

QUERCETIN-INDUCED CYTOTOXICITY IN RPMI 7951 HUMAN MELANOMA CELLS: AN *IN VITRO* STUDY

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Abstract

Melanoma remains one of the most aggressive forms of skin cancer and is frequently associated with treatment resistance. In recent years, natural compounds, particularly flavonoids, have gained increasing attention as potential candidates for complementary treatment strategies for melanoma. Among them, quercetin (QUE) has emerged as a compound of interest. In this context, the present study evaluated the cytotoxic effects of QUE (25 - 200 μ M) on the two-dimensional (2D) human melanoma model RPMI 7951. Cell viability was assessed using MTT and NRU assays, while morphological changes were examined by bright-field microscopy. Mitochondrial membrane potential was evaluated using the JC-1 assay, whereas nuclear morphology, F-actin organization, and tubulin distribution were analyzed by immunofluorescence. Pro-apoptotic/necrotic-like changes were assessed by AO/PI dual staining. Cytotoxic effects became more evident at 100 μ M, with concentration-dependent reductions in viability and NRU, mitochondrial depolarization, morphological alterations, and cytoskeletal disruption. AO/PI staining further supported apoptosis-associated and membrane-damage-related changes. However, these preliminary findings warrant further investigation in more complex melanoma models.

Rezumat

Acest studiu a evaluat efectele citotoxice ale QUE (25–200 μ M) asupra modelului bidimensional (2D) de melanom uman RPMI 7951. Viabilitatea celulară a fost evaluată prin testele MTT și Neutral Red uptake, iar modificările morfologice au fost analizate prin microscopie în câmp luminos. Potențialul membranei mitocondriale a fost evaluat prin testul JC-1, iar morfologia nucleară, organizarea filamentelor de F-actină și distribuția tubulinei au fost analizate prin imunofluorescență. Modificările de tip pro-apoptotice/necrotice au fost evaluate prin colorare duală cu AO/PI. Efectele citotoxice au devenit mai evidente începând cu concentrația de 100 μ M, fiind asociate cu reduceri dependente de concentrație ale viabilității celulare și ale captării de neutral red, depolarizarea membranei mitocondriale, modificări morfologice și perturbări ale organizării citoscheletului. Colorarea AO/PI a evidențiat suplimentar apariția unor modificări asociate apoptozei și pierderii integrității membranei celulare. Totuși, aceste rezultate preliminare necesită investigații suplimentare pe modele mai complexe de melanom.

Keywords: Quercetin, melanoma, RPMI 7951, cytotoxicity, *in vitro*

Introduction

Melanoma is a malignant neoplasm that arises from transformed melanin-producing melanocytes and is characterized by aggressive behavior, including

local invasion and an increased risk of recurrence and early metastasis (1). In addition, risk factors for melanoma include both genetic predisposition and environmental factors. Mutations in the BRAF,

NRAS, and KIT genes are important in tumor biology, and UV radiation exposure, skin phototype, and cumulative photodamage contribute to disease development (2).

Therapeutically, the treatment of melanoma is, from a therapeutic point of view, mainly based on immunotherapy and targeted treatments, which depend on the stage of the disease and the molecular characteristics of the tumor. Immunotherapy involves the use of immune checkpoint inhibitors (pembrolizumab, nivolumab and ipilimumab) alone or in combination. In BRAF-mutated melanoma, combinations of BRAF and MEK inhibitors are also used, dabrafenib–trametinib, vemurafenib–cobimetinib, or encorafenib–binimetinib (3). Nevertheless, although significant progress has been made in the management of melanoma in recent years, through improved surveillance and the development of systemic therapies, advanced forms of the disease continue to pose a therapeutic challenge (4). Moreover, even if targeted therapies and immunotherapy have improved the treatment of melanoma, the development of resistance remains a major problem. This resistance can be intrinsic or acquired and is driven by genetic alterations, signaling pathway adaptations, and tumor heterogeneity, which allow resistant cell populations to survive (5). In this context, research on natural compounds in melanoma is gaining increasing interest, largely because they can simultaneously modulate multiple processes involved in tumor progression. Preclinical studies have shown promising effects of substances and extracts such as curcumin, cynaropicrin, pine bark extract, and propolis, including induction of apoptosis, increased ROS production, and modulation of proteins involved in cell survival (6). Among these natural compounds, flavonoids have attracted considerable interest because they are widely distributed in plants and have diverse biological activities. One of the most extensively studied representatives of this class is quercetin (QUE).

QUE is characterized by the presence of five hydroxyl groups that are chiefly responsible for its biological activity. It is a yellow crystalline solid with low solubility in water and occurs in many plant sources including onions, apples, berries, capers, and many aromatic herbs (7). Reportedly, it inhibits the release of inflammatory mediators such as TNF- α and IL-1 β , and interferes with MAPK and JAK/STAT signaling. Its antiproliferative activity in cancer cells has also been associated with modulation of PI3K/Akt and Raf/MEK/ERK pathways, which are important regulators of cell growth and survival. QUE also modulates intracellular ROS levels and apoptosis, indicating that oxidative balance and mitochondrial-related mechanisms may play a role in its anticancer effects (8).

In this context, the present study was designed to evaluate the cytotoxic effects of QUE on human

melanoma RPMI 7951 cells. The study investigated its effects on cell viability, morphology, and lysosomal integrity, as well as its impact on mitochondrial membrane potential. Furthermore, changes in nuclear morphology, F-actin organization, and tubulin distribution were examined by fluorescence, together with cell death-associated changes assessed by AO/PI staining.

