



Antiproliferative Activity of Selected Triterpene Acids from Rosemary on Metastatic Melanoma Cell Line WM-266-4

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Abstract

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BACKGROUND: Natural products and their derivatives, particularly secondary metabolites, have been recognized for many years as an important source of therapeutic agents. In this context, pentacyclic triterpene acids including betulinic acid (BA), oleanolic acid (OA), and ursolic acid (UA) are highly valuable triterpenic acids because of their wide range of biological activities.

AIM: Therefore, the aim of our study was to investigate any potential effect of BA, UA, and OA on human melanoma WM-266-4 cells' proliferation activity.

METHODS: BA, UA, and OA have been prepared in dimethyl sulfoxide in concentration range from 0.002 to 200 μ M separately or in selected combination (UA+OA ratio 1:1 or 3.5:1), while cells in cell culture medium served as controls. The rapid colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was used to measure proliferation activity of the metastatic melanoma cell line WM-266-4 after being exposed to selected concentrations of BA, UA, OA, or UA+OA and during different time periods. Student's t-test was used for single statistical comparisons. Data were analyzed using IBM SPSS 25.0 (IBM Corp., Armonk, NY). To account for multiple comparisons bias, $p < 0.001$ was considered statistically significant.

RESULTS: Our results showed decreased cell proliferation activity after 4 h of incubation of WM-266-4 cells with BA, UA, OA, and UA+OA. The highest inhibitory effect was noted when cells were incubated with selected triterpenic acids and both combinations of UA+OA during the incubation period of 48 h. When compared to control cells, concentration of 2 μ M was the lowest concentration of BA that showed a significant decrease of the cells' proliferation activity regardless the incubation period (4 h, 24 h, and 48 h) ($p < 0.001$).

CONCLUSION: Our encouraging results could be a good starting point for further studies on possible use of BA, UA, and OA in prevention and treatment of metastatic melanoma.

Introduction

Malignant melanoma is skin cancer that has evolved to become a common malignancy and a major public health problem [1]. It originates from melanocytes, which are present in all areas of epidermis and in the parts of the eye and upper respiratory, gastrointestinal, and genitourinary tracts [2]. The incidence of melanoma has been steadily increasing during the past few decades in most fair-skinned populations and it is the fifth leading cancer in males and the seventh in females in the United States [3]. Increasing incidence rates have been observed also in Europe, with south/north and east/west variations [1]. Melanoma incidence has been more profoundly raised in North Europe, particularly in Scandinavian countries. Increases in incidence rates are not followed by a similar trend in mortality rates [4]. Mortality has decreased during the past few decades because of heightened awareness and screening programs resulting in

earlier detection. However, the treatment of melanoma remains difficult. Many of the chemotherapeutic agents used show a limited selectivity for tumor cells and cause adverse effects [5]. Furthermore, besides known adverse effects, resistance to chemotherapy is frequently observed in melanoma. Understanding skin cancer incidence is important for planning prevention and treatment strategies. It has been shown that many clinically approved anticancer drugs were of natural origin, that is, natural products, derivatives, or synthesized molecules based on natural product compound pharmacophores [6]. Moreover, well-known anti-cancer drugs include *Catharanthus* alkaloids, colchicine, etoposide, and Taxol [7]. In Europe, several of the traditionally used medicines have been developed into modern registered drugs, which have been studied in clinical trials [6], [7]. Furthermore, many of modern drugs have been developed from purified compounds, isolated from medicinal herbs and/or plants, based on their traditional pharmacological use [7]. There are more than 100,000 known secondary metabolites,

and for many of them, their bioactive properties and mechanisms of action are yet unknown [6]. Plant secondary metabolites such as polyphenol compounds, alkaloids, and terpenes possess diverse pharmacological properties, including cytotoxic and cancer chemopreventive effects [8], [9], [10]. The latter may act during different phases of the malignant tumor development and involve various mechanisms [1], [4]. During the past two decades, a growing number of studies which investigated the effects of polyphenols on different cancer cell lines have been observed. Many of them analyzed the antiproliferative and antimetastatic capacity of such compounds against melanoma cell lines [11], [12], [13]. In addition, triterpenoid-derived compounds (including triterpenic acids) isolated from different medicinal plants have been shown to possess significant antiproliferative activity against cancer cell lines with promising selectivity for malignant cells [5], [7], [14], [15].

