

Antimelanogenesis of quercetin-related compounds in B16 melanoma cells: the difference moieties of C-3 position and its effect on H₂O₂

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Research Article

Keywords: quercetin related compounds, antimelanogenesis, H₂O₂, B16 melanoma cells

Posted Date: August 31st, 2025

DOI: <https://doi.org/10.21203/rs.3.rs-7328612/v1>

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Additional Declarations: No competing interests reported.

Abstract

Quercetin (**compound 2**), isolated from the dried skin of *Allium cepa*, and 3-prenyl luteolin (**compound 6**), derived from the wood of *Artocarpus heterophyllus*, are polyphenolic compounds with demonstrated potential in regulating melanogenesis. This study investigated their anti-melanogenic activity and reactive oxygen species (ROS) scavenging effects, particularly against hydrogen peroxide (H_2O_2), in B16 melanoma cells. Both compounds significantly suppressed melanin synthesis, indicating potential as natural depigmenting agents. Quercetin (**2**) exhibited a half-maximal inhibitory concentration (IC_{50}) of $8.0\text{ }\mu\text{g/mL}$, while 3-prenyl luteolin (**6**) showed an IC_{50} of $20.1\text{ }\mu\text{g/mL}$. For comparison, compound 1 displayed the strongest activity with an IC_{50} of $3.0\text{ }\mu\text{g/mL}$, whereas compounds **3–5** demonstrated no appreciable inhibitory effects. Additionally, both compounds reduced intracellular H_2O_2 levels, supporting their antioxidative role in mitigating oxidative stress-induced melanogenesis. These findings highlight the potential application of quercetin and 3-prenyl luteolin as novel active ingredients in skin-whitening formulations through dual mechanisms of melanin synthesis inhibition and ROS reduction.

Introduction

Melanin is a complex, heterogeneous biopolymer synthesized through the enzymatic oxidation and subsequent polymerization of tyrosine-derived precursors within melanosomes, specialized organelles in melanocytes [1–3]. Serving as a critical photoprotective pigment, melanin effectively absorbs ultraviolet (UV) radiation, thereby mitigating UV-induced oxidative damage to skin cells and contributing to skin coloration and protection [4–6]. The melanogenesis pathway is tightly regulated, beginning with the rate-limiting enzyme tyrosinase catalyzing the hydroxylation of tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) and subsequent oxidation to dopaquinone. This intermediate undergoes multiple enzymatic transformations, involving tyrosinase-related proteins TRP-1 and TRP-2, which lead to the formation of eumelanin and pheomelanin pigments [7–9]. The microphthalmia-associated transcription factor (MITF) plays a pivotal role by regulating the expression of tyrosinase and related enzymes, thus ensuring coordinated melanocyte differentiation, survival, and pigment synthesis [7, 10–12].

Reactive oxygen species (ROS), particularly hydrogen peroxide (H_2O_2), are generated endogenously during melanin biosynthesis via tyrosinase-mediated oxidation of L-DOPA and are also produced exogenously following UV exposure [13–16]. At physiological concentrations ($\sim 50\text{--}300\text{ }\mu\text{M}$), H_2O_2 acts as a secondary messenger that promotes melanogenesis by activating melanocyte-specific transcription factors such as MITF through pathways involving cyclic AMP (cAMP)/protein kinase A (PKA) and the nuclear factor erythroid 2-related factor 2 (Nrf2), which regulates cellular antioxidant responses [17–23]. However, excessive or chronic accumulation of H_2O_2 can disrupt intracellular redox homeostasis, leading to deleterious effects such as endoplasmic reticulum (ER) stress, impaired trafficking and maturation of tyrosinase, melanocyte senescence, and ultimately pigmentary disorders including melasma, post-inflammatory hyperpigmentation, and premature skin aging [16, 24, 25]. These pathological conditions highlight the dual role of ROS in melanogenesis as both necessary signaling molecules and potential mediators of oxidative damage.

Melanocytes are equipped with robust endogenous antioxidant systems, including catalase, glutathione peroxidase, and thioredoxin reductase, which cooperatively degrade H_2O_2 to maintain redox balance and prevent oxidative stress [24, 26–31]. These antioxidant enzymes are transcriptionally regulated by the Nrf2 signaling pathway, which governs the expression of a battery of protective genes such as heme oxygenase-1 (HO-1), NAD(P)H quinone dehydrogenase 1 (NQO1), and enzymes involved in glutathione synthesis [21, 32–35]. Despite these sophisticated defenses, chronic oxidative stress and overwhelming ROS production can impair these protective mechanisms, resulting in protein misfolding, ER dilation, defective melanogenesis, and the exacerbation of hyperpigmentation disorders [16, 20, 36, 37]. Thus, controlling oxidative stress and ROS levels remains a critical strategy in the development of effective depigmenting agents.

Quercetin, a naturally abundant bioflavonoid in various fruits and vegetables, has emerged as a promising natural compound for inhibiting melanogenesis due to its potent antioxidant, anti-inflammatory, and enzyme inhibitory properties [38–40]. In vitro experiments in human melanocytes and murine B16F10 melanoma cells have demonstrated that quercetin, particularly at concentrations ranging from 20 to 50 μM , significantly inhibits tyrosinase activity and melanin synthesis [41]. This effect is mediated by downregulation of MITF and its downstream effectors, tyrosinase-related proteins TRP-1 and TRP-2, and suppression of the cAMP/PKA signaling cascade, all achieved without notable cytotoxicity [39, 42]. Additionally, quercetin mitigates H_2O_2 -induced ER stress, preserves ER structure, and supports proper trafficking and expression of melanogenic enzymes, thereby maintaining melanocyte functionality under oxidative stress conditions [43].

Mechanistically, quercetin scavenges various ROS species, including peroxynitrite and superoxide radicals, elevates intracellular glutathione levels, and modulates catalase and HO-1 activities to restore cellular redox balance and inhibit melanogenesis [40, 44–48]. Its derivatives have demonstrated enhanced anti-melanogenic effects by targeting multiple signaling pathways including cAMP/PKA/CREB/MITF and MAPK, further highlighting the therapeutic potential of this class of compounds [41]. Notably, quercetin's effects exhibit dose dependency, as lower concentrations ($< 20 \mu M$) may paradoxically stimulate melanogenesis through post-translational stabilization of tyrosinase and TRP-2 proteins, highlighting the necessity for precise dose optimization in therapeutic applications [41]. Despite encouraging in vitro and preclinical results, clinical evidence for quercetin's efficacy in treating hyperpigmentation remains insufficient. Challenges such as improving its bioavailability, developing optimized topical formulations, and conducting rigorous clinical trials comparing quercetin with established depigmenting agents like kojic acid, arbutin, and vitamin C are critical for its translation to clinical use [49, 50]. Addressing these gaps will be essential to fully harness the therapeutic potential of quercetin in oxidative stress-related pigmentary disorders.

The present study aims to investigate the anti-melanogenic effects of two compounds: quercetin (**2**), isolated from the dried skin of *Allium cepa* (onion), and 3-prenyl luteolin (**6**), isolated from the wood of *Artocarpus heterophyllus* (jackfruit). These compounds have been selected as novel candidates for anti-melanogenesis due to their unique structures and potential bioactivity. This research will explore their

inhibitory activity on melanin formation, revealing their potential as novel depigmenting agents for skin-whitening cosmetics. In addition, we will evaluate the capability of these compounds to reduce ROS, especially H₂O₂, in B16 melanoma cells, which represent a significant source of oxidative stress associated with melanin overproduction. By targeting the oxidative pathways contributing to melanogenesis, these compounds could serve as promising candidates for the development of effective skin whitening products through the reduction of melanin overproduction.

