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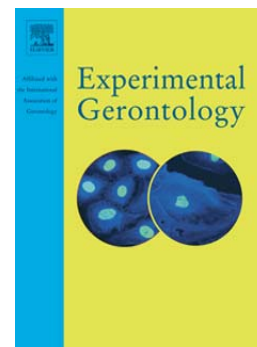
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Anti-ageing and rejuvenating effects of quercetin

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Abstract

Homeostasis is a key feature of the cellular lifespan. Its maintenance influences the rate of ageing and it is determined by several factors, including efficient proteolysis. The proteasome is the major cellular proteolytic machinery responsible for the degradation of both normal and damaged proteins. Alterations of proteasome function have been recorded in various biological phenomena including ageing and replicative senescence. Proteasome activities and function are decreased upon replicative senescence, whereas proteasome activation confers enhanced survival against oxidative stress, lifespan extension and maintenance of the young morphology for longer in human primary fibroblasts. Several natural compounds possess anti-ageing/anti-oxidant properties. In this study, we have identified quercetin (QUER) and its derivative, namely quercetin caprylate (QU-CAP) as a proteasome activator with antioxidant properties that consequently influence cellular lifespan, survival and viability of HFL-1 primary human fibroblasts. Moreover, when these compounds are supplemented to already senescent fibroblasts, a rejuvenating effect is observed. Finally, we show that these compounds promote physiological alterations when applied to cells (i.e. whitening effect). In summary, these data demonstrate the existence of naturally occurring anti-ageing products that can be effectively used through topical application.

Introduction

Free radicals are reactive molecules with one or more unpaired electron(s). Living organisms are constantly exposed to them as a consequence of aerobic life and physiology. In normal situations, the endogenous antioxidant network that comprises several primary and secondary antioxidant mechanisms provides sufficient protection against reactive species like ROS and RNS (Sen, 1995). However upon different situations an imbalance between oxidative load and anti-oxidation might appear leading to increased oxidative stress. Elevated levels of oxidative stress are related to various phenomena including ageing (Trouwakos et al., 2003).

Ageing is a natural, inevitable phenomenon that can be studied *in vivo* and *in vitro*. *In vitro* normal human fibroblasts undergo a limited number of divisions in culture and progressively they reach a state of irreversible growth arrest, a process termed as replicative senescence. Senescence is attributed to a combination of genetic and environmental factors (Petropoulou et al., 2000). Senescent fibroblasts are viable and fully functional with several alterations of morphological and biochemical characteristics as compared to their young / proliferating counterparts (Collado et al., 2007). Several cellular mechanisms malfunction upon senescence with the main secondary antioxidant mechanism, namely the proteasome, included (Chondrogianni and Gonos, 2005). The proteasome is the major cellular non-lysosomal threonine protease, implicated in the removal of normal as well as abnormal, denatured or otherwise damaged proteins (Navon and Ciechanover, 2009). The core particle, namely the 20S proteasome, is a barrel-shaped complex made of 28 subunits, 7 different α - and 7 different β - subunits, arranged as $\alpha 1-7 \beta 1-7 \beta 1-7 \alpha 1-7$ structure. The $\beta 5$, $\beta 1$ and $\beta 2$ subunits are the catalytic centers of chymotrypsin-like (CT-L), caspase-like/PGPH (peptidylglutamyl-peptide hydrolyzing) and trypsin like (T-L) activities, respectively, cleaving peptide bonds after hydrophobic, acidic and basic residues respectively (Tanaka, 1998). The 20S proteasome is also central to the ATP/ubiquitin dependent intracellular protein degradation pathway where it represents the proteolytic core of the 26S complex (Glickman and Ciechanover, 2002).

We and others (Chondrogianni and Gonos, 2005) have reported loss of proteasome function upon ageing of several human tissues as well as in senescent primary cultures. We have shown a reduction of proteasome activities and content upon replicative senescence of human embryonic fibroblast cultures (Chondrogianni et al., 2003) with an acceleration of senescence upon proteasome inhibition (Chondrogianni et al., 2008, Chondrogianni and Gonos, 2004,). Importantly, upon proteasome activation either through genetic means (Chondrogianni et al., 2005) or through natural means (Katsiki et al., 2007), a delay of senescence by approximately 15% is observed.

The biological process of ageing can be positively influenced by few environmental factors, such as natural antioxidants. Vitamin E (Packer et al., 1974), kinetin (Rattan and Clark, 1994), carnosine (McFarland and Holliday, 1994) and garlic (Svendsen et al., 1994) are few examples of natural compounds that have been shown to exert a pro-longevity effect on human primary cultures. More importantly, natural substances that possess proteasome activating properties like oleuropein (Katsiki et al., 2007) have been also shown to promote lifespan extension. Given these findings, we sought to investigate the role of QUER, a potent, natural anti-oxidant, on survival and lifespan of human fibroblasts along with its possible effects on the main secondary anti-oxidant mechanism, the proteasome.

Materials and Methods

Reagents and antibodies

QUER was purchased from Biospectrum, QU-CAP and caprylic/capric triglycerides (CAP) were obtained from BASF and dimethyl sulfoxide (DMSO) was obtained from Sigma. LLVY-AMC, MG132, as well as primary proteasomal antibodies against α_7 (PW8110; 30 kDa) and β_5 (PW8895; 23 kDa) subunits were purchased from BIOMOL. Primary antibodies against GAPDH (sc25778; 37 kDa) and secondary antibodies were purchased from Santa Cruz Biotechnology. Primary antibody against tyrosinase (#MS-800-P0, 70-80 kDa) was obtained from Thermo Scientific.

Cell lines and culture conditions

HFL-1 human embryonic fibroblasts (obtained by the European Collection of Cell Cultures) and mouse melanocytes (B10F16 cell line) were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% foetal bovine serum (v/v; Invitrogen), 2 mM glutamine, and 1% non-essential amino-acids. Cells were subcultured at 37°C, 5% CO₂ and 95% humidity, were fed approximately 16 h prior to each assay and cell number was determined in duplicates using a Coulter Z₂ counter (Beckman Coulter). HFL-1 cells were subcultured when they reached confluence at a split ratio 1:2 until they entered to senescence.

Proteasome peptidase assays

HFL-1 or B10F16 cells were seeded in petri dishes (5x10⁵ cells) and the following day they were treated with 2 µg/ml (for HFL-1 cells) or 5 µg/ml (for B10F16 cells) QUER, 0.5, 2 and 5 µg/ml (for HFL-1 cells) or 5 and 10 µg/ml (for B10F16 cells) QU-CAP or the diluents (0.1% DMSO or 0.1% CAP) for 24h. Proteins were then extracted and CT-L proteasome activity was assayed in crude extracts with the hydrolysis of the LLVY-AMC fluorogenic peptide at 37°C for 30 min, as

described previously (Chondrogianni et al., 2000). Proteasome activity was determined as the difference between the total activity of crude extracts and the remaining activity in the presence of 20 μM MG132. Fluorescence was measured using a VersaFluorTM fluorescence spectrophotometer (BioRad). Protein concentrations were determined using the Bradford method with bovine serum albumin (BSA) as standard.