Rosemary (*Rosmarinus officinalis* L.) is a medicinal plant that belongs to the *Lamiaceae* family, native of Mediterranean regions. The phytochemicals present in *R. officinalis* are rosmarinic acid, camphor, caffeic acid, ursolic acid (UA), betulinic acid (BA), carnosic acid, and carnosol [6], [15]. BA, UA, and oleanolic acid (OA) belong to the group of pentacyclic triterpenes [11], [13]. While BA and UA have been a subject of many research studies, OA has been studied to a lesser extent on cell cultures [11]. Previous results indicate that BA, UA, and OA have pleiotropic biologic activities such as anti-inflammatory, cytotoxic, and antiproliferative activities on cancer cells [8], [16]. BA and its derivatives have been extensively researched for their strong cytotoxic properties against melanoma [16], [17]. Recently, OA has attracted more attention due to its marked antitumor effects and pharmacological safety [18]. Furthermore, UA and OA were found as effective anti-hepatoma agents with marked anti-cancer activity. They also show protective effects against H₂O₂-induced DNA damage in leukemic L1210, K562, and HL-60 cells as well as significant antioxidant effects [18], [19], [20], [21], [22], [23]. In addition, three pentacyclic triterpenoids, 12-oleanen-27-oic acid derivatives, 3 β -hydroxy-12-oleanen-27-oic acid (ATA), 3 β , 6 β -dihydroxy-12-oleanen-27-oic acid (ATB), and 3 β -acetyl-12-oleanen-27-oic acid (ATC), isolated from the rhizomes of *Astilbe chinensis* (Maxim.) Franch. et Savat. (*Saxifragaceae*), have shown a strong cytotoxic activity toward human ovarian carcinoma HO-8910 cells, human cervical squamous carcinoma HeLa cells, and human leukemic HL60 cells [24].

According to the previous studies, the aim of our research was to investigate the possible antiproliferative effect of triterpenes BA, UA, OA, and combined effect of mixture of UA+OA (1:1 and 3.5:1) on cell proliferation in selected metastatic melanoma cell line.

Methods

Cell culture

Skin metastatic melanoma cell line WM-266-4 American Type Culture Collection (ATCC[®] CRL1676[™]) was purchased from (ATCC, Manassas, VA, USA). The cell line was isolated from lymph node metastasis of the 55-year-old female patient. The cells were grown to confluence in complete medium containing Eagle's Minimum Essential Medium (EMEM, ATCC[®] 30-2003[™]), supplemented with 1% fetal bovine serum (ATCC[®] 30-2021[™]) and 0.02% MycoZap Plus-CL (Lonza, Portsmouth, NH, USA) and incubated at 37°C in 5% CO₂ humidified ($\geq 90\%$ RH) atmosphere in tissue culture flasks. Complete medium was changed every 48 h. When the culture became confluent, the cells were subcultivated by trypsinization using DetachKit (catalog number C-41220, PromoCell, Heidelberg, Germany, EU) and replated.

Preparation of BA, UA, OA, and UA+OA solutions

BA, UA, and OA (analytical purity) were purchased from Fluka (Sigma-Aldrich, Steinheim, Germany). Stock solutions of BA, UA, OA, and UA+OA were prepared in sterilized dimethyl sulfoxide (DMSO; Sigma-Aldrich) and stored at 4°C. For cell proliferation assay, stock solutions of BA, UA, and OA were diluted in DMSO (0.002, 0.02, 0.2, 2, 10, 20, 40, and 200 μ M). To analyze a combined effect of UA and OA, these were dissolved in DMSO in two different ratios: 1:1 and 3.5:1.