Materials and Methods

Materials and equipment

Eagle's Minimum Essential Medium (EMEM), fetal bovine serum (FBS), penicillin/streptomycin solution (100 U/mL–100 μ g/mL), and trypsin-EDTA solution were obtained from PAN-Biotech GmbH (Aidenbach, Germany). Quercetin (QUE) (Quercetin hydrate catalog number: PHR1488-1G; purity: 95.0%), phosphate-buffered saline (PBS), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). For the MTT assay, the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reagent was supplied by Roche (United Kingdom). For the neutral red uptake assay, Neutral Red dye was obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). The mitochondrial membrane potential was detected by the JC-1 Mitochondrial Membrane Potential Assay Kit (E-CK-A301) (Elabscience, Houston, TX, USA) (E-CK-A301). Hoechst 33342 (62249), Texas RedTM-X phalloidin (T7471), alpha-tubulin monoclonal antibody (B5-1-2), and Alexa FluorTM 488-conjugated goat anti-mouse IgG (H+L) secondary antibody (A-11029) were purchased for fluorescence staining from Thermo Fisher Scientific (Waltham, MA, USA). Triton X-100 was bought from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany), 4% paraformaldehyde in PBS was purchased from Santa Cruz Biotechnology (Dallas, TX, USA), and bovine serum albumin (BSA) was purchased from Cell Signaling Technology (Danvers, MA, USA). Acridine Orange (AO) and Propidium Iodide (PI) for the AO/PI double staining were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). The absorbance and fluorescence were measured in a Cytation 5 multimode microplate reader, and the microscopic images were taken using a Lionheart FX Automated Microscope. Data acquisition and processing were carried out using Gen5TM Microplate Data Collection and Analysis Software (Version 3.14). All equipment and software were provided by BioTek Instruments (Winooski, VT, USA).

Experimental design

The human malignant melanoma cell line RPMI 7951 (ATCC[®] HTB-66TM) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). RPMI 7951 cells were cultured in Eagle's Minimum Essential Medium (EMEM)

supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin mixture (100 U/mL penicillin and 100 µg/mL streptomycin). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂ and routinely subcultured at approximately 70 % confluence using a split ratio of 1:4.

Cells were exposed to QUE for 24 h at concentrations of 25, 50, 100, 150, and 200 µM. A stock solution of QUE was prepared in DMSO and diluted in culture medium to achieve the desired working concentrations. The final concentration of DMSO in the culture medium did not exceed 0.5% (v/v). The control group was untreated cells. The effects of QUE were evaluated after treatment by complementary assays measuring cell viability, morphology, lysosomal integrity, mitochondrial membrane potential, cytoskeletal and nuclear organization, and cell death-associated changes.

MTT assay for cell viability analysis

The cell viability after QUE exposure was determined by the MTT assay. The RPMI 7951 melanoma cells were seeded in 96-well plates at a density of 1×10^4 cells/well and allowed to attach prior to treatment. Cells were then treated with QUE at 25, 50, 100, 150, and 200 µM for 24 h. At the end of the treatment period, the treatment medium was removed and replaced with fresh culture medium. Then, 10 µL/well MTT reagent was added to each well. Plates were incubated for 3 h at 37°C in a humidified atmosphere of 5% CO₂. Then 100 µL/well of solubilization solution was added, and the plates were kept at room temperature for 30 minutes. Absorbance was then read at 570 nm and 630 nm using Cytation 5. Cell viability was expressed as a relative percentage compared to the untreated control (9).

Microscopic assessment of cell morphology

The morphological changes of the cells related to the treatment were evaluated by bright-field microscopy. RPMI 7951 melanoma cells were seeded at a density of 1×10^4 cells/well in 96-well plates and were maintained at standard culture conditions of 37°C and 5% CO₂. After cell attachment for 24 h, the cells were treated with QUE (25, 50, 100, 150, and 200 µM). Untreated cells were used as a control. Representative brightfield images were taken at the end of the exposure period at 20× magnification (NA= 0.45) with the Lionheart FX automated microscope. Morphological characteristics were compared to the untreated control. Image acquisition and processing were performed using Gen5™ Microplate Data Collection and Analysis software.

Neutral red uptake assay

The neutral red uptake (NRU) assay was performed as described by Petroman *et al.* (10) with slight modifications in the experimental conditions used here. RPMI 7951 cells were plated in 96-well plates at 1×10^4 cells/well and allowed to attach. Then the cells were treated with QUE at 25, 50, 100, 150, and 200

µM for 24 h. At the end of treatment, the medium was removed, and 100 µL of NR working solution (40 µg/mL prepared in complete EMEM) was added to each well. The cells were incubated with the dye for 2 h at 37°C and 5% CO₂ and washed with PBS to remove unincorporated dye. Representative bright-field images were taken on the Lionheart FX automated microscope with a 20× objective (NA = 0.45). The intracellular dye was released for quantitative analysis by adding 150 µL of a decolorization solution containing 50% ethanol, 49% deionized water, and 1% glacial acetic acid. The plates were shaken for 10 min to facilitate dye extraction, and absorbance was measured at 540 nm on the Cytation 5. Cell viability assessed by NRU was expressed as a percentage relative to the untreated control (100%).

JC-1 staining assay of mitochondrial membrane potential ($\Delta\Psi_m$)

The effect of QUE on mitochondrial membrane potential ($\Delta\Psi_m$) was analyzed by JC-1 staining (11) as previously described. RPMI 7951 cells were plated at 1×10^4 cells/well in black-walled, clear-bottomed 96-well plates and allowed to attach before treatment. The cells were then treated with 25, 50, 100, 150, and 200 µM QUE for 24 h. After the treatment period, cells were washed with PBS and incubated with 100 µL/well JC-1 staining solution at a final concentration of 5 µM for 45 min at 37°C and 5% CO₂. The excess dye was removed by washing with PBS, and representative fluorescence images were then taken at 20× magnification (NA = 0.45) using the Lionheart FX automated microscope. Fluorescence intensities were measured using a Cytation 5 multimode microplate reader, with excitation/emission wavelengths of 485/530 nm for JC-1 monomers (green channel) and 540/590 nm for JC-1 aggregates (red channel). Excitation and emission bandwidths were set to 20 nm for both channels. For each well, the aggregate-to-monomer ratio was calculated by dividing the red fluorescence intensity by the green fluorescence intensity. The resulting ratios were normalized to the mean ratio of the untreated control group, which was set at 100%.

