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IMPROVEMENT OF NATURAL COMPOUNDS ANTITUMORAL PROPERTIES BY FUNCTIONALIZATION AS TARGETED NANOSTRUCTURES

ABSTRACT

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CONTENT

ABSTRACT

Skin conditions, from simple irritations to malignancies, presented diversified features in recent years, especially due to the environmental factors that have increased in number and in aggressiveness. Malignant melanoma is the type of skin cancer with the lowest incidence but the most aggressive, with an extremely low prognosis, being also responsible for the vast majority of skin cancer-related deaths. Despite the progress made in recent years in terms of both diagnostic methods and therapeutic approaches, there are still several drawbacks related to cancer chemotherapy resistance and to the severe adverse reactions exerted by classical therapies. To remedy these inconveniences, the natural compounds' research field is intensively explored to find novel effective and safe alternatives for cancer therapy. Plant-derived compounds possess multiple pharmacological effects becoming genuine candidates as antitumor agents. Rutin (glycosylated flavonoid) is a compound of natural origin, which due to its chemical structure, exerts a multitude of beneficial biological effects at cutaneous level, such as: antioxidant and anti-inflammatory activity, and also acts as a filter for ultraviolet radiation. Two other compounds of natural origin, betulin and betulinic acid (pentacyclic triterpenes) are being investigated for their apoptosis-inducing properties in various tumor cells.

A significantly increased concern for malignant diseases, especially those of the skin, lungs and liver exists worldwide and implicitly in our country. This can be attributed to the need for a prolonged life expectancy through appropriate treatments and the adoption of a lifestyle that diminishes the risk factors. A number of factors are associated with an increased risk of appearence and evolution of cancer, like: (i) genetic mutations, alcohol consumption, obesity and physical inactivity have all been linked to an increased risk of breast cancer, (ii) chronic viral hepatitis, cirrhosis, alcohol consumption and tobacco are some of the main causes leading to hepatocellular carcinoma, (iii) smoking, exposure to toxic compounds (arsenic, asbestos, etc.), environmental pollution, use of certain food supplements translate into lung cancer. The treatments used for each of these pathologies, present differences in terms of mechanism of action but share as common feature the occurrence of serious side effects. Most of the side effects following chemotherapy are determined by the lack of antitumor agents' specificity against tumor cells. These effects can be counteracted by establishing a personalized treatment and by manufacturing optimal formulations that ensure a targeted delivery of the active substance. Finding appropriate formulations that act as targeted carriers of the active substance and augment its beneficial properties is an expanding field of interest.

Reducing the side effects of classic chemotherapeutic drugs is a continuing challenge for researchers in the field. Currently, the development of formulations that increase the degree of stability and bioavailability, along with dose reduction, preservation of biological activity and

reduction of adverse effects is a topical issue. Despite the progress recorded in recent years, a lot of hard work is still needed to elucidate the mechanisms involved in various pathologies, new and old. Therefore, specialists in the field are constantly working on new and effective methods to increase life expectancy and life quality for cancer patients.

The main objectives established for the doctoral thesis were: (i) analysis of the cytotoxic effect exerted by natural compounds, rutin and betulinic acid, on three different types of melanoma cells (*in vitro* study on 2D cells) (ii) preparation of a rutin-based formulation for topical application and assessment of its safety profile by modern methods conducted on products with dermo-cosmetic applicability (*in vitro* study on reconstructed human tissue - 3D cells) and (iii) obtaining metal nanoparticles loaded with betulin and betulinic acid, with increased efficacy against melanoma cells (*in vivo* study - murine model of melanoma) and liver and lung tumor cells (*in vitro* 2D cell study).

The doctoral thesis is structured according to the methodological norms in four main parts: (i) the general part, (ii) the original research part, (iii) personal contributions and conclusions and (iv) the bibliography. The general part comprises two chapters that describe the current notions related to: (a) medicinal plants as sources of new compounds with therapeutic potential and (b) intelligent systems for functionalization and transport of biologically active compounds.