Evaluation of cell proliferation

The effects of BA, UA, OA, and UA+OA on proliferation activity of WM-244-6 cells were determined with MTT colorimetric assay (Sigma-Aldrich), following the manufacturer's instructions. Briefly, the cells (10⁴ cells/well) were incubated in 96-well microplates for 24 h to form monolayers. Afterward, BA, UA, OA, and UA+OA in selected concentrations were added to each well. The cells were thereafter incubated in a humidified atmosphere at 37°C in 5% CO₂ for 4 h, 24 h, or 48 h. After each period of incubation, 10 μ l of MTT/100 μ l of complete medium was added and plates were incubated at 37°C for 2 h. Then, 200 μ l of DMSO (Sigma-Aldrich) was added to each well to dissolve formed formazan crystals. Cells in cell culture complete medium (not treated) were used as negative controls. The extent of MTT reduction was determined spectrophotometrically at 570 nm (background signal at 600 nm) using Tecan plate reader (Infinite 200). All treatments were performed in pentaplicates.

In addition, cell morphology was observed with an inverted microscope (DM16000B, Leica) using

a digital camera (DFC365 FX Leica, Buffalo Grove IL, USA).

Statistical analysis

Student's t-test was used for single statistical comparisons. Results are presented as mean \pm SD. Data were analyzed using IBM SPSS 25.0 (IBM Corp., Armonk, NY). To account for multiple comparisons bias, $p < 0.001$ was considered statistically significant.

Results

As shown in Figure 1, the proliferation activity was decreased from 80% to 60% when WM-266-4 cells were treated with 0.002, 0.02, and 0.2 μ M BA. The only exception was 24 h incubation of WM-266-4 cells with 0.002 μ M BA when cells showed slightly increased proliferative activity when compared to control. The lowest concentration of BA that caused a significant decrease of cell proliferation activity was observed when WM-266-4 cells were treated with 2 μ M BA for 4 h, 24 h, or 48 h. Furthermore, higher concentrations of BA (>2 μ M) significantly inhibited the proliferation activity of WM-266-4 cells at all incubation times. In the latter concentration range, the cells' proliferation activity decreased even below 20%.

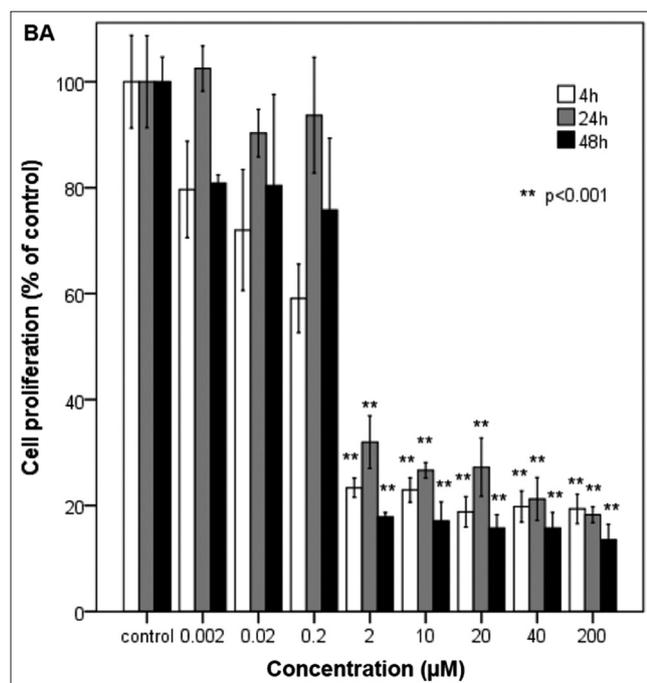


Figure 1: Inhibition of WM-266-4 cells' proliferation activity by BA. Data are presented as mean \pm SD. Significant results are assigned as ** $p < 0.001$ versus control. BA: Betulinic acid

