*https://doi.org/10.31925/farmacia.2023.6.21* **ORIGINAL ARTICLE**

# EVALUATION OF OLEANOLIC ACID, DOXORUBICIN AND THEIR ASSOCIATION IN THE TREATMENT OF MELANOMA: ENHANCED EFFICACY AND ANTIANGIOGENIC POTENTIAL

GEORGE PUENEA [1#], BOGDAN ALMĂJAN-GUȚĂ [2#], RAUL CHIOIBAȘ [1*], IOANA MACAȘOI [3,4], ANDREEA GEAMANTAN [3,4], ȘTEFANIA DINU [5,6], PETRU MERGHEȘ [7], EUGEN RADU BOIA [1], ALINA ANDREEA TISCHER [1], ANDRADA IFTODE [3,4]

[1]*Faculty of Medicine, "Victor Babeș" University of Medicine and Pharmacy from Timișoara, 2 Eftimie Murgu Square, 300041, Timișoara, Romania*
[2]*Faculty of Physical Education and Sport, West University of Timișoara, Vasile Pârvan Boulevard 4, 300223, Timișoara, Romania*
[3]*Faculty of Pharmacy, "Victor Babeș" University of Medicine and Pharmacy from Timișoara, 2 Eftimie Murgu Square, 300041, Timișoara, Romania*
[4]*Research Centre for Pharmaco-Toxicological Evaluations, Faculty of Pharmacy, "Victor Babeș" University of Medicine and Pharmacy from Timișoara, 2 Eftimie Murgu Square, 300041, Timișoara, Romania*
[5]*Faculty of Dental Medicine, "Victor Babeș" University of Medicine and Pharmacy from Timișoara, 9 Revoluției 1989 Boulevard, 300070, Timișoara, Romania*
[6]*Paediatric Dentistry Research Centre, Faculty of Dental Medicine, "Victor Babeș" University of Medicine and Pharmacy from Timișoara, 9 Revoluției 1989 Boulevard, 300041, Timișoara, Romania*
[7]*Department of Physical Education and Sport, "King Michael I" University of Life Sciences from Timișoara, 119 Calea Aradului Street, Timișoara, Romania*

**corresponding author: office@medcom.ro*
*[#]Authors with equal contribution.*

*Manuscript received: July 2023*

## Abstract

As one of the most aggressive types of skin cancer, melanoma, remains a challenging malignancy to treat, necessitating continued research for more effective therapeutic approaches. This study examined the impact of oleanolic acid and doxorubicin individually and in combination on the viability of cells, morphological changes and antiangiogenic properties. Oleanolic acid, doxorubicin and a combination of both were used to treat melanoma cells - A375. The viability of the cells was assessed using MTT assay, and phase-contrast microscopy was used to observe morphological changes. To assess apoptotic alterations in the nuclei, Hoechst 33342 staining was applied to the nuclei. The antiangiogenic potential was evaluated using the hen's egg chorioallantoic membrane as a biological model. Cell viability was significantly reduced by both oleanolic acid and doxorubicin while cell morphology revealed distinct changes in shape and adherence, which are common to apoptotic processes. Apoptosis was confirmed as the underlying mechanism by the presence of condensation and fragmentation of the nuclei. It should be noted that the combination of oleanolic acid and doxorubicin had a more pronounced effect on cell viability and morphological changes associated with apoptosis. Furthermore, the assessment of antiangiogenic potential demonstrated that oleanolic acid, along with doxorubicin, was capable to inhibit the formation of new blood vessels. According to this study, both oleanolic acid and doxorubicin exhibit promising anti-melanoma properties, including a reduction in cell viability and induction of apoptosis. These effects are enhanced by their association, indicating a potential synergistic effect. A valuable insight has been gained into the therapeutic potential of combining oleanolic acid and doxorubicin in the treatment of melanoma in the present study. Efforts should be made to elucidate the underlying mechanisms of action of this combination as well as evaluate its safety and efficacy in preclinical and clinical settings to improve therapeutic outcomes.

## Rezumat

Fiind unul dintre cele mai agresive tipuri de cancer de piele, melanomul rămâne o afecțiune malignă dificil de tratat, necesitând cercetări continue pentru abordări terapeutice mai eficiente. Acest studiu a examinat impactul acidului oleanolic și al doxorubicinei individual și în combinație în ceea ce privește impactul la nivelul viabilității celulelor, modificărilor morfologice și proprietăților antiangiogenice. Acidul oleanolic, doxorubicina și o combinație a ambelor au fost folosite pentru a trata celulele melanomului - A375. Viabilitatea celulelor a fost evaluată folosind testul MTT și a fost utilizată microscopia cu contrast de fază pentru a observa modificările morfologice. Pentru a evalua modificările apoptotice ale nucleilor, a fost aplicată colorarea Hoechst 33342. Potențialul antiangiogenic a fost evaluat utilizând membrana corioalantoică a oului de găină ca model biologic. Viabilitatea celulară a fost redusă semnificativ atât de acidul oleanolic, cât și de doxorubicină în timp ce morfologia celulară a evidențiat modificări distincte de formă și aderență, care sunt comune proceselor apoptotice. Apoptoza a fost confirmată ca mecanism de bază prin prezența condensării și fragmentării nucleilor. Combinația de acid oleanolic și doxorubicină a avut un efect mai pronunțat asupra viabilității celulare și asupra modificărilor morfologice asociate cu apoptoza. Mai mult, evaluarea potențialului

antiangiogenic a demonstrat că acidul oleanolic, împreună cu doxorubicina, a fost capabil să inhibe formarea de noi vase de sânge. Potrivit acestui studiu, atât acidul oleanolic, cât și doxorubicina prezintă proprietăți anti-melanom promițătoare, inclusiv o reducere a viabilității celulare și inducerea apoptozei. Aceste efecte sunt sporite de asocierea lor, indicând un potențial efect sinergic. În studiul de față s-a obținut o perspectivă valoroasă asupra potențialului terapeutic al combinării acidului oleanolic cu doxorubicina în tratamentul melanomului. Trebuie depuse eforturi pentru a elucida mecanismele de acțiune de bază ale acestei combinații, precum și pentru a evalua siguranța și eficacitatea acesteia în medii preclinice și clinice pentru a îmbunătăți rezultatele terapeutice.

**Keywords**: oleanolic acid, doxorubicin, synergism, melanoma

## Introduction

The melanoma is one of the most aggressive types of cancer, and it has a heterogeneous aetiology. Among the noteworthy attributes of this particular cancer are its escalating incidence rate, coupled with its pronounced aggressiveness and high fatality [5]. Thus, melanoma is responsible for over 80% of skin cancer deaths globally due to its high invasion and metastasis rates, as well as its ability to develop resistance to conventional treatments [12]. A major characteristic of melanoma is that, unlike other solid tumours, it primarily affects people between the ages of 25 and 50. Additionally, the female sex is more affected under the age of 50, whereas the male sex shows a higher incidence after the age of 55 [22]. In the early stages of melanoma, surgical intervention is the predominant method of treatment. However, some patients may experience relapses as a consequence of this therapeutic strategy [36]. For patients with advanced stages of cancer, conventional treatment involves chemotherapy as well as the use of RAF and MEK kinase inhibitors and immune checkpoint inhibitors. It is important to note that the main problem in this case is a failure to respond to treatment [25].