The original research part, that comprises the three experimental studies (objectives, methodology and results), shows: (a) the contributions related to the behavior of melanoma cell lines following stimulation with the compounds of natural origin, rutin and betulinic acid; (b) preparation of a proniosomal gel loaded with rutin with a toxicological safe profile for topical use in skin conditions and (c) theranostic systems of silver nanoparticles based on betulin and betulinic acid evaluated in terms of antimelanoma potential and cytotoxic effects exerted on other types of tumor cells (hepatocellular carcinoma and lung cancer). The final part of the thesis integrates the conclusions and personal contributions, and the current cited bibliographic references that were studied for background documentation, the selection of the research methods applied and for the interpretation of the obtained results. The experimental part of the thesis was conducted using standard internationally validated methods.

The first study was focused on the *in vitro* evaluation of two natural compounds, rutin (RUT) and betulinic acid (BA) in terms of cytotoxic potential in human healthy (HaCaT) and melanoma cells (A375, RPMI-7951 and SK-MEL-28). The cytotoxic potential of RUT and BA was assessed by monitoring specific parameters, as: cell viability, cellular and nuclear morphology. To check the impact of test compounds on cells' viability were applied two standardized colorimetric methods, MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and Alamar blue, assays that offer quantitative data regarding the percentage of viable metabolically active cells capable to convert MTT into formazan and resazurin to fluorescent resorufin, respectively (Figures

1 and 3). The changes induced by test compounds on cellular morphological features were monitored under bright field illumination and by taking pictures at 24 h posttreatment. The nuclear modifications following test compounds treatment (for 24 h) were highlighted by the means of Hoechst 33342 nuclear staining (figure 2). The pro-senescence inducing potential of RUT was verified by the means of a validated method, the X-Gal staining assay. The two compounds triggered a dose-dependent selective cytotoxic effect in melanoma cells, characterized by apoptotic features. In addition, RUT presented a senescence-inducing effect highlighted by the increased expression of SA-β-gal (senescent-associated beta-galactosidase) in SK-MEL-28 melanoma cells. Rutin exerts a selective cytotoxic profile *in vitro*, as follows: (i) no detrimental impact on healthy cells – human immortalized keratinocytes (HaCaT cells) in terms of cell viability, cellular and nuclear morphology at the concentrations tested (1-50 µM) and (ii) a cytotoxic effect in human melanoma cells (A375, RPMI-7951 and SK-MEL-28) dependent of concentration and cell type: A375 (IC_{50} = 8.601 µM) > SK-MEL-28 (IC_{50} = 47.44 µM) > RPMI-7951 (IC_{50} = 64.49 µM).

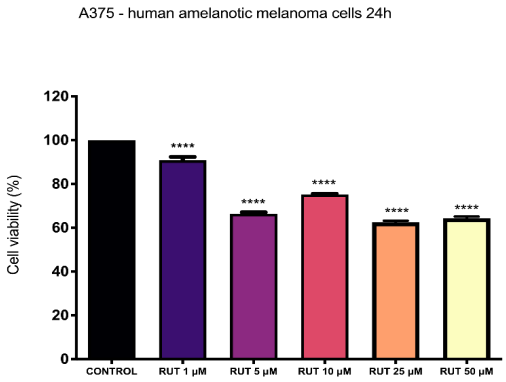

Figure 1. The *in vitro* assessment of rutin (RUT) activity on human melanoma cells (A375) viability after a 24-h treatment with five different concentrations (1, 5, 10, 25, and 50 µM) performed by Alamar Blue assay. The results are presented as percentage (%) of viable cells normalized to control cells and are expressed as mean values ± standard deviation (SD) of three independent tests conducted in triplicate. Statistical differences (between the untreated and the treated cells) were calculated by one-way ANOVA analysis, followed by Tukey's post-test (****$p < 0.0001$).