When WM-266-4 cells were treated with 0.002, 0.02, 0.2 μ M, and 2 μ M UA, the proliferation activity decreased up to 50%. The lowest concentration of

UA that caused a statistically significant decrease of WM-266-4 cells' proliferation activity was 10 μ M. The decrease in the cells' proliferation activity at 10 μ M UA was 25%, 10%, and 5%, after 4 h, 24 h, and 48 h, respectively. Higher concentrations of UA (>20 μ M) significantly inhibited the cells' proliferation activity regardless of the time of incubation (4 h, 24 h, or 48 h). In the latter concentration range, the cells' proliferation activity decreased below 5%. Details are evident from Figure 2.

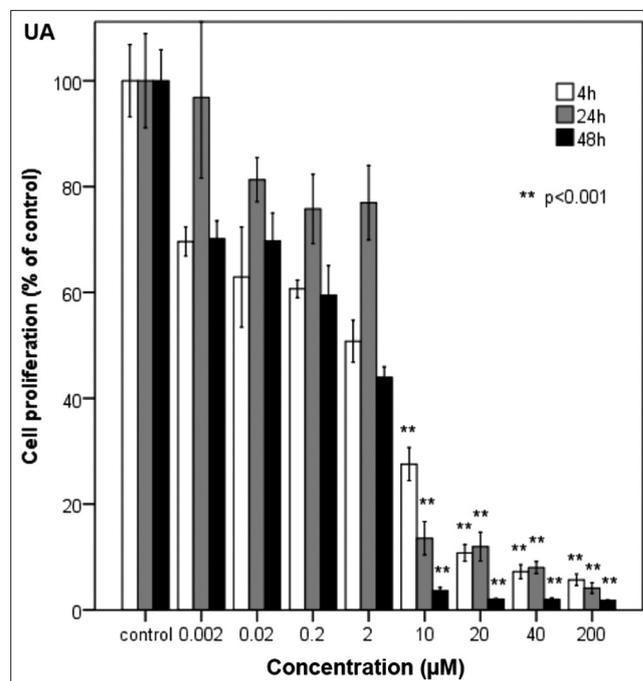


Figure 2: Inhibition of WM-266-4 cells' proliferation activity by UA. Data are presented as mean \pm SD. Significant results are assigned as ** $p < 0.001$ versus control. UA: Ursolic acid

As shown in Figure 3, the lowest OA concentration that caused a significant decrease of WM-266-4 cells' proliferation activity was 20 μ M (10% and 21% following 24 h and 48 h incubation, respectively). The most significant inhibition of the cells' proliferation was observed following 48 h incubation of WM-266-4 cells with 200 μ M OA.

Next, we investigated a combined effect of UA and OA on WM-266-4 cell proliferation activity in their two different ratios UA: OA (1:1 and 3.5:1). No significant influence on the proliferation activity was observed when concentrations of both UA and OA (1:1 and 3.5:1) were <10 μ M. However, when WM-266-4 cells were treated with 0.002 μ M UA+OA (1:1) during the period of 4 h a slight increase of proliferative activity when compared to control can be seen. The same effect can be seen when WM-266-4 cells were treated with UA+OA (3.5:1) for 24 h. Furthermore, the concentration of 10 μ M UA+OA (both 1:1 and 3.5:1) caused a significant decrease of the cells' proliferation to around 20%, 7%, and 6% following 4 h, 24 h, and 48 h incubation, respectively. Higher concentrations of UA+OA (>10 μ M) significantly inhibited the cells'

proliferation activity at 4 h, 24 h, or 48 h of incubation. Details are evident from Figure 4.

In Figure 5, images of WM-266-4 cells with formazan granules are presented. For each tested solution, two images are shown, that is, before and after significant inhibition of cell proliferation activity.

