰ As well, Urmiensolide B and Urmiensic acid isolated from *S. urmiensis* showed IC_{50} values of 2.8 and 1.6 μM against MCF-7 cells, respectively.²⁸ urs-12-ene-1 β ,3 β ,11 β ,22 α -tetraol isolated from *S. urmiensis* represented cytotoxicity with IC_{50} values of $88.35 \pm 0.09 \mu\text{M}$ toward MCF-7 cells.²⁹ In comparison with other ursane triterpenoids, Salvrurin A and B demonstrated desirable cytotoxic effects against A549 cells.

We investigated the mechanism via apoptosis and cell cycle arrest. Both compounds induced apoptosis on A549 cells and also, the apoptotic effects were dose dependent. Figure 5 illustrated apoptotic effects in A549 cells by various concentrations of Salvrurin A and Salvrurin B. In addition, we observed a rise in the proportion of G2/M cells by increasing the concentrations of the compounds in all treatments (Figure 6). In addition, the number of cells in

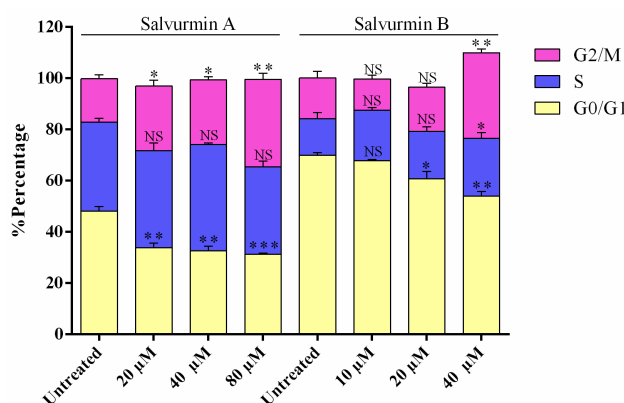


Figure 6. The effects of various concentrations of Salvrurin A and Salvrurin B on cell cycle in the A549 cell line after 72 hours. The data presented as mean \pm SD showed accumulation of the cells in G0/G1, S and G2/M phases of cell cycle. * $p < 0.01$, ** $p < 0.001$, and *** $p < 0.0001$ compared to negative control.

G0/G1 phases in A549 cells decreased in a dose-dependent manner (Figure 6). The comparison of these results with the results of the annexin V/7-AAD test, showed that both compounds were able to inhibit the cell cycle by inducing apoptosis in A549 cells. ANOVA with Tukey's post-hoc tests were applied to analyze the data.

Conclusion

In conclusion, Salvurmin A and Salvurmin B, two novel ursane triterpenoids of *S. urmiensis*, showed significant antiproliferative effects on human carcinoma cell lines (A549, HT29). In addition, both compounds showed a significant decrease in cytotoxic effect on the normal breast cells (MCF-10a) compared to the carcinoma breast cells (MCF-7) indicating their relative selectivity in growth inhibitory effect between tumorigenic and non-tumorigenic cell lines. Salvurmin B showed a better selectivity index than Salvurmin A and Cisplatin on cancerous and normal breast cells. In addition, in all studied carcinoma cell lines, Salvurmin B represented better antiproliferative activity than Salvurmin A. Both compounds were more potent against human lung carcinoma cell line (A549) compared to other cell lines. Moreover, these two compounds induced apoptosis and cell cycle arrest in the G2/M phase in A549 cell line in a dose-dependent manner. Therefore, Salvurmin B can be considered as a potential candidate for further studies against human lung carcinoma.

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Author Contributions

SH performed the biological experiments, analyzed the data and prepared the manuscript. HS supervised the analysis of data and revising the manuscript. RK contributed in biological assay and analyzing them and revising the manuscript. ARJ edited the manuscript. NE supervised the biological assay and revised the manuscript. All authors read and approved the final manuscript.

Conflict of Interest

The authors report no conflicts of interest.

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