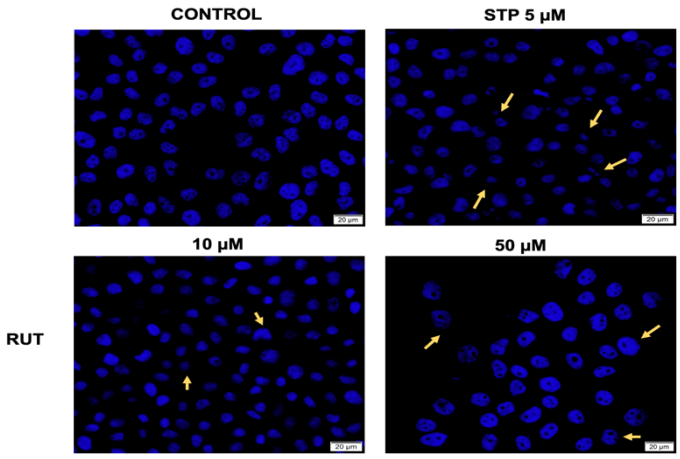

Figure 2. Nuclear labeling using Hoechst 33342 in human melanoma cells (A375) cells after treatment with rutin (RUT) at two different concentrations (10 and 50 µM) for 24 h. Positive control for apoptotic changes at nuclear level was Staurosporine (STP) 5 µM solution and the yellow arrows highlight the apoptotic cells with nuclear fragmentation. The scale bar was 20 µM.

The cytotoxic effect induced by rutin in the melanoma cells was characterized by the following features: a dose-dependent decrease of cell viability percentage, changes of cells' morphology (round shape, floating cells, loss of adherence and a reduced confluence) and nuclear specific apoptotic signs (chromatin condensation, nuclear fragmentation and blebbing). *In vitro* treatment with rutin triggers pro-senescence capacity in SK-MEL-28 human melanoma cells – a novel potential antimelanoma mechanism of action.

The highest concentrations of BA (25 and 50 µM) induced a reduction of human immortalized keratinocytes - HaCaT cells viability, changes in cells morphology (round and floating cells) and nuclear fragmentation. Betulinic acid proved a dose-dependent cytotoxic effect in A375 human melanoma cells (IC_{50} = 16.91 µM) characterized by a significant decrease of cells viability, morphological changes both at cellular (floating round cells, loss of cell adhesion, cell debris and a diminished confluence) and nuclear (chromatin condensation, nuclear fragmentation, and the presence of apoptotic bodies) level. Betulinic acid treatment triggered a similar dose-dependent cytotoxic effect in the other two types of human melanoma cells, RPMI-7951 and SK-MEL-28, the decrease in cells viability being more pronounced in the case of RPMI-7951 cells (figure 3).

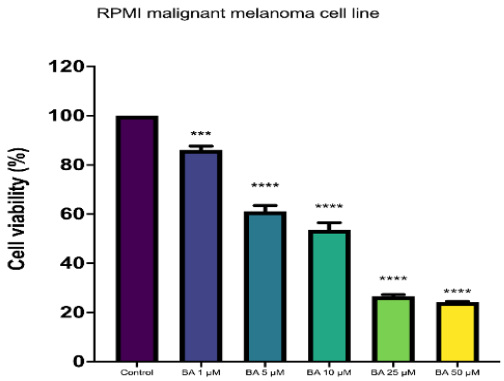

Figure 3. The *in vitro* assessment of betulinic acid (BA) activity on human melanoma cells (RPMI-7951) viability after a 24-h treatment with five different concentrations (1, 5, 10, 25, and 50 µM) performed by MTT assay. The results are presented as percentage (%) of viable cells normalized to control cells and are expressed as mean values ± standard deviation (SD) of three independent tests conducted in triplicate. Statistical differences (between the untreated and the treated cells) were calculated by one-way ANOVA analysis, followed by Tukey's post-test (*p < 0.1, **p < 0.01, ***p < 0.001, and ****p < 0.0001).

Rutin (RUT), a quercetin rhamnoglucosine, also known as sophorin, rutoside, or quercetin-3-rutinoside, can be found in nature (citrus, vegetables, black tea, apples, buckwheat, green tea, etc.) and based on its chemical structure meets the profile of a valuable antioxidant compound. RUT (hydrophobic polyphenolic molecule) is part of the vast class of flavonoids, one of the most studied class of plant-derived compounds due to their antioxidant properties. This small molecule gained its place in different studies that aimed to evaluate its potential as an active natural agent formulated both as pharmaceutical and cosmetic products due to its multispectral pharmacological and biochemical effects, especially antioxidant, anti-inflammatory and antitumoral (figure 4).

