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# **PhD THESIS**

## **EXPERIMENTAL EVALUATION OF NATURAL COMPOUNDS ON IN VITRO AND IN OVO MODELS**

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**Timișoara  
2023**

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## INTRODUCTION

Medicinal plants are important sources of phytochemicals with an extraordinary therapeutic spectrum. This phenomenon is also observed from the perspective of scientific publications, which have recently been intensely focused on the study of natural compounds. The most deeply studied are triterpenes and bioflavonoids [1]. One of the most researched diseases with a continuously rising mortality rate is cancer. Classical therapies evolve progressively, but insufficiently and with many adverse reactions, the most severe being high toxicity. For this reason, natural compounds are intensively studied to serve as alternatives or even anticancer treatments/co-treatments.

Lupeol is a triterpene representative with known antibacterial, antioxidant, anti-inflammatory and anticancer properties [2]. This natural compound is intensively studied on liver, breast, gastro-intestinal, prostate, head and neck and bladder cancer forms, and less studied on skin cancer forms, especially on malignant melanoma (MM) which represents the most life-threatening skin cancer worldwide, with an inefficient chemotherapeutic treatment [3].

Rutin is another important phytochemical, but from flavonoid class, frequently found in citrus, apples, green tea, buckwheat, black tea, and vegetables, with large spectrum of therapeutic potential, including antioxidant and antitumoral activities [4].

The aim of the present work is to analyze *in vitro* and *in vivo* the biological potential of lupeol and rutin and optimize their biocompatibility properties. The main objectives of this study are:

- a) analysis of the cytotoxic potential exerted by lupeol on human melanoma cells,
- b) analysis of the cytotoxic potential exerted by rutin on human melanoma cells,
- c) obtaining, characterizing and evaluating the cytotoxic potential of some innovative formulations based on metal nanoparticles loaded with lupeol,
- d) obtaining a formulation for topical application based on proniosomal gel loaded with rutin and evaluating biocompatibility.

The research was divided in three parts:

1. obtaining of green and synthetic metal nanoparticles uploaded with lupeol, characterization and biological evaluation
2. preparation of proniosomal gel for topical rutin administration, physicochemical characterization and in vitro toxicological profile analysis using 3D reconstructed human epidermal tissue and 2D cells
3. in vitro and in ovo investigation of lupeol as an anti-melanoma agent

The personal contribution consists of in vitro on and in ovo evaluation of lupeol and rutin, lupeol nanoparticles formulation and proniosomal gel with rutin preparation and characterization.

## RESULTS

In the first study, two different gold nanoparticles (AuNPs) were obtained by chemical and biochemical synthesis, respectively. The gold detection limit was obtained at 0.0036 µg/L and the gold concentration in AuNPs and lupeol gold nanoparticles (Lup\_AuNPs) obtained by ICP-MS was 29.8374 and 24.1642 µg Au /mL suspension. Gold nanoparticles obtained by chemical synthesis were charged with lupeol to see the activity following the association with this bioactive molecule. The data are presented in table 1 together with size and stability data.

Table 1. Characterization of gold nanoparticles: i) chemically synthesized, ii) loaded with lupeol and iii) biosynthesized

Sample	Concentration (µg / Au / mL)	Size (nm)	Zeta potential (mV)
AuNPs	29,486	38	-31
Lup_AuNPs	24,322	54	-38
B_AuNPs	31,485	82	-27

The of 3- (4,5-dimethylthiazol-2-yl) -2,5- bromide. diphenyltetrazolium (MTT) cell viability test was performed to assess the viability of chemical and biochemical gold nanoparticles, gold nanoparticles loaded with aqueous lupeol, lupeol, and lemon balm from the HT-29 and CaCo-2 colon cancer cell lines. Cells were stimulated with different concentrations of test compounds at 24-hour intervals. In

the case of lupeol (Lup) and Lup\_AuNPs, the data showed that a slight decrease in cell viability can be observed at low concentrations for all substances tested. In contrast, at high concentrations of Lup\_AuNPs the viability of HT-29 cells was approximately 76% (at 25  $\mu$ M) and approximately 68% (at 50  $\mu$ M), and at CaCo-2 cells it was approximately 85% (at 25  $\mu$ M) and about 78% (at 50  $\mu$ M), as can be seen in Figure 1.