On a large scale, doxorubicin (DOX) is one of the most widely used conventional chemotherapy agents. In spite of this, its application in the treatment of melanoma is limited due to a number of disadvantages [18, 23]. The therapeutic efficacy of this treatment is limited, due to the ability of melanoma tumour cells to develop resistance to it. Consequently, it is sometimes necessary to combine doxorubicin with other chemotherapeutic agents, and this therapeutic strategy is associated with an increased risk of toxicity [23]. Second, DOX is known to cause high levels of toxicity, particularly cardiotoxicity and myelosuppression [2, 38].

Since there are many disadvantages associated with anti-melanoma therapy, as well as toxic reactions, natural compounds have received considerable interest. Over the centuries, phytocompounds have proven their therapeutic effectiveness, having been used in a variety of pharmaceutical forms since ancient times. There is a growing interest in establishing doses and biological mechanisms for natural compounds that are potential anti-tumour agents [9]. In recent years, triterpenes have received increased attention because of their chemical diversity and their potential as anti-tumour agents. Several reports have described the molecular mechanisms by which phytocompounds are able to modulate targets such as oncogenes, cytokines, inflammatory enzymes, antiapoptotic proteins and transcription factors that induce apoptosis [17]. The preclinical studies conducted have revealed that both natural and synthetic triterpenoids are effective in inhibiting and preventing the growth of melanoma and other neoplasms [30].

The pentacyclic triterpenoid compound, oleanolic acid (OA), has a wide distribution in the plant kingdom. Oleanolic acid garners significant scientific interest owing to its notable biological efficacy against a spectrum of diseases [7]. The activity of OA has proven useful for treating a variety of neoplasia by altering several biological mechanisms, including inhibition of signalling pathways that are crucial for cell proliferation and survival, induction of apoptosis and stimulation of reactive oxygen species in cancer cells [1]. Oleanolic acid also has the advantage of being able to be used in conjunction with conventional therapies in order to enhance the therapeutic response and to reduce the accompanying toxic effects [32]. In terms of the therapeutic potential of OA for treating and preventing melanoma, there are not enough studies to demonstrate the effectiveness and the associated biological mechanisms. Accordingly, the purpose of this study was to evaluate the cytotoxic potential of oleanolic acid, doxorubicin and the combination of these two agents in human melanoma cells - A375, focusing on their impact on cell viability, morphology and nucleus structure. Furthermore, the antiangiogenic potential of the samples was evaluated *in ovo*, at the level of the chorioallantoic membrane.

## Materials and Methods

*Reagents*

In this study, oleanolic acid (OA), doxorubicin (DOX), dimethylsulfoxide (DMSO), phosphate-buffered saline, MTT [3-(4,5-dimethylthiazole-2-bromide)-yl)-2,5-diphenyltetrazolium)] kit and penicillin-streptomycin mixture were provided from Sigma-Aldrich, Merck KgaA (Darmstadt, Germany). Ultrapure distilled water and Hoechst 33342 were supplied from Invitrogen l Thermo Fisher Scientific, Inc. (Waltham, MA, USA); and the culture medium and supplements - Dulbecco's modified Eagle Medium (DMEM), fetal bovine serum (FBS) and trypsin-EDTA from PAN-Biotech GmbH (Aidenbach, Germany).

*Cell culture*

The cytotoxic potential of samples (OA, DOX and the combination of OA and DOX) was evaluated using A375 (CRL-1619) cell line, purchased from ATCC (American Type Culture Collection) in frozen vials. A375 cells were cultured in specific medium - DMEM (Dulbecco's modified Eagle Medium) containing 10% FBS (foetal bovine serum) and 1% antibiotic mixture (penicillin-streptomycin). The cells were incubated in standard conditions (37ºC and 5% $CO_2$) during the experiments.

*Cellular Viability Evaluation*

To assess the impact of the compounds (OA, DOX and the combination of OA and DOX) at the level of cell viability, the MTT method (tetrazolium colorimetric assay) was applied, adapted as described in the literature [15, 21]. Initially, the impact was assessed for OA (10, 25, 50, 75, 100 μM) and DOX (0.05, 0.1, 0.5, 1 and 5 μM) individually and subsequently cell viability was determined for OA (25, 50 and 75 μM) in combination with DOX (0.05, 0.1, 0.5, 1 and 5 μM). Cells were cultured in 96-well plates and allowed to attach to the plate. Upon reaching the desired confluency, cells were stimulated with OA, DOX, and the combination for 24 hours. After the incubation period with the test substances, the culture medium was changed with 100 μL/well of fresh medium and a volume of 10 μL of the MTT kit-1 was added to each well, then the plate was incubated for another 3 hours. After this time interval, 100 μL of kit 2-solubilization buffer solution was added to each well and stored at room temperature in a place away from light for 30 minutes. Finally, absorbance was measured at 570 nm using Cytation 5 (BioTek Instruments Inc., Winooski, VT, USA.) Using the absorbance of the samples, cell viability was calculated and compared with control cells (unstimulated). Data obtained was expressed as percentage (%) of viable cells normalized to control cells.

*Cellular Morphology*

A375 cells were cultured in 12-well plates to determine the impact on cell morphology. The cells were stimulated with the concentrations of OA, DOX and OA+DOX previously tested in the cell viability analysis after they reached an adequate confluency, approximately 90%. The morphological characteristics of the cells were evaluated using Cytation 1 (BioTek Instruments Inc., Winooski, VT, USA) by photographing them after 24 h of treatment.

*Immunofluorescence staining*

An immunofluorescence method was used to determine the cytotoxicity of samples by staining the nuclei with Hoechst 33342. A mixture of OA 50 μM and DOX 0.5 and 1 μM, and respectively, OA 75 μM and DOX 0.1 and 1 μM, was used to stimulate the cells grown in 12-well plates. The concentrations were selected based on the results obtained in the previous tests for evaluating cell viability and morphology. After 24 hours of stimulation with the compounds of interest, Hoechst 33342 solution (dilution 1:2000 in PBS) was added, and the plate was washed with PBS solution 3 times. Representative images of cell nuclei morphology were taken with Cytation 1 (BioTek Instruments Inc., Winooski, VT, USA).

*Chorioallantoic Membrane Assay (CAM assay)*

OA, DOX and their combination were evaluated at the chorioallantoic membrane level of the hen's egg to determine their effect on angiogenesis. Thus, disinfected and incubated until the 4$^{th}$ day when approximately 7 mL of albumen were extracted through a small hole made in the eggshell. On the fifth day, a window was cut into the top side of the egg to facilitate observation of the vascular plexus.

The experiment began on the seventh day of incubation with the application of a ring to the chorioallantoic membrane and the addition of samples in a volume of 10 μL daily for five days [26]. A microscopy and photographic examination of the blood vessels inside the ring was conducted on each day during the experiment in order to compare them with those outside the ring, as well as with the control blood vessels stimulated with water.