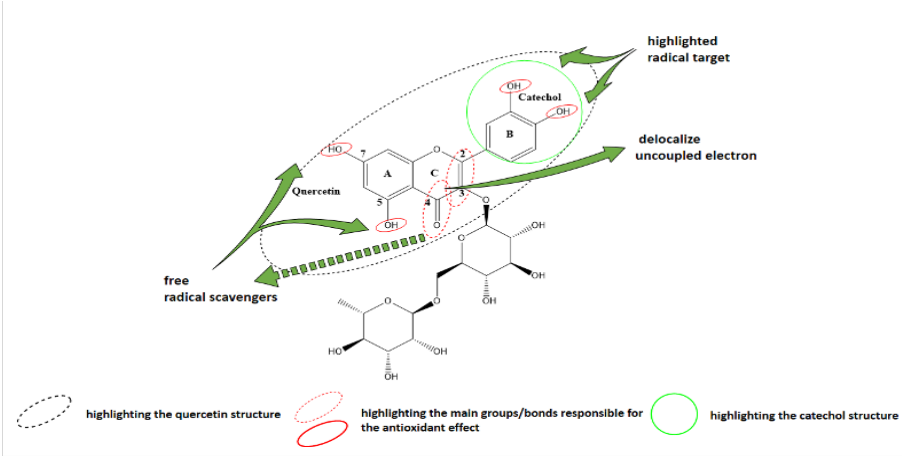

Figure 4. Chemical structure of rutin (RUT) highlighting the functional groups responsible for the antioxidant effect.

Despite the multiple pharmacological properties of RUT, its application *in vivo* is limited by the very low solubility in aqueous media and its decreased stability in biological fluids. To remedy these inconveniences related to RUT administration *in vivo*, the second study of the thesis

was oriented toward the obtention and characterization of an innovative nanoformulation of RUT for topical application – a proniosomal gel (figure 5).

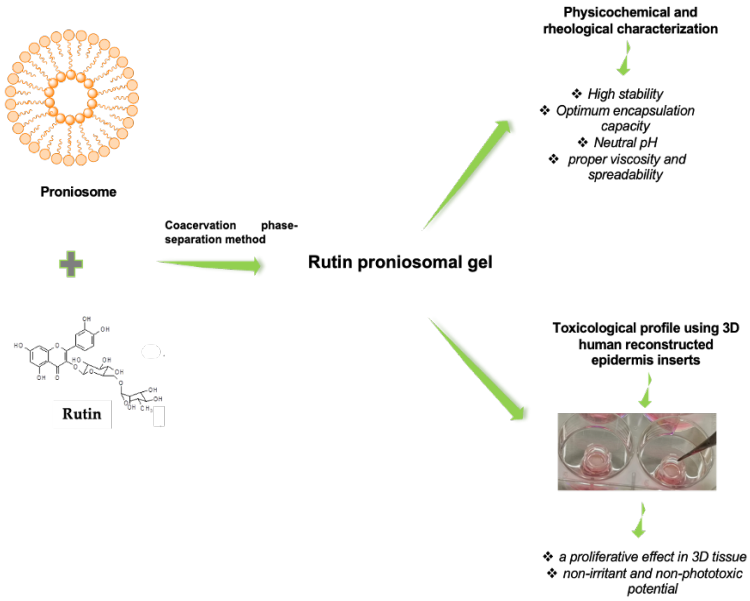

Figure 5. Schematic protocol of the study

The proniosomal gel was prepared by coacervation phase-separation method, a method frequently described in the literature for the preparation of proniosomal gels of different therapeutical agents. The obtained RUT-based proniosomal gel complies with the standard requirements in terms of particle size (140.5±2.56 nm), zeta potential (-27.33±0.09 mV), encapsulation capacity (> 50%), pH (7.002±0.18) and rheological properties (a thinning-thixotropic behavior, increased penetration capacity, optimal viscosity and consistency, and easiness in application) of semi-solid preparations for medical use. The toxicological assessment of the gel was performed on 3D human reconstructed epidermis tissues, an alternative *in vitro* method to *in vivo* studies, that was validated and included in the international guidelines for the evaluation of medical devices. The gel revealed a safety profile *in vitro* showing a high biocompatibility in the 3D reconstructed human epidermis model characterized by an increased viability of the cells and a lack of irritant and phototoxic potential.

The third study of the thesis consisted of the synthesis and characterization (physicochemical and toxicological) of silver nanoparticles and PEGylated silver nanoparticles as drug carriers for betulin (B) and betulinic acid (BA), two very active antitumoral natural compounds with a poor solubility in aqueous media (figure 6).