Immunoblot analysis and detection of oxidized proteins

Cells were harvested at indicated time points, lysed in reducing Laemmli buffer and proteins were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to standard procedures (Harlow and Lane, 1999). Proteins were then transferred to nitrocellulose membranes for probing with appropriate antibodies. Secondary antibodies conjugated with horseradish peroxidase and enhanced chemiluminescence were used to detect the bound primary antibodies. Equal protein loading was verified by reprobing each membrane with a GAPDH antibody. Immunoblot detection of carbonyl groups into proteins (oxidized proteins) was performed with “OxyblotTM protein oxidation kit” (Chemicon International) by using an anti-DNP antibody according to manufacturer’s instructions.

Measurement of reactive oxygen species

2', 7'-dichlorodihydrofluorescein diacetate H₂DCFDA (Molecular Probes, Invitrogen) was used for the detection of reactive oxygen species (ROS). HFL-1 cells treated with 2 $\mu\text{g}/\text{ml}$ QUER, 0.5, 2 and 5 $\mu\text{g}/\text{ml}$ QU-CAP or the diluents (0.1% DMSO or 0.1% CAP) for 24 h, were resuspended in PBS \pm the dye at a final concentration of 10 μM (loading buffer) and incubated at 37°C for 30 min. The loading buffer was then removed; cells were resuspended in prewarmed complete medium and incubated at 37°C for 5 min. The absorption and the emission of the oxidation product were measured at 493 and 520 nm respectively. Each sample was measured in triplicates. In replica cultures, cells were additionally incubated with 300 μM H₂O₂ in the presence or absence of the substances for 2.5 h and cells were then collected for detection of ROS.

Continuous cell treatment with QUER or QU-CAP

Five $\times 10^4$ young HFL-1 cells were treated with 2 $\mu\text{g}/\text{ml}$ QUER, 0.5, 2 and 5 $\mu\text{g}/\text{ml}$ QU-CAP or the diluents (0.1% DMSO or 0.1% CAP) every day until they entered senescence. Upon 90% confluence, cell number was determined in triplicates using a Coulter Z₂ counter and the performed population doublings were calculated using the formula: $\log(N_f / N_0) / \log 2$, where N_f is the number of cells that were measured when each culture reached confluence and N_0

represents the number of cells that was initially seeded. Photographs of the cells maintained in cell culture and treated with the substances for the same time period were taken. For rejuvenation assay of middle-aged (cpd 59) or terminally senescent cells (cpd 65), HFL-1 fibroblasts were subcultured when they reached confluence at a split ratio 1:2 until they entered to the requested cpd in the absence of the substances and were then subjected to treatment with 2 $\mu\text{g}/\text{ml}$ QUER, 0.5, 2 and 5 $\mu\text{g}/\text{ml}$ QU-CAP or the diluents (0.1% DMSO or 0.1% CAP) for 5 days (for middle-aged cells) and for 2 weeks (for terminally senescent cells) before being photographed, assayed for β -galactosidase activity or counted using a Coulter Z2 counter (Coulter Corporation). The cells were replenished with fresh media supplemented with the substances or the diluents every 24 h.

β - Galactosidase Staining

Staining for β -galactosidase activity was performed as described previously (Dimri et al., 1995). Briefly, 10^5 cells were seeded in 6-well plates. After 24 h cells were washed with PBS, fixed in 0.2% glutaraldehyde and 2% formaldehyde for 5 min, washed again with PBS and finally stained at 37 °C for 24 h in the absence of CO_2 , in staining solution (150 mM NaCl, 2 mM MgCl_2 , 5 mM $\text{K}_3\text{Fe}(\text{CN})_6$, 40 mM citric acid, and 12 mM sodium phosphate, pH 6.0) containing 1 mg/ml 5-bromo-4-chloro-3-indolyl- β -D-galactoside.

Survival assays

Cell survival was monitored by counting the number of cells following treatment with 300 μM H_2O_2 for 2.5 h. Briefly, 4×10^5 HFL-1 cells were plated in petri dishes and 24 h later they were treated with 2 $\mu\text{g}/\text{ml}$ QUER, 0.5, 2 and 5 $\mu\text{g}/\text{ml}$ QU-CAP or the diluents (0.1% DMSO or 0.1% CAP) for additional 24 h, followed by incubation with 300 μM hydrogen peroxide (H_2O_2) for 2.5 h. Cells were left to recover for 5 days and then their numbers were scored. Cell number was determined in triplicates using a Coulter Z₂ counter.

Whitening assay

10^5 B10F16 melanocytes were seeded in petri dishes and the following day they were treated with 5 $\mu\text{g}/\text{ml}$ QUER, 5 and 10 $\mu\text{g}/\text{ml}$ QU-CAP or the diluents (0.1% DMSO or 0.1% CAP) for 72 h. The media were replenished every 24 h. Cells were then collected and pelleted and the cell pellet was photographed and quantified. The same cell pellet was then used for protein extraction and immunoblot analysis as described above.

Statistical analysis

Statistics were performed by using Microsoft Excel software. Statistical significance was evaluated using the one-way analysis of variance (ANOVA). Results at $p < 0.05$ or $p < 0.01$ are denoted in graphs by a single (*) or double (**) asterisk, respectively. All values were reported as mean of three independent experiments \pm standard deviation, unless otherwise indicated.

Results

QUER and QU-CAP possess anti-ageing and rejuvenating properties.

Initially, we aimed to reveal potential anti-ageing properties of QUER (Figure 1A shows its chemical formula). Therefore, young HFL-1 cells were continuously treated with 2 $\mu\text{g/ml}$ QUER throughout their lifespan. Although the young morphology of the QUER-treated cells was maintained for longer, QUER promoted a growth retardation of the treated cultures. Consequently, we have tested a derivative of QUER, namely QU-CAP (Figure 1B shows its chemical formula) to examine whether this retarded phenotype could be alleviated while maintaining all the positive effects of QUER. Indeed, a minimal (less than 5%) but still statistically significant increase of cellular lifespan was scored upon QU-CAP treatment as compared to their control counterparts (data not shown). Moreover, the young morphology of these cells was maintained for longer as compared to the control cultures, further demonstrating the delay in the appearance of the senescent phenotype (Fig. 2Ai & Bi). Cells treated with the compounds were more elongated, growing in parallel arrays as compared to the control cultures that exhibited a higher number of flattened cells with more irregular shape. In support, cells treated with QUER and QU-CAP exhibited a lower percentage of β -galactosidase positive staining, as compared to the DMSO- and CAP-treated cells (Fig. 2Aii & Bii). Table 1 recapitulates the percentage of β -galactosidase positive cells following constant treatment with QUER and QU-CAP for 55 days.

