



Depigmenting effect of Xanthohumol from hop extract in MNT-1 human melanoma cells and normal human melanocytes

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ABSTRACT

Xanthohumol (XH) is the most abundant prenylated flavonoid found in the hop plant (*Humulus lupulus L.*) and has previously been shown to have depigmenting effects in B16F10 mouse melanoma cells; however, studies of its depigmenting efficacy in human melanocytes are still lacking. In this work, we explored the effects of XH on melanogenesis in MNT-1 human melanoma cells and normal human melanocytes from darkly-pigmented skin (HEM-DP). XH was screened for cytotoxicity over 48 h, and subsequently tested on melanogenesis in MNT-1 cells. XH was further tested in HEM-DP cells for melanin synthesis and melanosome export; dendricity was quantitated to assess effects on melanosome export. Melanosome degradation was studied in human keratinocytes (HaCaT). Our results showed that XH inhibited melanin synthesis in MNT-1 cells at 30 μ M but increased intracellular tyrosinase activity without affecting ROS levels. In HEM-DP cells, XH robustly suppressed cellular tyrosinase activity at nontoxic concentrations (2.5–5 μ M) without any effect on melanin synthesis. However, XH inhibited melanosome export by reducing dendrite number and total dendrite length. Further testing in HaCaT cells demonstrated that XH induced melanosome degradation at low micromolar concentrations without any cytotoxicity. In summary, our results demonstrate that XH at low micromolar concentrations might hold promise as a potent inhibitor of human pigmentation by primarily targeting melanin export and melanin degradation. Further studies to elucidate the signaling mechanisms of action of melanosome export inhibition by XH and *in vivo* efficacy are warranted.

1. Introduction

Ultraviolet radiation (UVR) activates the central neuroendocrine system to maintain body homeostasis and also induces upregulation of synthesis of melanin pigment [1]. The pigment melanin in the skin provides UV photoprotection, quenches free radicals, and chelates toxic ions, however an aberrant overproduction of melanin causes disorders of hyperpigmentation such as age spots, freckles, melasma, drug-induced pigmentation and post-inflammatory hyperpigmentation (PIH) [2,3]. These affect social life and pose cosmetic concerns, especially for female populations who desire a flawless complexion. Melanin is synthesized by neural-crest derived cells -melanocytes located at the basement membrane of the skin; inside melanocytes the pigment is synthesized in specialized organelles called melanosomes. Post-synthesis, melanin is exported from the melanocyte dendrite tips into keratinocytes; these cells then migrate towards the upper layers of

skin laden with melanosomes, causing the visual appearance of skin pigmentation [4]. Tyrosinase, a glycoprotein, is the key rate-limiting enzyme which catalyzes two early steps in the synthesis of melanin; it catalyzes the conversion of L-Tyrosine to L-DOPA, which is oxidized to Dopaquinone and polymerized to melanin [5]. In melanocytes, L-Tyrosine is transported into melanosomes from the extracellular space and initiates the melanogenic pathway *in vivo* [6]. Apart from regulating melanogenic pathway, L-Tyrosine and L-DOPA are also known to regulate cellular functions in a direct or indirect manner via non-receptor mediated processes [7].

Commercially used skin-whitening agents, such as hydroquinone, arbutin, and kojic acid have exhibited serious side-effects [8,9] and are no longer deemed safe. Additionally, rhododendrol, a natural skin-whitening cosmetic was recalled after it caused adverse reactions of leukoderma and vitiligo in the Japanese population [10]. Recently, natural bioactives have emerged which do not exhibit these serious side-effects; there have been reports of melanogenesis inhibition by

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List of abbreviations

XH	Xanthohumol
DMSO	Dimethyl Sulfoxide;
L-DOPA	3,4-Dihydroxy-L-phenylalanine;
DMEM	Dulbecco's Modified Eagle's Medium
PBS	Phosphate Buffered Saline;
MEM	Minimum Essential Medium
MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)
BCA	Bicinchoninic Acid
HI-FBS	Heat-Inactivated Fetal Bovine Serum
HMGs	Human Melanocyte Growth Supplement
DP	Darkly-Pigmented
TDL	Total Dendrite Length
PNG	P-Nitrophenyl- α -D-glucopyranoside;
ROS	Reactive Oxygen Species
HBSS	Hank's Buffered Salt Saline
DCF	Dichlorofluorescein

many natural product extracts or purified bioactives. Moreover, since the deadly skin cancer, melanoma, is characterized by a high melanin content [11,12], the identification of a natural compound that may inhibit melanogenesis is also significant as such compounds could be used as adjuvants in the therapy of melanotic melanomas. Melanogenesis inhibitors are not only limited to compounds which reduce melanin synthesis in cells, but also compounds which can suppress melanosome export, as after being synthesized in the cells, melanin is secreted from melanocytes and phagocytosed by keratinocytes via various pathways [13]. The traditional tyrosinase-based inhibitors of melanogenesis which function by reducing melanin accumulation in the cells are not very appealing, partly due to a rise in the search of novel compounds which can target different steps in melanogenesis pathway, in particular,

melanosome export. Furthermore, it has been documented that post-transfer, melanosomes are degraded in keratinocytes; melanosomes in keratinocytes from lightly-pigmented skin are degraded faster as compared to those from darkly-pigmented skin [14]. This has also spurred research into cosmetic agents which can target melanosome degradation in keratinocytes.