The images were taken and analysed using the Discovery v.8 stereomicroscope and the ZEN core 3.8 software.

*Statistical analysis*

Results are expressed as means ± SD (standard deviation), the one-way ANOVA test, followed by Dunett`s multiple comparison post-test being applied. The software used the statistical analysis was GraphPad Prism version 9.4.0 for Windows (GraphPad Soft-ware, San Diego, CA, USA, www.graphpad.com). The statistically significant differences between data are marked with * (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

## Results and Discussion

Despite significant advances in the treatment of melanoma, there are several challenges and problems associated with its management. Treatment resistance, limited treatment options for advanced stages of the disease, increased cost of treatment and toxic effects associated with conventional therapies are some of the most important factors to consider [13]. Exploring the potential of natural compounds as adjunctive therapies in melanoma treatment offers hope for a holistic approach to complement established medical interventions [14]. Unlocking the therapeutic potential of triterpenoids in melanoma treatment shows promise in targeting this aggressive cancer through natural compounds [44]. Oleanolic acid, a naturally occurring compound, holds potential for innovative approaches in melanoma treatment, offering new avenues for research and therapy [43]. As conventional treatment has low efficacy and increased toxicity, as well as the therapeutic potential of natural compounds, the present study was conducted

to evaluate oleanolic acid's antimelanoma potential both alone and in combination with a conventional chemotherapeutic drug, doxorubicin.

To assess the impact of the compounds on the viability of A375 cells, they were initially subjected to individual treatment with OA (10, 25, 50, 75, 100 µM) and DOX (0.05, 0.1, 0.5, 1, 5 µM) for 24 hours. According to Figure 1, viability decreases in a dose-dependent manner after treatment with OA and DOX, respectively, with the most potent effect observed at the highest concentrations tested. Thus, in the case of OA, cell viability reached values of approximately 92% and 86% at concentrations of 75 and 100 µM, and in the case of DOX, viability values were approximately 88% and 77% at concentrations of 1 and 5 µM. It is important to note that OA (10 and 25 µM) and DOX (0.05, 0.1 and 0.5 µM) increased cell viability compared to unstimulated control cells (Figure 1).

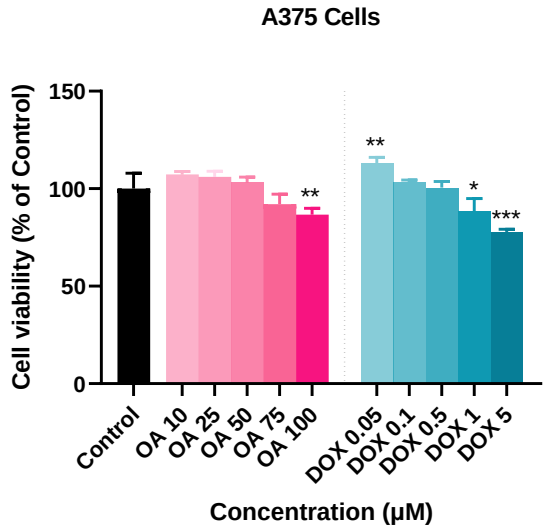


**Figure 1.**

Graphical representation of the impact of OA (10, 25, 50, 75, 100 µM) and DOX (0.05, 0.1, 0.5, 1, 5 µM) on the viability of A375 malignant melanoma cells after 24 h of treatment

Data are normalized to control (untreated cells) and presented as mean values ± standard deviation. For statistical analysis, One-way ANOVA followed by Dunett's test for multiple comparisons was applied (* $p < 0.5$; ** $p < 0.01$; *** $p < 0.001$)

Previous studies have examined the antimelanoma potential of oleanolic acid as well as its derivatives in A375 cells. Accordingly, George and colleagues tested concentrations of up to 200 µM of OA in A375 cells, and their study indicated that this compound has a highly cytotoxic and antiproliferative profile, especially at concentrations exceeding 50 µM [10]. Additionally, Ghosh *et al.* showed that OA extracted from *Phytolacca decandra* causes a significant decrease in cell viability in A375 cells. Furthermore, the researchers concluded that oleanolic acid's cytotoxic effects are a result of caspase-3-mediated activation of cell apoptosis [16]. Moreover, diverse oleanolic acid derivatives have demonstrated potential as therapeutic agents for melanoma. A study conducted by Bednarczyk-Cwynar and colleagues demonstrated that four semi-synthetic OA derivatives reduced A375 cell viability *in vitro* [1]. In a similar manner, Macasoi *et al.* evaluated the cytotoxicity of an oleanolic acid derivative conjugated with Rhodamine B at the level of several tumour cell lines, including A375. It was found that the OA derivative was strongly cytotoxic, even at nanomolar concentrations, due to a mitochondria-dependent mechanism, resulting in a marked decrease in cell viability [26].

OA has garnered significant attention for its potential synergistic effects when used in conjunction with conventional chemotherapy. This emerging field of research suggests that combining oleanolic acid with standard chemotherapy treatments may lead to enhanced therapeutic outcomes in cancer patients [45]. To assess the potential synergistic effect between the triterpene compound and the chemotherapeutic agent, cell viability was also determined after combining OA (25, 50, 75 µM) with DOX (0.05, 0.1, 0.5 and 1 µM).

OA potentiated the cytotoxic effect of DOX in A375 malignant melanoma cells. The results showed that the cell viability rate for the combination of the two compounds is reduced compared to the rates obtained with individual treatments. The effect was dependent on OA and DOX concentrations. Thus, OA at a concentration of 25 µM produced a decrease effect on cell viability (approximately 70%) when it was combined with DOX at a concentration of 1 µM. At the 50 µM concentration, OA associated with 0.5 and 1 µM DOX reduced cell viability to approximately 66% and 61%, while at the 75 µM concentration, OA significantly reduced cell viability following association with all concentrations of DOX- 0.05 µM (75%), 0.1 µM (74%), 0.5 µM (63%) and 1 µM (43%) (Figure 2). The use of doxorubicin in the treatment of melanoma is limited due to its resistance to the treatment and toxicological profile. To date, doxorubicin has been evaluated in various combinations *in vitro* for its antimelanoma properties [41]. A study conducted on A375 cells examined the potential therapeutic effects of cold atmospheric plasma technology and doxorubicin. As a result of the study, a decrease in cell viability was detected as a result of the synergistic effect of the association [46]. In a similar manner, doxorubicin was associated with hyperthermia and evaluated at the level of the human melanoma cell line A375. In the presence of this combination treatment, cell viability significantly decreased, but in a manner that was dependent upon the concentration of the drug and the duration of the thermal treatment [37].