Materials and Methods

Chemicals

Sodium hydroxide (NaOH), dimethyl sulfoxide (DMSO), and rutin (**compound 3**) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Ethylenediaminetetraacetic acid (EDTA) was purchased from Dojindo Laboratories (Kumamoto, Japan). The tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was sourced from Sigma-Aldrich (St. Louis, MO, USA), and Eagle's Minimum Essential Medium (EMEM) was obtained from Nissui Pharmaceutical Co., Ltd. (Osaka, Japan). Hyperin (**compound 4**) was supplied by Tokiwa Phytochemical Co. (Chiba, Japan), luteolin (**compound 1**) was purchased from Extrasynthese (Genay, France), and isoquercitrin (**compound 5**) was obtained from Fluka Chemie GmbH (Steinheim, Germany). Quercetin (**compound 2**) was isolated from the dried outer skin of *Allium cepa*, while 3-prenyl luteolin (**compound 6**) was purified from the wood of *Artocarpus heterophyllus*. All other reagents used were of analytical grade and sourced from standard commercial suppliers. The chemical structures of the isolated compounds are illustrated in Fig. 1.

Cell culture

The B16 murine melanoma cell line was obtained from the RIKEN Cell Bank (Tsukuba, Japan). Cells were cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% fetal bovine serum (FBS) and 0.09 mg/mL theophylline. Cell cultures were maintained at 37°C in a humidified incubator with 5% CO₂ atmosphere.

Inhibitory effect of melanin biosynthesis using cultured B16 melanoma cells

The assay was performed following the method described by Arung et al. (2007), with slight modifications. Briefly, confluent B16 melanoma cells were washed with phosphate-buffered saline (PBS) and detached using 0.25% trypsin-EDTA solution. The cells were seeded into two 24-well culture plates—one designated for melanin content analysis and the other for cell viability assessment—at a density of 1×10^5 cells per well. After 24 hours of incubation in complete medium to allow cell attachment, the culture medium was replaced with 998 μ L of fresh EMEM, and 2 μ L of dimethyl sulfoxide (DMSO), with or without the test compounds at various concentrations ($n = 3$), was added. Arbutin was included as a

positive control. Cells were incubated for an additional 48 hours, after which the medium was refreshed and the same concentrations of test compounds were reapplied. Following a further 24-hour incubation, adherent cells were subjected to melanin content and cell viability assays as described below.

Determination of melanin content in B16 melanoma cells

Melanin content in the treated B16 melanoma cells was quantitatively evaluated following compound exposure. After removing the culture medium, cells were washed thoroughly with phosphate-buffered saline (PBS) to eliminate residual media components. The adherent cells were then lysed by incubation in 1.0 mL of 1 N sodium hydroxide (NaOH), which solubilized intracellular melanin. The resulting lysates were transferred to a 96-well microplate, and absorbance was measured at 405 nm using a microplate reader (BioTek Instruments, Winooski, VT, USA). Melanin levels in the treated samples were normalized to those of the untreated control group and expressed as a percentage of control values to assess the inhibitory effects of the test compounds on melanin synthesis.

Cell Treated with H₂O₂

Cells were seeded at a density of 1×10^5 cells per well into three separate 24-well plates and incubated for 24 hours to allow adherence. Following incubation, the test compounds were administered at designated concentrations. To induce oxidative stress, hydrogen peroxide (H₂O₂) was added to each well at a final concentration of 0.25% at 1, 2, and 4 hours after compound treatment. Cell viability was subsequently assessed using the MTT assay as described below.

Cell viability

Cell viability was evaluated using the microculture tetrazolium (MTT) assay, which assesses mitochondrial metabolic activity based on the formation of formazan crystals in viable cells. B16 melanoma cells were seeded into 24-well plates at a density of 1×10^5 cells per well. Following the designated treatments and incubation periods, 50 μ L of MTT solution [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 5 mg/mL in PBS] was added to each well. Plates were incubated for 4 hours at 37°C in a humidified incubator with 5% CO₂ to allow for formazan formation. After incubation, the medium was aspirated, and 1.0 mL of isopropyl alcohol containing 0.04 N HCl was added to each well to dissolve the formazan crystals. Absorbance was measured at 570 nm with a reference wavelength of 630 nm using a microplate reader (BioTek Instruments, USA). The absorbance values were used to calculate cell viability relative to untreated controls.

Results and Discussion

Antimelanogenesis of quercetin-related compounds

The anti-melanogenic properties of all isolated constituents were assessed using B16 melanoma cell assays. Among the tested flavonoids, no statistically significant differences were observed in the melanin inhibition rates of rutin (**3**), hyperin (**4**), and isoquercetin (**5**). In contrast, compounds **1**, **2**, and **6** exhibited markedly higher inhibitory effects on melanogenesis compared to the remaining isolates and the reference agent, arbutin. A dose-dependent suppression of melanin synthesis and cell viability was observed with increasing concentrations of these compounds. Notably, compound **1**, structurally identified as luteolin, exerted the most potent melanogenesis-inhibitory effect, surpassing the activities of quercetin (**2**) and 3-prenyl luteolin (**6**), as illustrated in Figs. 2–7.

Luteolin demonstrated the capacity to inhibit melanin synthesis by over 50% at a concentration of 5 µg/mL. At 25 µg/mL, luteolin achieved more than 80% inhibition of melanin formation, concomitant with a reduction in cell viability to below 30% (Fig. 2). In contrast, quercetin inhibited melanin production by less than 50% at 5 µg/mL and exceeded 65% inhibition at 25 µg/mL, while maintaining cell viability above 70% (Fig. 3). The inhibitory profile of 3-prenyl luteolin differed from these compounds, with melanin synthesis suppressed by over 60% only at 30 µg/mL (Fig. 7). The IC₅₀ values determined for luteolin, quercetin, and 3-prenyl luteolin were 3.0 µg/mL, 8.0 µg/mL, and 20.1 µg/mL, respectively, indicating varying degrees of anti-melanogenic efficacy.

Luteolin, quercetin, and 3-prenyl luteolin have been classified as polyphenolic compounds, which are well-recognized for their significant roles and extensive applications in the cosmetic industry. Previous studies have demonstrated the anti-melanogenic properties of these compounds [48, 51, 52]. Among them, 3-prenyl luteolin exhibits lower anti-melanogenesis activity compared to luteolin and quercetin, consistent with previous research [48, 51]. This variation in activity is attributed to differences in functional groups, as prior research indicated that the presence of an isoprenoid moiety in 3-prenyl luteolin diminishes its inhibitory effect on melanin synthesis in B16 melanoma cells [51]. Additionally, other studies have highlighted that the anti-melanogenic efficacy is highly dependent on the specific position of functional groups within the molecular structure [53–55].