Xanthohumol (XH; chemical structure shown in Fig. 1A) is a bioactive prenylated flavonoid derived from the female inflorescence of the hop plant (*Humulus lupulus L.*) and constitutes up to 0.1–1% of dry weight of hops [15]. XH has been shown to exhibit a broad spectrum of biological activities such as anticancer activity [16], antibacterial activity [17], and anti-inflammatory activity; it inhibits both elastase and matrix metalloproteinase (MMP) activity [18]. The biological benefits of XH on skin health have been reviewed previously [19]. XH showed neuroprotective effects in ischemia-reperfusion injury model [20] and has recently demonstrated anti-Alzheimer's activity by inhibiting aggregation of amyloid- β [21] and tau proteins [22]. Furthermore, XH-enriched functional foods and extracts can reach pharmacologically relevant concentrations [23] and XH has been demonstrated to have a well-tolerated safety profile for *in vivo* consumption as reported in a previous study [24].

Previous studies have reported that XH inhibited mushroom tyrosinase activity in an acellular system [25] and inhibited melanin synthesis in B16F10 mouse melanoma cells. The authors concluded that XH is a skin-whitening agent [26]. However, in the latter study, the effect of XH on melanosome export was never reported. Moreover, at this time there are no reports if XH can inhibit melanogenesis in human melanocytes. Hence, in this work, we have studied the effects of XH on melanogenesis in MNT-1 human melanoma cells and primary human melanocytes and delineated a novel mechanism of anti-melanogenic activity of this compound at low micromolar concentrations. Our findings support the potential of XH as a skin-lightening agent for diminution of human melanogenesis.

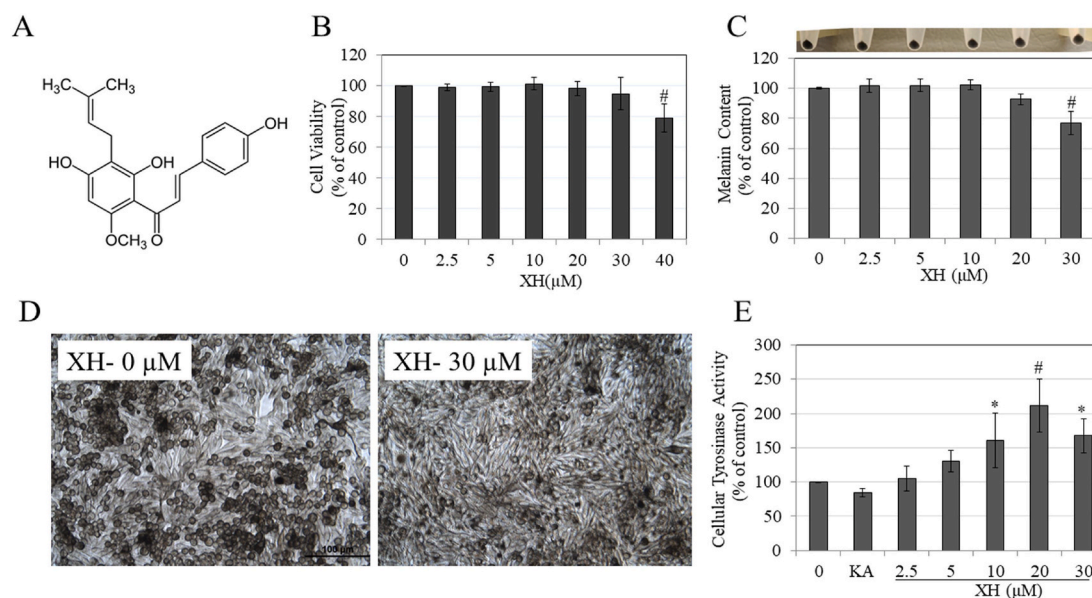


Fig. 1. A) Chemical structure of XH; B) Viability of MNT-1 human melanoma cells treated with XH at different concentrations for 48 h; #p < 0.01 vs. control; One-Way ANOVA with Dunnett's test; C) Melanin assay in MNT-1 human cells treated with XH for 48 h with corresponding panel showing photos of cell pellets; #p < 0.01 vs. control; One-Way ANOVA with Dunnett's test; D) Representative bright-field micrographs showing control and XH (30 μM) group. Scale bar corresponds to 100 μm; E) Cellular tyrosinase activity in lysates of MNT-1 cells treated with XH (0–30 μM) and Kojic acid (KA) at 1 mM as a positive control; *p < 0.05; #p < 0.0001 vs. control; One-Way ANOVA with Tukey's test; Data is Mean ± SD of at least three independent experiments for all except E) which is Mean ± SD of four independent experiments.

2. Materials and methods

2.1. Materials

XH (>98% purity) were purchased from Enzo Life Sciences (NY, USA). Tyrosinase from mushroom (Cat#: T3824-25KU), L-DOPA (Cat#: D9628) and synthetic melanin (Cat#: M8631) were purchased from Sigma-Aldrich (St. Louis, MO). 2', 7'-dichlorodihydrofluorescein diacetate (H2DCF-DA) reagent was purchased from Molecular Probes.