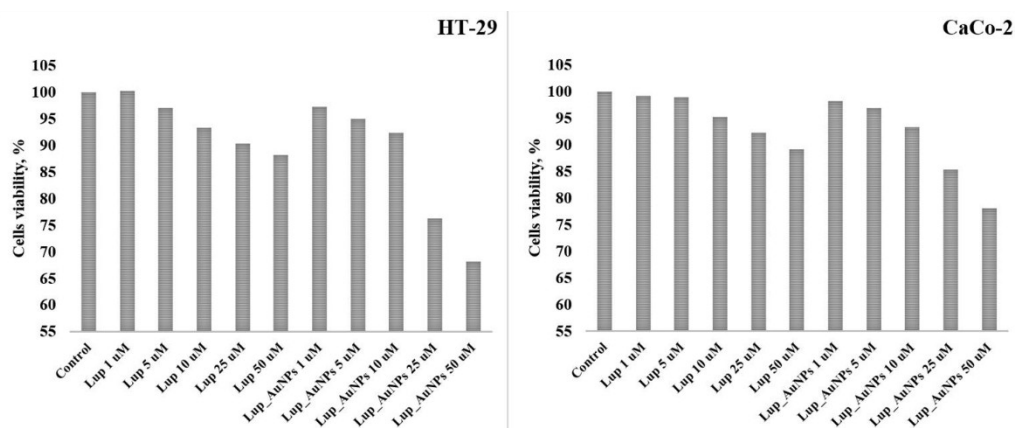


Figure 1. Viability of colorectal adenocarcinoma cells in the presence of lupeol and lupeol gold nanoparticles

When testing other types of lupeol formulations by MTT test did not show a sudden decrease in cell viability, and treatments with it and PLGL showed differences: when using lupeol, the viability was lower compared to PLGL nanoparticles [5]. Both aqueous lemon balm extract and biosynthesized gold nanoparticles (B\_AuNPs) were evaluated for cell viability. As shown in Figure 2 the lemon balm extract at the tested concentrations (between 10-500  $\mu$ g / mL) does not show a significant effect on the viability of HT-29 or CaCo-2 cells.

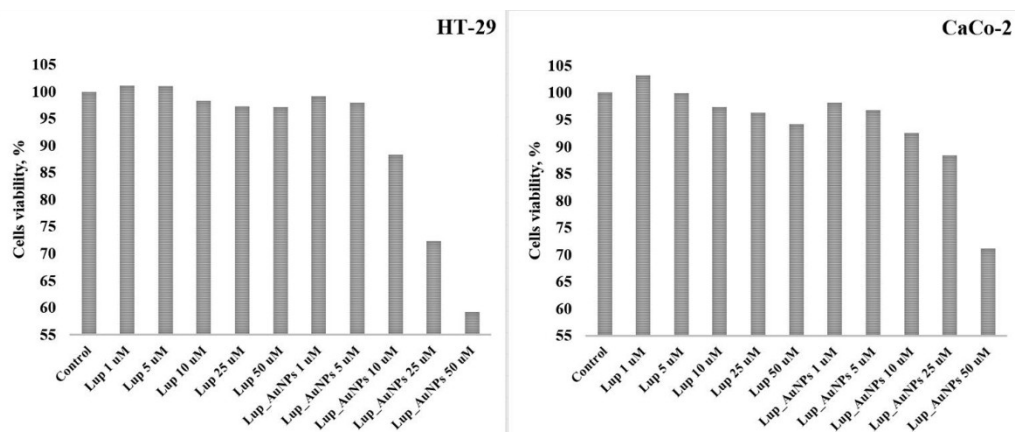


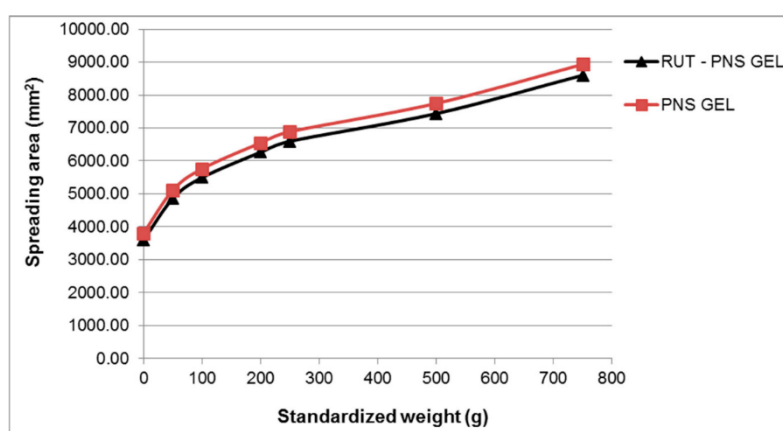
Figure 2. Viability of colorectal adenocarcinoma cells in the presence of aqueous lemon balm extract and biosynthesized gold nanoparticles