Anti-melanogenic agents exert their effects through multiple mechanisms, including inhibition of tyrosinase mRNA transcription, disruption of tyrosinase maturation, suppression of tyrosinase enzymatic activity, promotion of tyrosinase degradation, and indirect modulation of tyrosinase function [56]. Among these, tyrosinase inhibition is widely regarded as the primary target for reducing hyperpigmentation [57–59]. Luteolin and quercetin inhibit melanin synthesis via distinct pathways. Luteolin reduces melanin production in B16 melanoma cells by decreasing intracellular cyclic adenosine monophosphate (cAMP) signaling [52]. The downregulation of cAMP leads to dephosphorylation of PKA/CREB, resulting in diminished MITF expression, impaired tyrosinase regulation, and subsequent inhibition of melanin synthesis [39, 47, 60, 61]. In contrast, quercetin acts as a potent tyrosinase inhibitor, directly interfering with the enzyme's catalytic activity and downstream signaling pathways to prevent melanin formation [62].

Effect of quercetin related compounds on B16 melanoma cells treated with H₂O₂

Cell viability following hydrogen peroxide (H₂O₂) exposure was assessed using the MTT assay to establish an optimal concentration for inducing oxidative stress in B16 melanoma cells. Cells were treated with increasing concentrations of H₂O₂ (0.05%, 0.1%, 0.25%, and 0.5%), and a clear dose-dependent decrease in viability was observed (Fig. 8), consistent with prior reports demonstrating H₂O₂'s cytotoxicity through oxidative damage and mitochondrial dysfunction [2, 63–65]. Notably, there was no statistically significant difference in cell viability between 0.25% and 0.5% H₂O₂ treatments (Fig. 8), indicating a saturation effect at higher concentrations where additional oxidative burden did not further reduce viability [66]. Based on these results, 0.25% H₂O₂ was selected for subsequent assays, as it induced substantial oxidative stress while avoiding excessive cytotoxicity that could confound downstream analyses. Similar concentrations have been used in previous studies to model oxidative stress conditions in melanocytes and melanoma cells, validating its use as a physiologically relevant dose [20]. These doses effectively mimic oxidative environments that contribute to melanocyte damage, melanogenesis alterations, and melanoma cell death, supporting their physiological relevance.

The impact of quercetin-related compounds on B16 melanoma cell viability under oxidative stress is presented in Figs. 9 and 10. Notably, luteolin demonstrated a pronounced ability to enhance cell survival following hydrogen peroxide (H₂O₂) exposure, outperforming other tested compounds in mitigating oxidative damage. This suggests that luteolin exerts stronger cytoprotective effects, likely due to its potent antioxidant activity and modulation of intracellular signaling pathways involved in cell survival and melanogenesis regulation. Luteolin and its derivative luteolin 7-sulfate inhibit melanin synthesis by downregulating tyrosinase (TYR) expression through CREB- and MITF-mediated signaling pathways, which are key regulators of melanogenesis in melanoma cells. Moreover, luteolin reduces cellular reactive oxygen species (ROS) levels by directly inhibiting oxidative enzymes such as xanthine oxidase, thereby protecting cells from oxidative damage [44, 67]. Other quercetin derivatives also partially restored cell viability compromised by H₂O₂, indicating their potential to alleviate oxidative stress in melanocytes. These findings corroborate previous reports indicating that quercetin and its analogs reduce reactive oxygen species (ROS) levels and protect against oxidative injury by scavenging free radicals and activating endogenous antioxidant defenses [40, 47, 68, 69].

At a concentration of 5 ppm, the quercetin-related compounds exhibited lower cytotoxicity compared to quercetin 3-O-β-D-glucuronide (Q-3-G) and synthetic quercetin glycosides, which have shown dose-dependent cytotoxic effects in various cell lines including HaCaT keratinocytes and B16F10 melanoma cells at 20 μM [70, 71]. This differential toxicity profile highlights the advantage of naturally derived compounds, which may offer enhanced safety and efficacy for potential therapeutic or cosmetic applications targeting hyperpigmentation and oxidative stress-related skin disorders. Furthermore, luteolin protects cells through both direct antioxidant activity via the Nrf2/ARE pathway and by modulating melanogenesis-related signaling cascades such as CREB/MITF, making it a potent agent

against oxidative stress and hyperpigmentation [44, 67]. These pathways contribute to reducing oxidative damage and restoring melanocyte function, thereby inhibiting excessive melanogenesis.

In the current study, polyphenolic compounds including luteolin, isoquercetin, quercetin, and rutin demonstrated significant ROS-scavenging activity in B16 melanoma cells, with luteolin exerting the most pronounced protective effect against H₂O₂-induced cytotoxicity. This aligns with previous findings that highlight the superior antioxidant capacity of luteolin in skin cells through multiple mechanisms such as direct radical scavenging [72]. Quercetin's efficacy in mitigating ROS accumulation is consistent with its chemical structure, which features multiple hydroxyl groups facilitating electron donation and free radical neutralization [47, 68]. Furthermore, quercetin's metal-chelating properties inhibit Fenton and Haber-Weiss reactions, which otherwise generate highly reactive hydroxyl radicals from H₂O₂ and transition metals, thus breaking a critical cycle of ROS amplification [73–75].

Quercetin, a well-known flavonoid isolated from *Allium cepa* (onion) skin, exhibits potent antioxidant and antimelanogenic activities, largely attributed to its polyphenolic structure and multiple hydroxyl groups that efficiently scavenge reactive oxygen species (ROS) such as hydrogen peroxide (H₂O₂) and superoxide anions [48, 76]. The generation of ROS during UV exposure or cellular metabolism is a critical trigger for melanogenesis, often resulting in hyperpigmentation due to melanin overproduction [28].

Beyond antioxidative effects, quercetin's anti-inflammatory properties reduce melanocyte activation caused by inflammation, which is a key factor in post-inflammatory hyperpigmentation [71, 77]. However, challenges remain in improving its bioavailability and skin penetration for clinical applications. Nonetheless, the comprehensive inhibitory effects of quercetin on oxidative stress and melanogenesis highlight its potential as a natural, safe, and effective ingredient for skin-whitening and photoprotective cosmetics derived from *Allium cepa*.

This study uniquely isolates quercetin specifically from *Allium cepa* skin and comparatively evaluates it alongside 3-prenyl luteolin, a prenylated flavonoid derivative obtained from the wood of *Artocarpus heterophyllus*. Prenylation of flavonoids like luteolin is known to enhance their lipophilicity and cellular uptake, often improving bioavailability and biological activity [78]. This modification may increase the compound's antioxidant capacity and its interaction with key signaling pathways involved in melanogenesis and oxidative stress response. Our findings demonstrate that 3-prenyl luteolin exhibits significant inhibitory effects on melanin production and effectively reduces intracellular ROS levels, although its cytoprotective efficacy under H₂O₂-induced oxidative stress was somewhat lower compared to luteolin and quercetin. This comparative analysis provides novel insights into how structural modifications influence the bioactivity of flavonoids in melanocyte models. Furthermore, the study emphasizes sustainable utilization of agricultural by-products as valuable sources of natural bioactives, bridging environmental considerations with practical applications in the development of effective and safe skin-whitening agents. This integrative approach advances the understanding of quercetin-related compounds and prenylated flavonoids as multifunctional depigmenting agents, highlighting their potential for cosmeceutical innovation beyond what has been previously reported.

Conclusions

Quercetin (**compound 2**), isolated from the dried skin of *Allium cepa*, and 3-prenyl luteolin (**compound 6**), derived from the wood of *Artocarpus heterophyllus*, are promising natural polyphenolic compounds with potent antioxidant and antimelanogenic activities. Their ability to scavenge reactive oxygen species and reduce oxidative stress is likely a key mechanism underlying their inhibition of melanogenesis. These properties make them attractive candidates for developing novel skin-whitening and depigmenting agents. However, further studies focusing on safety and efficacy are essential before their application in humans can be realized. Overall, these compounds offer valuable potential for managing hyperpigmentation and oxidative stress-related skin disorders through natural, multifunctional mechanisms.