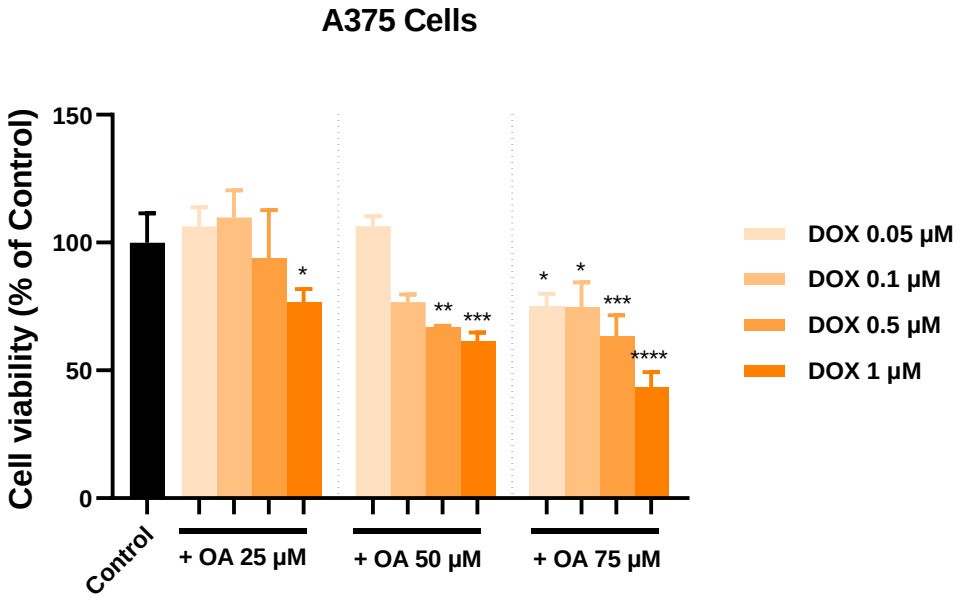


**Figure 2.**
Graphical representation of the impact of OA (25, 50, 75 µM) associated with DOX (0.05, 0.1, 0.5 and 1 µM) on the viability of A375 malignant melanoma cells after 24 h of treatment
Data are normalized to control (untreated cells) and presented as mean values ± standard deviation. For statistical analysis, One-way ANOVA followed by Dunett's test for multiple comparisons was applied
(* $p < 0.5$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$)

Subsequent of the analysis of the effects on cell viability, the next step in determining the cytotoxic profile of the compounds involved evaluation of the impact on cell morphology. During the evaluation of agents' cytotoxic profiles, microscopic scrutiny of cellular morphology yields valuable insights into both the potential mechanisms of action and the specific modes of cell death elicited by these substances [33].

As part of this study, the cells were stimulated with OA and DOX at the previously assessed concentrations. OA and DOX do not significantly reduce cell confluence, but induce morphological changes, such as the rounding of cells and their separation from the plate, especially at high concentrations of OA (100 µM) and DOX (5 µM) (Figure 3).

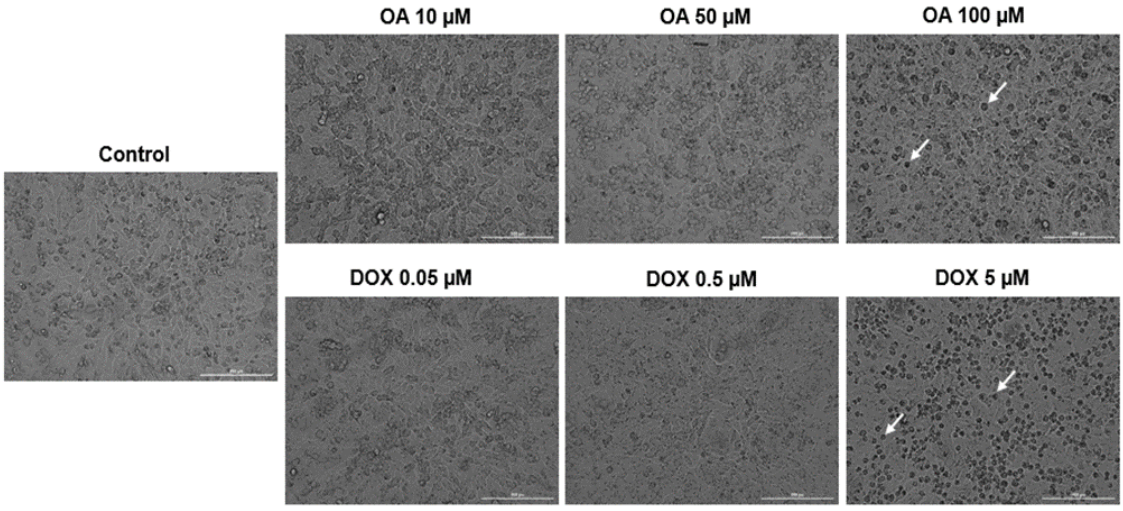


**Figure 3.**
Morphology and confluence of A375 malignant melanoma cells after 24 h of treatment with OA (10, 50, 100 µM) and DOX (0.05, 0.5, 5 µM)
Arrows indicate morphological changes at the cellular level; Scale indicates 200 µm

Previously, Bednarczyk-Cwynar *et al*. evaluated the effect of OA on the morphology of human melanoma cells - A375. During a time interval of 24 hours, they investigated similar concentrations to those used in

the present study. The results of the study indicated that OA had a substantial impact on cell morphology, causing dose-dependent changes such as loss of connections between cells, decrease in confluency, alterations characteristic of apoptotic cell death [10]. In addition to melanoma cells, oleanolic acid was also evaluated in terms of its effect on morphology in other types of tumour cells. Thus, Peng and colleagues examined the impact of oleanolic acid from the *Pyrus ussuriensis* on human hepatocarcinoma cells. Researchers have found that OA leads to significant apoptotic-like changes in the shape of cells [32]. In a similar study, Hassan and colleagues investigated the effect of oleanolic acid obtained from *Monotheca buxifolia* at the level of hepatocarcinoma cells, HepG2. Based on the findings, OA causes a significant decrease in cell confluency, resulting in major changes in cell shape, similar to those observed in the current study [20]. As a similar approach, Coricovac *et al*. examined betulinic acid (BA), a compound also belonging to the family of pentacyclic triterpenes like oleanolic acid in malignant melanoma cell line A375. BA at a concentration of 50 μM induced morphological changes of a significant order, including round floating cells, reduced confluence and cellular debris, clear signs of triterpene-induced cytotoxicity [11]. Bociort *et al*. evaluated lupeol as an anti-melanoma agent, noting that the compound induced morphological changes in A375 cancer cell lines at concentrations of 30 and 50 mM. As a result of these concentrations, rounding of cells was observed, which indicates the onset of cell death [3].

As for the combination of the two compounds, the concentrations that induced the greatest decline in cell viability were chosen (OA 50 μM + DOX 0.5 μM; OA 50 μM + DOX 1 μM; OA 75 μM + DOX 0.5 μM and OA 75 μM + DOX 1 μM). Similarly, in this case, the morphological changes observed were the rounding of the cells and the detachment from the plaque, which were most apparent at OA 75 μM + DOX 1 μM concentrations (Figure 4).