2.2. Cell culture

MNT-1 human melanoma cells were obtained as a kind gift from Dr. Michael Marks, University of Pennsylvania, and were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 18% heat-inactivated fetal bovine serum (HI-FBS), 1% minimum essential medium (MEM), 1% antibiotics (penicillin and streptomycin), and 10% AIM-V media (Invitrogen). Human primary epidermal melanocytes from darkly-pigmented (HEM-DP) neonatal donor was obtained from Cascade Biologics (Portland, OR) and were cultured in Medium 254 (Cascade Biologics) supplemented with 1% human melanocyte growth supplement (HMGS, Cascade Biologics) and 1% antibiotics. Human keratinocytes (HaCaT) were purchased from AddexBio (San Diego, CA) and cultured in DMEM medium supplemented with 1% antibiotics and 10% HI-FBS. All cells were cultured at 37 °C in a humidified atmosphere with 95% air and 5% CO₂.

2.3. Cytotoxicity assay in MNT-1 human melanoma cells

In order to test the effects of XH on melanogenesis, we first screened nontoxic concentrations of the compounds using MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) cytotoxicity assay. Briefly, MNT-1 cells (3×10^4 cells/well) were cultured in a 96-well plate for 24 h at 37 °C in 5% CO₂ incubator. After 24 h, the medium was replaced by fresh medium containing XH at various concentrations, and further maintained for 48 h. At the end of 48 h, the culture medium was aspirated and replaced by 100 µl of fresh medium with 20 µl of MTS reagent and incubated for 1 h. After that, the absorbance was read at 490 nm using a Versamax® microplate reader and cell viability was calculated from the absorbance values relative to control groups and expressed in %.

2.4. Melanin assay in MNT-1 cells

MNT-1 cells (2.3×10^5 cells/well) were seeded in 12-well plates and cultured for 48 h after which XH was added and cultures maintained for another 48 h. At the end of the treatments, cells were harvested, and cell pellets were washed in PBS. After aspiration, 250 µL of 1 N NaOH was added and heated to 70 °C for 45 min to solubilize melanin. Next, 200 µl aliquots of lysate were transferred to a 96-well plate and the absorbance was read at 475 nm using a microplate reader. A portion of the lysate was used to evaluate total protein content using BCA assay (Pierce BCA kit, Thermo Scientific). The absorbance was normalized to total protein contents and reported as relative melanin levels as % of control.

2.5. Tyrosinase assay in MNT-1 cells

We quantitated the cellular tyrosinase activity as L-DOPA oxidase activity which involves the conversion of DOPA to Dopachrome based on established method [2]. Briefly, MNT-1 cells were seeded in 12-well plates at a density of 2.3×10^5 cells/well. After 48 h, the medium was changed and XH was added, and further incubated for 48 h. At the end of treatments, cells were trypsinized and cell pellets were washed in PBS and lysed. 50 µl of lysates were then aliquoted in a 96-well microplate and 100 µl of 3 mM L-DOPA solution (freshly prepared in phosphate buffer, pH 6.5) was added. The absorbance was measured at 475 nm in

the kinetic mode every 30 s for 40 min at 30 °C using a microplate reader. The % tyrosinase activity was calculated from the slope of the linear range of the velocities of inhibition and was normalized by the total protein contents assayed by BCA kit.

2.6. Intracellular α -glucosidase activity in MNT-1 cells

Previous reports have established that the activity of α -glucosidase enzyme regulates the early stages of tyrosinase maturation [27–29]. In order to test if XH's anti-melanogenic activity might be explained, at least in part, by the inhibition of α -glucosidase activity, MNT-1 cellular lysates were tested with p-nitrophenyl- α -D-glucopyranoside (PNG) substrate. This substrate reacts with α -glucosidase enzyme to produce p-nitrophenol which can be measured spectrophotometrically. MNT-1 cells (4.5×10^5 cells/well) were grown in 6-well plates for 48 h followed by addition of XH at various concentrations and incubated for a further 48 h. Cells were harvested, lysed and 50 µl of lysates were aliquoted in a 96-well plate and 100 µl of 2 mM PNG substrate was added. The rate of the formation of the reaction product was monitored at 405 nm in the kinetic mode for 45 min at 37 °C in a microplate reader. The intracellular α -glucosidase activity (%) was calculated as: (rate of sample reaction/rate of control reaction) \times 100%.

2.7. Cellular ROS generation in MNT-1 cells

We next evaluated ROS production in MNT-1 cells treated with XH to test if reduced melanin production might be explained by a reduction in ROS generation since melanogenesis is accompanied by increased ROS generation and oxidative stress [30]. For this, we used the nonfluorescent H₂DCFDA probe which is converted to highly fluorescent 2', 7'-dichlorofluorescein (DCF) upon oxidation by ROS. Briefly, MNT-1 cells (1×10^5 cells/well) were cultured in a 24-well plate for 48 h followed by replacement of medium containing XH and incubated for another 48 h. At the end of treatment, the medium was aspirated, cells were washed with HBSS buffer and then incubated in 50 µM DCFH-DA dye solution (prepared in serum-free, pyruvate-free, phenol-red free DMEM medium) for 45 min at 37 °C. Subsequently, the medium was aspirated, cells washed in buffer and the plate was read in a fluorescence microplate reader at excitation/emission of 485/535 nm using the well-scan mode. Results are reported as % relative fluorescence intensity.