Declarations

Funding Declaration

This research was supported by the Japan Society for the Promotion of Science (JSPS) under the Postdoctoral Fellowship Program for Foreign Researchers. This research is also supported by the National Research and Innovation Agency (BRIN) and a research grant from BRIN (Grant number: B-1146/II.7/HK 01.00/5/2025) with the title “Pusat Kolaborasi Riset Kosmetik Berteknologi Nano Berbasis Biomassa”.

Author Contribution

NF, DJ, RAE, ETA : Conception or design of the work; NF, DJ, WF, RAE, YUK, KS, ETA; or the acquisition, analysis, and interpretation of data; NF, DJ, HI, WF, RAE, YUK, KS, ETA : drafted the work or revised it critically for important intellectual content; NF, DJ, HI, WF, RAE, YUK, KS, ETA : approved the version to be published

Acknowledgement

The authors acknowledge the support and facilities provided by the Japan Society for the Promotion of Science and the National Research and Innovation Agency (BRIN).

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Figures

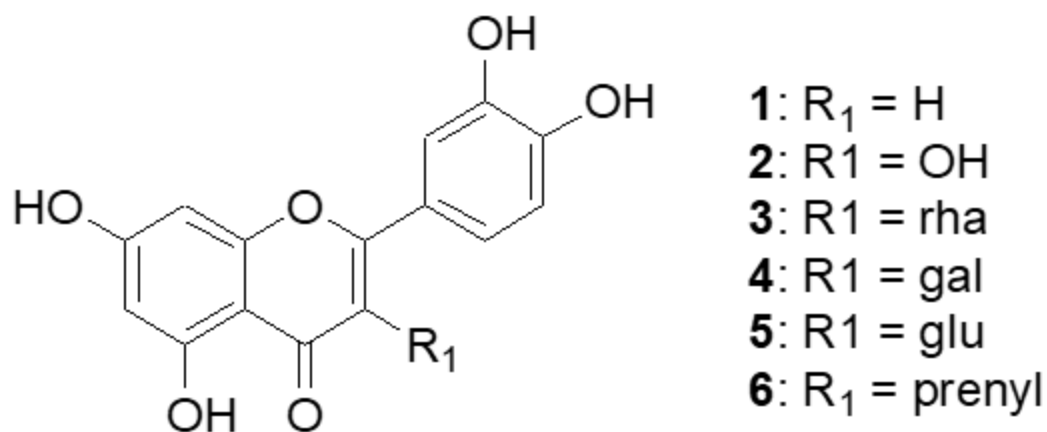


Figure 1

The structure of test compounds.