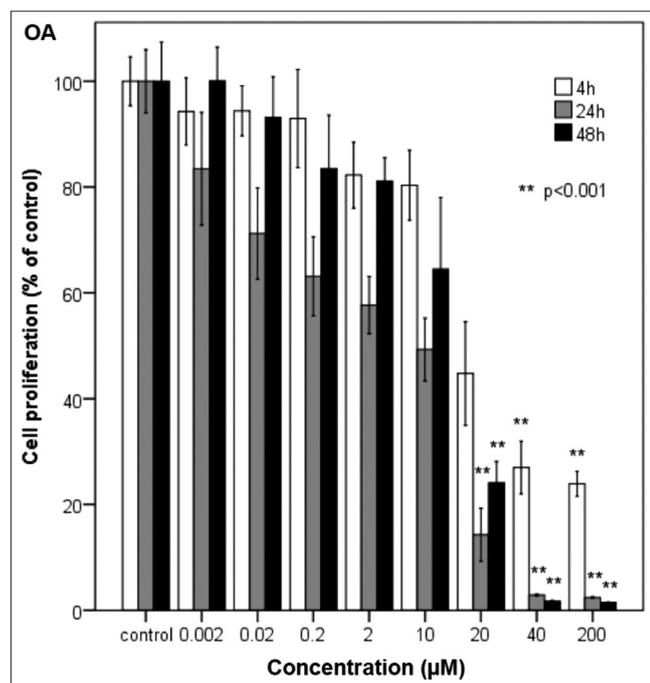


Figure 3: Inhibition of WM-266-4 cells' proliferation activity by OA. Data are presented as mean±SD. Significant results are assigned as ** $p < 0.001$ versus control. OA: Oleanolic acid

Discussion

Triterpenic compounds are considered today as important constituents of the human diet regarding their anticancer properties. Using human and murine melanoma cell lines, several mechanisms have been proposed to explain their anticancer activity including cytotoxicity, inhibition of cell proliferation, as well as their effect on cell differentiation and angiogenesis processes [5], [16].

The present study focused on the effect of three triterpenic acids, that is, BA, UA, and OA on the proliferative activity of metastatic melanoma cells. Metastatic form of the melanoma remains difficult and presents a great clinical challenge to treat. The treatment of malignant melanoma usually includes surgery, radiotherapy, and chemotherapy, all of which show a limited selectivity for tumor cells at the same time causing adverse effects to other living cells in the body [14], [16]. For that reason, many studies have focused on using natural products to prevent and/or treat cancer [8], [16], [17], [18], [19], [20]. Herbs and plants have been a source of prophylaxis and medicinal treatments for thousands of years, although their use was diminished when synthetic drugs were introduced. However, herbal medicine still plays an essential role in the primary health care of 80% of the world's underdeveloped and developing countries [6]. Furthermore, plants present a good source of a variety of bioactive compounds, among which, an important group is triterpenes that can be found in fruit peel,