We were then asked whether the positive effects of QUER and QU-CAP also apply on middle-aged and terminally senescent cells. Therefore, middle-aged cells (~1 population doubling per 1 week) and terminally senescent cells (0 population doublings per 2 weeks) were treated with 2 $\mu\text{g/ml}$ QUER, 0.5, 2 and 5 $\mu\text{g/ml}$ QU-CAP or the diluents (0.1% DMSO or 0.1% CAP) for 5 consecutive days (for middle-aged cells) and for 2 weeks (for terminally senescent cells) and they were then counted. As shown in Fig. 3, both cultures exhibited a higher proliferation rate in the presence of QUER as compared to the control cultures. Regarding QU-CAP, all three concentrations promoted a higher rate of proliferation in middle-aged cells and only 2 $\mu\text{g/ml}$ QU-CAP had a significantly positive effect on proliferation of terminally senescent cells. On top of that, cells treated with the compounds exhibited a rejuvenated phenotype with more elongated

morphology and lower numbers of β -galactosidase positive cells (data not shown). Thus, QUER and QU-CAP appear to exert a positive effect on proliferation and morphology of cells of different *in vitro* ages.

Cytoprotective effects of QUER and QU-CAP.

Next, we investigated the QUER and QU-CAP effects on cellular survival following oxidative stress and consequently on the intracellular ROS levels and the oxidative protein load. As shown in Fig. 4, cells that were treated with QUER or QU-CAP exhibited higher survival rates ranging from 5.4% to 7.5% as compared to the cells that were treated with the diluents. Additionally, cells that were treated with QUER or QU-CAP and were then subjected to oxidative stress exhibited a higher survival rate reaching to 14.5% as compared to the survival rate of the relative control cultures. In support, as shown in Fig. 5, we have observed reduced levels of both ROS and oxidized proteins following cell treatment with QUER or QU-CAP for 24 h under normal conditions or increased oxidative stress via H₂O₂-treatment. To this end, QUER and QU-CAP possess cytoprotective properties against oxidative stress.

Proteasome activation by QUER and QU-CAP.

As QUER and QU-CAP have been related to mechanisms against oxidative stress, we then addressed the question whether these substances have an effect on the main secondary anti-oxidant mechanism of the cell, the proteasome. Young HFL-1 cells were treated with 2 μ g/ml QUER, 0.5, 2 and 5 μ g/ml QU-CAP or the diluents (0.1% DMSO or 0.1% CAP) for 24 h and the CT-L proteasome activity was measured. As shown in Fig. 6A, the activity was significantly induced following cell treatment with either of the substances. CT-L activity was increased from ~1.5 (for QU-CAP) to ~2.4 fold (for QUER). Moreover we have also detected enhanced protein expression levels of representative proteasome subunits in the same cells and experimental conditions (Fig. 6B), thus further verifying proteasome activation by QUER and QU-CAP.

Whitening properties of QUER and QU-CAP.

We were then asked whether this activated proteasomal pathway has physiological effects on cellular characteristics. Skin pigmentation is a characteristic that accompanies skin ageing (Ortonne, 2001). Tyrosinase, the main metalloglycoprotein that catalyzes several steps in the melanin pigment biosynthetic pathway that is mainly responsible for the age spots, has been shown to be a proteasome substrate (Ando et al., 2004), and proteasome-mediated tyrosinase degradation has been shown to promote skin de-pigmentation (Ando et al., 2004, 2009). Moreover, increasing number of studies is dealing with the identification of natural compounds

that may act as de-pigmenting agents (Smit et al., 2009). Consequently, the whitening properties of our compounds were also tested. Initially, we tested whether QUER and QU-CAP exert proteasome activating effects on melanocytes. Therefore, mouse melanocytes (B10F16 cell line) were treated with 5 µg/ml QUER or 5 and 10 µg/ml QU-CAP (we note that for this cell line and the relative assays a higher concentration of the compounds was necessary) for 24 h. As shown in Fig. 7A, CT-L activity is stimulated in melanocytes by both QUER and QU-CAP. Mouse melanocytes were then constantly treated with 5 µg/ml QUER or 5 and 10 µg/ml QU-CAP for 72 h and they were then collected, pelleted and photographed. As shown in Fig. 7B, treatment with QUER and QU-CAP revealed whitening potential. This was further verified when the protein expression levels of tyrosinase of treated cells were revealed. In the presence of QUER and QU-CAP reduced levels of tyrosinase were detected coinciding with the observed whitening effect (Fig. 7C).

Discussion

In this study, we have identified QUER and its derivative, namely QU-CAP as a potent proteasome activator with antioxidant properties that consequently influence cellular lifespan, survival and viability. Moreover, we reveal a rejuvenating effect of these compounds on middle-aged and terminally senescent primary fibroblasts. Although the anti-oxidant properties of QUER are already known, this is the first demonstration of its anti-ageing and rejuvenating effects in *in vitro* studies that link it with the proteasome and its activation.

Flavonoids are a group of antioxidants that are good candidates for antioxidant therapy. They constitute a class of naturally occurring polyphenolic compounds, ubiquitously present in photosynthesizing cells (Saito, 1974). They are present in fruits, vegetables, nuts and plant-derived beverages such as tea and wine (Kuhnau, 1976). Moreover, herb-containing medicaments and antioxidant supplements contain high doses of flavonoids. QUER is the main flavonol present in our diet. It is the aglycon form of a number of other flavonoid (flavonol) glycosides such as rutin (rhamnoglucoside of QUER –rutinoside) and quercitrin (glucoside of QUER). It is the most active flavonoid with the highest reported anti-oxidant activity and multiple mechanisms of anti-oxidative action. Natural sources rich in QUER and its derivatives, include Oak tree (*Quercus sp.*), capers (*Capparis spinosa*), red grapes (*Vitis vinifera*), apples (*Pyrus malus*), onions (*Allium cepa*), tea (*Camellia sinensis*), Citrus fruits (*Citrus sp.*), lovage, brocoli, other leafy green vegetables, cherries and many species of berries. Therefore, QUER is reported as the most consumed flavonoid present in fruits and vegetables.