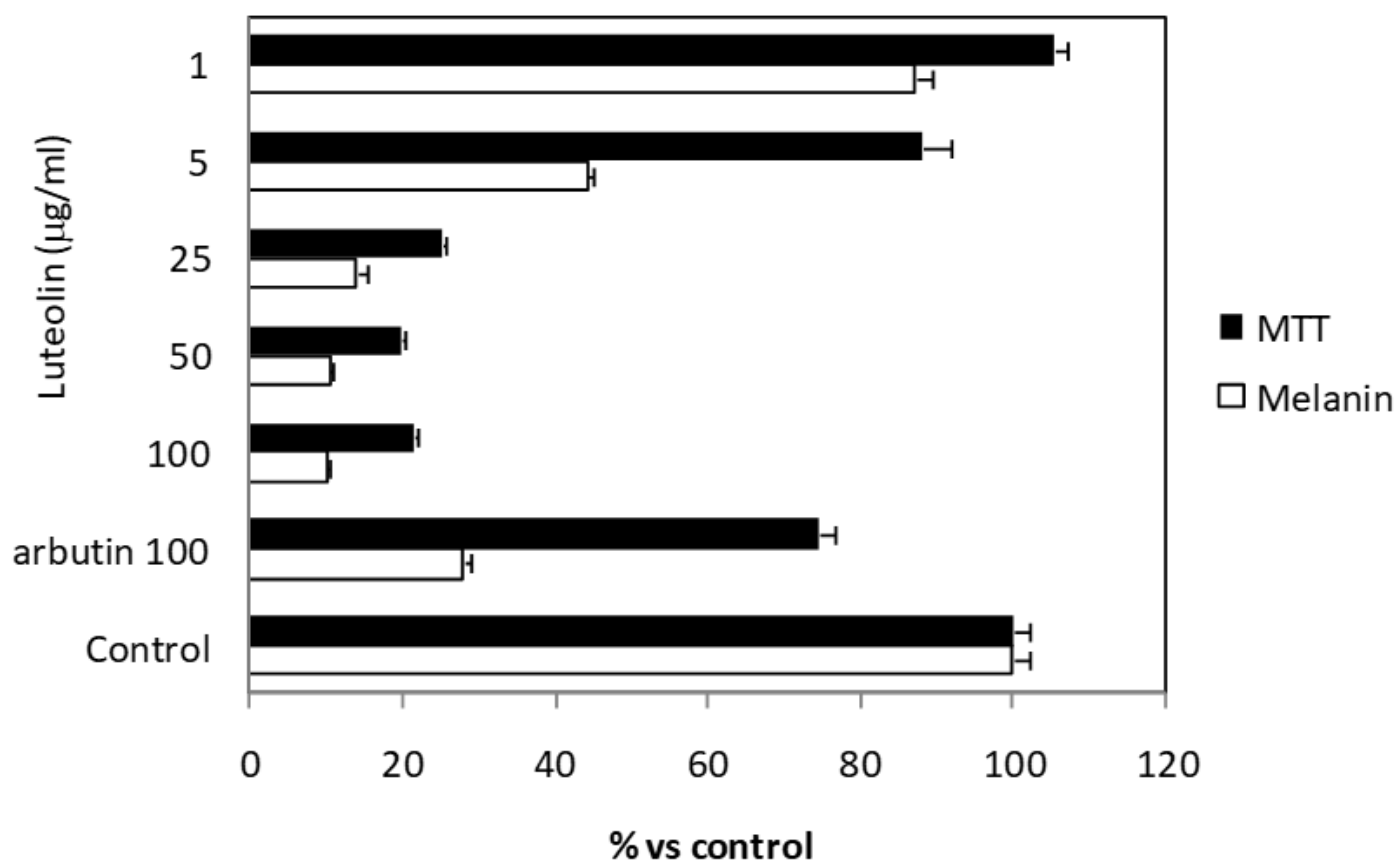


Figure 2

Antimelanogenesis effect of luteolin in B16 melanoma cells

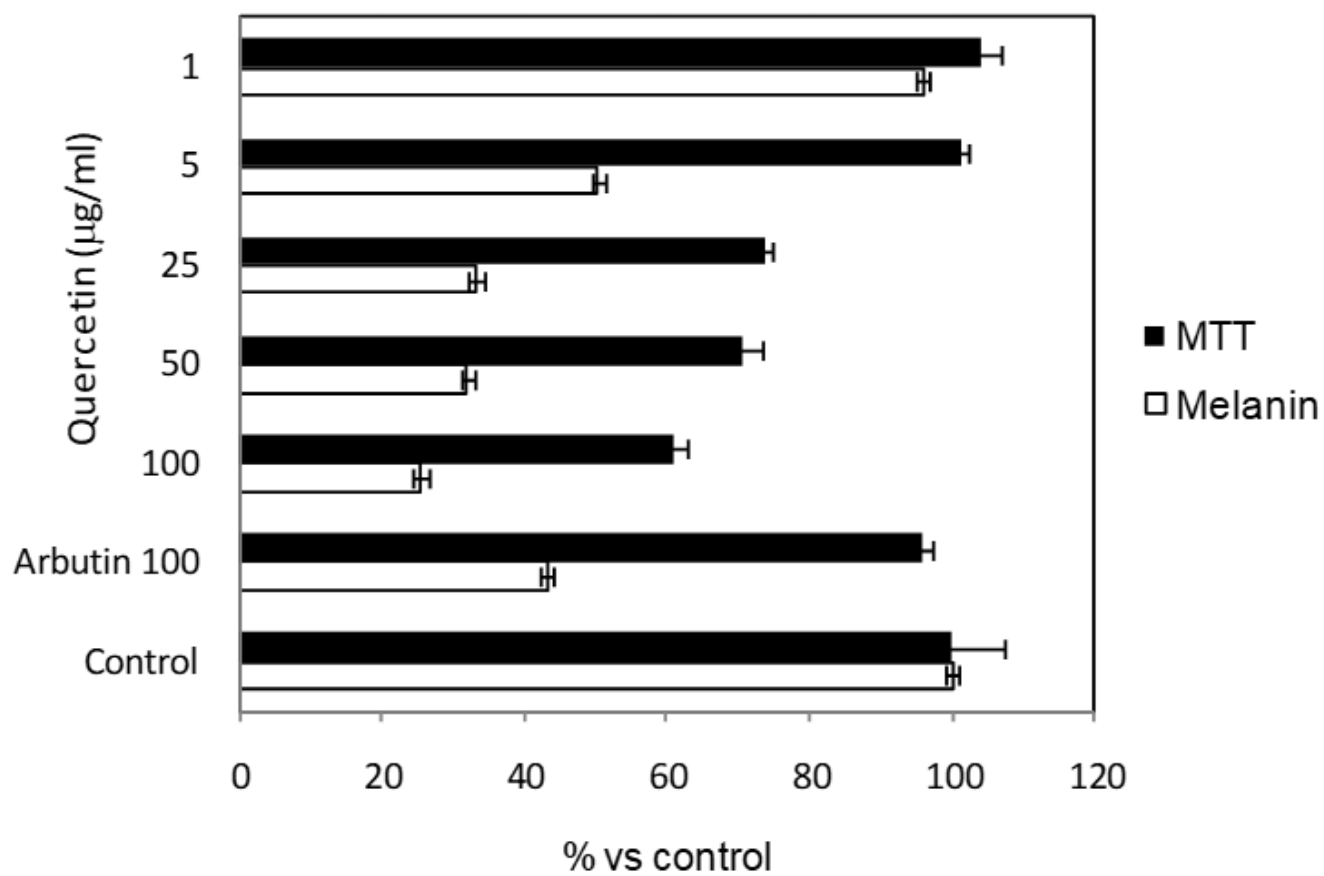


Figure 3

Antimelanogenesis effect of quercetin in B16 melanoma cells

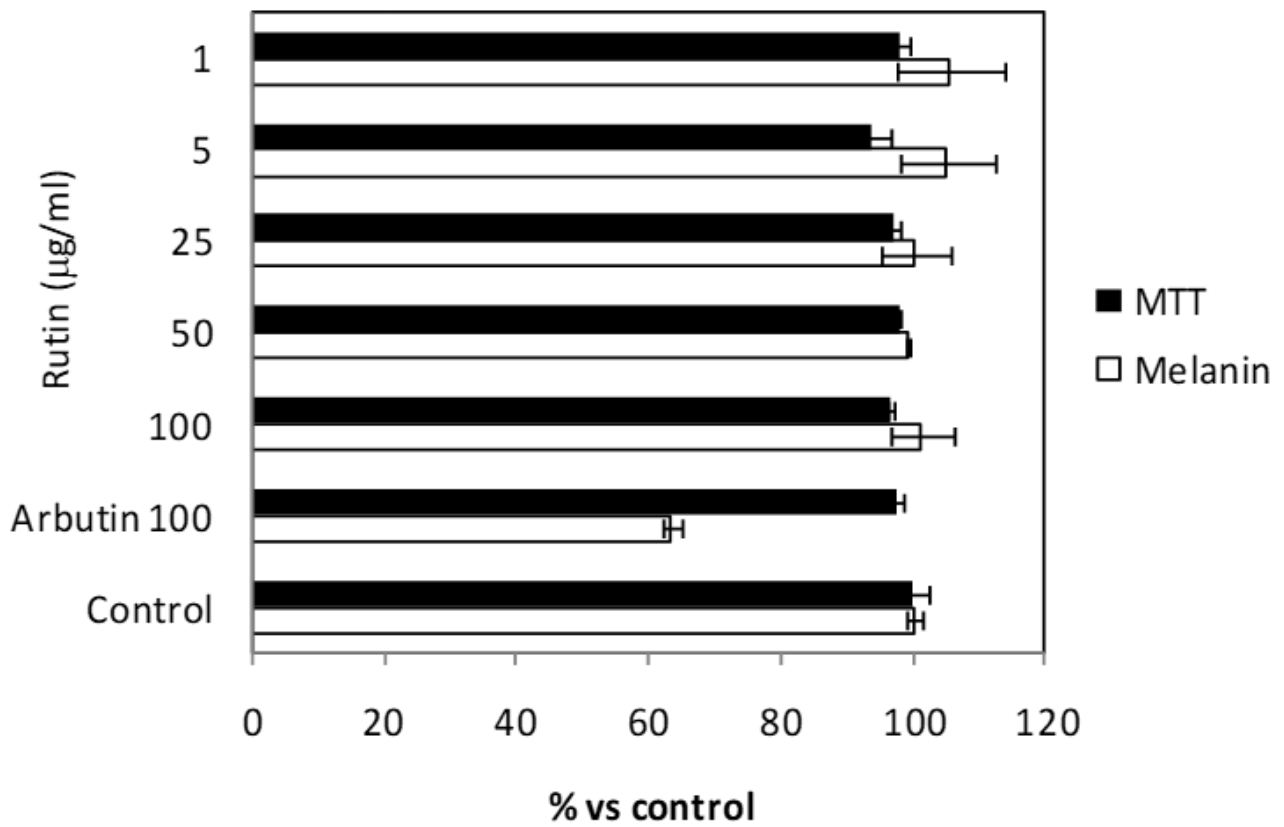