2.8. Cytotoxicity in HEM-DP cells

MTS assay was used to screen XH for cytotoxicity in HEM-DP cells. Briefly, 3.2×10^4 cells/well were inoculated in a 48-well plate for 24 h and then XH was added and cultures were maintained for 48 h. MTS assay was conducted similar to method reported earlier, and cell viability reported as % normalized to negative control after blank subtractions.

2.9. Melanin and tyrosinase activity assay in HEM-DP cells

We next assayed the effects of XH on melanin synthesis levels and intracellular tyrosinase activity in HEM-DP cells similar to the method outlined before. For melanin assay, HEM-DP cells (1.9×10^5 cells/well) were seeded in a 12-well plate and cultured for 48 h followed by addition of XH and cultured maintained for 48 h. Cells were harvested, washed, and solubilized in 200 µl of NaOH at 70 °C and then 150 µl were aliquoted in a 96-well plate and absorbance was read at 475 nm; the absorbance was normalized to the total protein content and reported as % of control.

For assaying cellular tyrosinase activity, HEM-DP cells were cultured in 12-well plates (1.3×10^5 cells/well) and after 48 h, the medium was replaced with fresh medium containing various concentrations of XH and cultured for another 48 h. After the treatment, the cells were

harvested, lysed, and 25 μ l of lysates were incubated with 75 μ l of 3 mM L-DOPA and the absorbance was measured at 475 nm for 20 min (with reading every 30 s at 30 °C) using a microplate reader. The % tyrosinase activity was calculated from the slope of the linear range of the velocities of inhibition and was normalized to the total protein contents assayed by BCA kit.

2.10. Dendricity analysis in HEM-DP cells

Melanocyte cytosolic projections known as dendrites are critical conduits for pigment export to keratinocytes and quantification of dendricity parameters were based on the parameters reported previously [31]. HEM-DP cells were cultured in 12-well plates at 5×10^4 cells/well for 24 h and then XH were added for 48 h. At the end of experiments, 5–6 random microscopic fields were imaged at $20\times$ objective magnification and analyzed using a Nikon Labphot microscope with digital camera and computer-interfaced NIS Elements 5.0 imaging software. Dendrite lengths were measured from the center of each cell to the tip and these parameters were computed: *i*) Total dendrite length; *ii*) Number of dendrites; and *iii*) % of cells >3 dendrites.

2.11. Cytotoxicity assay in HaCaT cells and melanosome degradation assay in HaCaT

We first tested XH in HaCaT cells over a duration of 48 h for cytotoxicity before conducting the melanosome degradation assay. Briefly, 2×10^4 HaCaT cells were seeded in a 96-well plate for 24 h followed by replacement of medium with XH at various doses and incubation for another 48 h. At the end of treatments, MTS assay was conducted with similar protocol as outlined earlier with an incubation of 90 min.

2.12. Melanosome degradation assay in HaCaT

The degradation of melanosomes in keratinocytes was studied using a previously reported method with some modifications [32]. Briefly, HaCaT cells were seeded in 6-well plates and grown for 48 h followed by the addition of synthetic melanin and incubation was continued for another 48 h. Post incubations, the wells were extensively washed in HBSS to remove unbound melanin and XH was added at various doses and the cultures were maintained for 48 h. At the end of treatment, melanin content was analyzed using method outlined earlier and the results were normalized to total protein content and were reported as % of control after subtraction of background absorbance of negative control which consisted of cells without melanin.

2.13. Statistical analysis

One-way analysis of variance (ANOVA) with Dunnett's or Tukey's post-hoc test was run using GraphPad Prism software (version 8.0; La Jolla, California). Differences were considered statistically significant at $p < 0.05$. All data are reported as Mean \pm SD.

3. Results

3.1. Effects of XH on MNT-1 cell viability

The results of viability of MNT-1 cells upon treatment with XH are summarized in Fig. 1B. XH was nontoxic in the concentration range 2.5–30 μ M, but induced significant toxicity at 40 μ M ($p < 0.01$). Hence, in subsequent experiments, we selected a concentration range of XH over 2.5–30 μ M to test for effects on melanogenesis.

3.2. Effects of XH on melanin synthesis in MNT-1 cells

After screening nontoxic concentrations of XH, we next tested its effects on melanogenesis in MNT-1 cells over a 48 h duration; our results

are summarized in Fig. 1C. Visual examination of cell pellets showed that the pellet of group treated with XH at 30 μ M showed a slightly less intense coloration compared to untreated control group. Quantitation of melanin showed that XH significantly inhibited melanin synthesis by 23% ($p < 0.01$) at 30 μ M. The images of cells showed no change in morphology after treatment with XH at 30 μ M, except a visible reduction in intracellular melanin pigment was noted (Fig. 1D).

3.3. Effects of XH on intracellular tyrosinase activity in MNT-1 cells

Tyrosinase activity was assessed in cellular lysates of MNT-1 cells to study if reduction in melanogenesis could be explained, at least in part, by a reduction in the activity of tyrosinase enzyme. Our results showed that XH unexpectedly stimulated intracellular tyrosinase activity over a concentration range of 5–30 μ M, with a peak at 20 μ M (Fig. 1E). Tyrosinase activity was upregulated by 30.34%, 60.81%, 112% and 67.85%, by XH at concentrations of 5, 10, 20 and 30 μ M, respectively; significance was achieved for concentrations 10, 20 and 30 μ M. These results indicate that alterations in tyrosinase activity are not related to the anti-melanogenic action of XH. The auto-oxidation of DOPA was not corrected in control groups since that found to be negligible (Fig. S1).