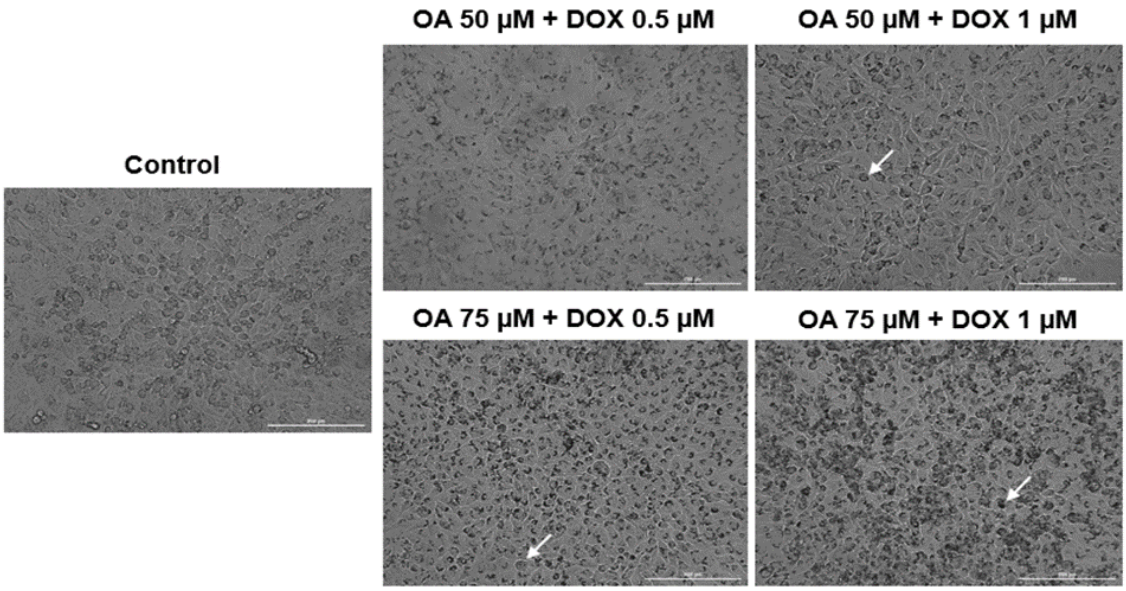


**Figure 4.**
ViscosiMorphology and confluence of A375 malignant melanoma cells after 24 h of treatment with different combinations of OA and DOX:
OA 50 μM + DOX 0.5 μM; OA 50 μM + DOX 1 μM; OA 75 μM + DOX 0.5 μM; OA 75 μM + DOX 1 μM
Arrows indicate morphological changes at the cellular level; Scale indicates 200 μm

Furthermore, doxorubicin has been reported to be cytotoxic in numerous cell lines, proving its effectiveness as a chemotherapeutic agent. As part of their study, Diana Salvador and her colleagues investigated doxorubicin alone and in conjunction with hyperthermia in melanoma cell lines and also examined the morphological effects. In the A375 cell line, DOX produced cellular stretching and flattening, while hyperthermia produced the same effect, along with some rounding of the cells. Nevertheless, the associated treatment increased the number of cells in suspension as well as the roundness of the cells [37]. According to previous studies, oleanolic acid may act synergistically with a variety of cancer chemotherapeutics. OA combined with 5-fluorouracil was investigated by Jianteng Wei and colleagues on the pancreatic cancer cell line PANC-28. The results indicated that OA potentiates the effects of 5-fluorouracil, and that the cytotoxic effects are more evident when these two compounds are combined [42]. There is intense research being conducted on the potential of pentacyclic triterpene compounds in combination with other recognized

chemotherapeutics that are currently being used in therapeutics. The effects of ursolic acid have been studied both individually and in combination with paclitaxel, a taxane-derived chemotherapeutic agent used in the treatment of various cancers, on TE-8 and TE-12 cell lines, esophageal cancer lines. In accordance with the results obtained, most of the changes leading to the inhibition of cancer cells were also revealed by their association [28].

As a result of the observation that the two compounds in combination have a major impact on malignant melanoma, the next step was to examine the structure of the nucleus of the cell. The application of Hoechst 33342 is a technique that is used in various fields such as cell and molecular biology or pathology, in order to study the structure and behaviour of cell nuclei. A key advantage of this method is its specificity for DNA, emitting blue fluorescence, as well as its economic value - it is not an expensive procedure. Moreover, the method can be applied to live or fixed cells and is also used for analysing cell cycles and monitoring condensation. Due to its additional ethyl group, Hoechst 33342 is more lipophilic and thus more permeable than Hoechst 33258 [24]. Hoechst 33332's binding to DNA induces minimal cytotoxicity, a feature that is exploited and a major advantage of the method in experimental studies [6].

It was chosen for this study that the combination of OA and DOX be administered at concentrations of OA 50 µM plus DOX 0.5 and 1 µM and OA 75 µM with DOX 0.5 and 1 µM. Control cells nuclei show normal morphology (they are round or oval) and show no signs of fragmentation or condensation. As shown in Figure 5, the nuclei show frequent irregularities in their shape (highlighted by arrows), they are degraded, condensed and fragmented and also apoptotic bodies are observed -indicating the presence of cell death by apoptosis (especially for OA 75 µM + DOX 0,5 µM; OA 75 µM + DOX 1 µM) (Figure 5).

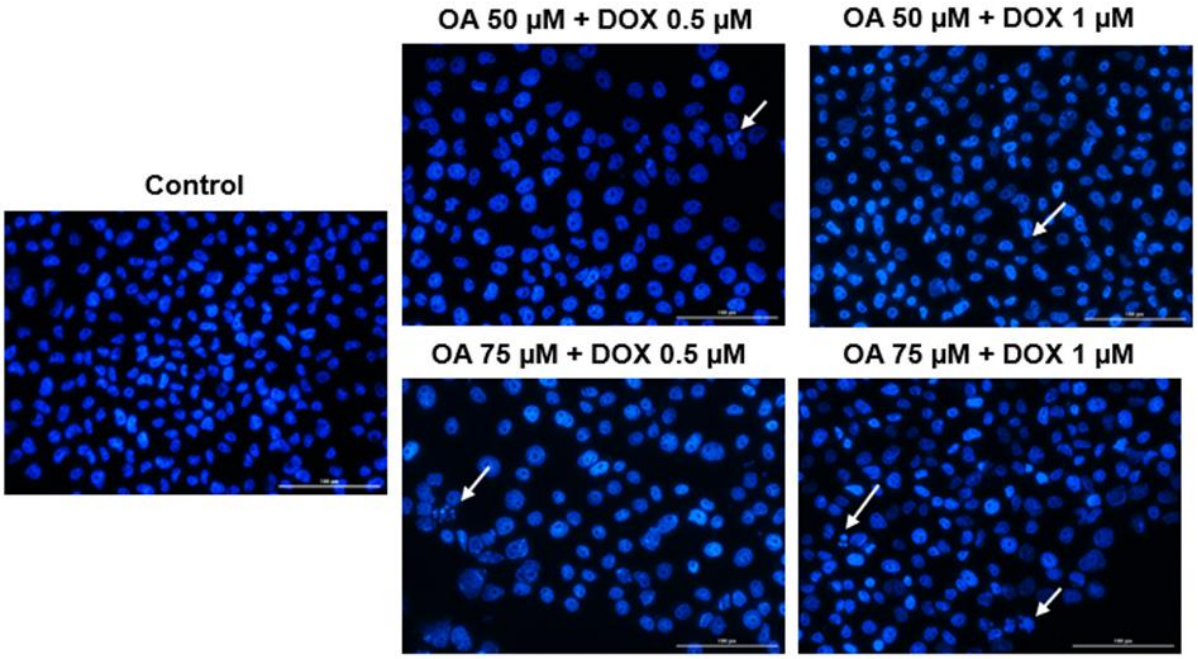


**Figure 5.**
Hoechst 33342 cell nuclei morphology highlighting after 24 h treatment of A375 malignant melanoma cells with OA 50 µM + DOX 0.5 µM; OA 50 µM + DOX 1 µM; OA 75 µM + DOX 0.5 µM; OA 75 µM + DOX 1 µM
Arrows indicate nuclei showing morphological changes; Scale indicates 100 µm