Figure 4

Antimelanogenesis effect of rutin in B16 melanoma cells

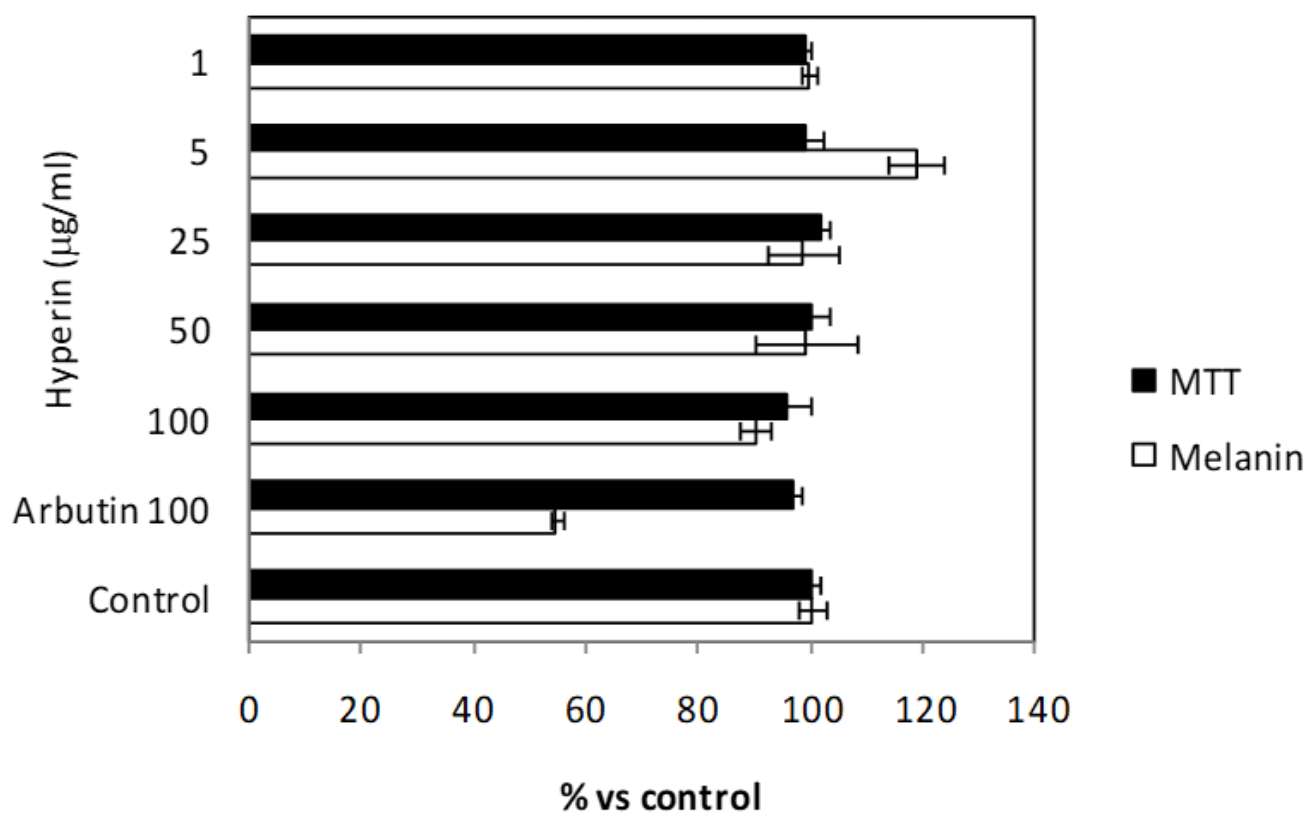


Figure 5

Antimelanogenesis effect of hyperin in B16 melanoma cells

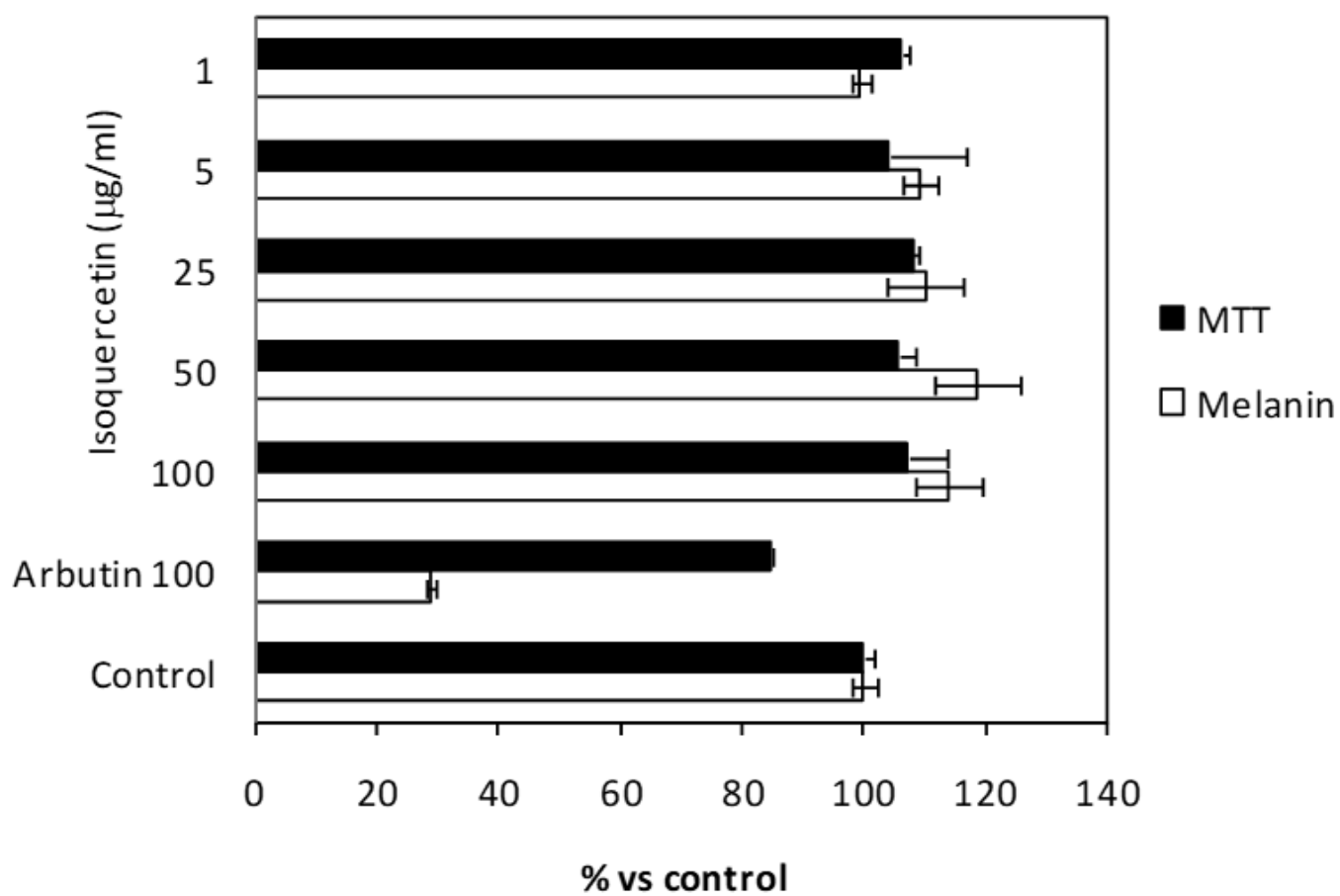


Figure 6

Antimelanogenesis effect of isoquercitrin in B16 melanoma cells