Fluorescent detection of cell nuclei and cytoskeletal filaments

After 24 h of treatment, the nuclear morphology and organization of the cytoskeleton were assessed by fluorescence staining of nuclei, F-actin, and α -tubulin. The effects of QUE (25, 50, 100, 150 and 200 µM) were evaluated. RPMI 7951 cells were plated at a density of 1×10^4 cells/well in clear-bottom black 96-well plates and left to adhere before treatment. When the cells reached a sufficient degree of confluence, they were treated with the selected QUE concentrations for 24 h. Cells were fixed in 4% paraformaldehyde for 15 min at room temperature after treatment and then washed twice with PBS. Cells were permeabilized with 50 µL/well of 0.1% Triton

X-100 for 15 min at room temperature and then washed with PBS twice. To reduce nonspecific antibody binding, cells were incubated with 100 μ L/well of 1% BSA for 30 min at room temperature. Cells were incubated for 3 h at room temperature with a 1:500 dilution of a stock concentration of 0.5 mg/mL of anti- α -tubulin monoclonal antibody (B-5-1-2) in 1 % BSA, washed once with PBS, followed by a 1:500 dilution of a stock concentration of 4 μ g/mL of Alexa Fluor™ 488-conjugated goat anti-mouse IgG (H+L) secondary antibody for 45 min to visualize the microtubule network. After two PBS washes, F-actin and nuclei were counterstained with a mixture of Texas Red™-X phalloidin (1:200) and Hoechst 33342 stock concentration 20 mM (dilution 1:2000) in a final volume of 50 μ L/well. The staining step was performed for 30 min at room temperature in the dark. Cells were then washed twice with PBS, and fluorescence images were acquired using the Lionheart FX Automated Microscope with 20 $\times$ magnification (NA = 0.45). Image acquisition and analysis were performed using Gen5™ Microplate Data Collection and Analysis Software, version 3.14. The staining procedure was adapted from a previously reported protocol by Dahma *et al.* (12).

Acridine Orange/Propidium Iodide (AO/PI) assay

Cellular alterations associated with apoptotic and necrotic-like processes were investigated in RPMI 7951 cells by dual fluorescence staining with acridine orange (AO) and propidium iodide (PI). RPMI 7951 cells were plated in 96-well plates at a density of 1×10^4 cells *per* well and subsequently treated for 24 h with QUE (25, 50, 100, 150, and 200 μ M). After the completion of the exposure time, 100 μ L containing a 10% mixture of complete culture medium containing AO (10 μ g/mL) and PI (10 μ g/mL) was added to each well. The cells were maintained with the staining mixture for 10 min at room temperature while protected from light, and subsequently washed once with PBS before imaging. Viable cells were identified by uniform green nuclear fluorescence, while early apoptotic cells exhibited bright green fluorescence with condensed or fragmented chromatin. Late apoptotic/necrotic cells displayed orange-red fluorescence with condensed or fragmented nuclei. Fluorescence pictures were then obtained using a Lionheart™ FX Automated Microscope with 20 $\times$ magnification (NA = 0.45), and image processing was performed with Gen5™ Microplate Data Collection and Analysis Software. The experimental procedure was adapted from the AO/PI staining method previously reported by Alsaeyd Ahmad *et al.* (13).

Statistical analysis

All *in vitro* assays were conducted as three independent biological experiments, with three technical replicates included in each experiment. Results are expressed as mean $\pm$ standard deviation (SD). The Shapiro–Wilk test was applied to assess data

distribution. Differences between untreated cells and the various QUE-treated groups were analyzed by one-way ANOVA followed by Dunnett's post hoc test. Statistical analyses were carried out using GraphPad Prism 11.0.2 (GraphPad Software, San Diego, CA, USA). Significance levels were reported as * $p < 0.1$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

Results and Discussion

Melanoma remains one of the most aggressive forms of skin cancer, and its prognosis is influenced by the stage of the disease at the time of diagnosis. Although the localized form of the disease is associated with a high 5-year survival rate, the prognosis becomes significantly less favorable once distant metastases appear (14).

Melanoma cell lines are widely used to study tumor behavior and response to treatment; therefore, selecting an appropriate model is an important part of experimental design (15). RPMI 7951 is a malignant melanoma cell line with an epithelial morphology and adherent growth. They contain melanosome granules and is capable of synthesizing melanin *in vitro* and forming pigmented tumors *in vivo*. According to the ATCC characterization, this cell line exhibits alterations in the BRAF, PTEN, and TP53 genes, making it a relevant experimental model for studying melanoma biology and response to treatment (16).

To evaluate the effects of QUE, the concentration range selected for QUE treatment of RPMI 7951 cells was based on previous studies evaluating its effects in melanoma models. For example, concentrations in the range of 50 - 200 μ M have been tested for 24 h in SK-MEL-28 cells (17) and in the range of 5 - 50 μ M in RPMI 7951 cells (18). In addition, concentrations ranging from 5 to 100 μ M have been studied in MEL-202 human uveal melanoma cells (19). Based on these findings, the selected concentration range was extended up to 200 μ M to investigate the effects of QUE at concentrations higher than those previously tested in RPMI 7951 cells and to obtain a broader understanding of its concentration-dependent cytotoxic effects.

Cytotoxic effect of QUE on RPMI 7951 melanoma cells

The first step consisted of the evaluation of cell viability after 24 h of treatment in an effort to evaluate the cytotoxic effect of QUE on RPMI 7951 melanoma cells. As shown in Figure 1, QUE induced a concentration-dependent decrease in cell viability (95.14% at 25 μ M to 89.89% at 50 μ M, 64.92% at 100 μ M, 56.39% at 150 μ M, and 34.94% at 200 μ M). A more pronounced cytotoxic effect was observed from 100 μ M and upwards, with the highest reduction at 200 μ M, whereas only minor modifications were observed at the two lowest concentrations.

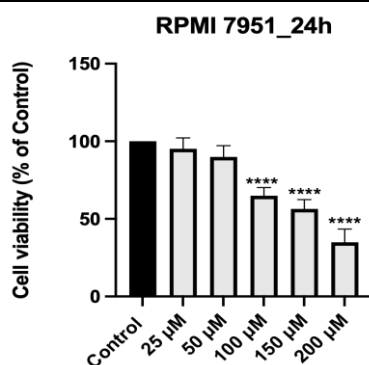


Figure 1.

Effects of 24 h exposure to quercetin (QUE; 25 - 200 µM) on the viability of RPMI 7951 human malignant melanoma cells. Cell viability was assessed using the MTT assay and expressed as a percentage relative to the untreated control, which was assigned a value of 100%. Data are presented as mean ± standard deviation (SD) from three independent experiments, each performed in triplicate. Statistical differences between treated groups and the untreated control were assessed using one-way ANOVA followed by Dunnett's multiple comparisons test.