In the second study, in case of proniosomes, it was observed a negative zeta potential, and the rutin loading led to a slight change in the zeta potential to a value more negative. Regarding the average size and distribution of these types of biodegradable polymers, an increase in the size of proniosomes on a rutin basis can be observed compared to the size of control proniosomes. Table 2 lists the apparent viscosity and thixotropy values obtained for medicated proniosomal gel formulations and pH values.

**Table 2. Characteristics of proniosomal gels: viscosity, thixotropy, penetration values and pH**

Cod gel	Viscosity (Step)	Thixotropy (Pa / s)	Penetration value (mm)	PH value	Size (nm)	$\zeta$ Potential (mV)	EE (%)
PNS GEL	$0.502 \pm 0.24$	2567	$233.00 \pm 1.15$	$7.105 \pm 0.09$	$116.2 \pm 1.13$	$25.53 \pm 0.2$	-
RUT_PNS GEL	$0.488 \pm 0.62$	2930	$249.00 \pm 0.82$	$7.002 \pm 0.18$	$140.5 \pm 2.56$	$27.33 \pm 0.09$	$59.6 \pm 4.8$

The results of the spread test are shown in Figure 3 as extensiometric profiles. The area of spread increased progressively with increasing weight applied, and for all applied weights, the proniosomal gel containing 0.3% rutin showed larger areas of spread. The results of the assessment of consistency by penetrometry and the spread test were consistent with those obtained from the steady-state flow test, suggesting the influence of the chemical properties of the incorporated drug molecules (rutin).



**Figure 3. Extensiometric curves of experimental proniosomal gel containing 0.3% rutin and control formulations**



The viability of the reconstituted human epidermis (RhE) was achieved using the MTT test after 18 hours of exposure, and the ultrapure water and Triton X-100 1% were used as negative control and positive control according to Organisation for Economic Co-operation and Development (OECD) recommendations. The viability of RhE after exposure to positive control and the incubation period of 18 hours after application decreased to about 5% (Figure 4). Rutin and proniosomal gels (control and with rutin loaded) showed significantly higher viability than the positive control, but also compared to the negative control.

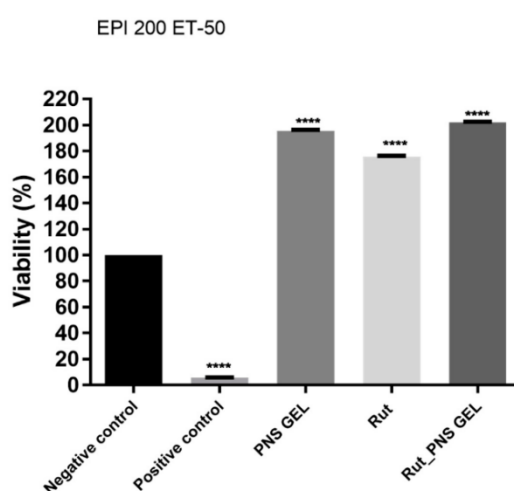


Figure 4. Percentage of viability of EpiDerm skin insert (EPI 200 ET-50) at 18 hours with the tested samples (proniosomal control gel, rutin and rutin proniosomal gel).

Another cell line used in the present study as an in vitro experimental model to establish the toxicological profile of Ruth was A375 - the human melanoma cell line. To determine cell viability, cells were treated with different concentrations of Ruth (1, 2.5, 5, 7.5, 10, 20, 25, 50 and 75  $\mu$ M) for 24 hours. The results showed signs of cytotoxicity in A375 cells starting with the lowest concentration - 1  $\mu$ M, but the most significant decrease in the percentage of viable cells was observed at the highest tested concentrations (25, 50 and 75  $\mu$ M): 53.19 %, 58.12% and 49.32% respectively (Figure 5). The  $IC_{50}$  value calculated for Ruth is 8,601  $\mu$ M.

