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1 = Untreated control, **2** = Umbelliferone, **3** = 7-Isopentenyloxycoumarin, **4** = Auraptene, **5** = Umbelliprenin (Melan-a- cells)

Natural oxyprenylated coumarins are modulators of melanogenesis

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Abstract. Naturally occurring coumarins 7-isopentenyloxycoumarin, auraptene, and umbelliprenin are able to modulate the biosynthesis of melanin in murine Melan-a cells probably through the interaction with selected biological targets like estrogen receptor β and aryl hydrocarbon receptor. Such a modulation strictly depends on the individual structure of the coumarin: the presence of a 3,3-dimethylallyloxy side chain is a structural determinant for tanning activation whereas a farnesyl one leads to the opposite effect. The parent compound with a free OH group, umbelliferone, did not provide any interaction. Other coumarins assayed, having shorter chains and/or being substituted in other positions, and prenyloxypsoralens, were not active or not further investigated in this context being cytotoxic at low doses.

Keywords. Auraptene, *Citrus* spp., 7-Isopentenyloxycoumarin, Melanogenesis, Oxyprenylated phenylpropanoids, Umbelliprenin.

List of abbreviations

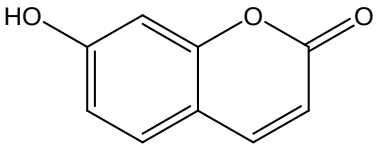
AMP	adenosinemonophosphate
AhR	aryl hydrocarbon receptor

CREB	cAMP-responsive element binding protein
DOPA	3,4-dihhydroxyphenylalanine
ER	estrogen receptor
MITF	microphthalmia-associated transcription factor
α-MSH	α-melanocyte stimulating hormone
TRP	tyrosinase-related protein
UV	ultraviolet

Introduction

Pigmentation of skin, eyes, and hair in humans and other mammals is due to and regulated by the biosynthesis and distribution of melanin. This mediator plays a pivotal role in protecting skin tissues from the hazardous effects of UV radiations. The melanin biosynthetic machinery comprises three enzymes: tyrosinase (polyphenol oxidase, EC 1.14.18.10), TRP-1, and TRP-2. The first is the enzyme catalyzing the rate-limiting step of the whole melanogenesis and is able to promote two different reactions: the hydroxylation of tyrosine to DOPA and the oxidation of this latter to DOPAquinone [1,2]. The overall machinery is transcriptionally modulated by the MITF that in turn is sensitive to the α-MSH through a cascade of events, the last of which consists in the phosphorylation of the CREB that is able to bind to the CRE motif of MITF, thus stimulating melanin biosynthesis [3]. On the other hand, melanogesis is inhibited by the activation of the extracellular signal-regulated kinase that leads to the phosphorylation and degradation of MITF [4]. Disorders in melanin biosynthesis may provide abnormal pigmentation of the skin. Massive production of such a mediator causes its accumulation in the epidermis leading to melisma, freckles, and senile lentigines [5,6]. On the other hand, hypopigmentation, associated to a decrease in melanin biosynthesis, may lead to vitiligo, albinism, Griscelli syndrome, and scalp problems (dandruff, lice, cradle cap, and ringworms) [7]. Different remedies based on the use of natural products and/or plant extracts have been proposed for the treatment of both disorders. Compounds

