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Cytotoxic, apoptotic and cell migration inhibitory effects of atranorin on SPC212 mesothelioma cells

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ABSTRACT

Objective: To investigate the effects of atranorin, a lichen secondary metabolite, on SPC212 malignant mesothelioma cells *in vitro*.

Methods: SPC212 malignant mesothelioma cell line was used. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was used to evaluate cytotoxic effects of atranorin and cisplatin at 24, 48 and 72 h. Hematoxylin-eosin staining and 4',6-diamidino-2-phenylindole, dihydrochloride staining were used for determining cell and nucleus morphology, respectively. Wound healing assay was used for investigating cell migration. The xCELLigence real-time cell analysis system was used for determining cell proliferation.

Results: Atranorin at 5-450 μM decreased cell viability at 24, 48 and 72 h. IC_{50} values of atranorin were 300.94, 292.6 and 278.02 μM at 24, 48 and 72 h, respectively; meanwhile, the IC_{50} values of cisplatin were 128.00, 34.37 and 17.05 μM at 24, 48 and 72 h, respectively. Furthermore, atranorin disrupted cell and nuclear morphology with increasing concentrations. Atranorin significantly reduced cell migration by 38%, 37% and 35% at 300, 250 and 200 μM, respectively (P<0.000). Atranorin at 160-450 μM decreased cell proliferation at 72 h (P<0.000).

Conclusions: Atranorin has cytotoxic, antiproliferative, apoptotic and cell migration inhibitory effects on SPC212 malignant mesothelioma cancer cells.

1. Introduction

Some plants are used worldwide for the treatment of various diseases. In Turkey, it is indicated that around 500 and that 200 kinds of plants have therapeutic potential. According to World Health Organization data, there are 1 900 herbal therapeutic agents in the world[1]. Considering the richness of the vegetation in our country, studies on widespread use of plants in treatment of diseases will be very beneficial in terms of economic returns.

Lichens are in a mutualistic life form formed by the combination of at least one fungus and photosynthetic green algae and/or cyanobacteria. In this symbiotic life form, algae provide water and mineral source for fungus photosynthesis, so the fungus can survive, grow and reproduce *via* photosynthesis products[2].

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Lichens are organisms spreading worldwide and there are many species in Turkey. They synthesize secondary metabolites to adapt to environment and living conditions. These metabolites are highly valuable. They cannot be isolated separately from each organism that forms the lichen, and can only be obtained from the whole lichen life form. These isolated secondary metabolites have been found to exhibit vasodilating, inhibitory, anti-inflammatory, antioxidant, anti-bacterial and cytotoxic effects on end-products of advanced glycation[3,4]. More than one thousands of secondary metabolites have been isolated from lichens[5]. The most frequently studied lichen secondary metabolites are atranorin, physodic acid, (+)-usnic acid, (-)-usnic acid, vulpinic acid, gyrophoric acid, olivetoric acid, stictic acid and salazinic acid[5].

Atranorin, a depsid derivative, is the most studied metabolite among all other secondary metabolites. Reports on anti-cancer properties of atranorin are increasing day by day. The effects of atranorin on melanoma, leukemia, colorectal, lung, prostate, breast, ovarian, and cervical cancers were investigated *in vitro* and promising results were obtained[6,7]. According to our knowledge, there is a lack of studies on its effects on malignant mesothelioma, which is a very insidious and rapidly progressing cancer type.

Malignant mesothelioma is an aggressive cancer resulting from mesothelial cells that form the lining of body cavities, characterized by rapid progression, late metastasis and poor prognosis. Although it may originate from all body cavities, it is mostly seen in the pleura and peritoneum, and less frequently in the tunica vaginalis of the pericardium and testis[8]. In this study, SPC212 malignant pleural mesothelioma (MPM) cells were used. MPM is a common disease in Turkey due to environmental asbestos and erionite exposure, especially in Eskisehir, Nevsehir, and Diyarbakır in Turkey. It is estimated that 500-600 people every year in Turkey have been diagnosed as MPM[9]. The treatment is quite difficult and the survival rates are low. Therefore, there is a great need for new therapeutic agents and strategies.

The aim of this study was to investigate the effects of atranorin, a lichen secondary metabolite, on SPC212 MPM cells *in vitro*.

2. Materials and methods

2.1. Collection of plant material

Tepromela atra (Huds.) Hafellner was collected at Bozda Mountain, southwest of Tandır village, Eski ehir province in Turkey at 1 400 m on 10th of October 2018 and on siliceous rocks. Collected lichens were identified by using standard keys[10,11]. Herbarium sample of the material is stored at the Herbarium of Eskisehir Technical University in the Department of Biology.