OA has previously been evaluated in MCF-7 breast carcinoma cells for its effect on nuclear structure. According to the results of the study, oleanolic acid causes strong chromatin condensation and the formation of apoptotic bodies, which were also observed in the present study [34]. In addition, derivatives of oleanolic acid were evaluated from the perspective of their impact on the nucleus. As shown by Macasoi *et al*., RhodOA (oleanolic acid-rhodamine B derivatives) induced condensation of nuclei in the same cell lines as in the present study (A375); RhodOA exhibited the greatest effect at a concentration of 100 nM [26]. An important indicator of the type of cell death incriminated is the structure of the nucleus. Therefore, activating the pathways involved in cell apoptosis can result in morphological changes, particularly at the nucleus level. They are characterized by chromatin condensation, nuclear contraction and the formation of apoptotic bodies [27]. These changes were observed both in the present study and in the other studies discussed, suggesting that combining oleanolic acid with doxorubicin causes an apoptotic-like effect.

Angiogenesis, the growth of new blood vessels, is a critical hallmark in the cancer process, as it provides tumours with the nourishment they need to thrive and spread. Comprehending and therapeutically addressing angiogenesis presents a promising approach in melanoma treatment, given that interference with the tumour's vascular supply can effectively deprive it of essential nutrients and impede its advancement [35].

The chorioallantoic membrane, with its remarkable transparency and robust angiogenic properties, serves as an invaluable model for advancing our understanding of angiogenesis in various research studies [29]. As a final step of the study, CAM from chicken eggs were used to evaluate the effect of OA (50 µM), DOX (0.5 µM) and their combination on angiogenesis. In terms of the effect of OA on blood vessels, a reduction in the number and a decrease in their diameter were observed when compared with the control. The concentration of 0.5 µM of DOX had no significant effect on the vascular plexus, even after 5 days of application of the sample, as the formation of blood vessels was similar to the control group. However, the combination of the two compounds resulted in the appearance of meshes in the network of vessels and a significant reduction of blood vessel formation compared to untreated areas. Additionally, none of the applied samples caused irritating effects at this level, no signs of coagulation or haemorrhage were noted (Figure 6).

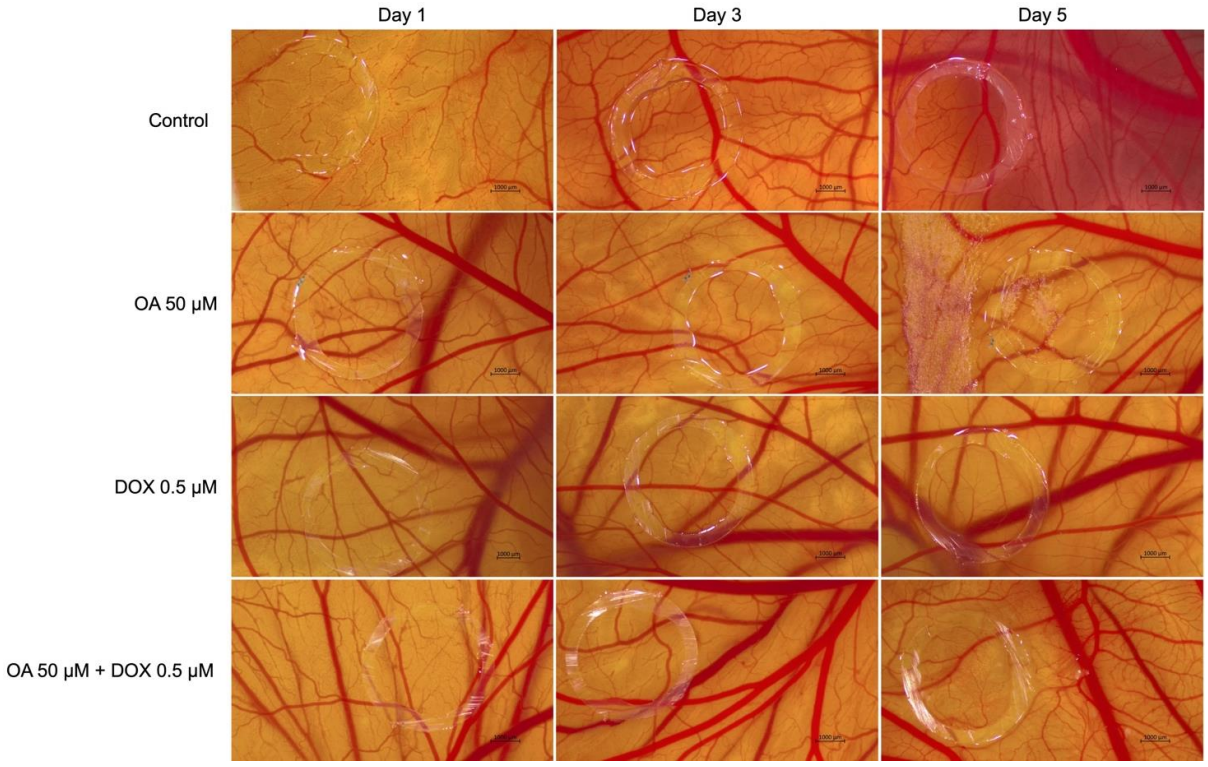


**Figure 6.**
Representative stereomicroscopic images of CAM for the antiangiogenic effect of OA 50 µM, DOX 0.5 µM and the combination of the two after 5 days of treatment

Various studies have investigated the antiangiogenic effects of triterpenes, including oleanolic acid, at the level of the chorioallantoic membrane. As a result, Sohn *et al.* conducted one of the first studies evaluating the effects of ursolic acid and oleanolic acid on the vascular plexus. Based on the findings of the study, both compounds possess significant antiangiogenic properties [39]. Additionally, Caunii *et al.* studied the effects of oleanolic acid at the level of the vascular plexus of the chorioallantoic membrane, emphasizing its importance in inhibiting blood vessel formation [8]. The exact mechanisms underlying the effects of oleanolic acid on angiogenesis are not fully understood, but some research suggests that it may modulate various molecular pathways involved in angiogenic processes. These pathways may include vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and various signalling cascades [31]. Doxorubicin showed anti-angiogenic effects in certain situations. By interfering with the growth of new blood vessels, doxorubicin can help reduce the blood supply to tumours [4]. In addition, the association of doxorubicin with other compounds of natural origin increased the antiangiogenic activity of the drug and prevented the development of treatment resistance [19, 40]. All the aforementioned investigations substantiate the findings of the current study. Nonetheless, to the best of our knowledge, no prior research has been conducted concerning the

synergistic interaction between oleanolic acid and doxorubicin as a potential therapeutic approach for this cell type. This aspect constitutes the novelty of the present study.