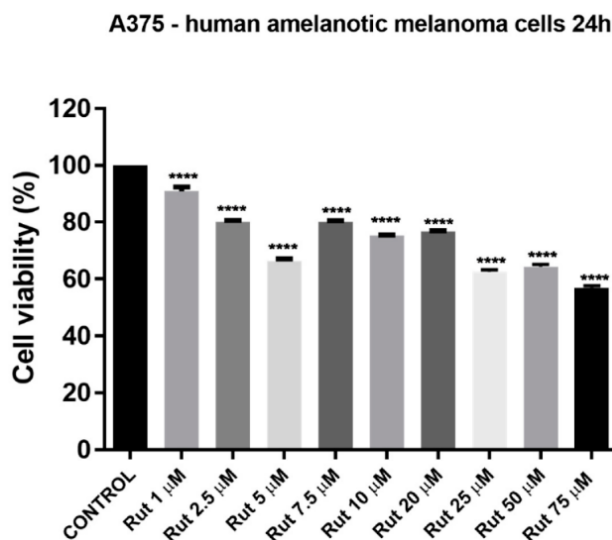
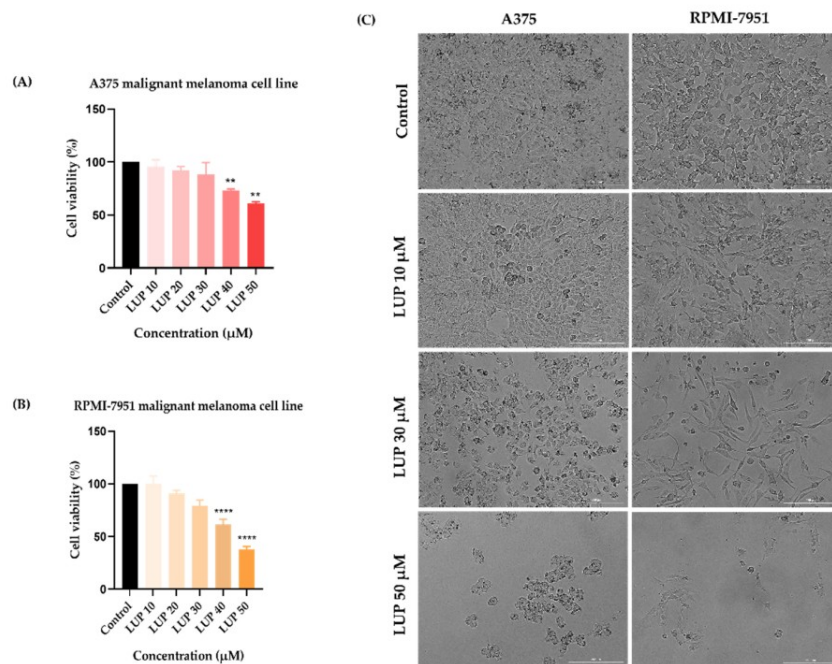


Figure 5. Evaluation of the in vitro effect of rutin (Ruth; 1, 2.5, 5, 7.5, 10, 20, 50 and 75  $\mu\text{M}$ ) on the viability of human melanoma cells (A375) after a 24-hour treatment by applying alamarBlue test.

In case of lupeol, the anti-melanoma effect was evaluated on two lines of human malignant melanoma: A375 and RPMI-7951. The safety of LUP was evaluated on two healthy skin cell types (human keratinocytes - HaCaT and human fibroblasts - 1BR3).

The cells were evaluated following 24-hour treatment with increasing concentrations (10-50  $\mu\text{M}$ ) of LUP. The results indicated a dose-dependent cytotoxic activity of LUP on both cell lines (Figure 6A, B), with the highest inhibition of cell viability being recorded at the highest concentration tested (50  $\mu\text{M}$ ) when the percentages reached 61, 29% for A375 and 37.78% for RPMI-7951, respectively.



**Figure 6.** In vitro evaluation of the effect of LUP (10, 20, 30, 40 and 50  $\mu\text{M}$ ) on the viability (and (B) of RPMI-7951 MM cells after 24 hours of treatment by applying the MTT test and morphology (C) and confluence of MM cells after 24-hour treatment with LUP 10, 30 and 50  $\mu\text{M}$

Regarding the LUP-induced apoptosis effect, no significant changes were detected in A375 cells at 10  $\mu\text{M}$  compared to the control (untreated cells), while at higher concentrations specific apoptotic characteristics (indicated by arrows) can be observed, such as nuclear fragmentation, and apoptotic bodies (LUP 20  $\mu\text{M}$ ), membrane blebbing and chromatin condensation (LUP 50  $\mu\text{M}$ ). In the case of RPMI-7951 cells, signs of apoptosis (membrane filling, fragmentation of cell nuclei) were recorded at all concentrations (indicated by arrows). The results are shown in Figure 7.