QUER has been shown to be a powerful *in vitro* anti-oxidant (Hanasaki et al., 1994, van Acker et al., 1995). It is a potent scavenger of ROS and RNS, including $O_2^{\cdot-}$, NO and ONOO⁻ (Boots et al., 2008). This enhanced capacity is related to the optimal configuration of two pharmacophores within the molecule (Heijnen et al., 2002). Furthermore, various *in vitro* studies have shown that QUER possesses a wide range of pharmacologic and health promoting properties including anti-microbial (Cushnie and Lamb, 2005, Sen et al., 2007), anti-inflammatory (Orsolic et al., 2004, Read, 1995) and anti-tumorigenic/anti-proliferative (Gulati et al., 2006, Kuo, 1996, Landis-Piwowar et al., 2008, Orsolic et al., 2004, Zamin et al., 2009) effects accompanied by preventing and protecting properties against several pathological situations like heart diseases (Perez-Vizcaino et al., 2009) and cognitive dysfunctions (Singh et al., 2003). However, it is noteworthy that most of these studies have been performed in cell models of immortalized or cancer cells or in the mouse model that poses difficulties to extrapolate to the *in vivo* human situation. There are however two studies regarding the *in vivo* effect of QUER supplementation of patients suffering from diseases that are associated with oxidative stress. The first study reports the beneficial effects of QUER supplementation on the blood pressure of pre-hypertensive and stage 1 hypertensive patients (Edwards et al., 2007) while the second study examines QUER's positive effects on patients with a chronic inflammatory lung disease, sarcoidosis (Baugman et al., 2003). More *in vivo* studies regarding QUER supplementation have been performed but lack of positive effects was reported (Conquer et al., 1998, Wilms et al., 2005). This however could be explained by the fact that the latter studies involved study of healthy volunteers and not subjects under increased oxidative stress where extra anti-oxidative defense is required. Our *in vitro* results show for the first time the anti-ageing and rejuvenating results of QUER and QU-CAP in human cells and indicate the possibility of direct epidermal application in humans. Our preliminary *in vivo* studies that include topical application of QUER and QU-CAP on human volunteers in the form of emulsion enriched in these compounds, exhibit positive results as regards to elasticity, moisturization and depth of wrinkles, but further controls are needed (data not shown). Nevertheless, there are reports of other groups in the mouse model where a protective effect of topical formulations containing QUER against UVB-induced oxidative stress in hairless mice was reported (Casagrande et al., 2006). The possible explanation for the observed positive results could be that our skin is constantly under stress due to UV/light exposure.

Given the anti-oxidant properties of QUER and the link between ageing and oxidative stress (Trogakos et al., 2003), the QUER-mediated protection against ageing has been also studied. QUER has been previously shown to exert protective effects on ageing of specific tissues and functions in various mouse and cellular models. For example, upon supplementation with QUER,

enhancement of exploratory behavior, spatial learning and memory has been revealed in aged mice (Liu et al., 2006), a protective effect on immune cell functions has been shown in prematurely senescent mice (Alvarez et al., 2006), whereas a reversal of ageing and chronic ethanol-induced cognitive dysfunction (Singh et al., 2003) and a protective effect in primary neurons against Abeta(1-42) (Ansari et al., 2009) have been also reported. Additionally, QUER has been shown to increase oxidative stress resistance and longevity in *Saccharomyces cerevisiae* (Belinha et al., 2007) and to extend lifespan in *C. elegans* (Kampkötter et al., 2007, Pietsch et al., 2009, Saul et al., 2008). We are the first to present anti-ageing affects of QUER in human primary fibroblasts along with the rejuvenating effects on senescent cells.

The proteasome represents the main secondary antioxidant mechanism (Davies, 1986, Grune et al., 1997) that becomes activated upon oxidative challenge. It is responsible for the cellular clearance from damaged and oxidized proteins that may cause deleterious side effects (Friguet et al., 2000) upon lack of proteolysis and aggregation. Given that we have already shown that the proteasome can be activated either through genetic means (Chondrogianni et al., 2005) or through natural means (Katsiki et al., 2007) endowing the cells with an enhanced survival capacity against oxidative stress and an extended lifespan, we have examined whether QUER as a potent antioxidant agent may stimulate the proteasome function. We show that CT-L proteasome activity and content are induced upon QUER treatment in HFL-1 human primary cells. QUER is a known enhancer of Nrf2 transcription factor (Tanigawa et al., 2007) and Nrf2 has been shown to regulate genes forming the 26S proteasome complex (Kwak et al., 2003). Therefore, the QUER-mediated proteasome activation that we report could be mediated via the Nrf2 pathway. This could additionally explain the increased survival ability of cells following treatment with QUER and its derivatives since activation of Nrf2 pathway enhances the cellular antioxidant capacity through the transcription of a panel of antioxidant and anti-inflammatory genes (Kensler et al., 2007, Zhang, 2006). It is also worth noting that previous reports have shown inhibitory effects of QUER on CT-L proteasome activity but these studies were performed either in cell-free systems or in tumor cells and the used concentrations were constantly over 25 μ M (Chen et al., 2005) in contrast to our effective concentration that reaches 6.6 μ M (2 μ g/ml). Moreover, the same group reports the selective inhibition of proteasome activity in tumor cells but not in non-transformed cells concluding that specific flavonoids might have the potential to be developed into cancer-preventative agents that function through the mechanism of proteasome inhibition selectively in cancer over normal cells. Finally, the positive effect of QUER and QU-CAP on pigmentation that we show may be at least partially linked to proteasome activation given that a link between the ubiquitin-proteasome system and the regulation of skin pigmentation has already been revealed in

melanocytes (Ando et al., 2004, 2009). In conclusion, it is worth noting that given the multiple pathways that the proteasome is implicated along with the various functions that an anti-oxidant can affect, we have to keep in mind that proteasome stimulation might represent just one possible explanation among others for the reported anti-ageing and rejuvenating effects of QUER and QU-CAP. We believe that it is the combination of pure anti-oxidant properties with the proteasome activating properties of QUER and QU-CAP that lead to the described protective effects.

Ageing is an unavoidable biological phenomenon influenced by both genetic and environmental factors (Trogakos et al., 2003). Many attempts are made in the marathon towards healthy ageing and/or longevity. Although research has shown that genetic manipulations are usually more potent with stronger effects, they provide minimal help for future anti-ageing intervention due to obvious bioethical and practical restrains. In this regard, natural compounds exerting protective effects against diseases but also normally occurring situations like failure of homeostasis and ageing are the bet of nutraceutical but also cosmeceutical fields. QUER and its derivatives represent strong players in the anti-oxidation battle with expansion to various oxidation-related situations like diseases and ageing of various tissues, including skin while their reported rejuvenating effects place them among the runner-ups in the quest for natural anti-ageing products.