able to inhibit melanin production include arbutin and its aglycone derived from bearberry, cranberries, mulberries, blueberries, and several other plant species, kojic acid, a by-product of the fermentation process of malting rice, azelaic acid, a component of wheat, rye, and barley, flavonoids like luteolin and quercetin, phytochemicals from licorice, alisol B, a triterpene isolated from *Alisma orientale* (Sam.) Juz (Alismataceae), *O*-methylflavones from *Scutellaria baicalensis* Georgi (Lamiaceae), protocatechuic acid, and several other natural products, the effectiveness of which has been recently reviewed [8]. Tanning activators include furanocoumarins like psoralen, bergamottin, and bergaptene, all components of bergamot essential oil [9], pratol, a methoxyflavone isolated from *Trifolium pratense* L. (Fabaceae) [10], flavonoids like sinensetin, tangeretin, and nobiletin, all widespread in the genus *Citrus* [11], ferulic acid [12], and 6,7-dimethoxycoumarin [13]. However, the use of some of the above listed active principles for cosmetic and/or therapeutic purposes is associated to severe side effects. For example, arbutin and hydroquinone are able to evoke skin irritation and contact dermatitis [14], kojic acid causes allergic reactions and sensitization [15], and finally psoralen and related furanocoumarins are suspected to possess a great photocarcinogenic potential [16], because of which psoralens have been banned as sunscreen ingredients in several countries worldwide. Therefore, the search for novel natural remedies to generally modulate melanin production for therapeutic and cosmetic purposes is a field of current and stimulating interest. In this context we wish to describe herein for the first time in the literature the interaction of oxyprenylated umbelliferone derivatives, namely 7-isopentenyloxycoumarin **2**, auraptene **3**, and umbelliprenin **4**, with the melanin biosynthetic machinery in non-tumorigenic murine melanocyte Melan-a cell line. Also other naturally occurring coumarins, having a different pattern of substitution respect to compounds **2-4**, have been tested and these include collinin **5**, lacinartin **6**, 7-geranyloxy-6-methoxycoumarin **7**, 5-geranyloxy-7-methoxycoumarin **8**, and psoralens 8-geranyloxypsoralen **9**, imperatorin **10**, isoimperatorin **11**, and bergamottin **12** (Figure 1).



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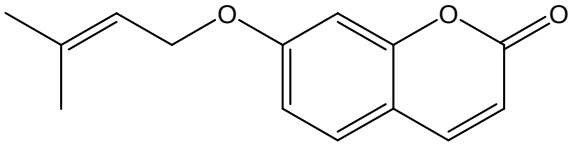

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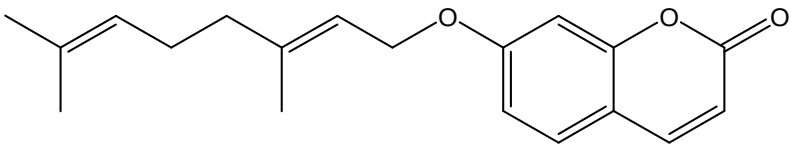

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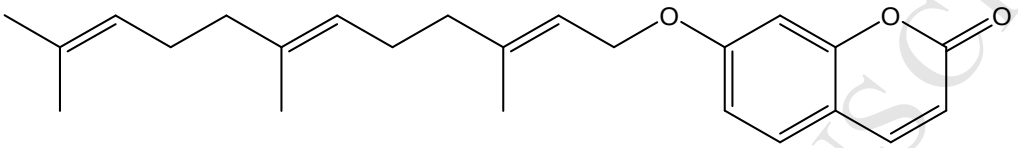

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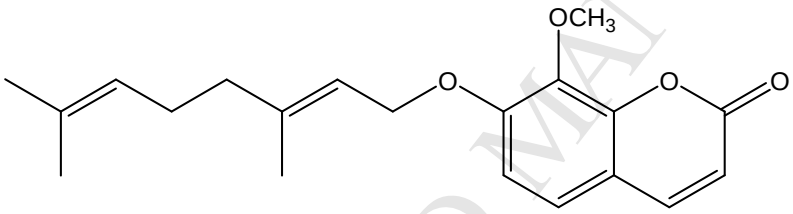

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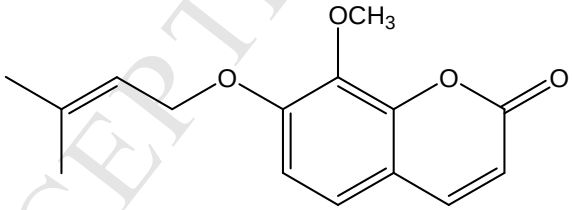