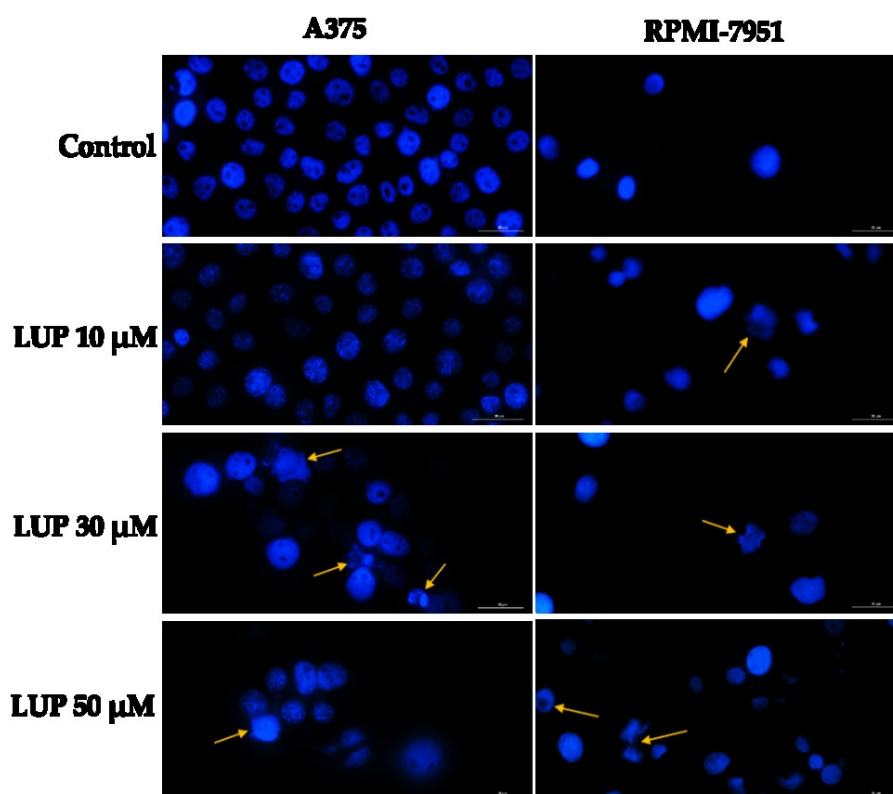


Figure 7. Staining of cell nuclei using Hoechst 33342 in A375 and RPMI-7951 malignant melanoma cells after 24-hour treatment with LUP 10, 30 and 50  $\mu\text{M}$ . Staurosporine (STP) 5  $\mu\text{M}$  was selected as a positive control for apoptosis. Arrows indicate apoptotic nuclei that express specific characteristics. The ladder bars represent 30  $\mu\text{m}$

In order to obtain a complete perspective on the impact of LUP on the morphology of melanoma cells A375 and RPMI-7951, a fluorescence immunochemistry technique was applied which highlights the changes that took place in the distribution of cytoskeletal components (actin filaments - red and microtubules - green) after 24 hours of treatment. In addition, cell nuclei were counter-stained with DAPI. At the lowest tested concentration (10  $\mu\text{M}$ ) LUP did not induce significant changes in the cytoskeleton appearance of A375 and RPMI-7951 cells. However, at higher concentrations (30 and 50  $\mu\text{M}$ ), some changes in the organization of AF and MT (indicated by arrows) can be observed, as follows: (i) in A375 cells (Figure 8) - condensation of actin bundles and reorganization of MT into a cortical ring leading to cell rounding; and (ii) in RPMI-7951 cells (Figure 9) - actin condensation, perinuclear distribution of MT and shortened longitudinal axis of cells.

