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Original Research

Cytotoxicity and Apoptosis Inducing Effects of Some Lathyrane and Tigliane Diterpenes against Breast Cancer Cell Lines

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Abstract

Natural compounds and especially herbal medicine are of great interest due to their various biological effects and their potential to act as a drug for the treatment of various neoplasms especially breast cancer that we are facing with its increasing prevalence around the world. The aim of this study was to evaluate the cytotoxic and cell death mechanism of some diterpenoids (Lathyrane or Tigliane) extracted from the *Euphorbia sogdiana* Popov against two breast cancer cell lines, MCF-7 and 4T1. Determination the cytotoxic effects of four various diterpenoids was performed using MTT assay against MCF-7, 4T1, and HUVEC cell lines. The IC50 of each compound against cell lines was determined by drawing the dose-response graph using graph Pad prism software. Finally, the apoptotic effects of compound with the most cytotoxic effects was determined by flow cytometry assay for 24 hrs of incubation in IC50 concentration. Statistical analysis confirmed compound (3) with the most cytotoxicity against both cancer cell lines. The IC50 of compound (3) was determined as 10.1 ± 5, 28 ± 5, and 50 ± 3 µg/ml, for MCF-7, 4T1, and HUVEC cells, respectively. Furthermore, the cells treated with 5 and 10 µg/ml of compound (3) for 24 hrs, showed 49 and 57% of apoptosis, respectively. So, these surveyed compounds have the potential to be considered as useful anti-breast cancer agents due to the great cytotoxicity and apoptotic effects against related cancer cell lines and safety profile according to their rational selectivity index.

Keywords: *Euphorbia sogdiana* Popov; Lathyrane; Tigliane

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Introduction

Breast cancer is the most common malignancy in women around the world [1]. The statics in 2012 showed 1,671,149 new cases and 521,907 cases of deaths due to breast cancer in the world [2]. Because of the considerable side effects of routine chemotherapeutic agents in the treatment of breast cancer, nowadays, the using of natural products, have attracted much attentions to reduce the adverse effects of these agents [3]. Natural compounds are of great interest due to their various pharmacological and biological effects [4]. These products either can play an important role in the discovery and development of new drugs, or can be directly used as a drug or as a primary structure in the synthesis of other compounds [5].

Herbal compounds showed therapeutic effects against bacteria, fungi, viruses, and especially cancer cells. In this regard, 67 % of anti-cancer agents are in natural origin [5].

Terpenoids, the largest and most diverse family of natural compounds, are containing a large number of 5-carbon structures, so that about 5% of the known compounds are in this category. Diterpenes, the most diverse terpenoids, composed with five carbon units, have linear or single- to four-rings in their structure. Different species of *Euphorbia sp*., contain various diterpene esters with various biological activity; the most important of which are Lathyrane and Tigliane [6].

Lathyranes are one of the largest classes of tricyclic diterpenes [7]. Various studies have investigated the cytotoxic and anti-tumor effects of these compounds; for example, two Lathyrane compounds isolated from *Euphorbia lathyris* were investigated against different cancer cell lines of breast, liver, and lung [8]. Furthermore, the aerial part of *Euphorbia helioscopia* containing a Lathyrane diterpene showed specific cytotoxic effects against MCF-7 and PANC-1 cell lines [9].

Tiglianes are another group of diterpenes with 4 rings in their structure [10]. Previous studies have investigated the cytotoxic effects of Tiglianes against C8166 PBMC cells [11]. On the other hands, the cytotoxic effects of six extracted Tigliane-like diterpenoids from *Euphorbia fischeriana* on MDA-MB-231 and HepG2 cells have been proven [12]. Finally, the cytotoxicity evaluation of *Euphorbia poisonii* latex contains Tigliane diterpene was established against A-498 cell line [13]. Based on these studies, the aim of the present study was to investigate the cytotoxic effects and cell death mechanism of three new Lathyrane and a new Tigliane extracted from the *Euphorbia sogdiana* Popov against two breast cancer cell lines as well as evaluating their safety profile in a normal cell line as HUVECs.