Statistical significance is indicated as follows:

****p < 0.0001

The MTT assay is a colorimetric method commonly used to estimate cell viability by measuring NAD(P)H-dependent metabolic activity. Metabolically active cells reduce the yellow tetrazolium salt MTT to purple, insoluble formazan crystals through

NAD(P)H-dependent oxidoreductases, including those located outside mitochondria. The resulting crystals are then solubilized and quantified spectrophotometrically (20). In our study, QUE inhibited viability of RPMI 7951 cells in a concentration-dependent manner, showing minor effects at concentrations of 25 - 50 µM and a more pronounced reduction starting at 100 µM. Similar results have been shown for other melanoma cell lines. QUE decreased the viability of SK-MEL-28 cells from approximately 74% at 50 µM to 47% at 200 µM after a 24-h treatment (17). Similarly, in A375SM melanoma cells, a concentration-dependent effect was observed after 24 h, with cell viability decreasing from 89% at 40 µM to 32% at 200 µM (21).

Morphological changes induced by QUE in RPMI 7951 melanoma cells

The next step in the evaluation of the cytotoxic effect of QUE was the analysis of the morphological changes induced by the treatment after 24 h of exposure (Figure 2). At lower concentrations of 25 and 50 µM, RPMI 7951 cells mostly preserved a morphology similar to that of the untreated control; however, a slight decrease in cell confluence was noticed. Morphological changes were more pronounced and increased in a dose-dependent manner starting at 100 µM. A marked decrease in cell density was observed at 100 - 200 µM, with cells appearing more rounded and shrunken. These changes were most pronounced at the highest concentration tested (200 µM), where the loss of confluence and changes in cell shape were most noticeable.

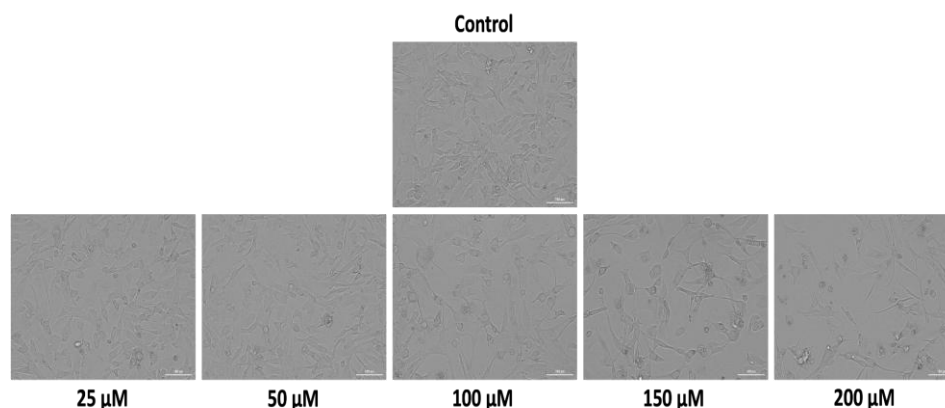


Figure 2.

Morphological appearance of RPMI 7951 human melanoma cells following 24 h exposure to quercetin (QUE; 25 - 200 µM). Representative bright-field micrographs illustrate treatment-associated changes in cell density, shape, and adherence compared with the untreated control. The scale bar indicates 100 µm

Brightfield microscopy is a label-free imaging modality that enables direct monitoring of treatment-induced changes in cell morphology such as changes in cell shape, culture organization, cell-cell contacts and confluence. These observations can be used in combination with cytotoxicity assays to give an

indication of the predominant mode of cell death. Apoptotic cells are usually recognized by shrinkage, chromatin condensation, nuclear fragmentation and membrane blebbing, while necrotic cells are mainly distinguished by cell swelling, cytoplasmic vacuolization and loss of plasma membrane integrity. Thus,

morphological evaluation may provide additional information on treatment-related cellular responses and features consistent with apoptotic or necrotic processes (22, 23).

In the present study, treatment induced apparent morphological changes when compared with the characteristic morphology of RPMI 7951 cells, which show an elongated, epithelial-like morphology under normal conditions. These changes were mostly demonstrated as a decrease in cell size and a reduction in cell confluence, with the most pronounced effects observed at the concentration of 200 μM QUE. The literature also reports similar treatment-induced morphological changes in other melanoma models. For instance, the significant changes in the morphology of SK-MEL-28 and G-361 melanoma cells were observed after 24 h of exposure to 100 - 300 μM of QUE. The major changes were similar to those observed in the present study, including reduced cell confluence, smaller cell size, and a more rounded cellular morphology (24). Hussein *et al.* reported that treatment of A375 melanoma cells with QUE concentrations from 10 to 100 μM induced morphological changes. The major changes observed after 48 h of exposure were a reduction in cell confluence and an increase in cell detachment from the culture surface (25). These results, taken

together, indicate that the morphological changes observed in RPMI 7951 cells treated with QUE are similar to those reported previously in other melanoma cell lines, supporting a concentration-dependent effect of QUE on melanoma cell morphology and growth.

Effects of QUE on Cell Viability Assessed by Neutral Red Uptake in RPMI 7951 Melanoma Cells

To further characterize the cytotoxic effect of QUE, cell viability was evaluated additionally by employing the neutral red uptake (NRU) assay after 24 h of treatment. While the MTT assay reflects NAD(P)H-dependent metabolic activity, the NRU assay evaluates cell viability based on the ability of viable cells to accumulate NR within lysosomes. Figure 3A shows that NR accumulation in cells treated with 25 and 50 μM QUE was similar to that of the untreated control. A gradual decrease in dye uptake was observed at 100 μM , and the lowest accumulation was at 200 μM . These microscopic observations are consistent with the quantitative results (Figure 3B) showing NRU values of 90.01%, 85.50%, 73.21%, 60.95% and 38.71% after exposure to 25, 50, 100, 150 and 200 μM QUE, respectively. Overall, the decrease in NRU supports a concentration-dependent reduction in cell viability, particularly at higher QUE concentrations.