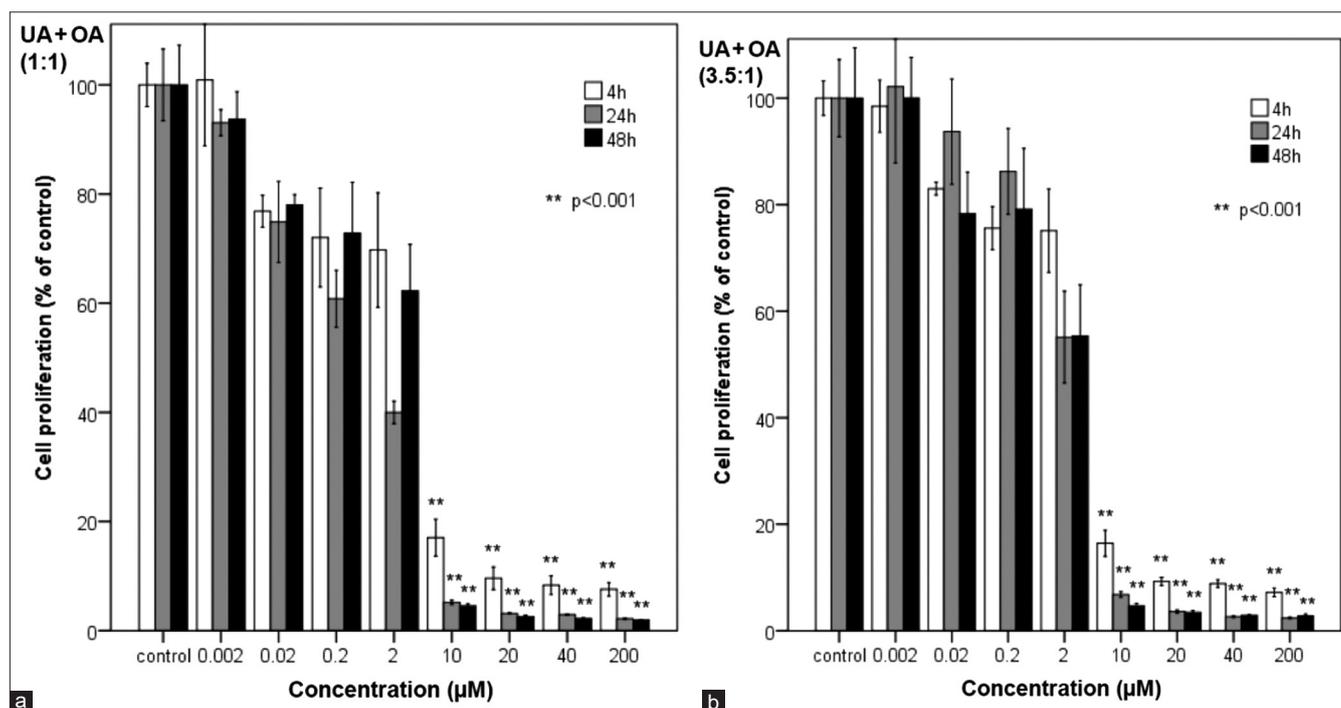


Figure 4: (a) Inhibition of the WM-266-4 cells' proliferation activity by combination of UA+OA (1:1). Data are presented as mean±SD. Significant results are assigned as ** $p < 0.001$ versus control. (b) Inhibition of the WM-266-4 cells' proliferation activity by combination of UA+OA (3.5:1). Data are presented as mean±SD. Significant results are assigned as ** $p < 0.001$ versus control. UA: Ursolic acid, OA: Oleanolic acid