3.4. Effects of XH on intracellular α -glucosidase activity

Since tyrosinase maturation is regulated by the activity of α -glucosidase enzyme, we next tested if the inhibition of melanin synthesis by XH could be explained, in part, by inhibition of intracellular glucosidase activity. The results showed that XH did not affect intracellular α -glucosidase activity at any concentration (Fig. 2A).

3.5. Effects of XH on intracellular ROS generation in MNT-1 cells

Next, we estimated ROS generation in MNT-1 cells to evaluate if the anti-melanogenic activity of XH (30 μ M) could be related to a reduction of intracellular ROS generation; however our results showed that XH had no effect on ROS levels as compared to control (Fig. 2B); fluorescent images also showed no changes in signal intensity (Fig. 2C).

3.6. Effects of XH on viability, melanin synthesis and cellular tyrosinase activity in HEM-DP cells

In order to confirm if XH can display anti-melanogenic activity in normal melanocytes, our next step consisted of testing XH for cytotoxicity in HEM-DP cells. As shown in Fig. 3A, XH induced significant cytotoxicity at 10 μ M, with a 65.97% reduction of viability, while concentrations <10 μ M were nontoxic and were thus selected for further study.

XH inhibited tyrosinase activity in a dose-dependent manner by 36.57% and 47.61% at 2.5 and 5 μ M, respectively ($p < 0.01$, Fig. 3B). However, XH did not affect melanin synthesis in cells at any concentration as detected visually or after quantitation (Fig. 3C).

3.7. Effects of XH on dendricity in HEM-DP cells

Since XH did not alter melanin biosynthesis in HEM-DP cells, we next evaluated if XH may inhibit melanosome export, which was evaluated by quantitation of dendricity. Cells treated with XH at concentrations of 2.5 and 5 μ M showed an altered cellular morphology with less branched dendrites as compared to control cells which were multi-dendritic (Fig. 4A). For cells treated with XH at concentration of 2.5 and 5 μ M, the dendrites appeared elongated, however the number of dendrites was visibly diminished. We next quantified the dendricity parameters to elucidate if XH had any inhibitory effect on melanosome export by inhibiting any or all of the three parameters. Our results showed that XH significantly reduced the dendrite number by 25.22% ($p < 0.01$) and 27.78% ($p < 0.01$) at 2.5 and 5 μ M as compared to control, respectively

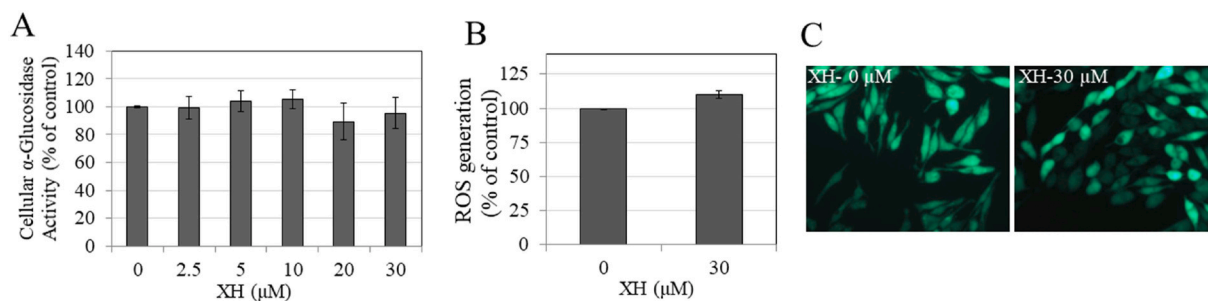


Fig. 2. A) Intracellular α -glucosidase activity in MNT-1 cells treated with XH at various doses for 48 h; B) Intracellular ROS generation levels in MNT-1 cells and; C) Representative fluorescent images showing cells from control and XH (30 μ M) group; All Data is Mean \pm SD of at least two independent experiments.

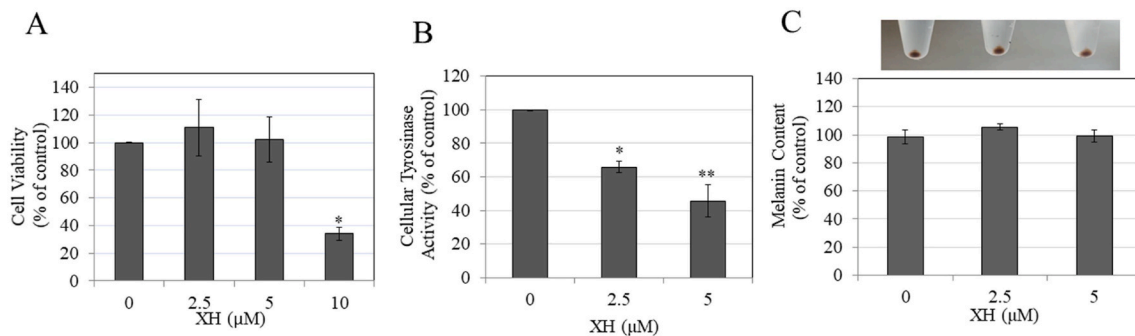


Fig. 3. A) Viability of HEM-DP cells in the presence of different concentrations of XH for 48 h measured by MTS assay; Data is Mean \pm SD of at least two independent experiments; B) Cellular tyrosinase activity in HEM-DP treated with XH for 48 h; * p < 0.05; ** p < 0.01 vs. control; One-way ANOVA with Dunnett's test; Data is Mean \pm SD of at least two independent experiments; C) Melanin content assay in cultures of HEM-DP cells treated for 48 h with different concentrations of XH with corresponding panel showing photos of cell pellets (n = 4 per group).