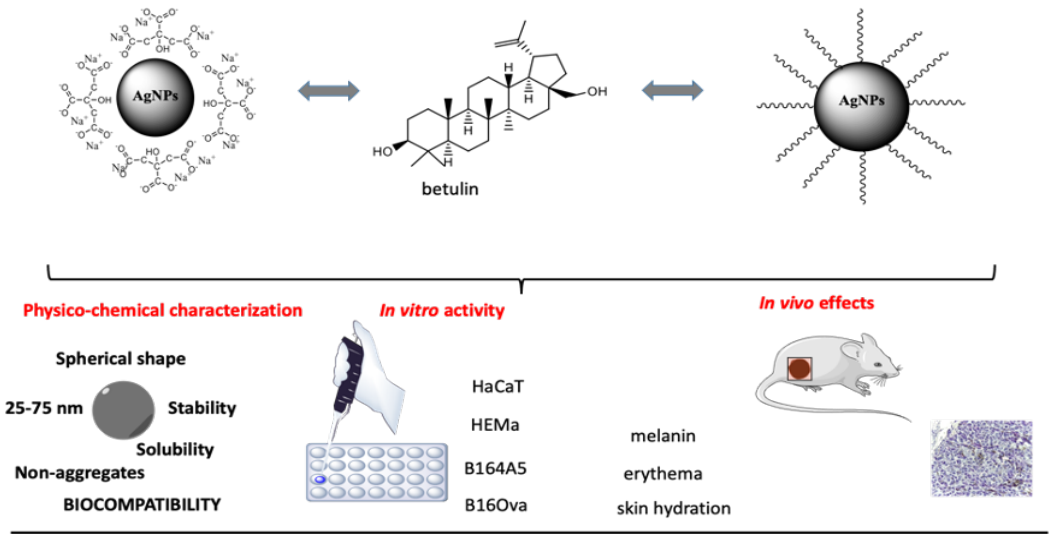

Figure 6. Schematic protocol of the *in vivo* study

The hollow and loaded with active compounds silver nanoparticles were synthetized by applying a consecrated method – the Turkevich procedure and were characterized using standard techniques as TEM, SEM, UV-VIS, DLS, etc. The obtained nanoparticles were stable, presented spherical shapes and mean hydrodynamic diameters in the range of 25 nm and 75 nm.

The toxicological profile of betulin-loaded AgNPs and PEG_AgNPs was analyzed both *in vitro* and *in vivo.* For the *in vitro* assessment, the experiments were conducted on human healthy cells (HaCaT – human immortalized keratinocytes and HEMa - primary human melanocytes) and on murine melanoma cells (B164A5 and B16Ova) using MTT method and Annexin V-FTIC assay (apoptosis detection kit).

Hollow AgNPs proved to be highly cytotoxic *in vitro* after a 72 h treatment on both healthy (human keratinocytes - HaCaT and primary human melanocytes - HEMa) and murine melanoma cells (B164A5 and B16Ova) in a dose and cell type-dependent manner, as follows: HEMa (IC_{50} = 26 μM) > HaCaT (IC_{50} = 40 μM) > B164A5 (IC_{50} = 70.81 μM) > B16Ova (IC_{50} = 73.07 μM).

Hollow PEG_AgNPs proved to be more cytotoxic after the 72 h treatment on murine melanoma cells (B164A5 and B16Ova) as compared to AgNPs and less cytotoxic on healthy (human keratinocytes - HaCaT and primary human melanocytes - HEMa) cells, what shows a selective toxic behavior oriented toward tumor cells: B16Ova (IC_{50} = 17 μM) > B164A5 (IC_{50} = 28.44 μM) > HaCaT (IC_{50} = 31.77 μM) > HEMa (IC_{50} = 39.73 μM).

PEG_AgNPs loaded with betulin exhibited the safest in *vitro* toxicological profile for healthy (human keratinocytes - HaCaT and primary human melanocytes - HEMa) cells and a strong cytotoxic effect on murine melanoma cells as compared to AgNPs loaded with betulin and betulin. PEG_AgNPs loaded with betulin exerted a dose-dependent pro-apoptotic effect inducing late apoptosis and necrosis in murine melanoma cells after the 72 h treatment. In addition, PEG_AgNPs

loaded with betulin significantly decreased human lung carcinoma – A549 and hepatocellular carcinoma – HepG2 cells viability after a 24 and 48 h treatment with 10 µM as compared to AgNPs loaded with betulin and betulin.