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Abbreviations

The abbreviations used are:

BSA, bovine serum albumin; CAP, caprylic/capric triglycerides; CT-L, chymotrypsin-like; DMEM, Dulbecco’s modified Eagle’s medium; DMSO, dimethyl-sulfoxide; H₂DCFDA, 2’, 7’-dichlorodihydrofluorescein diacetate; LLVY-AMC, Suc-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin; MG132, Z-Leu-Leu-Leu-H; Nrf2, Nuclear-erythroid factor 2; PBS, phosphate buffer saline; PGPH, peptidylglutamyl - peptide hydrolyzing; QU-CAP, quercetin caprylate; QUER, quercetin, RNS, reactive nitrogen species; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; T-L trypsin like.

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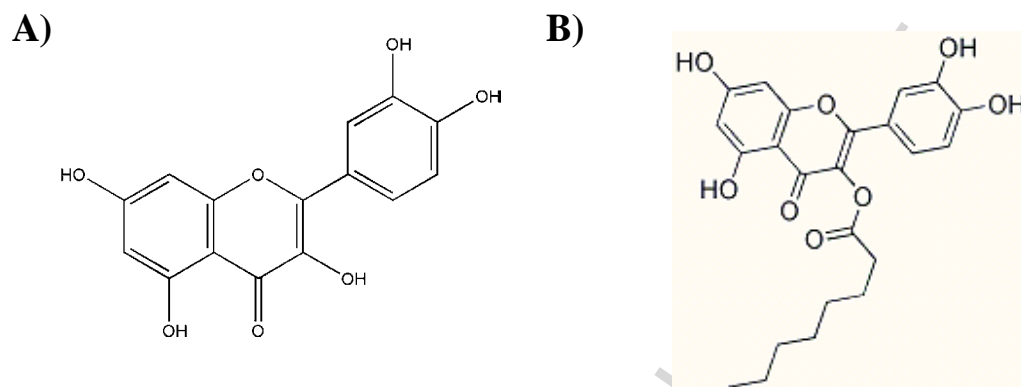
Figure legends**Figure 1: Chemical formula of A) QUER and B) QU-CAP.****Figure 1**

Figure 2: Anti-ageing effects of QUER and QU-CAP.

Photographs of (Ai, Bi) HFL-1 cells and (Aii, Bii) HFL-1 cells following staining for β -galactosidase activity that were treated with (Ai, Aii) 2 $\mu\text{g/ml}$ QUER, (Bi, Bii) 0.5, 2 and 5 $\mu\text{g/ml}$ QU-CAP or the diluents [(Ai, Aii) 0.1% DMSO or (Bi, Bii) 0.1% CAP] for the same time period (55 days of constant treatment). Arrows in control cultures show characteristic morphology of senescent cells. Cells positive for β -galactosidase activity appear in blue color.

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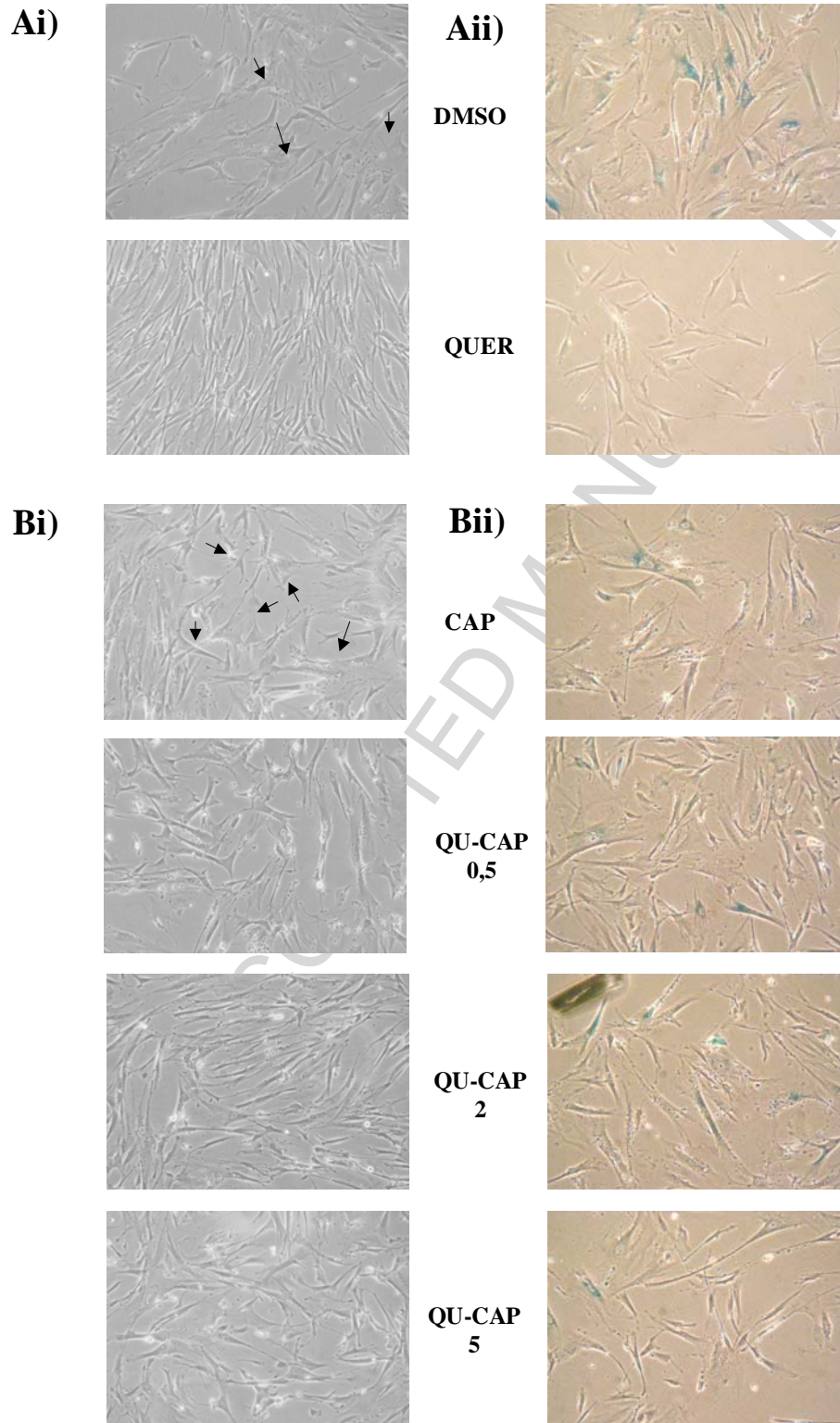


Figure 2

Figure 3: Reversal of senescent phenotype by QUER and QU-CAP.

Number of (A) middle aged cells and (B) terminally senescent cells that were counted following 5 days (for middle-aged cells) or 2 weeks (for terminally senescent cells) treatment with 2 $\mu\text{g/ml}$ QUER, 0.5, 2 and 5 $\mu\text{g/ml}$ QU-CAP or the diluents (0.1% DMSO or 0.1% CAP). Results at $p < 0.01$ are denoted in graphs by double (**) asterisks.