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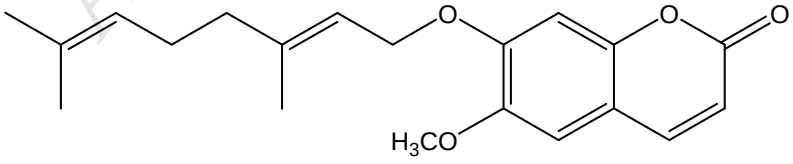

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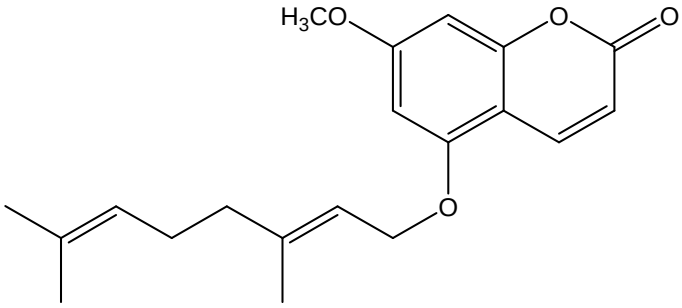

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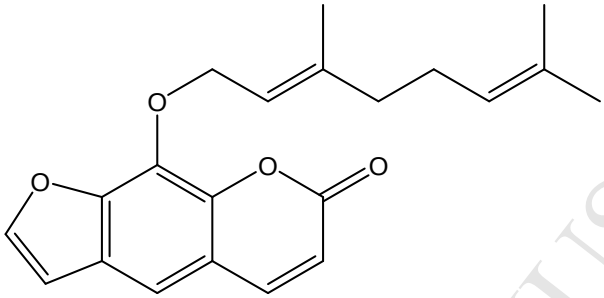

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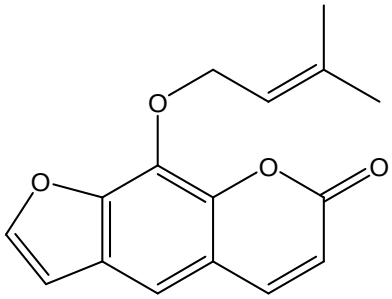

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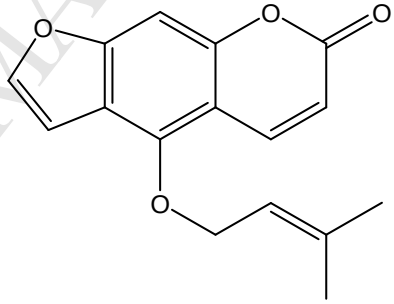

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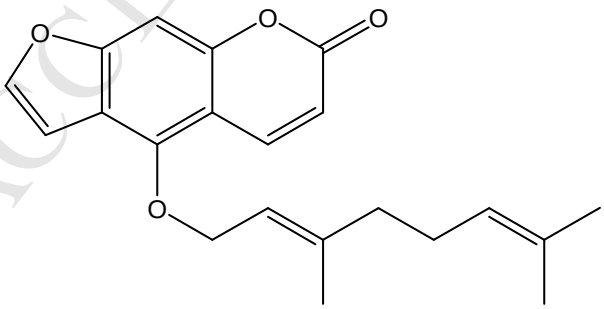

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Figure 1. Chemical structures of umbelliferone **1**, 7-isopentenyloxycoumarin **2**, auraptene, umbelliprenin **4**, collinin **5**, lacinartin **6**, 7-geranyloxy-6-methoxycoumarin **7**, 5-geranyloxy-7-methoxycoumarin **8**, and psoralens 8-geranyloxypsoralen **9**, imperatorin **10**, isoimperatorin **11**, and bergamottin **12.**

Materials and Methods

Chemistry. All oxyprenylated coumarins have been synthesized from parent commercially available hydroxylated coumarins by a single-step Williamson reaction using 3,3-dimethyallyl, geranyl, and all *trans* farnesyl bromides as the alkylating agents as already reported [17]. Their purity (> 97.6 %) was assessed by and HPLC, following the well validated analytical method we set up for the qualitative and quantitative analysis of oxyprenylated coumarins [18,19]. The same general procedure for NMR and elemental analysis experiments as already reported was followed [17]. Analyses indicated by the symbols of the elements or functions were within ± 0.4 % of the theoretical values. UV light experiments have been performed with a UV lamp 254 nm (EMD Millipore, Burlington, MA, USA).