The *in vivo* antitumor potential of PEG_AgNPs loaded with betulin was verified in a mouse model of murine melanoma using non-invasive techniques for the surveillance of the physiological skin parameters changes and histopathological examination of the harvested organs.

A reproducible murine melanoma animal model was developed using C57BL/6J female mice and a suspension of B164A5 cells inoculated subcutaneously. PEG_AgNPs loaded with betulin showed antimelanoma potential *in vivo* since the treated group of mice (group D) presented reduced tumor masses, the presence of large areas of necrosis, changed phenotype of tumor cells (from aggressive epithelioid cells to less invasive ones with fusiform shape) and an elevated quantity of intracytoplasmic melanin as sign of a good response to the therapy. No signs of metastasis were detected in the treated group of mice (PEG_AgNPs loaded with betulin – group D). The *in vitro* and *in vivo* results support the safe use of the PEG_AgNPs loaded with betulin obtained in the described experimental conditions.

The physicochemical characterization of BA silver nanoparticles (bare and PEGylated) showed the following results: the newly synthetized AgNPs and PEG_AgNPs presented a spherical shape and optimal nano-sizes, in the range of 21 – 68 nm for hollow and loaded AgNPs and between 53 – 75 nm for PEG_AgNPs.

In the case of BA loaded silver nanoparticles the biological evaluation was conducted on two *in vitro* experimental models—hepatocellular carcinoma (HepG2) and lung cancer (A549) cell lines using Alamar blue assay for the assessment of nanoparticles impact on cells' viability.

BA-loaded AgNPs proved to be more cytotoxic *in vitro* at a concentration of 10 µM (after 24 and 48 h) for human hepatocellular (HepG2) and lung (A549) carcinoma cells as compared to BA-loaded PEG_AgNPs and betulinic acid.

The *in vitro* assessment revealed a cell type- and time-dependent cytotoxic effect characterized by a decrease in cell viability as follows: (i) AgNPs_BA (66.44%) < PEG_AgNPs_BA (72.05%) < BA_DMSO (75.30%) in HepG2 cells, and (ii) AgNPs_BA (75.28%) < PEG_AgNPs_BA (86.80%) < BA_DMSO (87.99%) in A549 cells. The novel silver nanocolloids loaded with BA induced an augmented anticancer effect as compared to BA alone.

FINAL CONCLUSIONS

BEHAVIOR OF MELANOMA CELLS IN THE PRESENCE OF RUTIN AND BETULINIC ACID – IN VITRO STUDY ON 2D CELLS

1. Rutin exerts a selective cytotoxic profile *in vitro*, as follows: (i) no detrimental impact on healthy cells – human immortalized keratinocytes (HaCaT cells) in terms of cell viability, cellular and nuclear morphology at the concentrations tested (1-50 μM) and (ii) a cytotoxic effect in human melanoma cells (A375, RPMI-7951 and SK-MEL-28) dependent of concentration and cell type: A375 (IC_{50} = 8.601 μM) > SK-MEL-28 (IC_{50} = 47.44 μM) > RPMI-7951 (IC_{50} = 64.49 μM).
2. The cytotoxic effect induced by rutin in the melanoma cells was characterized by the following features: a dose-dependent decrease of cell viability percentage, changes of cells' morphology (round shape, floating cells, loss of adherence and a reduced confluence) and nuclear specific apoptotic signs (chromatin condensation, nuclear fragmentation and blebbing).
3. *In vitro* treatment with rutin triggers pro-senescence capacity in SK-MEL-28 human melanoma cells – **a novel potential antimelanoma mechanism of action.**
4. The highest concentrations of BA (25 and 50 μM) induced a reduction of human immortalized keratinocytes - HaCaT cells viability, changes in cells morphology (round and floating cells) and nuclear fragmentation.
5. Betulinic acid proved a dose-dependent cytotoxic effect in A375 human melanoma cells (IC_{50} = 16.91 μM) characterized by a significant decrease of cells viability, morphological changes both at cellular (floating round cells, loss of cell adhesion, cell debris and a diminished confluence) and nuclear (chromatin condensation, nuclear fragmentation, and the presence of apoptotic bodies) level.
6. Betulinic acid treatment triggered a similar dose-dependent cytotoxic effect in the other two types of human melanoma cells, RPMI-7951 and SK-MEL-28, the decrease in cells viability being more pronounced in the case of RPMI-7951 cells.