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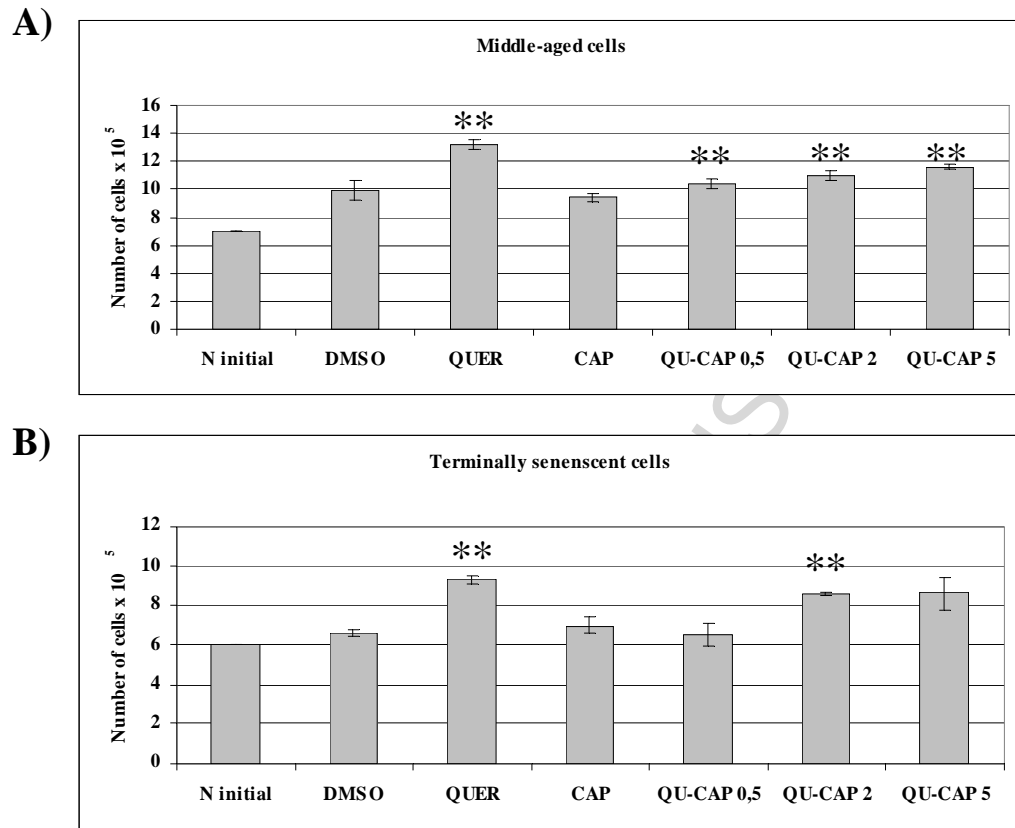


Figure 3

Figure 4: Increased survival cellular ability by QUER and QU-CAP.

Percentage of cell survival following treatment with 2 $\mu\text{g/ml}$ QUER, 0.5, 2 and 5 $\mu\text{g/ml}$ QU-CAP or the diluents (0.1% DMSO or 0.1% CAP) with (STRESS) or without (SUBSTANCE) being subjected to oxidative stress (300 μM H_2O_2 , 2.5h) and maintained for recovery for 5 days. Results at $p < 0.05$ or $p < 0.01$ are denoted in graphs by a single (*) or double (**) asterisk, respectively.

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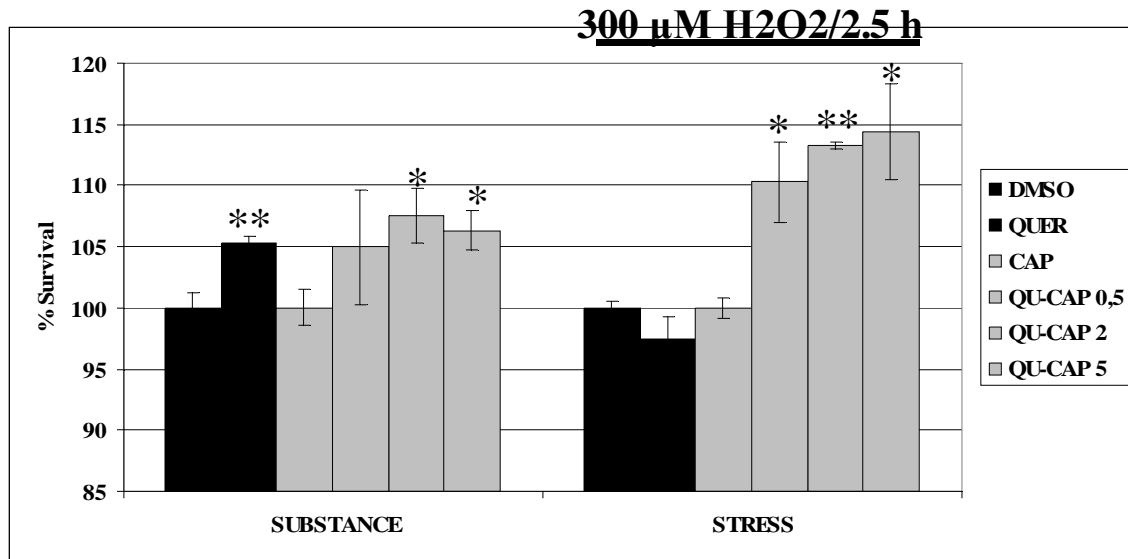


Figure 4

Figure 5: Cytoprotective effect of QUER and QU-CAP against oxidative stress.

(A) ROS levels in HFL-1 cells treated with 2 $\mu\text{g/ml}$ QUER, 0.5, 2 and 5 $\mu\text{g/ml}$ QU-CAP or the diluents (0.1% DMSO or 0.1% CAP) with (STRESS) or without (SUBSTANCE) being subjected to oxidative stress (300 μM H_2O_2 , 2.5h). Levels of ROS in DMSO- and CAP-treated cells were arbitrary set to 1. (B) Immunoblot analysis and the relative quantification of oxidized proteins in HFL-1 cells treated with 2 $\mu\text{g/ml}$ QUER, 0.5, 2 and 5 $\mu\text{g/ml}$ QU-CAP or the diluents (0.1% DMSO or 0.1% CAP). GAPDH levels were used as loading control. Results at $p < 0.05$ or $p < 0.01$ are denoted in graphs by a single (*) or double (**) asterisk, respectively.