2.2. Isolation and characterization of atranorin

The lichen samples were first crushed, and then 10 g portion of sample was added to 100 mL dichloromethane. The mixture was left at room temperature overnight and filtered. The crude extract residue in filtrate was obtained after removal of solvent using a rotary evaporator. Atranorin was isolated from the crude extract using a silica packed column chromatography [using a modified solvent G system (toluene + ethyl acetate + formic acid) (70:21:2 v/v/v)]. Atranorin fragment was obtained as sticky oil after the evaporation of column solvent. The pure product was obtained upon crystallization from acetone. The characterization of atranorin was done based on melting point and comparison R_f values in A, C and G eluent systems with those in literature[12,13]. The melting point was determined using a Mettler Toledo MP90 apparatus and was uncorrected. The melting point of atranorin was recorded as 190–194 ℃(decomp.) (lit 196 ℃).

2.3. Cell culture

SPC212 malignant pleural mesothelioma cell line was purchased from the cell bank of the European Collection of Authenticated Cell Cultures (Public Health England, Porton Down, Salisbury, SP4 0JG, UK). Cells were incubated in flasks with DMEM: Ham's F12 (1:1) media containing 15% inactive fetal bovine serum, 2 mM glutamine and 1% penicillin/streptomycin in an incubator with 5% CO_2 at 37 ℃ in accordance with the recommendation of the bank.

2.4. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Effects of atranorin on cell viability were determined by MTT assay. Atranorin was dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 0.1% and cisplatin dissolved in 0.9% sodium chloride solution. Atranorin was freshly prepared at the concentrations of 5, 20, 40, 80, 100, 160, 200, 250, 300, 350, 400 and 450 μM in the media before each experiment. A control group (untreated group) was used in all experiments. In order to determine the effects of atranorin and cisplatin on cell viability, cells were seeded at 5×10^3 per well in 96-well cell culture plates. The cells were incubated at 37 ℃ for 24 h for adhesion. At the end of the incubation period, the media of the adhered cells was removed by inverting the plates. The cells were then incubated for 24, 48 and 72 h at different concentrations of atranorin and cisplatin (5, 10, 20, 40, 80, 160, 200, 250, 300, 350 and 400 μM). Media with atranorin and cisplatin were removed at the end of the incubation periods. Cells were incubated with 0.5 mg/mL of MTT solution for 2 h. At the end of this period, MTT was removed from the wells. About 0.1 mL of DMSO was added to each well to dissolve the formazan salts generated by living cells. The absorbance of the wells was read at 570 nm wavelength using ELISA microplate reader. Untreated cell viability rate was

assumed 100% and the survival rate of the test cells was expressed as a percentage. MTT test was repeated 3 times in all groups independently.

2.5. Hematoxylin-eosin (H&E) staining

H&E staining was performed to examine cell morphology. SPC212 cells seeded on sterile coverslips that were placed on six-well plates were incubated in 5% CO_2 at 37 ℃ for 24 h. The media was then removed and the cells were incubated for 24 h at different concentrations of atranorin (Untreated, 3, 160, 200, 250 and 300 μM of atranorin). After 24 h, atranorin media was removed. At the end of the experiment, the cells adhered to the coverslips were fixed with 3.7% formaldehyde solution in phosphate buffer saline (PBS) for 15 min at 37 ℃. After fixation, the coverslips were washed 3 times with PBS. Cells were incubated with Triton-X 100 (0.2%) for 5 min and then washed 3 times with PBS. The coverslips were stained with hematoxylin for 5 min and then washed under running water. After 5 min of staining with eosin, washing coverslips under running water was repeated. The stained coverslips were passed through a series of alcohol solutions in increasing concentrations, and incubated in xylene for 20 min. The coverslips were closed and cells were examined under a binocular light microscope (Olympus BX51) to obtain appropriate images.

2.6. 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) fluorescent staining

DAPI is a fluorescent dye that binds to DNA. SPC212 cells seeded on sterile coverslips that were placed on six-well plates were incubated in 5% CO_2 at 37 ℃ for 24 h. The media was then removed and the cells were incubated for 24 h at different concentrations of atranorin (Untreated, 3, 160, 200, 250 and 300 μM of atranorin). After 24 h, atranorin media was removed. At the end of the experiment, the cells adhered to the coverslips were fixed with 3.7% formaldehyde solution in PBS for 15 min at 37 ℃. After fixation, the coverslips were washed 3 times with PBS. Cells were incubated with Triton-X 100 (0.2%) for 5 min and then washed 3 times with PBS. After washing with PBS, the coverslips were incubated in the dark with 1 mg/mL DAPI at 37 ℃ for 30 min. The coverslips were then washed with PBS and examined under fluorescence microscopy and photographed.