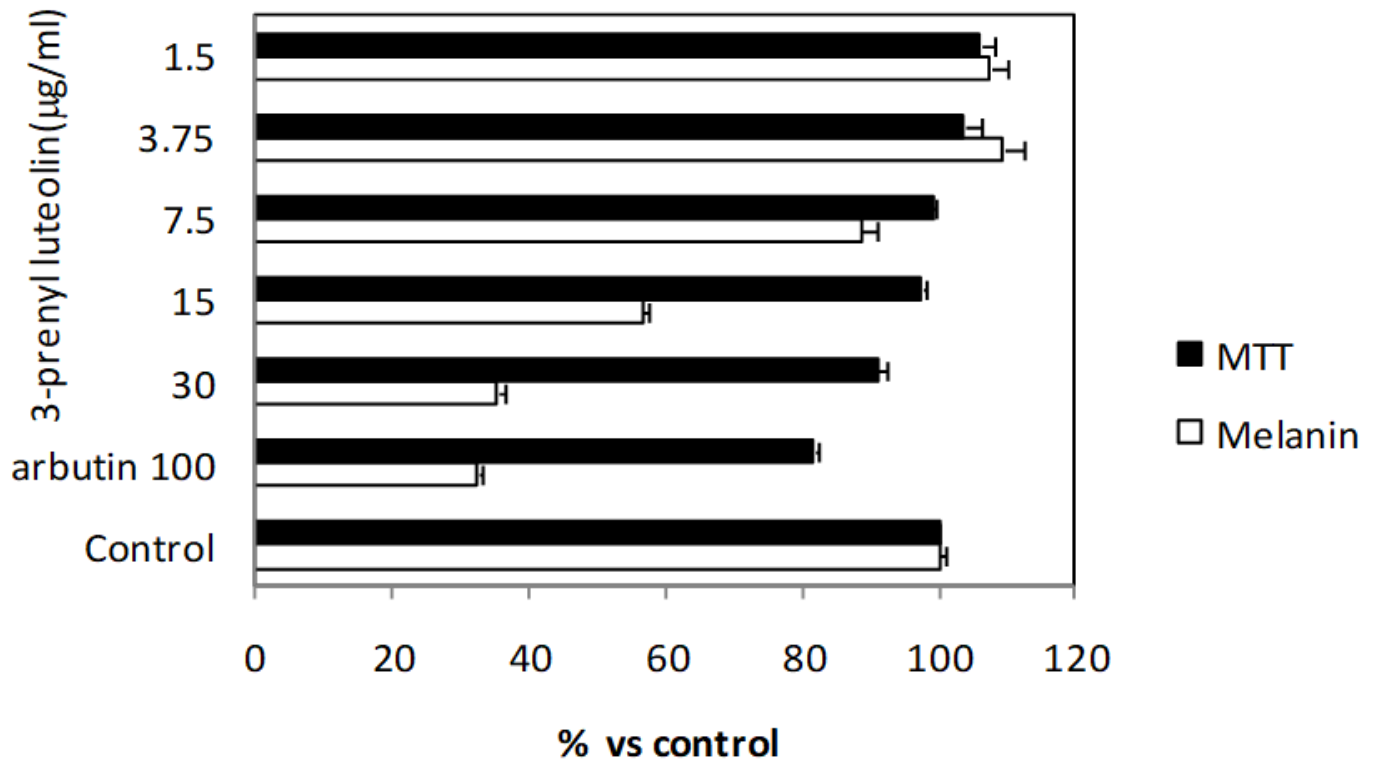


Figure 7

Antimelanogenesis effect of 3-prenyl luteolin in B16 melanoma cells

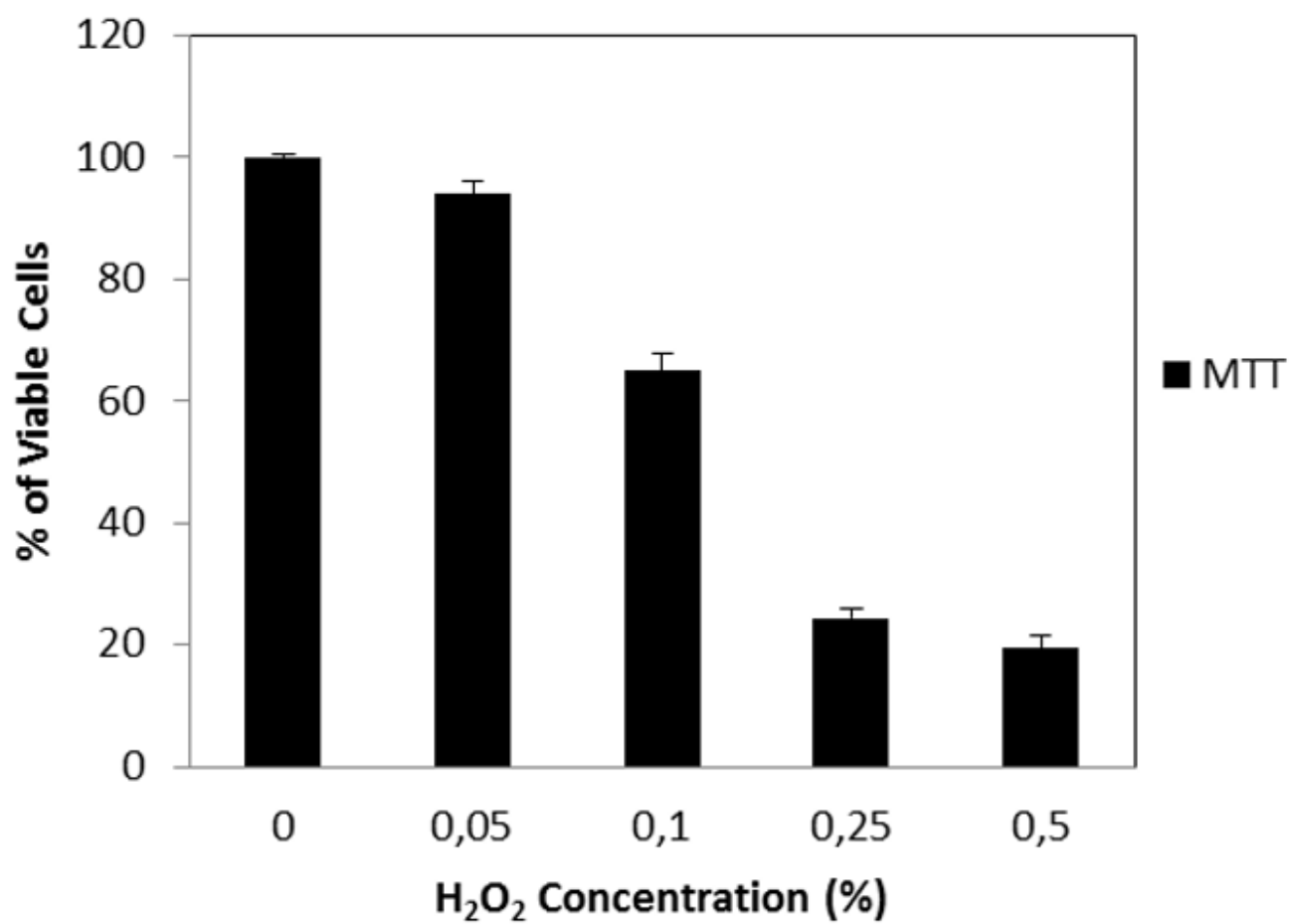


Figure 8

Effect of H_2O_2 in B16 melanoma cells

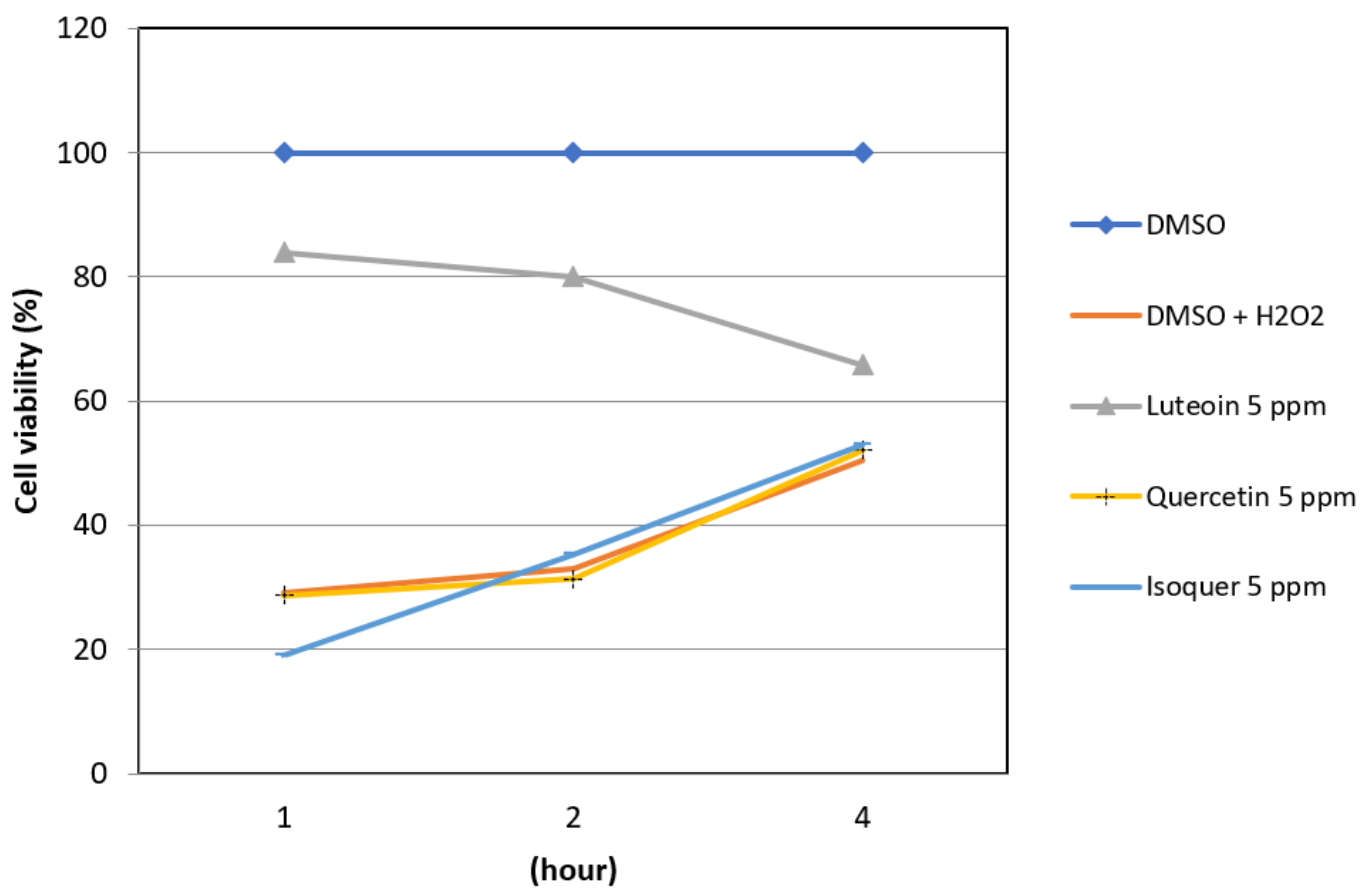