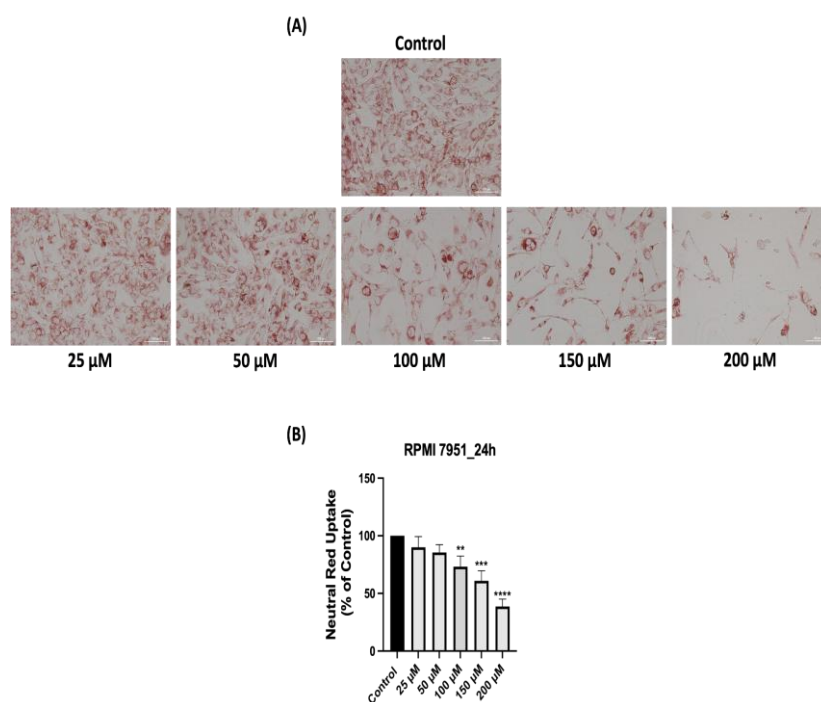


Figure 3.

NRU in RPMI 7951 melanoma cells after 24 h exposure to quercetin (QUE; 25 - 200 μM). (A) Representative bright-field micrographs illustrating changes in intracellular neutral red retention following treatment. The scale bar indicates 100 μm . (B) Relative NRU expressed as a percentage of the untreated control (100%). Results are shown as mean $\pm$ SD from three independent experiments performed in triplicate. Statistical significance versus the control group was evaluated by one-way ANOVA with Dunnett's post hoc test: ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$

In melanoma, lysosomes are increasingly recognized as active contributors to tumor cell survival and progression rather than solely as degradative organelles. Their role in autophagy enables melanoma cells to recycle cellular components and survive better metabolic stress, nutrient deprivation, and oxidative conditions in the tumor microenvironment. Lysosomal signaling is also involved in the pathways controlling cellular growth and proliferation. For example, the lysosomal Ca^{2+} channel MCOLN1 has been implicated in melanoma cell viability through regulation of MAPK- and mTORC1-related signaling. MITF also controls a large repertoire of genes involved in endolysosomal function, thus linking lysosomal homeostasis with melanoma biology. Disturbances in these regulatory mechanisms may favor tumor maintenance and disease progression (26).

The NRU assay assesses cell viability based on the ability of viable cells to accumulate the dye in lysosomes. After absorption, the dye is extracted and quantified spectrophotometrically, providing an indirect measure of lysosomal integrity and cell viability. The method is sensitive, easy to quantify, and inexpensive (27). In the present study, at low concentrations of QUE, the NRU was only slightly affected, whereas higher concentrations, particularly 100 μM or more, clearly reduced dye accumulation and suggested the presence of lysosomal damage. Nevertheless, the use of the NRU assay as a complementary method to MTT for evaluating cytotoxic potential is a common practice in the literature. For example, Jakimiuk *et al.* studied the cytotoxic effects of 37 flavonoids on two melanoma cell lines (A375 and C32) with up to 72 h of exposure and concentrations of up to 200 μM . Several of these compounds also demonstrated cytotoxic effects when assessed using the NRU assay (28).

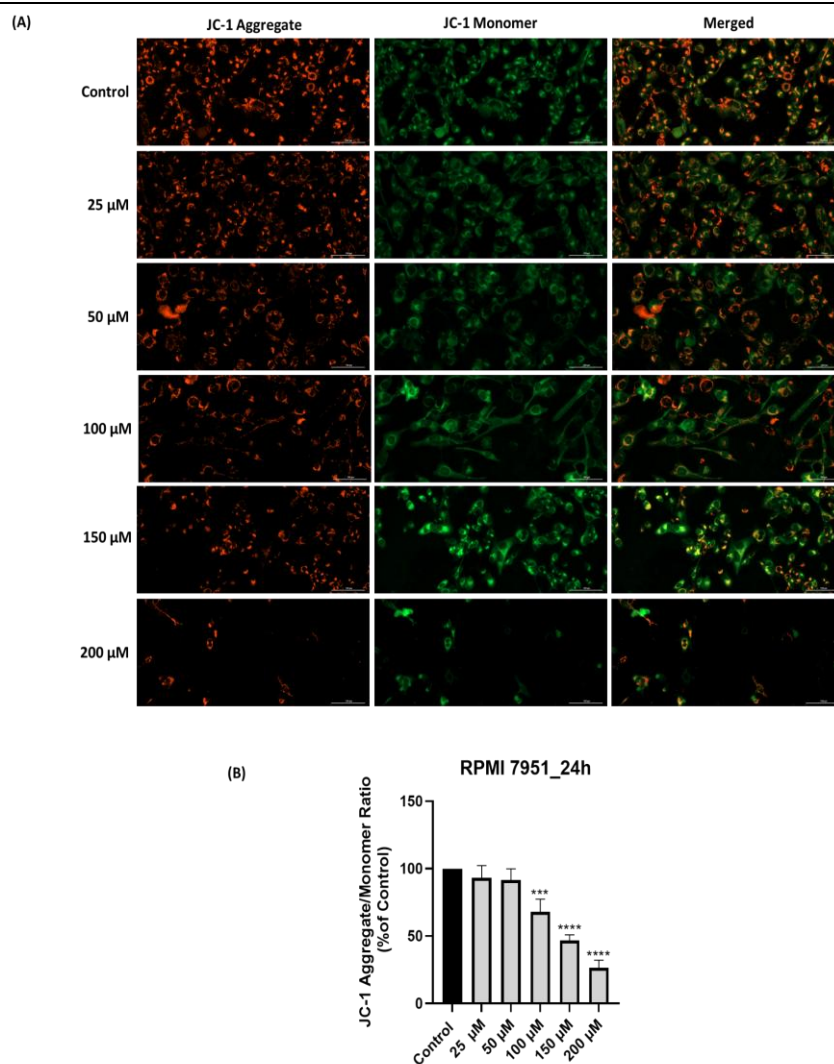
Alterations in mitochondrial membrane potential induced by QUE in RPMI 7951 melanoma cells