## Conclusions

Since melanoma is on the rise in incidence and mortality, the main objective of the present study was to evaluate the anti-melanoma potential of oleanolic acid in combination with doxorubicin. As part of this research, oleanolic acid, doxorubicin and their association were evaluated *in vitro* on human melanoma cells - A375, to determine their effects on viability, morphology and nucleus structure. Additionally, the antiangiogenic effect of the compounds was evaluated *in ovo* using the chorioallantoic membrane. Results of the study indicate that both compounds are cytotoxic *in vitro*, but their association causes a more profound reduction in viability and induces morphological and nuclear changes characteristic of cell apoptosis. Further, the association between OA and DOX caused a visible reduction in the formation of blood vessels and a thinning of their walls at the level of the chorioallantoic membrane vascular plexus. As a result of these studies, oleanolic acid was found to increase the antitumour activity of doxorubicin synergistically through its association with this drug. Further studies are required to determine the biological mechanisms and safety profile.

## Conflict of interest

The authors declare no conflict of interest.

## References


1. Bednarczyk-Cwynar B, Leśków A, Szczuka I, Zaprutko L, Diakowska D, The Effect of Oleanolic Acid and Its Four New Semisynthetic Derivatives on Human MeWo and A375 Melanoma Cell Lines. *Pharmaceuticals*, 2023; 16(5): 746
2. Bhinge KN, Gupta V, Hosain SB, Satyanarayanajois SD, Meyer SA, Blaylock B, Zhang QJ, Liu YY, The opposite effects of doxorubicin on bone marrow stem cells *versus* breast cancer stem cells depend on glucosylceramide synthase. *Int J Biochem Cell Biol.*, 2012;44(11):1770-1778.
3. Bociort F, Macasoi IG, Marcovici I, Motoc A, Grosu C, Pinzaru I, Petean C, Avram S, Dehelean CA, Investigation of lupeol as anti-melanoma agent: An in vitro-in ovo perspective. *Current Oncology*, 2021; 28(6): 5054-5066.
4. Borgström P, Gold DP, Hillan KJ, Ferrara N. Importance of VEGF for breast cancer angiogenesis *in vivo*: implications from intravital microscopy of combination treatments with an anti-VEGF neutralizing monoclonal antibody and doxorubicin. *Anticancer Res.*, 1999; 19(5B): 4203-4214.
5. Bratu LM, Marcovici I, Macasoi I, Manea A, Niculescu B, Olaru F, Heghes A, Dehelean C, Coricovac D, *In vitro* insights regarding the role of melanin in melanoma cells' response to betulinic acid treatment. *Farmacia*, 2022; 70(1): 8-16.
6. Bucevičius J, Lukinavičius G, Gerasimaite R, The Use of Hoechst Dyes for DNA Staining and Beyond. *Chemosensors*, 2018; 6(2): 18.
7. Castellano JM, Ramos-Romero S, Perona JS, Oleanolic Acid: Extraction, Characterization and Biological Activity. *Nutrients*, 2022; 14(3): 623.
8. Caunii A, Oprean C, Cristea M, Ivan A, Danciu C, Tatu C, Paunescu V, Marti D, Tzanakakis G, Spandidos DA, Tsatsakis A, Susan R, Soica C, Avram S, Effects of ursolic and oleanolic on SK-MEL-2 melanoma cells: *In vitro* and *in vivo* assays. *Int J Oncol.*, 2017; 51(6): 1651-1660.
9. Chinembiri TN, du Plessis LH, Gerber M, Hamman JH, du Plessis J, Review of natural compounds for potential skin cancer treatment. *Molecules*, 2014; 19(8): 11679-11721.
10. Cijo George V, Naveen Kumar DR, Suresh PK, Kumar RA, Oleanolic acid inhibits cell growth and induces apoptosis in A375 melanoma cells. *Biomedicine Preventive Nutrition*, 2014; 4(2): 95-99.
11. Coricovac D, Dehelean CA, Pinzaru I, Mioc A, Aburel OM, Macasoi I, Draghici GA, Petean C, Soica C, Boruga M, Vlaicu B, Muntean MD, Assessment of Betulinic Acid Cytotoxicity and Mitochondrial Metabolism Impairment in a Human Melanoma Cell Line. *Int J Mol Sci.*, 2021; 22(9): 4870.
12. Dhanyamraju PK, Patel TN, Melanoma therapeutics: a literature review. *J Biomed Res.*, 2022; 36(2): 77-97.
13. Domingues B, Lopes JM, Soares P, Pópulo H, Melanoma treatment in review. *Immunotargets Ther.*, 2018; 7: 35-49.
14. Fontana F, Raimondi M, Di Domizio A, Moretti RM, Montagnani Marelli M, Limonta P, Unraveling the molecular mechanisms and the potential chemopreventive/ therapeutic properties of natural compounds in melanoma. *Semin Cancer Biol.*, 2019; 59: 266-282.
15. Gag O, Macasoi I, Pinzaru I, Dinu S, Popovici R, Cosoroaba M-R, Buzatu R, Cabuta M, Chiriac SD, *In Vitro* Assessment of the Impact of Ultraviolet B Radiation on Oral Healthy and Tumor Cells. *Photonics*, 2023; 10(4): 464.
16. Ghosh S, Bishayee K, Khuda-Bukhsh AR, Oleanolic acid isolated from ethanolic extract of *Phytolacca decandra* induces apoptosis in A375 skin melanoma cells: drug-DNA interaction and signaling cascade. *J Integr Med.*, 2014; 12(2): 102-114.
17. Gill BS, Kumar S, Navgeet, Triterpenes in cancer: significance and their influence. *Molecular Biology Reports*., 2016;43(9):881-96.
18. Guo M, Zhou G, Liu Z, Liu J, Tang J, Xiao Y, Xu W, Liu Y, Chen C, Direct site-specific treatment of skin cancer using doxorubicin-loaded nanofibrous membranes. *Sci Bull* (Beijing), 2018; 63(2): 92-100.
19. Gurel-Gurevin E, Tuba Kiyan H, Esener OBB, Aydinlik S, Uvez A, Ulukaya E, Dimas K, Armutak EI, Chloroquine used in combination with chemotherapy synergistically suppresses growth and angiogenesis *in vitro* and *in vivo*. *Anticancer Res.*, 2018;38(7): 4011-4020.
20. Hassan S, Ahmad B, Khan MW, Shah ZA, Ullah A, Ullah S, Khan D, Rizwan M, Ahmad A, Ali Q, Kaushik