2.7. Cell migration assay

SPC212 cells were seeded into each well with equal numbers. Cells were incubated to reach 90% confluency. The media was then removed from the wells. Cells on the previously marked area on the plate base were linearly scraped with a 200 μL pipette tip. The cells were washed 2 times with PBS for 1 min. After washing, cells were incubated with media containing different concentrations of atranorin (Untreated, 3, 160, 200, 250 and 300 μM of atranorin). The images of the scraped area were examined at 0 and 24 h *via* an inverted microscope with a 10 × objective lens and photographed. The wound area of the images taken in μm^2 was measured *via* microscope attached image analysis software (BABsoft Bs200pro, Ankara, Turkey) and the measurements were converted to percentage values according to control group (untreated group).

2.8. xCELLigence real-time cell analysis system

xCELLigence system (ACEA Biosciences, San Diego, CA 92121 USA) is a real-time analysis system that used to determine the physiological changes in cells without any staining or labelling. In this analysis method, special 96-well plates (E-plate 96) with microelectrodes at bases of wells are used. This system performs an electrical impedance measurement that will provide quantitative information about the physiological changes such as the number, viability, morphology and movement of cells on the electrodes. The impedance measurement is determined by the ion concentration between electrodes. The higher number of cells adhering to the electrodes, the higher impedance is obtained. The impedance value increases with the number of cells adhering to the electrode surface[14].

In order to determine the effects of atranorin and cisplatin on cell proliferation, cells were seeded at 5×10^3 per well in E-plate 96. The cells were incubated at 37 ℃ for 19 h for adhesion. The cells were then incubated for 72 h at different concentrations of atranorin (20, 40, 80, 100, 160, 200, 250, 300, 350, 400 and 450 μM) and cisplatin (5, 10, 15, 20, 40, 50, 75, 100, 125, 150 and 175 μM). Impedance measurements were taken every 1 h for 72 h. Analysis of the data was expressed as cell index units.

2.9. Statistical analysis

All experiments were repeated 3 times. SPSS software (Statistics for Windows, Version 21.0, Armonk, New York, USA) was used to calculate the data. *P* values less than 0.05 were considered statistically significant. Shapiro-Wilk test was performed to examine whether the data were normally distributed. One-way ANOVA followed by Tukey's multiple comparison test were performed on normal dividing data.

3. Results

3.1. MTT results

After 24, 48 and 72 h, all concentrations of atranorin showed decreasing effect on the cell viability. After 24 h, atranorin at 100-450 μM significantly decreased the viability when compared to the control group ($P<0.000$). Cell viability was 6.7% at 450 μM (Table

1), and the IC_{50} concentration was 300.94 μM at 24 h. After 48 h and 72 h, atranorin at 20-450 μM significantly decreased the cell viability compared to the control (P<0.000), and cell viability was 0% at 400 and 450 μM (Table 1). The IC_{50} concentration was 292.6 μM at 48 h, and was 278.02 μM at 72 h.

After 24, 48 and 72 h, cisplatin at all concentrations showed decreasing effect on the cell viability. IC_{50} values of cisplatin were 128.00, 34.37 and 17.05 μM at 24, 48 and 72 h, respectively (Table 2).

Table 1. MTT results of atranorin-treated SPC212 cancer cells at different concentrations at 24, 48 and 72 h.

Groups	Cell viability (% Control)		
	24 h	48 h	72 h
Control	100.0 ± 2.7	100.0 ± 4.8	100.0 ± 3.6
DMSO control	100.0 ± 2.9	100.0 ± 5.1	100.0 ± 6.4
5 μM	96.8 ± 1.2	92.8 ± 2.3	89.6 ± 1.6
20 μM	95.0 ± 2.9	88.5 ± 2.3*	87.5 ± 3.1*
40 μM	94.0 ± 2.0	86.9 ± 2.5*	85.9 ± 4.1*
80 μM	93.4 ± 2.9	85.2 ± 2.4*	83.4 ± 3*
100 μM	90.2 ± 5.7*	82.3 ± 2.5*	79.5 ± 2.1*
160 μM	85.4 ± 5.2*	80.7 ± 2.6*	75.3 ± 9.8*
200 μM	80.4 ± 4.5*	79.0 ± 6.0*	70.2 ± 4.3*
250 μM	76.8 ± 1.9*	71.6 ± 0.8*	65.4 ± 3.1*
300 μM	52.5 ± 4.5*	39.0 ± 4.3*	30.7 ± 8.3*
350 μM	31.3 ± 3.3*	29.9 ± 2.0*	4.9 ± 0.8*
400 μM	12.9 ± 2.7*	0	0
450 μM	6.7 ± 0.3*	0	0

*: P<0.05, compared with the control group.