*7-Isopentenyloxycoumarin (**2**)*. ^{1}H NMR δ 1.66 (s, 3H), 1.72 (s, 3H), 4.55-4.60 (m, 2H), 5.44-5.49 (m, 1H), 6.29 (d, 1H, J = 9.6 Hz), 6.55-7.07 (m, 3H), 7.15 (d, 1H, J = 9.6 Hz); ^{13}C NMR δ 18.2, 25.7, 65.3, 101.5, 112.3, 112.9, 113.1, 118.5, 128.6, 139.2, 143.4, 155.8, 161.2. Anal. Calcd for $C_{14}H_{14}O_3$: C, 73.03; H, 6.13; O, 20.85. Found: C, 72.98; H, 6.09, O, 20.82

*Auraptene (**3**)*. ^{1}H NMR δ 1.38 (s, 3H), 1.42 (s, 3H), 1.72 (s, 3H), 1.85-2.28 (m, 4H), 4.89-4.96 (m, 2H), 5.02-5.11 (m, 1H), 5.44-5.69 (m, 1H), 6.22 (d, 1H, J = 9.6 Hz), 6.54-7.09 (m, 3H), 7.18 (d, 1H, J = 9.6 Hz); ^{13}C NMR δ 14.4, 16.9, 25.8, 26.6, 39.1, 65.7, 108.1, 114.4, 114.6, 120.5, 122.7, 123.8, 131.9, 134.4, 142.5, 143.2, 148.9, 156.7, 160.4. Anal. Calcd for $C_{19}H_{22}O_3$: C, 76.48; H, 7.43; O, 16.09. Found: C, 76.42; H, 7.37, O, 16.02

*Umbelliprenin (**4**)*. ^{1}H NMR δ 1.56 (s, 3H), 1.602 (s, 3H), 1.67 (s, 3H), 1.76 (s, 3H), 1.89-2.42 (m, 8H), 4.62-4.67 (m, 2H), 5.03-5.09 (m, 1H), 5.11-5.17 (m, 1H), 5.49-5.55 (m, 1H), 6.52 (d, 1H, J =

9.5 Hz), 6.85-7.13 (m, 3H), 7.17 (d, 1H, J = 9.5 Hz); ^{13}C NMR δ 16.0, 16.7, 17.6, 25.6, 26.1, 26.6, 39.1, 39.6, 65.4, 101.5, 112.4, 112.9, 113.2, 118.4, 123.4, 124.2, 128.6, 131.3, 135.5, 142.3, 143.4, 155.8, 161.2. Anal. Calcd for $C_{24}H_{30}O_3$: C, 78.65; H, 8.25; O, 13.10. Found: C, 78.61; H, 8.29, O, 13.04.

*Collinin (**5**)* ^{1}H NMR δ 1.35 (s, 3H), 1.44 (s, 3H), 1.81 (s, 3H), 1.87-2.25 (m, 4H), 3.64 (s, 3H), 4.88-4.95 (m, 2H), 5.01-5.10 (m, 1H), 5.45-5.68 (m, 1H), 6.19 (d, 1H, J = 9.4 Hz), 6.28 (d, 1H, J = 4.4 Hz), 6.92 (δ (d, 1H, J = 4.4 Hz), 7.15 (d, 1H, J = 9.4 Hz); ^{13}C NMR δ 14.1, 16.4, 25.6, 26.4, 39.6, 55.9. 69.8, 108.3, 113.3, 113.6, 120.1, 123.0, 123.9. 131.6, 134.7, 142.7, 143.6, 148.5, 156.1, 160.5. Anal. Calcd for $C_{20}H_{24}O_4$: C, 73.15; H, 7.37; O, 19.49. Found: C, 73.12; H, 7.33, O, 19.44.