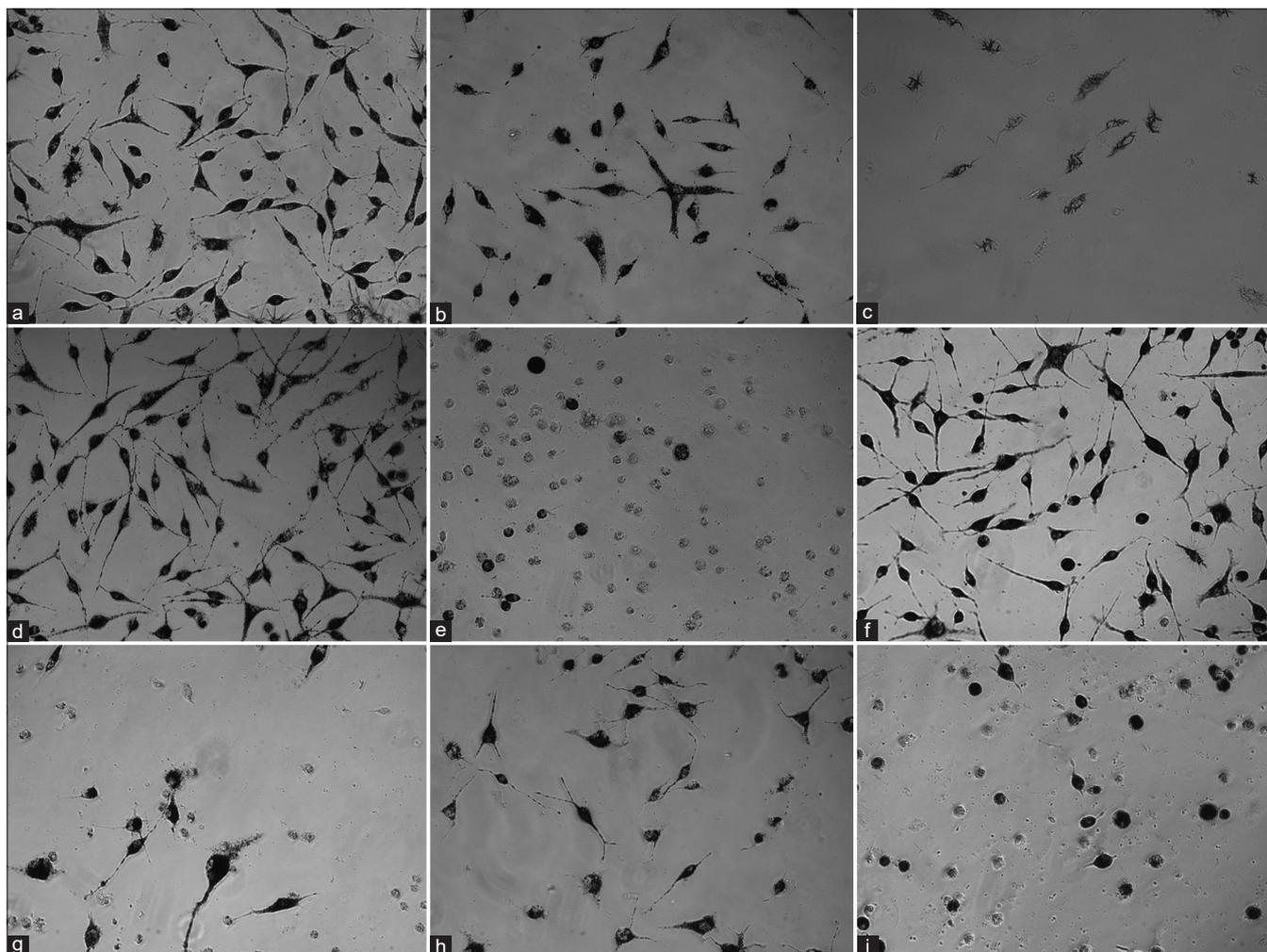


Figure 5: WM-266-4 cells with MTT formazan granule (24 h exposure time): Control (a). 0.2 μM BA (b) and 2 μM BA (c). 2 μM UA (d) and 10 μM UA (e). 10 μM OA (f), 20 μM OA (g). 2 μM OA+UA (1:1) (h) and 10 μM OA+UA (3.5:1) (i). BA: Betulinic acid, OA: Oleanolic acid, UA: Ursolic acid

leaves, and stem bark [16], [17]. Among these, BA, UA, and OA have been exploited recently due to their pleiotropic biological activities [17], [18]. Particularly, their antitumor potential against various cancers has been described in several recently published articles [17], [18], [19], [20], [21], [22].

In our study, we investigated the influence of the latter compounds on the WM-266-4 metastatic melanoma cells' proliferation. Selected concentrations were similar to those used in the previous studies, although a wider range of concentrations was used in the present study (0.002–200 μM). The three acids are poorly soluble in water due to their cholesterol-like chemical structure and for that reason they were dissolved in DMSO.

BA exhibits potent anti-tumor activities by triggering the mitochondrial path to apoptosis [17]. It showed cytotoxic activity on different cancer cell lines, including neuroblastoma, melanoma, glioblastoma, head-and-neck cancer, ovarian and cervix carcinoma, lung carcinoma, and leukemia [17], [25], [26]. Our results demonstrated good antiproliferative activity of BA against WM-266-4 cells confirming previous

observations. In our study, BA also proved to be more effective at lower concentrations than UA, OA, or UA+OA and inhibition of proliferation was rather constant over the concentration range (2–200 μM) regardless of the time of incubation.