Table 2. MTT results of cisplatin-treated SPC212 cancer cells at different concentrations at 24, 48 and 72 h.

Groups	Cell viability (% Control)		
	24 h	48 h	72 h
Control	100.0 ± 1.3	100.0 ± 2.6	100.0 ± 2.1
5 μM	94.4 ± 2.6*	84.9 ± 1.5*	70.4 ± 2.5*
10 μM	86.2 ± 1.7*	75.9 ± 1.6*	57.9 ± 1.3*
20 μM	65.7 ± 3.1*	65.2 ± 1.8*	36.3 ± 1.2*
40 μM	61.8 ± 2.5*	24.7 ± 2.6*	7.4 ± 1.6*
80 μM	60.3 ± 1.4*	7.8 ± 2.5*	0
160 μM	56.2 ± 1.9*	0	0
200 μM	40.8 ± 2.6*	0	0
250 μM	0	0	0
300 μM	0	0	0
350 μM	0	0	0
400 μM	0	0	0

*: P<0.05, compared with the control group.

3.2. H&E results

According to H&E staining data, in the control group and in the group treated with 3 μM of atranorin, the cell cytoplasm was eosinophilic and the nuclei were central and euchromatic, similarly. Cell density in these two groups was higher than the other groups. Atranorin at 3 μM did not change cell morphology. Atranorin caused an increased disruption of cell morphology at concentrations of 160, 200, 250 and 300 μM in a concentration-dependent manner. It was also observed that the cells became smaller in size. Nucleus condensation was common in those groups (Figure 1).

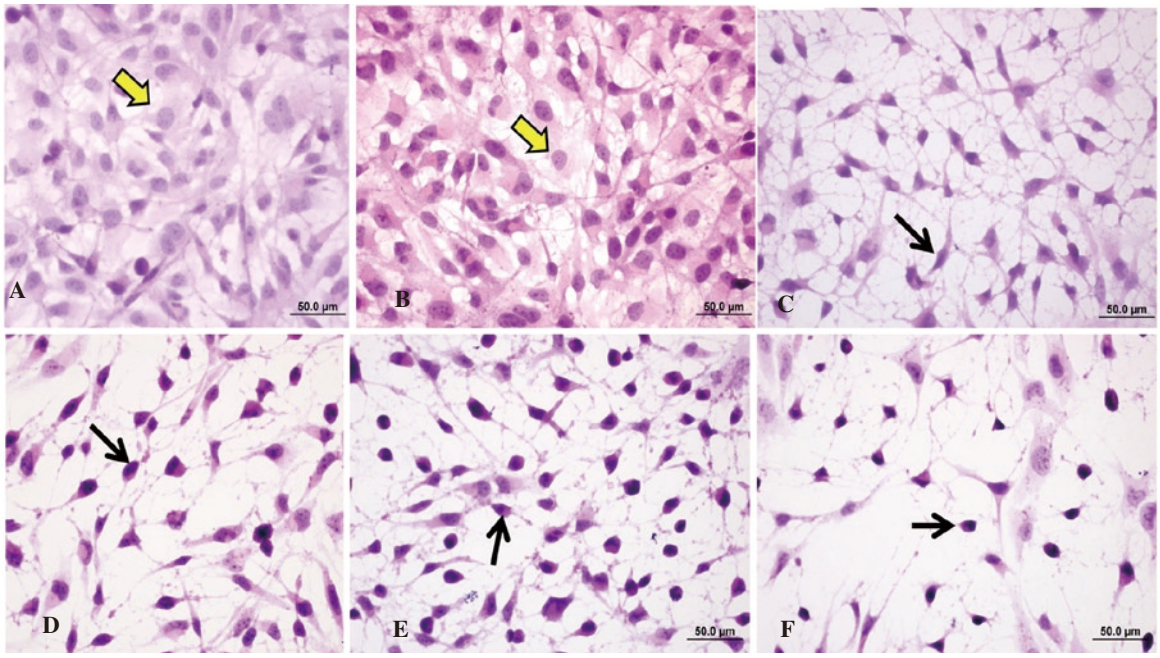

Figure 1. Hematoxylin-eosin staining images of SPC212 cancer cells treated with atranorin at different concentrations. The control group (A) and the group treated with 3 μM of atranorin (B) are similar in terms of cell morphology. The cells in both groups have normal morphology (thick arrow). Atranorin at 160 (C), 200 (D), 250 (E) and 300 (F) μM disrupts cell morphology in a dose dependent manner. Cells shrink in volume and their nuclei are condensed (thin arrow). All pictures are taken at 40× magnification. Scale bars are 50 μm in all figures.