*Lacinartin (**6**).* ^{1}H NMR δ 1.63 (s, 3H), 1.71 (s, 3H), 3.66 (s, 3H), 4.51-4.57 (m, 2H), 5.42-5.49 (m, 1H), 6.27 (d, 1H, J = 9.5 Hz), 6.32 (d, 1H, J = 4.2 Hz), 6.88 (δ (d, 1H, J = 4.2 Hz), 7.12 (d, 1H, J = 9.5 Hz); ^{13}C NMR δ 18.4, 25.4, 55.9, 65.2, 101.6, 112.2, 112.8, 113.8, 119.0, 138.6, 139.1, 143.9, 155.9, 160.0. Anal. Calcd for $C_{15}H_{16}O_4$: C, 69.22; H, 6.20; O, 24.59. Found: C, 69.26; H, 6.25, O, 24.63.

*7-Geranyloxy-6-methoxycoumarin (**7**)* ^{1}H NMR δ 1.39 (s, 3H), 1.48 (s, 3H), 1.77 (s, 3H), 1.83-2.21 (m, 4H), 3.72 (s, 3H), 4.91-4.97 (m, 2H), 5.05-5.12 (m, 1H), 5.41-5.55 (m, 1H), 6.23 (d, 1H, J = 9.5 Hz), 6.33 (s, 1H), 6.52 (δ (s, 1H), 7.09 (d, 1H, J = 9.5 Hz); ^{13}C NMR δ 14.3, 16.6, 24.9, 26.9, 39.9, 56.7, 69.2, 108.1, 113.2, 113.9, 120.2, 123.7, 123.8, 131.9, 134.1, 142.1, 143.2, 148.2, 156.8, 160.1. Anal. Calcd for $C_{20}H_{24}O_4$: C, 73.15; H, 7.37; O, 19.49. Found: C, 73.19; H, 7.32, O, 19.46.

*5-Geranyloxy-7-methoxycoumarin (**8**)* ^{1}H NMR δ 1.47 (s, 3H), 1.55 (s, 3H), 1.89 (s, 3H), 1.92-2.33 (m, 4H), 3.42 (s, 3H), 4.95-5.03 (m, 2H), 5.09-5.14 (m, 1H), 5.43-5.59 (m, 1H), 6.12 (d, 1H, J = 9.6 Hz), 6.15 (d, 1H, J = 1.0 Hz), 6.72 (d, 1H, J = 1.0 Hz), 7.22 (d, 1H, J = 9.6 Hz); ^{13}C NMR δ 15.3, 16.9, 24.6, 26.9, 39.7, 55.8, 69.0, 108.8, 113.1, 113.5, 120.1, 123.7, 123.9, 131.0, 134.0, 142.8, 143.4, 148.6, 156.3, 160.1. Anal. Calcd for $C_{20}H_{24}O_4$: C, 73.15; H, 7.37; O, 19.49. Found: C, 73.18; H, 7.35, O, 19.44

*8-Geranyloxypsoralen (**9**).* ^{1}H NMR δ 1.74 (s, 3H), 1.76 (s, 3H), 1.88 (s, 3H), 1.95-2.10 (m, 4H), 5.00-5.09 (m, 3H), 5.58-5.62 (m, 1H), 6.33 (d, 1H, d, *J* = 9.2 Hz), 6.88 (d, 1H, J = 2.2 Hz), 7.39 (s, 1H), 7.65 (d, 1H, J = 2.2 Hz), 7.79 (1H, d, *J* = 9.2 Hz); ^{13}C NMR δ 16.6, 18.4, 24.8, 26.1, 26.7, 39.8, 70.5, 108.0, 115.1, 116.9, 120.2, 123.6, 123.9, 132.1, 140.1, 144.3, 144.7, 147.0, 149.0, 155.0, 160.9. Anal. Calcd for $C_{21}H_{22}O_4$: C, 74.54; H, 6.55; O, 18.91. Found: C, 74.56; H, 6.50, O, 18.91