PRONIOSOMAL GEL FOR TOPICAL DELIVERY OF RUTIN – IN VITRO STUDY ON 3D CELLS

1. Rutin was loaded into an innovative formulation for topical application – a proniosomal gel obtained by coacervation phase-separation method.
2. The obtained rutin-based proniosomal gel exhibited physicochemical and rheological properties optimal for semi-solid formulations used for biomedical purposes, as: a neutral pH, a high stability, a thinning-thixotropic behavior, increased penetration capacity, optimal viscosity and consistency, and easiness in application.
3. Assessment of rutin-based proniosomal gel toxicological profile using 3D human reconstructed epidermis tissued indicated a safety profile for the novel formulation of RUT

for topical application since no impairment was noticed in terms of tissues' viability and a lack of irritant and phototoxic potential.

BIOACTIVE COMPOUNDS SILVER-BASED NANOPARTICLES ACTIVE IN TUMORAL CELLS – IN VIVO STUDY ON ANIMAL MODEL

1. Stable hollow and loaded with betulin and betulinic acid AgNPs and PEG_AgNPs were synthetized by adapted Turkevich's method.
2. The newly synthetized AgNPs and PEG_AgNPs presented a spherical shape and optimal nano-sizes, in the range of 21 – 68 nm for hollow and loaded AgNPs and between 53 – 75 nm for PEG_AgNPs.
3. Hollow AgNPs proved to be highly cytotoxic *in vitro* after a 72 h treatment on both healthy (human keratinocytes - HaCaT and primary human melanocytes - HEMa) and murine melanoma cells (B164A5 and B16Ova) in a dose and cell type-dependent manner, as follows: HEMa (IC_{50} = 26 μM) > HaCaT (IC_{50} = 40 μM) > B164A5 (IC_{50} = 70.81 μM) > B16Ova (IC_{50} = 73.07 μM).
4. Hollow PEG_AgNPs proved to be more cytotoxic after the 72 h treatment on murine melanoma cells (B164A5 and B16Ova) as compared to AgNPs and less cytotoxic on healthy (human keratinocytes - HaCaT and primary human melanocytes - HEMa) cells, what shows a selective toxic behavior oriented toward tumor cells: B16Ova (IC_{50} = 17 μM) > B164A5 (IC_{50} = 28.44 μM) > HaCaT (IC_{50} = 31.77 μM) > HEMa (IC_{50} = 39.73 μM).
5. PEG_AgNPs loaded with betulin exhibited the safest in *vitro* toxicological profile for healthy (human keratinocytes - HaCaT and primary human melanocytes - HEMa) cells and a strong cytotoxic effect on murine melanoma cells as compared to AgNPs loaded with betulin and betulin.
6. PEG_AgNPs loaded with betulin exerted a dose-dependent pro-apoptotic effect inducing late apoptosis and necrosis in murine melanoma cells after the 72 h treatment.
7. PEG_AgNPs loaded with betulin significantly decreased human lung carcinoma – A549 and hepatocellular carcinoma – HepG2 cells viability after a 24 and 48 h treatment with 10 μM as compared to AgNPs loaded with betulin and betulin.
8. A reproducible murine melanoma animal model was developed using C57BL/6J female mice and B164A5 cells inoculated subcutaneously.
9. PEG_AgNPs loaded with betulin showed antimelanoma potential *in vivo* since the treated group of mice (group D) presented reduced tumor masses, the presence of large areas of necrosis, changed phenotype of tumor cells (from aggressive epithelioid cells to less invasive ones with fusiform shape) and an elevated quantity of intracytoplasmic melanin as sign of a good response to the therapy.

10. No signs of metastasis were detected in the treated group of mice (PEG_AgNPs loaded with betulin – group D).
11. The *in vitro* and *in vivo* results support the safe use of the PEG_AgNPs loaded with betulin obtained in the described experimental conditions.
12. BA-loaded AgNPs proved to be more cytotoxic *in vitro* at a concentration of 10 μM (after 24 and 48 h) for human hepatocellular (HepG2) and lung (A549) carcinoma cells as compared to BA-loaded PEG_AgNPs and betulinic acid.