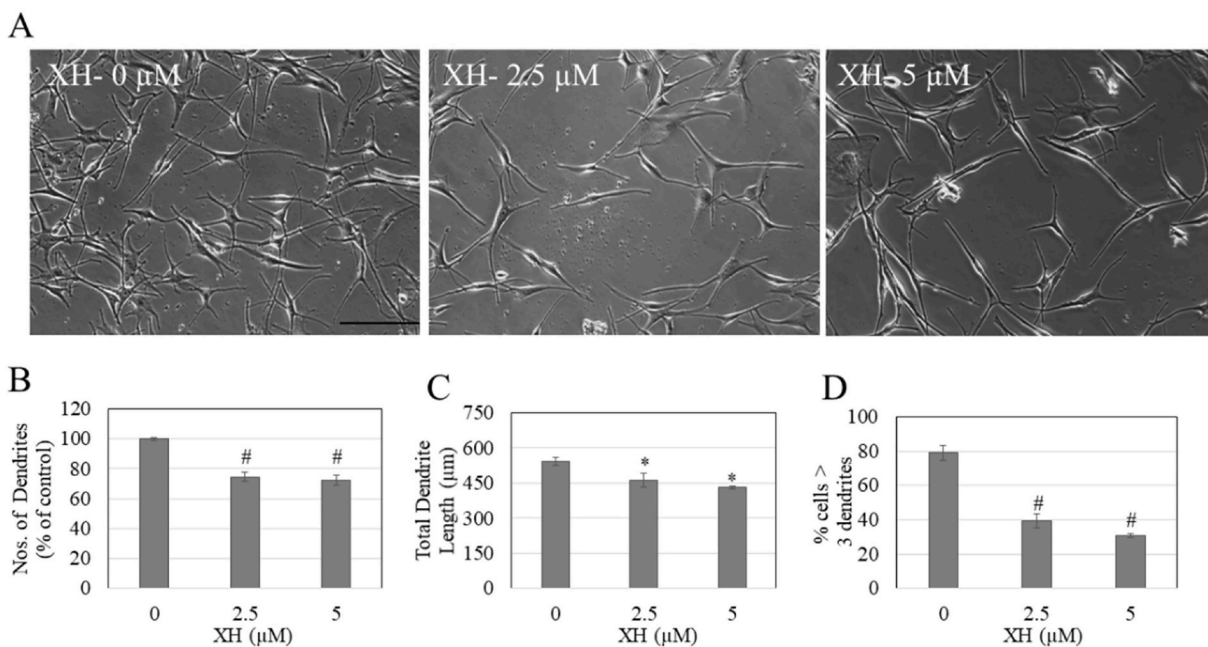


Fig. 4. HEM-DP cell morphology and dendricity measurement. A) Representative phase-contrast micrographs of HEM-DP cells treated with XH at concentrations 0–5 μ M; Scale bar corresponds to 100 μ m; Quantification of dendricity by B) Number of dendrites; C) Total dendrite length; D) % of cells > 3 dendrites; A total of \sim 60 cells were counted from duplicate wells for each treatment group; * p < 0.05; # p < 0.01; One-way ANOVA with Tukey's post-hoc test.

(Fig. 4B). Similarly, both concentrations of XH showed a significant reduction in total dendrite length (TDL), where 2.5 μ M reduced the TDL by 15.09% (p < 0.05) and 5 μ M reduced the TDL by 20.5% (p < 0.05) (Fig. 4C). In the control group, 78.95% of cells were multi-dendritic (>3

dendrites); this percentage was significantly reduced by 39% and 48% by XH at concentrations of 2.5 μ M and 5 μ M, respectively (p < 0.01, Fig. 4D).

Taken together, the results demonstrate that XH effectively inhibited

melanosome export by inhibiting dendricity parameters, without affecting melanin synthesis; this points to a novel mechanism of action of XH in human melanocytes.

3.8. Effects of XH on cell viability and melanosome degradation in HaCaT cells

Upon secretion of melanosomes from the melanocytes, they are phagocytosed by keratinocytes to form a perinuclear cap around the nucleus of keratinocytes and eventually undergo degradation based on skin type [14]. An increase in melanosome degradation is a novel mode of action of melanogenesis inhibition. We first screened XH for cytotoxicity over a duration of 48 h in human keratinocytes (HaCaT) using MTS assay in order to exclude any contribution of cytotoxicity on melanosome degradation assay. Our results showed that XH significantly stimulated keratinocyte proliferation at 2.5, 5 and 10 μM by 9.75%, 12.66% and 15.11%, respectively. However, XH induced significant toxicity at higher concentrations of 20 μM and 30 μM ($p < 0.01$; Fig. 5A), hence concentrations $< 20 \mu\text{M}$ were selected for further testing.