Figure 9

Effect of Quercetin related compounds in B16 melanoma cells treated with H₂O₂ (concentration : 0.25%)

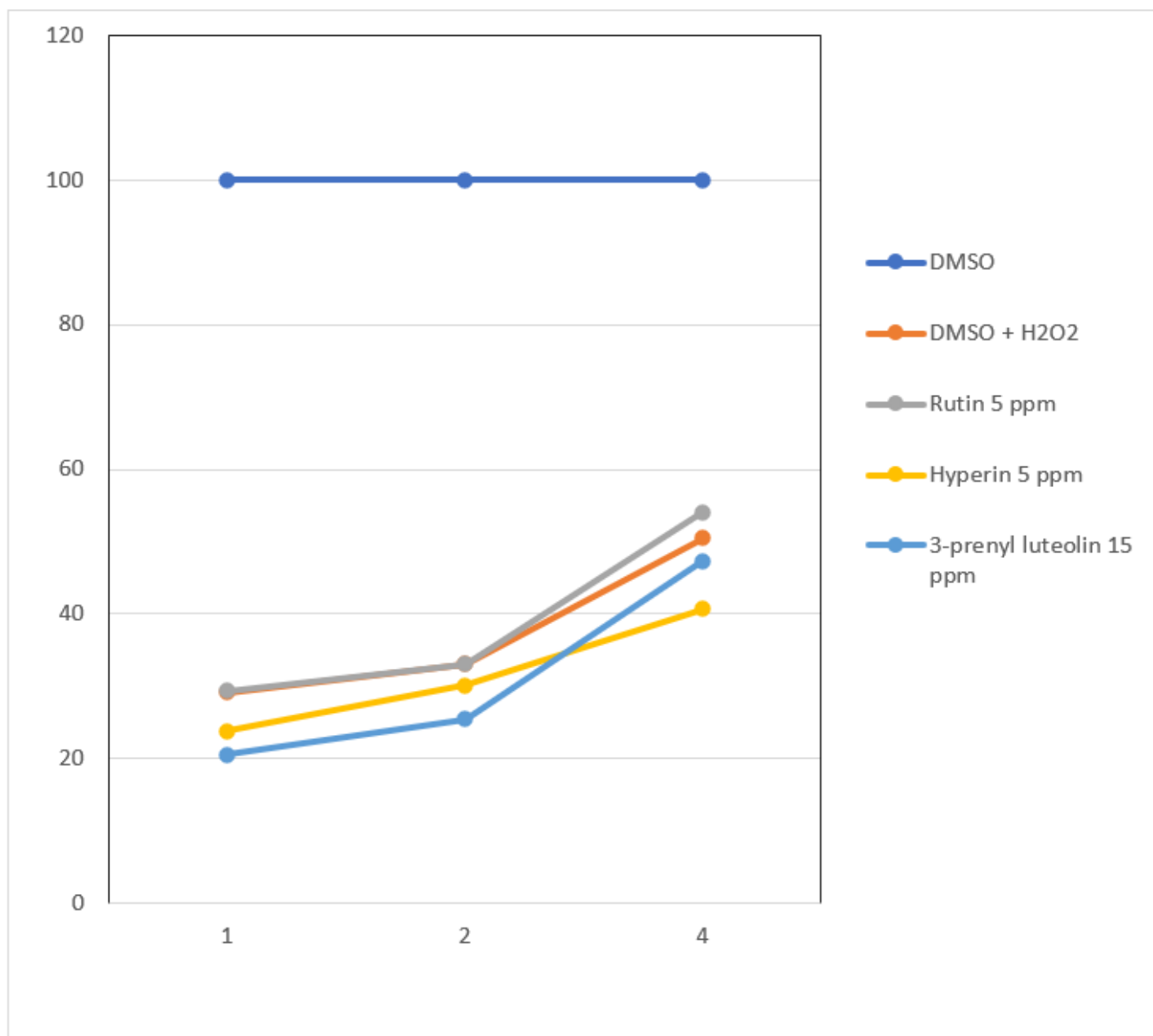


Figure 10

Effect of Quercetin related compounds in B16 melanoma cells treated with H₂O₂ (concentration : 0.25%)