Materials and Methods

Cells, compounds, and reagents

The purified Lathyrane diterpenes including 2,5,15-O-riacetyl-3-O-nicotinyl-14-oxo-12-ene-6(17)-epoxylathyrane(1), 2,3-O-dinicotinyl-5,15-O-diacetyl-14-oxo-12-ene-6(17)-epoxylathyrane (2), and 2,15-dihydroxy-3,5-O-diacetyl-14-oxo-12-ene-6(17)-epoxylathyrane (3), as well as a novel Tigliane diterpenes named

as 4-deoxy-4α-phorbol-12-(2,3-dimethyl) butyrate-13-isobutyrate (4) were provided from previous studies as explained bellow [14]. 4T1, HUVEC, and MCF-7 cell lines were purchased from National Cell Bank of Iran (Pasteur Institute, Tehran, Iran). Annexin-V-FLUOS Staining Kit was obtained from Invitrogen Company. All other chemicals were obtained from known commercial sources (Biosera, France) for cell culture tests.

Sample preparation for in vitro assays

The extraction of *E. sogdiana* Popov and fractionation was performed as described in our pervious study [14]. Compounds (1) to (4) were dissolved in 1 ml PBS (with 10 % of total DMSO) and serially diluted by PBS to produce various concentrations including 500, 250, 125, 62.5, and 31.25 µg/ml and used for biological tests after the filtration.

Cytotoxicity assay

In order to determine the cytotoxic effects of the mentioned purified compounds, MTT assay was used. Cell suspension with 2×10^4 cells/ml concentration of 4T1, MCF-7, and HUVEC cells in RPMI 1640 in final volume 180 µl, was seeded to each well of a 96 well-plate and incubated at 37 ºC in a CO2 incubator. In the next day, 20 µl of various concentrations of compounds (final concentrations: 50, 25, 12.5, 6.25, and 3.125 µg/ml) was added to each well. After 48 hrs of incubation, 20 µl of MTT solution (5 mg/ml) was added to each well, and the plate was further incubated for 3 hrs. Finally, the formazan crystals were dissolved in 150 µl DMSO, and the plate was subjected to absorbance read at 570 nm using a microplate reader.

Flow cytometry analysis

About 5×10^5 cells/well of MCF-7 cells was cultured in a 6-well microplate. After 24 hrs, the cells were incubated with the IC50 and sub-toxic concentration of compound with the most toxicity, for 24 hrs and subsequently subjected to flow cytometry analysis. Briefly, all cells were collected, washed with PBS and then incubated with annexin-V-FITC and propidium iodide according to the manufacturer's instructions (Invitrogen, US) for 15-20 min. Finally, the cells were centrifuged at 300 ×g and washed using PBS and binding buffer (1X), suspended in 200 µl binding buffer (1X) and analyzed by flow cytometry on a BD FACSCalibur (BD, USA).

Statistical analyses

Cytotoxicity assay was performed in three independent experiments and four replicate wells for each concentration of compounds. PBS-treated cells were considered as negative control and results were expressed as cell viability % ± SD. SPSS 23 software was used for statistical analysis. Analysis of variance (ANOVA) followed by post hoc (Tukey's test) was used to distinguish the differences between groups. The significance was assumed as $p < 0.05$. The IC50 of each structure was determined by drawing the graph of cell survival percent against concentration using GraphPad Prism 7.0 software. Finally, the selectivity index for each compound against two type of cancerous cell line was calculated by dividing the IC50 value against HU-

VEC cells to the IC50 of MCF-7 or 4T1 cells.

Results

Cytotoxicity of diterpene flavonoids against breast cancer and normal cells

Cytotoxicity evaluation of Tigliane or Lathyrane diterpenes was performed using MTT assay and the results showed that the cytotoxic effects of all compounds is concentration dependent; increasing in concentration led to more cell killing either for cancerous or normal cells.

According to the calculated IC50 for each compound against 4T1 and MCF-7, it was showed that the cytotoxicity of all compounds was statistically different for MCF-7 and 4T1 except for compound (2) with similar cytotoxic effects against two cell lines (P value = 0.89). For other diterpene flavonoids, MCF-7 showed more susceptibility than 4T1.

In comparison the observed cytotoxicity of cancer and normal cells, it was showed that there were significant differences between cytotoxicity of MCF-7 and HUVEC cells. In other words, the similar concentrations of each compound, showed significant toxicity for MCF-7 but not for HUVEC in all concentrations (P value < 0.05).