UA is a pentacyclic triterpenoid derived from berries, fruits, leaves, and flowers of medicinal plants, such as *R. officinalis* [15]. UA has been shown to inhibit tumorigenesis, tumor promotion and suppress angiogenesis. Scientists found that UA inhibits cell proliferation and induces apoptosis through both mitochondrial death pathway and extrinsic death receptor-dependent pathway in human breast cancer cell line, MDA-MB-231 [27]. The antiproliferative activity of UA has been demonstrated in a wide variety of cancer cell lines [19], [20]. Baishya *et al.* (2016) demonstrated cytotoxic activity of UA using B16F10 human melanoma cell line [11]. Our results are in accordance with the results of the previous studies. In our study, proliferation assay showed that UA exhibited strong antiproliferative activity against WM-266-4 cells. In our study, UA was more efficient on WM-266-4 cell line than BA; however, BA was more potent (more effective at lower concentration than UA).

OA was also chosen for the antiproliferative activity assay as it was already shown that it exhibited cytotoxic activity against many cancer cell lines [21], [20]. In addition, UA and OA exhibited good cytotoxic activity against the A549 cell line in the previous studies [18], [28]. The mechanism of apoptotic effect induced by OA has been studied in different types of cancer cell lines. Recent studies revealed that OA induces apoptosis through the mitochondrial pathway, with the generation of reactive oxygen species and mitochondrial fatty acid oxidation [13], [18]. In our study, proliferation activity of OA treated cells was significantly decreased at concentrations of 20 μM and higher after incubation of 24 h and 48 h. However, OA was less effective in inhibiting WM-266-4 cells' proliferation activity than BA and UA.

In attempt to improve antiproliferative activity of OA and with the aim to test if OA and UA synergistically interact in their antiproliferative activity, two mixtures of UA+OA were prepared in ratios – UA: OA 1:1 and UA: OA 3.5:1. The latter ratio more reflects the ratios in which both acids can be naturally found in rosemary. Our results showed that exposure of WM-266-4 cells to concentrations of UA+OA 10 μM and higher caused a significant loss of the cells' proliferation activity, more pronounced with longer incubation period. However, the combined UA+OA antiproliferative effects on WM-266-4 melanoma cell line did not differ significantly compared to the effects of UA exposure separately. Moreover, OA is less potent in inhibiting the WM-266-4 proliferation activity than UA. It could be speculated that OA effects are masked by UA effects and that the two tested substances do not interact significantly. Therefore, our results cannot confirm the results of Soica *et al.* who demonstrated a synergistic anti-tumor effect of UA and OA over the concentration range 40–100 μM using A375 human melanoma cell line [29].

Overall, the use of BA, UA, OA, and their both combinations resulted in a decreased proliferative activity of WM-266-4 cells. However, a concentration 0.002 μM during different incubation periods resulted in slightly increased proliferative activity (i.e. 24 h of incubation for both BA and UA+OA (3.5:1), and the same concentration of UA+OA (1:1)) during the shorter incubation period of 4 h. Such an increase of proliferative activity might reflect cellular protective or adaptation mechanisms as a response to an adverse change in the environment.

Conclusion

Many studies have recently described the numerous biological activities of terpenoids, with particular interest in their anti-tumor activity. Triterpenoids act at various stages of tumor

development and their anti-tumor properties were demonstrated against various types of cancer cells. We report promising inhibitory effect of the three selected triterpenoid acids, BA, UA, and OA on WM-266-4 metastatic melanoma cells' proliferation activity. However, further studies are necessary to elucidate the mechanisms of the antiproliferative activity. The effectiveness of pharmacokinetic properties of pure triterpenic acids BA, UA, and OA which we used in our study could most likely be improved with the use of their more potent derivatives. A significant disadvantage in terms of bioavailability of BA, UA, and OA is their low water solubility. Further studies will be necessary to overcome this issue and to improve antitumor drug delivery with possible use of different inclusion complexes, liposomes, or nanotechnology.

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