3.3. DAPI results

Nucleus morphologies were in round and equal sizes in the control group and in the group treated with 3 μM of atranorin. It was observed that atranorin disrupted the nucleus morphology and the nuclei were condensed and shrunk with increasing concentrations (Figure 2).

3.4. Cell migration assay results

The result showed that the cells migrated the wound area within 24 h in the control, 3 μM and 160 μM atranorin groups. Thus, it was indicated that concentrations of 3 μM and 160 μM of atranorin did not decrease cell migration. The concentrations of 200, 250 and 300 μM of atranorin significantly reduced cell migration by 35%, 37% and 38%, respectively (P<0.000) (Figure 3).

3.5. xCELLigence real-time cell analysis system results

The effects of atranorin on cell proliferation were investigated by xCELLigence real-time cell analysis system. After 72 h, atranorin significantly decreased cell proliferation at the concentrations of 160-450 μM when compared to the control group (P<0.000). At 300-450 μM, the proliferation was gradually decreased to 0 after 72 h. Atranorin had no effect on cell proliferation at 20-100 μM (Figure 4A).

After 72 h, cisplatin at all concentrations showed decreasing effect on the cell proliferation (P<0.000) (Figure 4B).

4. Discussion

This study has shown that atranorin, a lichen secondary metabolite, has cytotoxic, anti-proliferative, apoptotic and cell migration inhibitory effects on SPC212 malignant mesothelioma cells. Although the anti-cancer and apoptotic effects of atranorin have been previously demonstrated in many cancers[6,15], the effects of atranorin on malignant mesothelioma cells have not been investigated up to this study. In this respect, this is an original study.

Many lichen acids can be isolated as secondary metabolites from lichens and each of them has different biological effects. Galanty *et al*. showed that usnic acid, a lichen acid, had a stronger anti-carcinogenic effect on prostate and melanoma cells when compared to atranorin[15]. In our study, we used atranorin and mesothelioma cells. It is found that concentration of 3 μM of atranorin did not cause cytotoxicity in mesothelioma cells at 24, 48 and 72 h, while concentrations above 5 μM had cytotoxic effects. It has been demonstrated previously that 5 μM of atranorin is not cytotoxic at 48 h on A549 lung cancer cells[16]. According to the results of our current study and of Zhou *et al*.[16], different results were obtained at the same concentrations of atranorin, which may be due to the different types of cancer cells used. Bačkorová *et al*. reported that IC_{50} values of atranorin at 72 h were 197.9 μM in A2780 human ovarian cancer cells, >200 μM in HeLa cervical cancer cells, >200 μM in MCF-7 in breast cancer cells, >200 μM in SK-BR-3 breast cancer cells, >200 μM in HT-29 colon cancer cells, >200 μM in HCT-116 p53 +/+ colon cancer cells, 197.5 μM in HCT-116 p53 -/- colon cancer cells, 93.5 μM in HL-60 leukemia cells and 181.6 μM in Jurkat leukemia cells[6]. In our study, the IC_{50} value at 72 h was 278.02 μM, which is slightly higher than the results of Bačkorová