Next, we investigated whether 24 h exposure to QUE induced alterations in mitochondrial membrane potential using the JC-1 assay (Figure 4). Images from the fluorescence microscope showed that the treatment resulted in a dose-dependent increase in green fluorescence, which represents the monomeric form of JC-1, and a progressive decrease in red fluorescence, which is related to the JC-1 aggregates. These changes indicate a depolarization of the mitochondrial membrane, which became more pronounced starting from 100 μM . This finding was further confirmed by quantification of the JC-1 aggregate/monomer ratio. The JC-1 aggregate/monomer ratio

decreased to 93.14%, 91.55%, 67.92%, 46.73% and 26.61% of the untreated control at QUE concentrations of 25, 50, 100, 150 and 200 μM , respectively, suggesting a concentration-dependent loss of mitochondrial membrane polarization.

In melanoma, mitochondrial function can also be regarded as a potential vulnerability of tumor cells, as their survival and ability to adapt to stressful conditions are closely dependent on mitochondrial metabolic and bioenergetic flexibility (29). Previous Studies have reported that melanoma cells may generally exhibit a more pronounced hyperpolarization of the mitochondrial membrane potential compared with normal cells, suggesting that this feature could represent a potential target for investigating the antimelanoma effects of bioactive compounds (30). Furthermore, perturbation of mitochondrial membrane potential, especially mitochondrial depolarization, is accompanied by impaired cellular respiration, increased ROS production, and ATP depletion. Moreover, mitochondrial bioenergetic disruption has also been associated with reduced responsiveness to certain antimelanoma therapies, including BRAF-targeted therapy (31, 32). These results emphasize the significance of the mitochondrial membrane potential assessment when considering the cytotoxic effects of potential antimelanoma agents. Earlier studies showed accumulation of QUE in mitochondria of cancer cells, indicating this organelle could be an important intracellular target for its biological activity. Interestingly, mitochondrial stress appears to enhance intracellular QUE accumulation, with a fraction of the accumulated compound being localized within mitochondria (33).

The cationic fluorescent dye JC-1 is commonly used for the assessment of mitochondrial membrane potential ($\Delta\Psi\text{m}$). In polarized mitochondria, JC-1 forms red-fluorescent aggregates, while mitochondrial depolarization favors the monomeric green-fluorescent form. Thus, the ratio of red to green fluorescence is a marker of changes in the mitochondrial membrane potential (34). In our study, QUE gradually influenced $\Delta\Psi\text{m}$ with increasing concentration, and the changes were more obvious at 100 μM and most obvious at 200 μM . However, previous studies also reported the ability of QUE to induce alterations in the mitochondrial membrane potential of melanoma cells. Seo *et al.* examined the effects of QUE on SK-MEL-28 and G-361 melanoma cells at concentrations of 100 to 300 μM . After 24 h of exposure, JC-1 staining revealed mitochondrial membrane depolarization in both cell lines (24).

**Figure 4.**

JC-1 assay in RPMI 7951 melanoma cells after 24 h exposure to quercetin (QUE; 25 - 200 μ M). (A) Representative fluorescence micrographs showing changes in JC-1 red and green fluorescence following treatment. The scale bar indicates 100 μ m. (B) JC-1 aggregate/monomer fluorescence ratio expressed as a percentage of the untreated control (100%). Results are shown as mean $\pm$ SD from three independent experiments performed in triplicate. Statistical significance versus the control group was evaluated by one-way ANOVA with Dunnett's post hoc test: *** p < 0.001, and **** p < 0.0001

Cytoskeletal and nuclear alterations induced by QUE in RPMI 7951 melanoma cells

Next, we evaluated the effect of 24 h treatment with QUE at concentrations ranging from 25 to 200 μ M on the morphology of important cellular components such as the nuclei, F-actin filaments, and the network of microtubules (Figure 5). At 25 and 50 μ M QUE, the morphology of cells was mostly similar to the untreated control, with relatively uniform nuclei and maintenance of the extended organization of both actin filaments and the tubulin network. However, at 100 μ M, clear morphological changes were observed, with decreased cell confluence, decreased extension, and disorganization of the F-actin and microtubule networks, nuclear rounding, and

chromatin condensation. At 150 μ M, these changes were more pronounced, with progressive loss of normal cellular morphology and further disruption of cytoskeletal organization. The most significant effects were observed at 200 μ M QUE, where marked disorganization of both the actin filament and tubulin networks was evident, together with pronounced nuclear morphological alterations, including nuclear rounding and chromatin condensation. At this concentration, a marked decrease in cell confluence was also observed. Altogether, these results demonstrate a dose-dependent change in cell morphology and cytoskeletal organization, clearly evident at concentrations of 100 μ M and above.