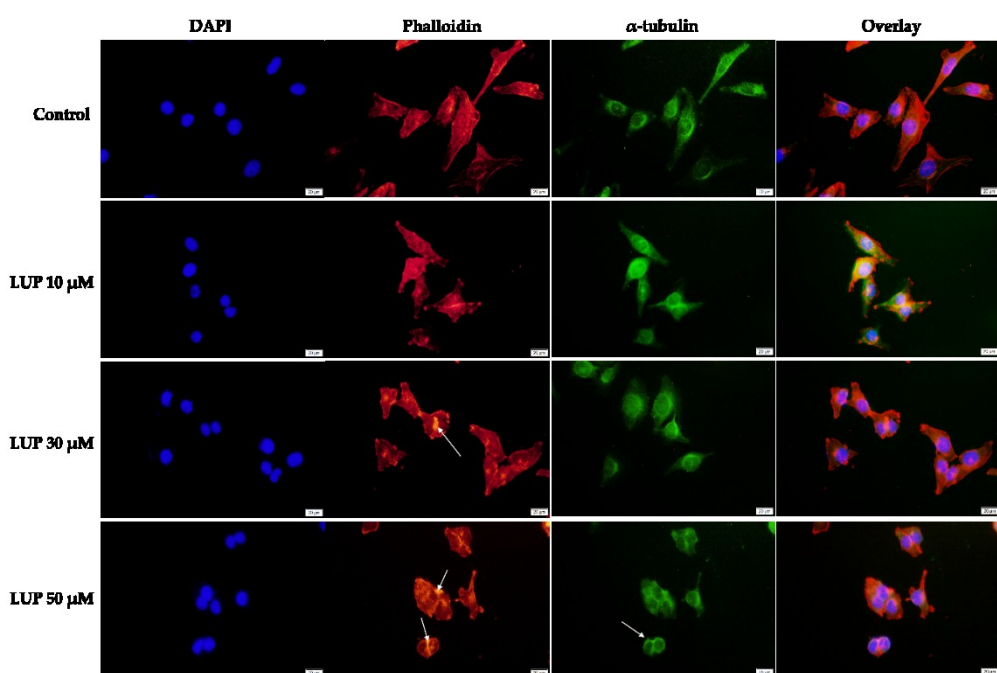


Figure 8. Human melanoma cells (A375) visualized by fluorescence microscopy after 24-hour treatment with LUP (10, 30 and 50  $\mu\text{M}$ ). Cell nuclei (DAPI), actin filaments (phalloidin) and microtubules ( $\alpha$ -tubulin) are presented individually and also combined (overlapping). Ladder bars indicate 20  $\mu\text{m}$ .

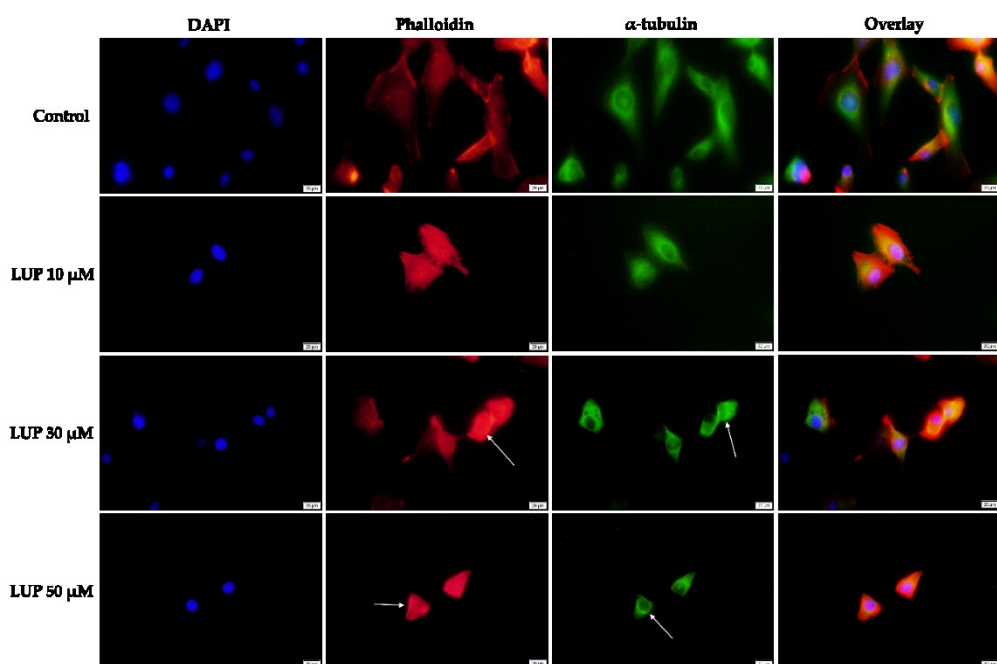


Figure 9. Human melanoma cells (RPMI-7951) visualized by fluorescence microscopy after 24-hour treatment with LUP (10, 30 and 50  $\mu\text{M}$ ). Cell nuclei (DAPI), actin filaments (phalloidin) and microtubules ( $\alpha$ -tubulin) are presented individually and combined (overlapping). Ladder bars indicate 20  $\mu\text{m}$ .