For 4T1 cell line, the cytotoxic effects of four compounds didn't show any significant difference according to the calculated IC50 for each compound (P value = 0.71). Except this fact that compound (1) showed the most cytotoxic effects against 4T1 in comparison to the other diterpene flavonoids (P value = 0.038) (Figure 1, a).

For MCF-7 cell line, on the other hand, there was significant difference between the cytotoxic effects of compound (2) and (3) (P value = 0.002); compound (3) with the most cytotoxicity and compound (2) with the less cytotoxic effects. However, there was no statistically significant difference between the cytotoxic effects of compounds (1), (3), and (4) (Figure 1, b).

For HUVECs, as the normal cell line, there was no significant cytotoxic differences between four compounds. Actually, various investigated diterpenes showed similar cytotoxic effects against these cells in the same concentration (P value > 0.05) (Figure 1, c).

Finally, based on drawn graphs of concentrations versus cell survival percent, the IC50 of compound (1) to (4) for MCF-7 was determined as 12 ± 4, 25 ± 4, 10.1 ± 5, and 10.3 ± 7 µg/ml, respectively. These data for 4T1 cell line, on the other hands, for all four compounds was 16 ± 4, 25 ± 6, 28 ± 5, and 25 ± 1 µg/ml, respectively. Lastly, For HUVEC cell line, the IC50 value was calculated as 50 ± 2, 48 ± 4, 50 ± 0.6, and 50 ± 0.1 µg/ml, for compound (1) to (4), respectively. The calculated selectivity index for each breast cancer cell line was summarized in table 1.

Table 1. Selectivity index of compound (1) to (4)

compound	MCF-7	4T1
(1)	4.17	3.13
(2)	1.92	1.92
(3)	4.95	1.79
(4)	4.85	2

Cell death mechanism detection of lathyrane using flow cytometry

In order to determine the cell death mechanism of compound (3) against MCF-7, flow cytometry assay was used. The results of this test showed that compound (3) in concentration of

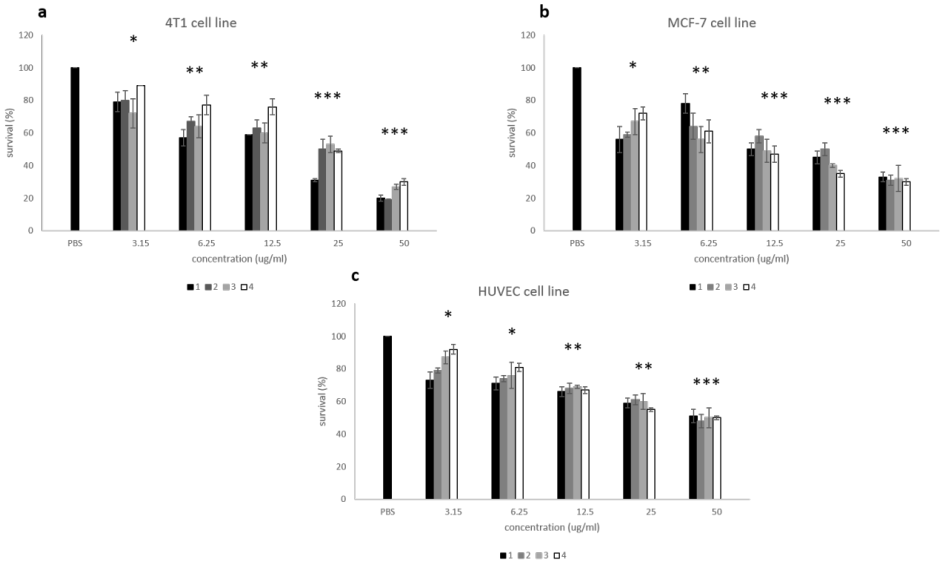

Figure 1. Cytotoxic effects of various diterpenoid (three lathyrane and a tigliane compound) against different cell lines. a: Treatment of 4T1 cells, showed significant toxicity for all concentrations of compounds (1) to (3) and concentrations of 6.25, 12.5, 25 and 50 µg/ml for compound 4 toward the negative control (PBS treated cells). b: Treatment of MCF-7 cells, showed significant cytotoxic effects in all concentrations of all compounds in comparison to the negative control. c: Treatment of HUVEC cells, showed significant cytotoxic effects in concentrations 6.25 µg/ml of all compounds in comparison to the negative control. Data represent the mean percent of three independent experiments of triplicates. Error bars represent SD. Stars showed the comparison of cytotoxic effects of compound (3) to the negative control.