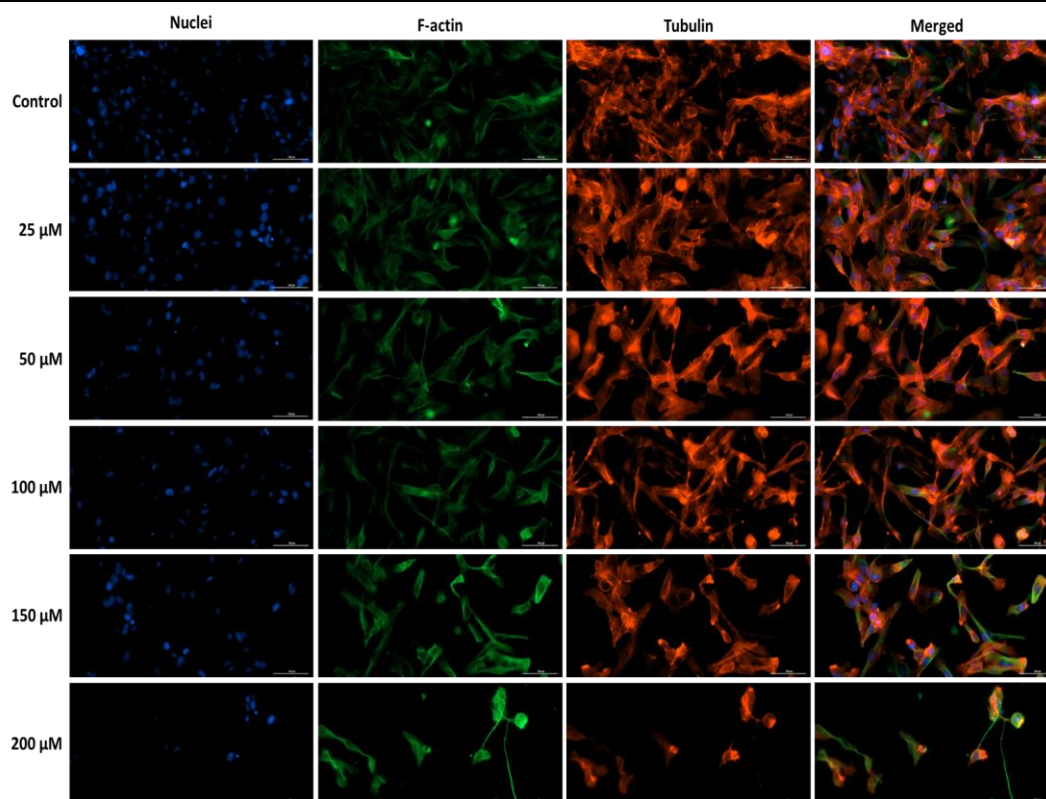


Figure 5.

Fluorescence microscopy images showing nuclear morphology, F-actin organization, and tubulin architecture in RPMI 7951 melanoma cells following 24 h treatment with quercetin (QUE; 25 - 200 μM). The scale bar indicates 100 μm

Nuclear morphology was examined by Hoechst 33342 staining, which allowed the identification of characteristic apoptotic features, particularly chromatin condensation and nuclear fragmentation (35). Cytoskeletal changes were investigated separately by fluorescence analysis of actin filaments and the tubulin network. Monitoring the integrity and organization of these structures is relevant for the evaluation of anticancer effects, as the cytoskeleton participates in the regulation of cell shape, proliferation, motility, and the cellular response to environmental or treatment-related stress (36). The treatment changed the morphology of these cellular frameworks beginning at 100 μM QUE, and the strongest effects were observed at the highest concentration used (200 μM).

Roman *et al.* used the same experimental approach as in the present study to study the effect of QUE on A375 melanoma cells, both as a single treatment with 10 μM and in combination with different concentrations of 5-fluorouracil (5-FU). After 24 h of exposure, QUE alone induced evident changes in nuclear morphology, including chromatin condensation and nuclear shrinkage. No significant changes were seen in the organization of actin filaments or in the tubulin network following treatment with QUE

alone. However, the association of QUE and 10 μM 5-FU potentiated the cytotoxic effects of 5-FU on the cytoskeleton, leading to a more prominent tubulin condensation and constriction of actin filaments. The combined treatment also intensified the nuclear alterations compared with either treatment alone (18). Furthermore, analogous nuclear alterations were also reported by Seo *et al.* in the SK-MEL-28 and G-361 melanoma cell lines following 24 h exposure to QUE at concentrations of up to 300 μM (24).

Microscopic evaluation of cell death-associated changes induced by QUE in RPMI 7951 melanoma cells

The final step consisted of the microscopic evaluation of the ability of QUE (25 - 200 μM) to induce apoptotic- or necrotic-like changes (Figure 6). PI-positive cells, identified by the appearance of red fluorescence, increased progressively with increasing QUE concentration, indicating a concentration-dependent loss of plasma membrane integrity. In addition, features consistent with early apoptosis became evident from 100 μM onward. These were characterized by chromatin condensation, cell rounding, and the appearance of membrane blebbing, which were mostly observed in AO-positive cells with green fluorescence.