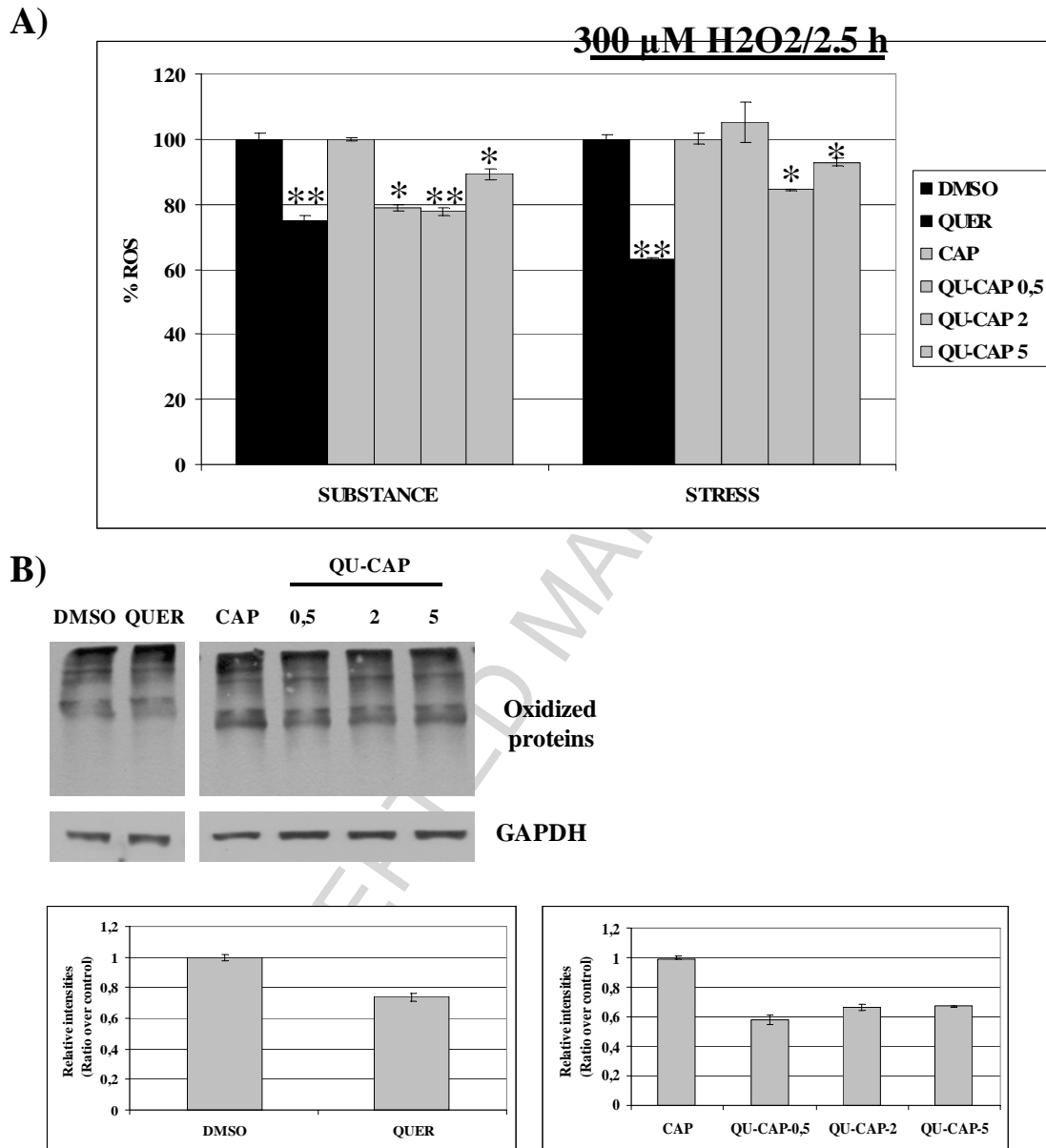
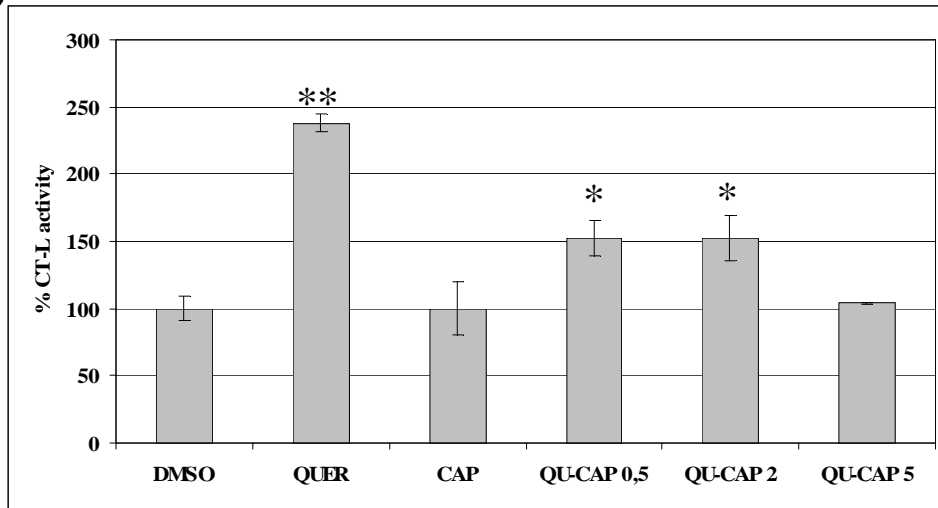


Figure 5

Figure 6: Proteasome activation following QUER and QU-CAP treatment.

(A) Levels of CT-L proteasome activity in HFL-1 cells treated with 2 $\mu\text{g/ml}$ QUER, 0.5, 2 and 5 $\mu\text{g/ml}$ QU-CAP or the diluents (0.1% DMSO or 0.1% CAP) for 24 h. Recorded levels in DMSO- and CAP-treated cells were arbitrary set to 100%. Results at $p < 0.05$ or $p < 0.01$ are denoted in graphs by a single (*) or double (**) asterisk, respectively. (B) Immunoblot analysis and the relative quantification of β_5 and α_7 proteasome subunits in HFL-1 cells treated with 2 $\mu\text{g/ml}$ QUER, 0.5, 2 and 5 $\mu\text{g/ml}$ QU-CAP or the diluents (0.1% DMSO or 0.1% CAP) for 24 h. GAPDH levels were used as loading control.