10 µg/ml (as IC50) induces apoptosis as 57 % after 24 hrs of treatment. The percent of apoptotic cells treated with the lower concentration of compound (3) (5 µg/ml) at the same time (24 hrs) was determined as 49 %. In the same condition, the live cell percent for cells treated with PBS was determined as 69 %. Furthermore, the PBS treated cells showed apoptosis as 30 %. These data confirmed the apoptosis induction ability of (3) with the most cytotoxic effects (Figure 2).

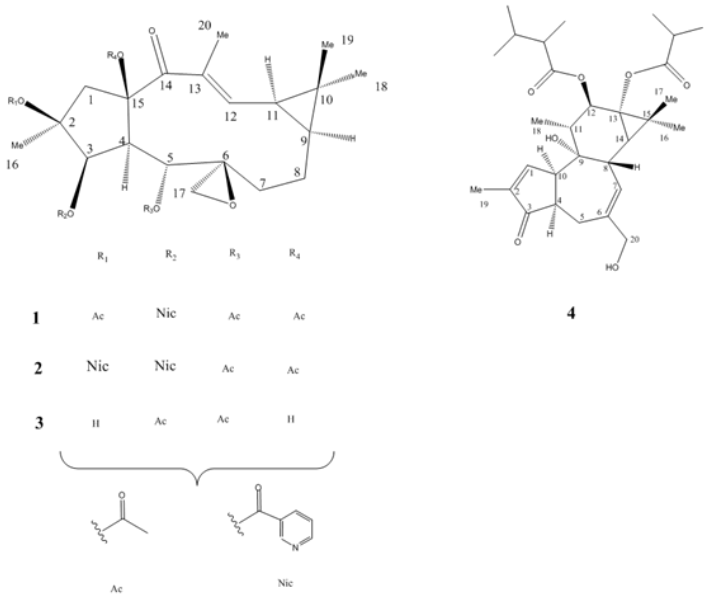

Figure 2. Chemical structures of the studied compounds

Discussion

The aim of the present study was to evaluate the cytotoxic effects of several diterpene flavonoids as Tigliane or Lathyrane extracted and purified from *Euphorbia sogdiana* Popov. The results of this study confirmed compound (3) (as a lathyrane diterpene) with IC50 of about 10.1 and compound (4) (as a tigliane terpenoid) with similar toxic effects against MCF-7 cells.

The cytotoxic effects observed in this project are consistent with the results of other studies on *Euphorbia sogdiana* Popov; for example in one study, the cytotoxic effects of several diterpenes was investigated against Jurkat and EJ-138 cell lines. The results of the mentioned study showed that the IC50 of these compounds against jurkate is in range 4-40 and for EJ-138 cell line as 30-50 µM [14]. The authors attributed the cytotoxic effects of these diterpenes to the functional groups in their structure. In the mentioned study, the more cytotoxic effects of compound (3) was attributed to its more hydrophilic properties [14]. Furthermore, for compound (1) and (2) further indicates that a nicotinyl residue at C-2 in the former, instead of an acetyl in compound (2), is a detrimental effect on the bioactivity. In our study, the cytotoxic effects of mentioned compounds showed similar pattern. Compound (3) showed the most cytotoxic effects due to its more hydrophylicity. Compound 1 resulted in more cytotoxic effects than compound (2) (similar to the mentioned study) which is attributed to the presence of a nicotinyl residue in compound (1) [14].

In Ayatollahi et al study, the Lathyrane compound (1) and (2) were extracted from Euphorbia aellenii and the cytotoxic effects of fraction containing these compounds was evaluated. The IC50 of this fraction was determined as 177.06 µg/ml [15]. In the case of compound (4) as a Tigliane terpenoid, extracted from *Euphorbia aellenii* and tested for its anti-proliferative effects against peripheral human blood lymphocytes, as a non-cancerous cells, the IC50 was calculated as above 50 µg/ml which is higher than the IC50 determined against breast cancer cells in our study [10]. So, it seems that these compounds showed more cytotoxic effects against solid tumor cell lines. Furthermore, in Betancur-Galvis et al study, several Tigliane compounds were isolated from the latex of *Euphorbia* obtusifolia and investigated for their inhibitory effects in mammalian mitochondrial respiratory chain via determining the NADH oxidase activity. The results of the present study showed that these compounds inhibited the activity of NADH oxidase with IC50 of about 2.5-19 µM [16].