XH robustly suppressed phagocytosed melanin levels in keratinocytes in a dose-dependent manner with visibly lighter color of pellets (image panel, Fig. 5B). Quantitation of melanin levels showed reductions of 21.36% ($p < 0.05$), 30.42% ($p < 0.01$) and 41.44% ($p < 0.001$) at concentrations of 2.5, 5 and 10 μM , respectively (Fig. 5B). These results highlight that XH might induce degradation of phagocytosed melanosomes, which in turn, could contribute to the reduced melanin content in keratinocytes.

4. Discussion

XH has previously been reported to possess inhibitory effects on melanogenesis in B16F10 mouse melanoma cells [26] while it inhibits activity of mushroom tyrosinase in a cell-free system [25]. However, to date, there are no reports on the effects of XH on melanogenesis in human melanocytes; this is a critical issue since most depigmenting candidates in the flavonoid group have shown opposite effects on melanogenesis in mouse and human cells in earlier studies [33,34]. Herein, we demonstrated that XH exhibits anti-melanogenic activity in human MNT-1 cells and primary melanocytes (HEM-DP) and that XH specifically; *i*) inhibited melanogenesis in MNT-1 human melanoma cells but unexpectedly stimulated cellular tyrosinase activity; *ii*) inhibited melanocyte dendricity (inhibited both the total dendrite length and dendrite number in HEM-DP cells, while it inhibited cellular tyrosinase activity without affecting melanin synthesis and; *iii*) induced melanosome degradation in keratinocytes, which is indicative of its capacity to target later steps in the melanogenesis cascade. To the best of our knowledge, the inhibition of melanosome export and degradation has not been reported yet for XH as a mechanism of anti-melanogenic activity.

Unexpectedly, our results of the effects of XH on cellular tyrosinase

activity in MNT-1 cells and HEM-DP were contradictory; XH increased tyrosinase activity in MNT-1 cells but decreased tyrosinase activity in HEM-DP cells. This result may be attributable to the intrinsic differences in both cells; MNT-1 are immortalized human melanoma cells while HEM-DP are normal melanocytes. It can be speculated that this may also be a compound-specific effect which may differ in normal and malignant cells; however, that hypothesis warrants further testing. We also evaluated if XH might downregulate expression of tyrosinase protein, however our results (Fig. S2) showed that XH did not affect tyrosinase protein expression up to concentrations of 20 μM , but significantly stimulated protein levels at 30 μM (where it augmented cellular tyrosinase activity but inhibited melanin synthesis). Overall, it appears that in MNT-1 cells, the effects of XH on cellular tyrosinase activity and protein levels cannot explain the reduction in melanin synthesis at 30 μM . Previous reports have described that flavonoids can interfere in dopa-oxidase assay due to their chemical structure and redox activity [35]. A typical flavonoid, quercetin, has been shown to partially form adducts with dopaquinone via a Michael-type addition, which was identified by HPLC [36]. We speculate that XH might similarly form intermediate adducts since it also shares a Michael-type group in its structure; such an intermediate might be formed only in MNT-1 cells (in contrast to HEM-DP cells) due to presence of some endogenous activators. Identification of this apparent discrepancy was not further pursued as it was not the focus of this work, however, further investigation of mechanisms and study of intermediates formed can be achieved by employing HPLC method.

Since XH displayed anti-melanogenic activity in both types of cells, it can be concluded that the inhibitory effects of XH on melanin synthesis in MNT-1 cells are unrelated to tyrosinase activity and involve other pathways. XH has been shown to be an effective inhibitor of both monophenolase and diphenolase activity of mushroom tyrosinase enzyme in a cell-free system: the authors reported the IC_{50} values for anti-monophenolase and anti-diphenolase activity inhibition to be 15.4 μM and 37.1 μM , respectively [25]. In the present study, we did not test XH for any effects on mushroom tyrosinase activity as mushroom tyrosinase differs from human tyrosinase in several aspects [37] and thus is not a true indicator of response from cellular melanin levels; this was also corroborated in another study where plasma-activated compounds inhibited mushroom tyrosinase activity but stimulated melanogenesis [38]. XH is a potent inhibitor of direct α -glucosidase activity in vitro [39], hence the inhibition of melanogenesis may be mediated, at least partially, by the direct inhibition of activity of α -glucosidase, which is involved in glycosylation and maturation of tyrosinase [29,40]. Interestingly, the structure of XH contains α - β unsaturated keto group which is a Michael acceptor group reminiscent of the structure of curcumin which inhibits melanogenesis as demonstrated previously [41]. The mechanism of melanin inhibition by XH in MNT-1 human melanoma cells does not involve inhibition of intracellular tyrosinase activity since that was significantly higher at concentrations $< 30 \mu\text{M}$ where no change in melanin levels were noted. In our earlier report, we noted a