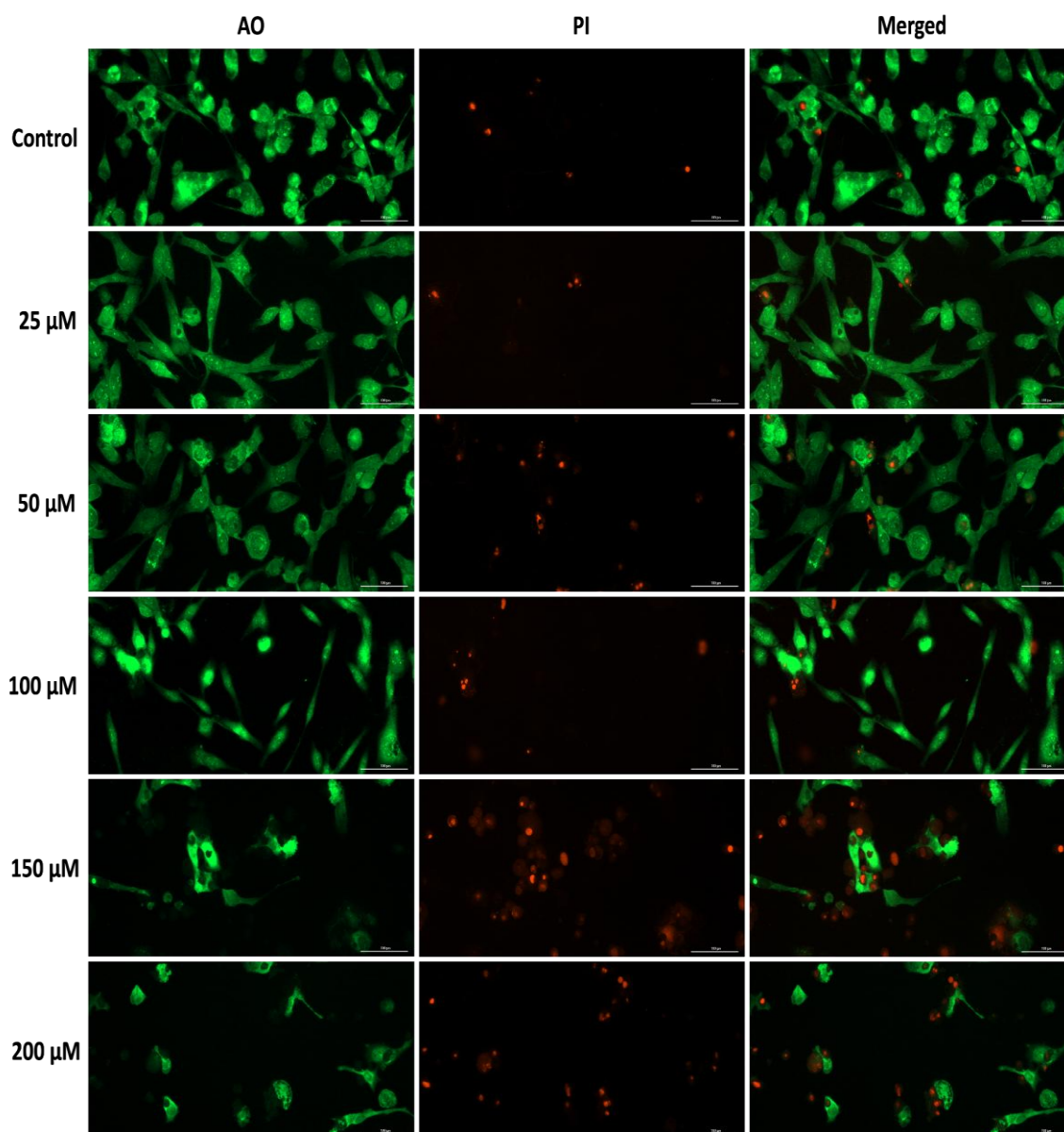


Figure 6.

AO/PI fluorescence staining of RPMI 7951 melanoma cells following 24 h exposure to quercetin (QUE; 25 - 200 μ M). Representative fluorescence images show treatment-related changes in cell morphology and membrane integrity. Acridine orange (AO, green fluorescence) labels nucleic acids in cells with preserved membrane integrity, whereas propidium iodide (PI, red fluorescence) is taken up by cells with loss of plasma membrane integrity. The scale bar indicates 100 μ m.

Changes associated with cell death are commonly visualized by AO/PI staining. Cells with intact membranes fluoresce green with AO; cells with damaged membranes take up PI and fluoresce red (37). Concentration-dependent cell death was induced by QUE, with an increase in PI positivity and loss of membrane integrity at higher concentrations. Starting at 100 μ M, chromatin condensation, cell rounding, and the formation of membrane vesicles were also observed.

Previous results in melanoma models are also in agreement with the pro-apoptotic morphological changes observed in the present study. In the study

by Rafiq *et al.*, the combination of UVB radiation and QUE at 20 and 40 μ M produced significant pro-apoptotic effects in B16F10 melanoma cells, as determined by Annexin V/propidium iodide double staining. Furthermore, QUE improved the apoptotic response induced by UVB irradiation after 24 h treatment (38). Furthermore, a similar approach, acridine orange/ethidium bromide (AO/EtBr) dual staining, was applied by Paneva *et al.* to assess the ability of an electrospun fiber-based delivery system for rutin, as well as free rutin, to induce apoptotic changes in the SH-4 melanoma cell line after 24 h of exposure. The rutin-loaded delivery system induced

more pronounced apoptotic features compared with free rutin or the fibrous material alone (39).

Taken together, the present findings indicate that QUE exerts cytotoxic effects on RPMI 7951 melanoma cells, particularly at concentrations ranging from 100 to 200 μM . However, these findings should be considered preliminary and interpreted within the limitations of the concentration range and exposure conditions investigated in the present study.

However, several limitations of the current study should be noted. The experiments were first performed on a single 2D melanoma cell model, without validation in other melanoma cell lines or in more complex 3D models that may better represent the structural and microenvironmental features of tumors *in vivo*. Additionally, the effects of QUE were assessed at one exposure time, which limits the evaluation of the temporal evolution and persistence of the observed responses. Another important limitation is the lack of a non-malignant cellular model tested in identical experimental conditions, which precludes a more thorough evaluation of the selectivity and safety profile of QUE. Furthermore, the current study did not explore the molecular mechanisms responsible for the observed cytotoxic effects and the influence of QUE on major signaling pathways involved in melanoma cell survival, proliferation, and progression. Another limitation of this study is that JC-1 fluorescence ratios were not additionally normalized to cell number. Consequently, the potential influence of treatment-related changes in cell density on whole-well measurements cannot be excluded, and the results should be interpreted alongside the viability and morphological findings. In addition, QUE may undergo auto-oxidation in the culture medium, generating H_2O_2 that could contribute to the cytotoxic effects observed at higher concentrations (40). Since catalase co-treatment was not performed, the possible contribution of this mechanism cannot be excluded.

Future studies should therefore extend these observations in further 2D and 3D melanoma models and include the appropriate non-malignant cellular counterparts for a more detailed assessment of the safety and selectivity of QUE. Longer exposure times should also be studied to see if the effects observed are persistent or change over time. Finally, additional mechanistic studies are required to identify the molecular targets and signaling pathways involved in the cytotoxic activity of QUE in melanoma cells. In this context, previous research in colorectal cancer cells has investigated apoptosis- and proliferation-related markers, including BAX, BCL-2, CASP3, and Ki-67 (41). Similar molecular investigations could help clarify the mechanisms underlying the cytotoxic effects of QUE in melanoma cells.

Conclusions

Despite considerable progress in the management of melanoma in the last few years, treatment resistance and the aggressive nature of the disease still highlight the necessity to explore new strategies to complement current treatment approaches. Here, we evaluated the effects of QUE at concentrations ranging from 25 to 200 μM after 24 h of exposure in RPMI 7951 melanoma cells. The results showed a more pronounced cytotoxic effect starting from 100 μM , with decreased cell viability and lysosomal integrity, decreased mitochondrial membrane potential, and marked morphological changes in important cellular components, including nuclei, actin filaments, and the tubulin network. In addition, the pro-apoptotic potential of QUE was further verified by microscopic evaluation using AO/PI dual staining. Taken together, these preliminary results provide a rationale for further investigation of QUE as a potential adjunct to current melanoma treatment strategies. However, additional studies are needed to clarify its underlying mechanisms of action, to determine its safety profile and to find the most appropriate concentration ranges and conditions of exposure.

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Availability of data and materials

The data generated in the present study are included in the figures and/or tables of this article.

Ethics approval and consent to participate

Not applicable.

AI use disclosure

During the preparation of this manuscript, the authors used QuillBot for language improvement, including spelling, grammar, clarity, and readability. The authors reviewed and revised all outputs from these tools and take full responsibility for the final content of the work.

Conflict of interest

The authors declare no conflict of interest.

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