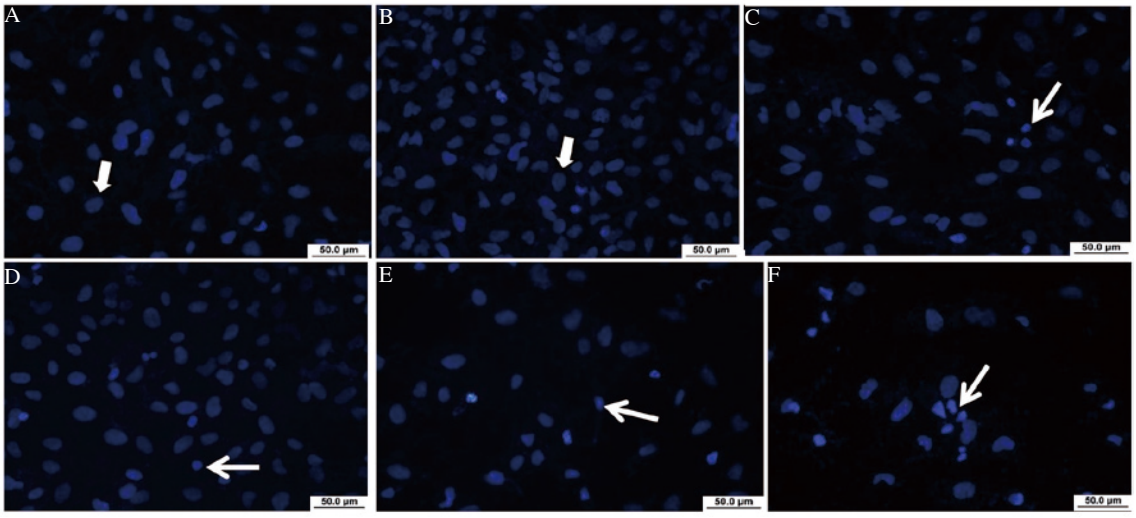

Figure 2. DAPI staining images of SPC212 cancer cells treated with atranorin at different concentrations. The control group (A) and the group treated with 3 μM of atranorin (B) are similar in terms of nucleus morphology. The nuclei in both groups have normal morphology (thick arrow). Atranorin at 160 (C), 200 (D), 250 (E) and 300 (F) μM disrupts nucleus morphology in a dose-dependent manner. The nuclei are smaller in volume and condensed (thin arrow). All pictures are taken at 40× magnification Scale bars are 50 μm in all figures.