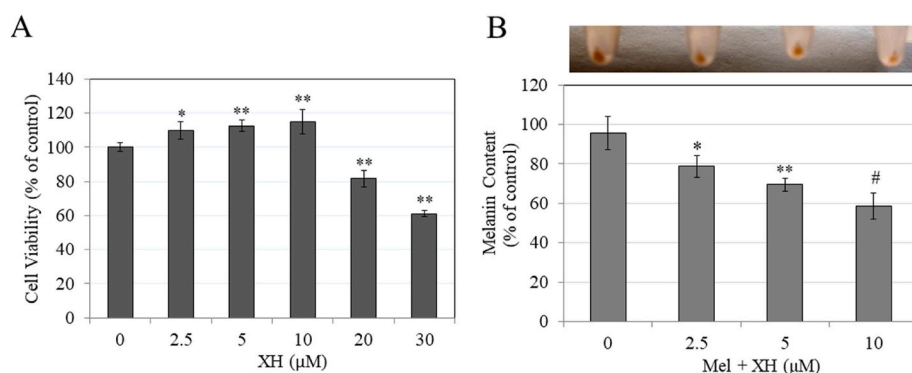


Fig. 5. A) Viability of HaCaT cells after treatment with XH for a duration of 48 h; B) Melanosome degradation assay in HaCaT cells treated with synthetic melanin (for 48 h) followed by treatment with XH at various nontoxic concentrations for another 48 h with corresponding panel showing photos of cell pellets; * $p < 0.05$; ** $p < 0.01$ and # $p < 0.001$ compared to control; One-way ANOVA followed by Dunnett's test; All data is representative of Mean \pm SD of one out of two independent experiments with $n = 3$ per group.

similar finding with the compound casuarictin which inhibited cellular tyrosinase activity without having any effect on melanin production levels [42]. Even though tyrosinase is the rate-limiting step in melanogenesis, there are other pathways distal to tyrosinase which may account for the mechanisms of melanogenesis inhibition.

Melanocyte dendrites transfer melanin granules into keratinocytes by mechanisms which are hitherto unclarified. XH did not inhibit melanin synthesis in HEM-DP cells at the tested concentrations (2.5–5 μ M); however, it significantly attenuated dendricity parameters, which suggests that the depigmenting action of XH is primarily attributable to steps in the melanogenesis pathway distal from melanin synthesis. In a previous study conducted in B16F10 cells [26], XH downregulated protein and mRNA expression of Microphthalmia Transcription Factor (MITF) which has a critical role in melanosome export and dendricity [43]. We thus speculate that a possible mechanism of reduced dendricity might be attributable to a reduction in MITF expression in HEM-DP cells; however, that putative mode of action needs further exploration. Our results on reduction of dendricity in the absence of any change in melanin synthesis in HEM-DP cells are somewhat reminiscent of the actions of piperine derivatives which were shown in a study on pro-melanogenic compounds to target a similar step in melanogenesis; in that study, the authors found that these derivatives stimulated dendricity but did not affect melanin content in the cell [44]. Several recent studies have started to exploit melanocyte dendricity as a novel target for skin-lightening agents which has appeal for the cosmetics market in addition to clinical usage. We did not test for the inhibition of melanosome export by XH in MNT-1 cells since they are not amenable to dendrite analysis due to their morphology: they do not possess the physiological dendrite network characteristic of primary human melanocytes from dark skin. The number of dendrites in darkly-pigmented (DP) melanocytes usually ranges from 3 to 10 per cell, which can extend hundreds of microns. In contrast, MNT-1 cells are bipolar (2 dendrites), and the dendrite length is similar to length of the cell body which is few microns. To the best of our knowledge, this is the first study to report inhibition of melanosome export by XH due to effects on dendricity.

Autophagy has been demonstrated to regulate skin pigmentation via degradation of melanosomes in keratinocytes [45,46]. Our results showed that XH also targets a further step in the melanogenesis pathway by inducing degradation of melanosomes in keratinocytes. Isoiquiritigenin, a dietary flavonoid which has a chalcone structure similar to XH has been shown to induce melanosome degradation in keratinocytes and the authors further assayed for activation of autophagy-related proteins [32]. We did not test if melanosome degradation may be attributable to autophagy but XH has shown to induce autophagy in cancer cells in earlier study [47]; hence it can be speculated that autophagy may be responsible for melanosome degradation, however the study of autophagy in detail and elucidation of proteins involved in autophagy are a subject of future research. We conducted our experiments on melanin degradation on HaCaT cells instead of primary keratinocytes, since the latter are known to exhibit limitations in terms of donor-dependent variability and difficulty to passage for longer period cultures. Although HaCaT cells are immortalized cells, they have been shown to mimic the functions of primary keratinocytes [48] and have been used as a convenient model of primary keratinocytes in several studies [49,50]. Nevertheless, future studies to evaluate XH's melanin degradation capacity in primary keratinocytes are warranted.

5. Conclusions

In summary, our results provide a proof-of-principle for the capacity of XH, a prenylated chalcone found in hop extract, to inhibit melanogenesis in human melanocytes at low micromolar concentrations. Our results further establish that XH inhibits melanogenesis by a dual-action mechanism of targeting melanin export in human melanocytes and melanin degradation in keratinocytes; these studies demonstrate that

XH is an inhibitor of melanin export in the absence of effects on melanin synthesis. Additionally, our results indicate that XH may hold promise as a pigmentation inhibitor for treatment of hyperpigmentation disorders for cosmetic and clinical use that could be included in oral or topical formulations. Future studies to elucidate the molecular signaling mechanisms of melanogenesis inhibition and test the efficacy of XH in a human skin model are warranted.

Author contributions

SG conceptualized the idea, designed, and performed the experiments, analyzed the data, and wrote and edited the manuscript. SRS provided resources and both authors approved the final manuscript.

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Declaration of competing interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Appendix A. Supplementary data

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