The second part of this study was to determine the cell death mechanism induced by these terpenoid compounds. Our data presented in the results section, showed that compound (3), with the most cytotoxic effects against MCF-7 cell line, induced apoptosis in these cells in sub-toxic concentration.

In Shekofteh et al study, it was showed that the hexane extract of three *Euphorbia species* including *E. microciadia Boiss*, *E. osyridea Boiss*, and *E. heteradenia* Jaub induced apoptosis in more than 60% of treated cells in concentration of 50 µg/ml of all three extract [17]. However, they did not identify the compounds responsible

for the induction of apoptosis by the extracts.

In another study, it was showed that Jolkinolide B diterpenoid isolated from the roots of *Euphorbia fischeriana Steud* showed apoptotic effects against various cancer cells [18]. In Wang et al study, enhancing the apoptosis of U937 cells in concentration of 50 µg/ml of Jolkinolide was established against B16F10 via down regulation of PI3K/Akt and IAP proteins [18]. Furthermore, the activation of caspase-3 and -9 was also seen in the mentioned study [18]. However, in our study, the apoptosis was seen in concentrations of 5 and 10 µg/ml of (3) diterpenoid against MCF-7 cells.

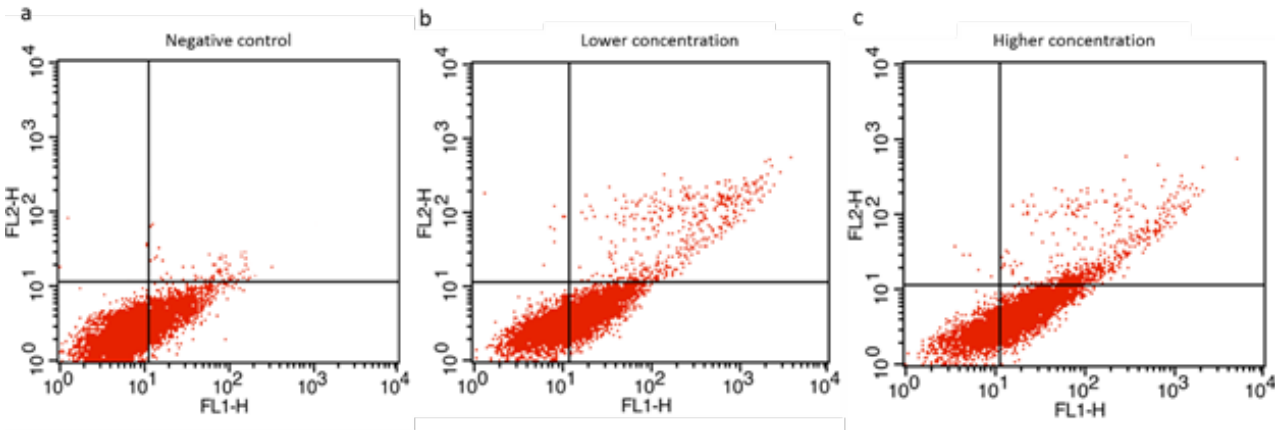

Figure 3. Determination of cell death mechanism of compound (3) by flow cytometry after 24 hrs of incubation. a: cells treated with 5 µg/ml of compound 3. b: cells treated with 10 µg/ml of compound (3) for 12 hrs. c: un-treated control cells. Lower left chamber: live cells (annexin V^-/ PI^-); Lower right chamber: early apoptotic cells (annexin V^+/ PI^-); Upper left chamber: dead cells (annexin V^-/ PI^+); Upper right chamber: late apoptotic cells (annexin V^+/ PI^+).

Conclusion

This project was the cytotoxicity evaluation and determination the cell death mechanism of Lathyrane and Tigliane type diterpenes against two breast cancer cell lines. The cytotoxic effects of compound (3) as a Lathyrane compound was more and this compound showed apoptotic effects against MCF-7 cell line. Because of no significant cytotoxicity of these structures against HUVC cells and rational selectivity index against MCF-7, in comparison to the routine chemotherapeutic agent such as paclitaxel, these compounds have the potential to be considered as useful anticancer agents.

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Conflict of interest

The authors declared that they have no conflicts of interest.

Ethics approval

Not required

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