A)



QU-CAP

B)

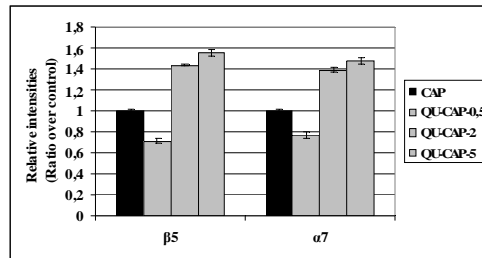
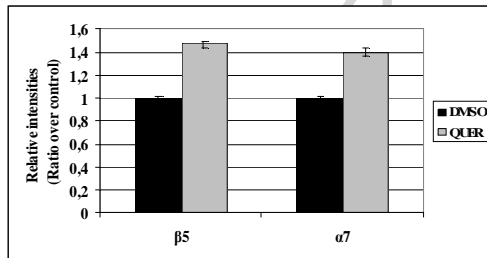
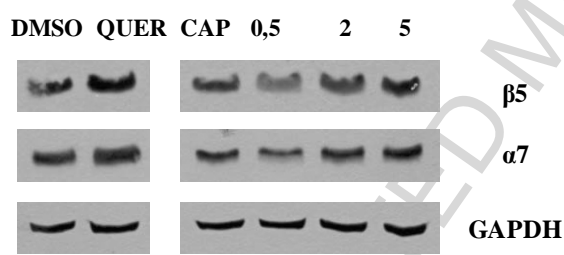


Figure 6

Figure 7: Cellular whitening of melanocytes following QUER and QU-CAP treatment.

(A) Levels of CT-L proteasome activity in B10F16 melanocytes treated with 5 µg/ml QUER, 5 and 10 µg/ml QU-CAP or the diluents (0.1% DMSO or 0.1% CAP) for 24 h. Recorded levels in DMSO- and CAP-treated cells were arbitrary set to 100%. Results at $p < 0.01$ are denoted in graphs by double (**) asterisks. (B) Photograph of the pellet and the relative quantification of melanocytes treated with (i) 5 µg/ml QUER and (ii) 5 and 10 µg/ml QU-CAP or the diluents [(Bi) 0.1% DMSO or (Bii) 0.1% CAP] for 72 h. (C) Immunoblot analysis and the relative quantification of tyrosinase in HFL-1 cells treated with 5 µg/ml QUER or 5 and 10 µg/ml QU-CAP or the diluents (0.1% DMSO or 0.1% CAP) for 24 h. GAPDH levels were used as loading control.

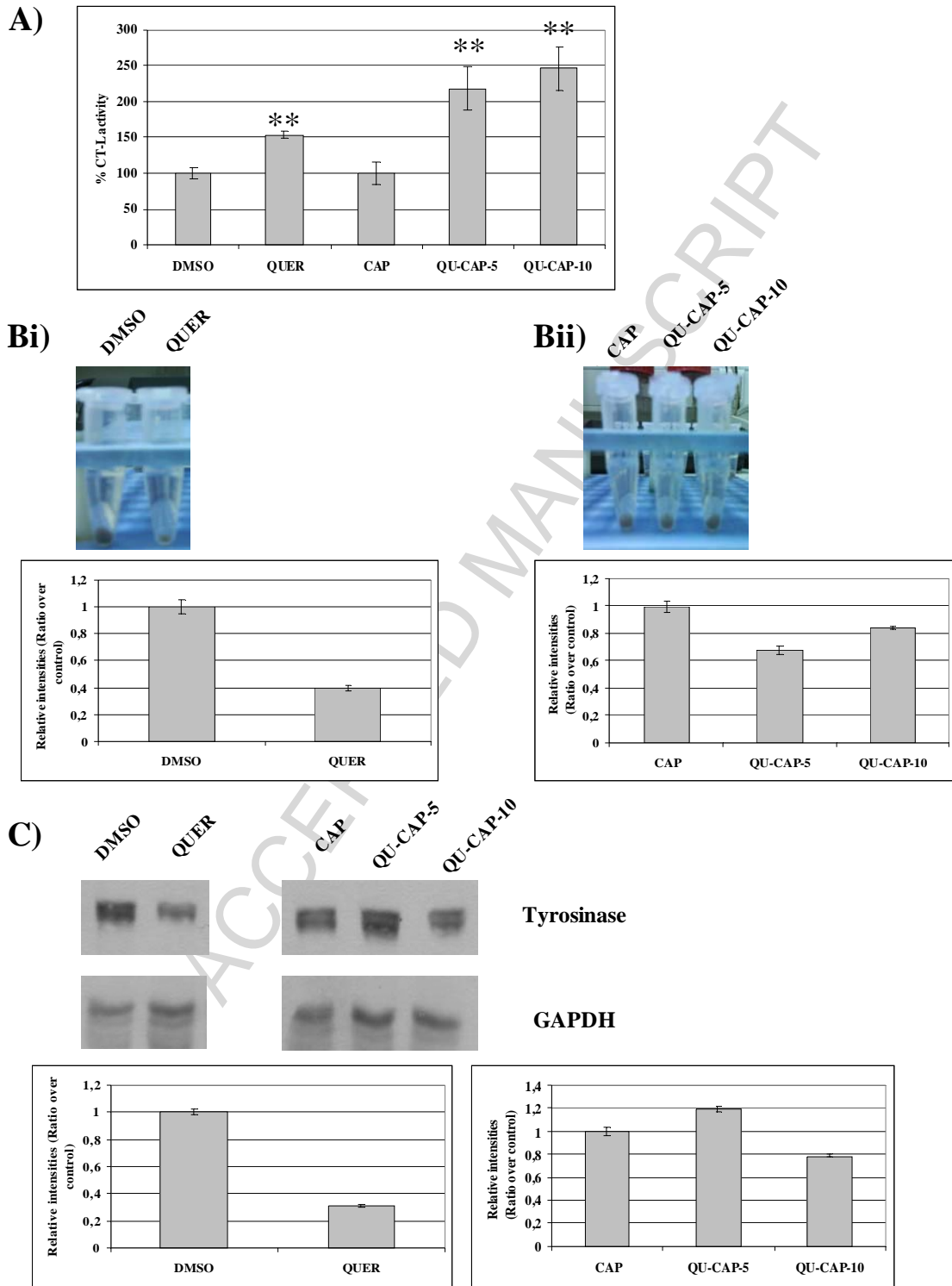


Figure 7

Table 1: Maintenance of young morphology following treatment with QUER and QU-CAP.

Percentage of β -galactosidase positive HFL-1 cells treated with 2 $\mu\text{g/ml}$ QUER, 0.5, 2 and 5 $\mu\text{g/ml}$ QU-CAP or the diluents (0.1% DMSO or 0.1% CAP) for the same time period (55 days of constant treatment). The performed cpds at this time point are also stated.

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SUBSTANCE	cpd	% β -galactosidase positive cells
DMSO	64,1	77
QUER	54,4	13,5
CAP	62,9	75
QU-CAP 0,5 μ g/ml	65,1	36,6
QU-CAP 2 μ g/ml	64,9	32,3
QU-CAP 5 μ g/ml	64	47

Table 1