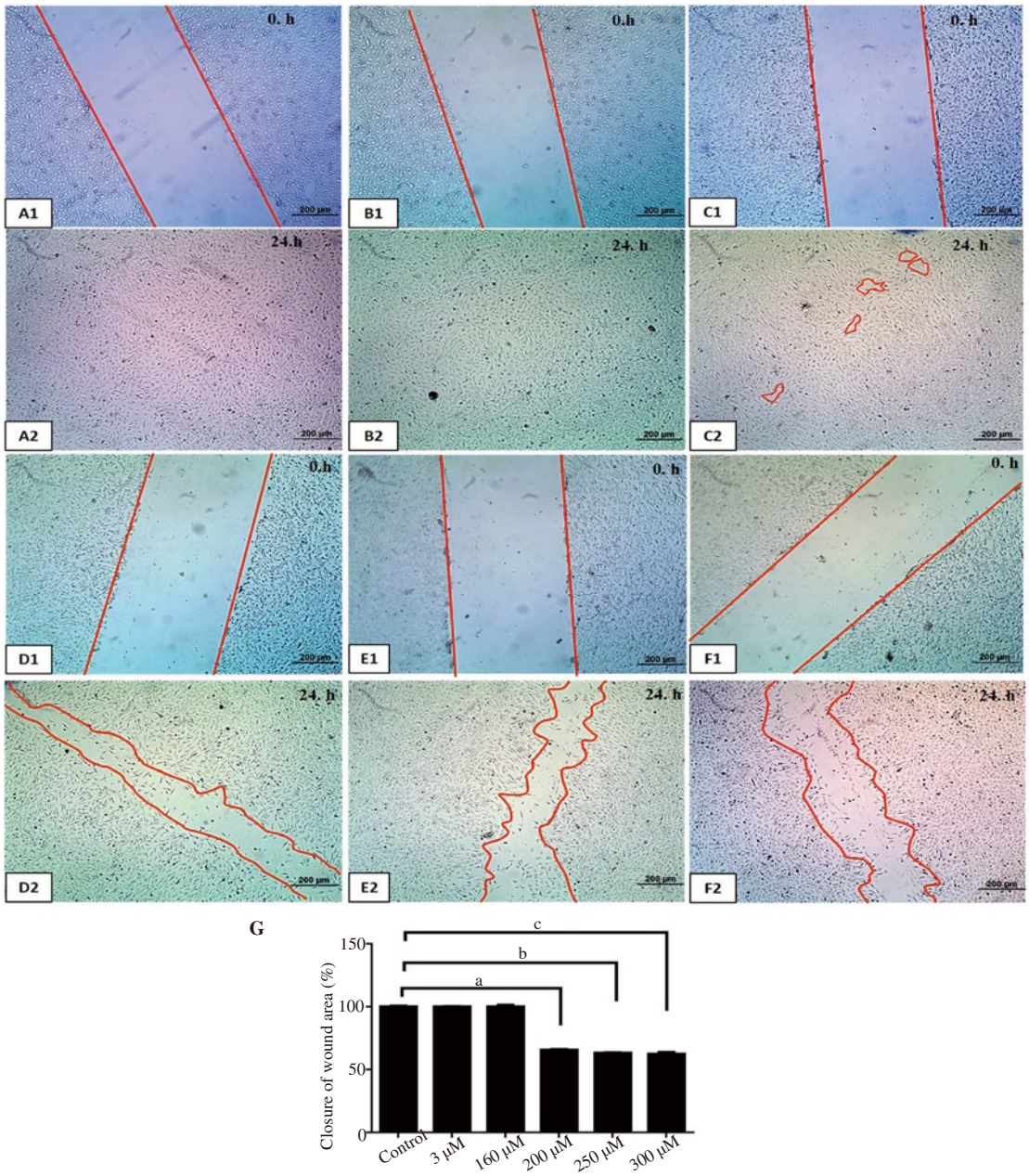

Figure 3. Results of cell migration of SPC212 cells treated with atranorin. In control (A1-A2), 3 (B1-B2) and 160 (C1-C2) μM of atranorin-treated groups, the cells closed 100% of the wound area at 24 h; meanwhile, 200 μM (D1-D2), 250 μM (E1-E2) and 300 μM (F1-F2) of atranorin closed the wound area by 65%, 63%, and 62%, respectively. The difference was significant between groups a: Control-200 μM of atranorin, b: Control-250 μM of atranorin, and c: Control-300 μM of atranorin (P<0.000). Scale bars are 200 μm in all figures.

et al and is close to IC_{50} values of cervix, breast and colon cancer cells. It has been reported that 75 μM of atranorin has a proapoptotic and colony inhibitory effects on 4T1 breast cancer cells at 48 h[17]. In our study, it was also observed that atranorin administration at 24 and 48 h had both cytotoxic effect and disruptive effect on cell nucleus. Russo *et al*. showed that 50 μM of atranorin significantly decreased cell viability in LNCaP and DU-145 prostate cancer cells at 72 h by 70% and 40%, respectively, when compared to control[18]. In our study, the cell viability decreased to 83.4% after 80 μM of atranorin administration at 72 h. This study demonstrated that SPC212 malignant mesothelioma cells are more resistant to atranorin, compared to LNCaP and DU-145 prostate cancer cells. Our study showed different cell viability and proliferation results. The cell viability significantly decreased between the concentrations of 20-450 μM of atranorin, while the cell proliferation decreased significantly between the concentrations of 160-450 μM. It indicated that atranorin may be more effective on cell viability. Considering that MTT assay measures cell viability through mitochondria, atranorin may act on mitochondria physiology.

The studies of the effects of atranorin on cell morphology are limited. de Melo *et al*. showed that atranorin did not cause any pathological changes in renal histology of rats at doses of 50, 100 and 200 mg/kg[19]. In our study, H&E staining showed that 3 μM of atranorin did not cause any change in cell morphology but increasing