P, Yilmaz S, Cytotoxic Activity of Phytoconstituents Isolated from *Monotheca buxifolia* against Hepatocellular Carcinoma Cell Line HepG2: *In Vitro* and Molecular Docking Studies. *ACS Omega*, 2023; 8(37): 33572-33579.
21. Ivănescu B, Pop CE, Vlase L, Corciovă A, Gherghel D, Vochita G, Tuchiluș C, Mardari C, Teodor CM, Cytotoxic effect of chloroform extracts from *Tanacetum vulgare*, *T. macrophyllum* and *T. corymbosum* on Hela, A375 and V79 cell lines. *Farmacia*, 2021; 69(1): 12-20.
22. Leonardi GC, Falzone L, Salemi R, Zanghì A, Spandidos DA, Mccubrey JA, Candido S, Libra M, Cutaneous melanoma: From pathogenesis to therapy (Review). *Int J Oncol.*, 2018; 52(4): 1071-1080.
23. Licarete E, Rauca VF, Luput L, Drotar D, Stejerean I, Patras L, Dume B, Toma VA, Porfire A, Gherman C, Sesarman A, Banciu M, Overcoming Intrinsic Doxorubicin Resistance in Melanoma by Anti-Angiogenic and Anti-Metastatic Effects of Liposomal Prednisolone Phosphate on Tumor Microenvironment. *Int J Mol Sci.*, 2020; 21(8): 2968.
24. Lin KK, Goodell MA, Detection of Hematopoietic Stem Cells by Flow Cytometry. *Methods Cell Biol.*, 2011; 103: 21-30.
25. Luke JJ, Flaherty KT, Ribas A, Long GV, Targeted agents and immunotherapies: optimizing outcomes in melanoma. *Nat Rev Clin Oncol.*, 2017; 14(8): 463-482.
26. Macașoi I, Pavel IZ, Moacă AE, Avram Ștefana, David VL, Coricovac D, Mioc A, Spandidos DA, Tsatsakis A, Șoica C, Dumitrașcu V, Dehelean C, Mechanistic investigations of antitumor activity of a Rhodamine B oleanolic acid derivative bioconjugate. *Oncol Rep.*, 2020; 44(3): 1169-1183.
27. Martelli AM, Zweyer M, Ochs RL, Tazzari PL, Tabellini G, Narducci P, Bortul R, Nuclear apoptotic changes: An overview. *J Cell Biochem.*, 2001; 82(4): 634-646.
28. Meng RY, Jin H, Nguyen TV, Chai OH, Park BH, Kim SM, Ursolic Acid Accelerates Paclitaxel-Induced Cell Death in Esophageal Cancer Cells by Suppressing Akt/FOXM1 Signaling Cascade. *Int J Mol Sci.*, 2021; 22(21): 11486.
29. Merckx G, Tay H, Lo Monaco M, van Zandvoort M, De Spiegelaere W, Lambrichts I, Bronckaers A2, Chorioallantoic Membrane Assay as Model for Angiogenesis in Tissue Engineering: Focus on Stem Cells. *Tissue Eng Part B Rev.*, 2020; 26(6): 519-539.
30. M.R. Patlolla J, V. Rao C, Triterpenoids for Cancer Prevention and Treatment: Current Status and Future Prospects. *Curr Pharm Biotechnol.*, 2011; 13(1): 147-155.
31. Niu G, Sun L, Pei Y, Wang D, Oleanolic Acid Inhibits Colorectal Cancer Angiogenesis by Blocking the VEGFR2 Signaling Pathway. *Anticancer Agents Med Chem.*, 2018; 18(4): 583-590.
32. Peng F, Li G, Xie Y, Yin HY, Li XJ, Yang YD, Compositional characterization of *Pyrus ussuriensis* Maxim and their antioxidant activities and induction of apoptosis in Bel-7402 cell. *J Food Biochem.*, 2020; 44(6): e13222.
33. Prasad A, Alizadeh E, Cell Form and Function: Interpreting and Controlling the Shape of Adherent Cells. *Trends Biotechnol.*, 2019; 37(4): 347-357.
34. Qamar KA, Farooq AD, Siddiqui BS, Kabir N, Begum S, Antiproliferative Effects of *Ocimum basilicum* Methanolic Extract and Fractions, Oleanolic Acid and 3-epi-Ursolic Acid. *Current Traditional Medicine*, 2019; 6(2): 134-146.
35. Rajabi M, Mousa SA, The Role of Angiogenesis in Cancer Treatment. *Biomedicines*, 2017; 5(2): 34.
36. Ross MI, Gershenwald JE, Evidence-based treatment of early-stage melanoma. *J Surg Oncol.*, 2011; 104(4): 341-353.
37. Salvador D, Bastos V, Oliveira H, Hyperthermia enhances doxorubicin therapeutic efficacy against A375 and MNT-1 melanoma cells. *Int J Mol Sci.*, 2022; 23(1): 35.
38. Santos, dos Silva D, dos Santos Goldenberg RC, Doxorubicin-Induced Cardiotoxicity: From Mechanisms to Development of Efficient Therapy. In Cardiotoxicity, InTech, 2018.
39. Sohn KH, Lee HY, Chung HY, Young HS, Yi SY, Kim KW, Anti-angiogenic activity of triterpene acids. *Cancer Lett.*, 1995; 94(2): 213-218.
40. Uvez A, Aydinlik S, Esener OBB, Erkisa M, Karakus D, Armutak EI, Synergistic interactions between resveratrol and doxorubicin inhibit angiogenesis both *in vitro* and *in vivo*. *Pol J Vet Sci.*, 2020; 23(4): 571-580.
41. Vydra N, Toma A, Glowala-Kosinska M, Gogler-Piglowska A, Widlak W, Overexpression of Heat Shock Transcription Factor 1 enhances the resistance of melanoma cells to doxorubicin and paclitaxel. *BMC Cancer*, 2013; 13: 504.
42. Wei J, Liu H, Liu M, Wu N, Zhao J, Xiao L, Han L, Chu E, Lin X, Oleanolic acid potentiates the antitumor activity of 5-fluorouracil in pancreatic cancer cells. *Oncol Rep.*, 2012; 28(4): 1339-1345.
43. Woo JS, Yoo ES, Kim SH, Lee JH, Han SH, Jung SH, Jung GH, Jung JY, Anticancer effects of oleanolic acid on human melanoma cells. *Chem Biol Interact.*, 2021; 347: 109619.
44. Wróblewska-Łuczka P, Cabaj J, Bargieł J, Łuszczki JJ, Anticancer effect of terpenes: focus on malignant melanoma. *Pharmacol Rep.*, 2023; 75(5): 1115-1125.
45. Xu AL, Xue YY, Tao WT, Wang SQ, Xu HQ, Oleanolic acid combined with olaparib enhances radiosensitization in triple negative breast cancer and hypoxia imaging with 18F-FETNIM micro PET/CT. *Biomed Pharmacother.*, 2022; 150: 113007.
46. Zhang H, Xu S, Zhang J, Li B, Liu D, Guo L, Liu Z, Xu D, Synergistic anticancer effects of different combinations of He+$O_2$ plasma jet and doxorubicin on A375 melanoma cells. *Plasma Processes and Polymers*, 2021; 18(6): 2000239.