To observe the effect of LUP on the development of blood vessels, the in ovo method was applied using the CAM test. The potential antiangiogenic profile of LUP at the highest concentration tested in vitro (50  $\mu\text{M}$ ) was examined for 24 hours. After this time, a number of changes were observed in the vascular plexus, such as a

decrease in capillary density, some areas of the chorioallantoic membrane being devoid of vascularization. In addition, it is worth noting that the blood vessels formed are devoid of branches, which is a crucial aspect in terms of angiogenesis in the tumor process. Moreover, at the level of the chorioallantoic membrane, a microhemorrhage can be observed, which after the treatment with LUP was prevented from evolving (Figure 10).

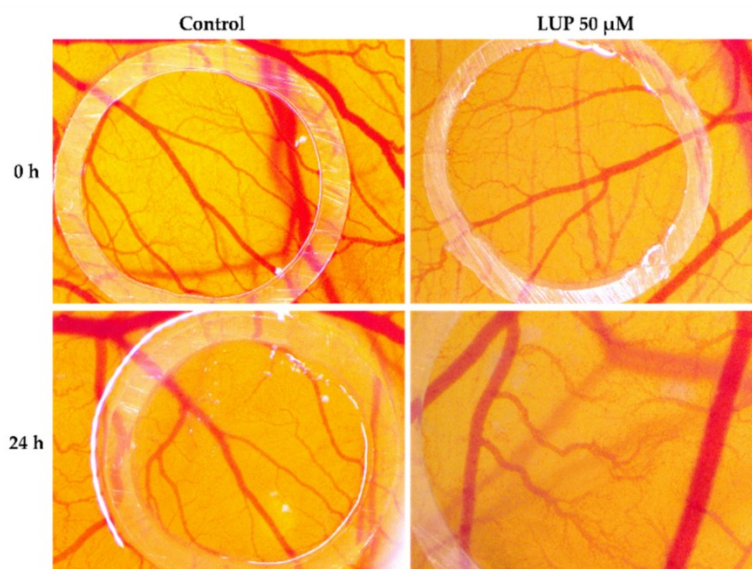


Figure 10. Stereomicroscopic imaging of the chorioallantoic membrane highlighting the angiogenic process after treatment for 24 hours with LUP 50  $\mu\text{M}$ .

## CONCLUSIONS

The obtained data revealed an effective and selective anti-melanoma effect exerted by lupeol at well-established concentrations against both types of human melanoma cells used. It was noticed:

- a) a dose-dependent decrease of viable cells and also of cell confluence,
- b) the appearance of specific apoptotic nuclear characteristics following the use of lupeol,
- c) reorganization of cytoskeletal components of relevant importance in apoptotic processes
- d) inhibition of the ability of cells to migrate in the presence of lupeol

In case of lupeol nanoparticles formulation, the following conclusions were drawn:

- e) the formation of the metallic nanoparticles was confirmed by UV-Vis spectroscopy, their sizes were in the range required for use in biological environments (38-82 nm) and the stability was good referring to the zeta potential values recorded.
- f) to evaluate the cytotoxic effect on tumor cells (colon adenocarcinoma), the loading of metallic nanoparticles with lupeol was realized; at the same time blank nanoparticles (unloaded) and nanoparticles obtained by green synthesis using plant extract were also tested.
- g) a more pronounced cytotoxic activity on tumor cells was observed for gold nanoparticles loaded with lupeol.

In the study with rutin gel, it was highlighted:

- a) the formulation presented characteristics for biological applicability (size, zeta potential, degree of encapsulation, pH, rheological properties).
- b) biocompatibility study on reconstructed human epidermal tissue revealed lack of toxicity correlated with viability, irritant potential and phototoxicity.
- c) rutin exerted toxic effects on tumor cells (human melanoma) expressed by decreasing cell viability, inducing nuclear fragmentation and changing cell morphology without being toxic on human keratinocytes.

In conclusion, experimental studies reflect original contributions that mainly complete the existing data regarding the therapeutic profile of naturally occurring compounds and innovative formulations.