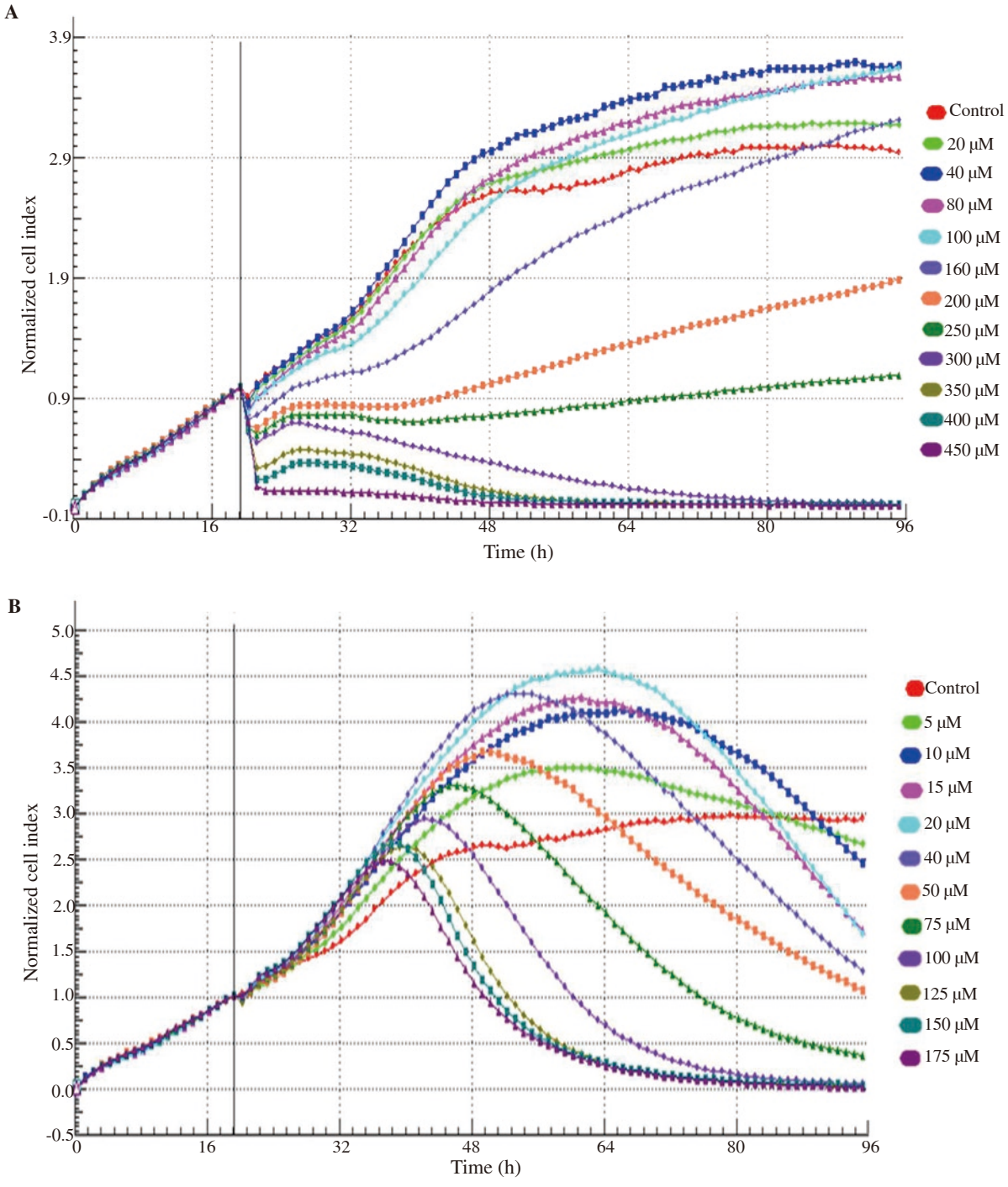

Figure 4. xCELLigence real-time cell analysis system results of different atranorin (A) and cisplatin (B) concentrations at 72 h.

doses changed cell morphology.

Bačkorová *et al*. showed that atranorin (100 and 200 μM) has proapoptotic effects on many cancer types at 48 and 72 h *via* DAPI fluorescence staining[6]. Our study showed that nuclear morphology of atranorin-treated malignant mesothelioma cells was disrupted, condensed and shrunk at the doses of 160, 200, 250 and 300 μM *via* DAPI fluorescence staining. Nuclear condensation is regarded as one of the apoptosis indicators[20]. In this respect, it can be said that atranorin has shown apoptotic effects at high concentrations. The study of HT-29 colon cancer cells by Ren *et al*. showed that lichen extract from *Lethariella zahlbruckneri*, which contains atranorin, caused cytotoxicity and fragmentation of the nucleus at the concentrations of 10, 30, 50 and 100 μM after 24, 48 and 72 h[21]. The results of our study were similar to results of Ren and his colleagues. Varol *et al*. showed that 800 μM of atranorin produced apoptotic bodies in keratinocyte cells[22]. The highest nucleus

condensation was observed in the group treated with 300 μM of atranorin also in our study.

The study of A549 lung cancer cells by Zhou *et al*. demonstrated that 5 μM atranorin reduced the expression of many proteins related to cell migration[16]. Yang *et al*. found that physciosporine, another lichen acid, had anti-metastatic properties on A549 lung cancer cells at a concentration of 5 μg/mL[23]. In our study, 3 and 160 μM of atranorin had no effect on cell migration and 200, 250 and 300 μM of atranorin reduced cell migration.

In conclusion, this study has been shown that atranorin has a cytotoxic, anti-proliferative, apoptotic and cell migration inhibitory effects on SPC212 malignant mesatelyoma cancer cells. New medicine and treatment approaches need to be developed for cancer and its treatment, which is the biggest medical problem of our time, and will be savior for the coming years. Secondary metabolites isolated from lichens have great potential to be new therapeutic agents. New and in-depth studies on these agents should be carried out.

Conflict of interest statement

Authors declare that there are no competing interests.

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