*Imperatorin (**10**)* ^{1}H NMR δ 1.74 (s, 3H), 1.76 (s, 3H), 5.02-5.07 (m, 2H), 5.60-5.63 (m, 1H), 6.37 (d, 1H, d, *J* = 9.5 Hz), 6.83 (d, 1H, J = 2.1 Hz), 7.36 (s, 1H), 7.70 (d, 1H, J = 2.1 Hz), 7.76 (1H, d, *J* = 9.5 Hz); ^{13}C NMR δ 18.5, 26.2, 70.5, 107.4, 115.1, 116.9, 120.2, 132.1, 140.1, 144.3, 144.7, 147.0, 149.0, 160.9. Anal. Calcd for $C_{16}H_{14}O_4$: C, 71.10; H, 5.22; O, 23.68. Found: C, 71.12; H, 5.27, O, 23.64

*Isoimperatorin (**11**)* ^{1}H NMR δ 1.72 (s, 3H), 1.82 (s, 3H), 4.94-4.99 (m, 2H), 5.51-5.56 (m, 1H), 6.29 (d, 1H, J = 9.6 Hz), 6.97 (d, 1H, J = 2.1 Hz), 7.17 (s, 1H), 7.61 (d, 1H, J = 2.1 Hz), 8.17 (1H, J = 9.6 Hz); ^{13}C NMR δ 18.6, 26.2, 70.2, 94.7, 105.4,108.0, 113.0, 114.7, 119.5, 139.9, 140.2, 145.3, 149.4, 153.1, 158.5, 161.7. Anal. Calcd for $C_{16}H_{14}O_4$: C, 71.10; H, 5.22; O, 23.68. Found: C, 71.14; H, 5.25, O, 23.69

*Bergamottin (**12**).* ^{1}H NMR δ 1.60 (s, 3H), 1.68 (s, 3H), 1.69 (s, 3H), 1.94-2.13 (m, 4H), 4.93-4.98 (m, 2H), 5.01-5.04 (m, 1H), 5.52-5.56 (m, 1H), 6.33 (d, 1H, d, *J* = 9.6 Hz), 6.96 (d, 1H, J = 2.3 Hz), 7.16 (s, 1H), 7.58 (d, 1H, J = 2.3 Hz), 7.99 (1H, d, *J* = 9.6 Hz); ^{13}C NMR δ 16.8, 17.8, 25.8, 26.3, 39.6, 70.0, 94.4, 105.2, 107.7, 112.7, 114.4, 119.0, 123.6, 132.2, 139.8, 143.2, 145.0, 149.1, 152.9, 158.3, 161.3. Anal. Calcd for $C_{21}H_{22}O_4$: C, 74.54; H, 6.55; O, 18.91. Found: C, 74.51; H, 6.52, O, 18.90.

Cell culture

Melan-a cells, an immortalized mouse melanocyte cell line, were obtained from the Wellcome Trust Functional Genomics Cell Bank. Cells were maintained in RPMI 1640 (Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum, 50 U/mL penicillin, 50 U/mL streptomycin (PS Lonza)

and 200 nM PMA (phorbol 12-myristate 13-acetate; Sigma). Cells were incubated at 37°C in a humidified 5% CO_2/air atmosphere. The stock solutions of isopentenyloxycoumarin, auraptene, and umbelliprenin were prepared in DMSO (1000X) and were stored at −20 °C until use. The concentrations used for the study were 1, 10, 20, and 40 μM, which were freshly prepared for each experiment with a final DMSO concentration of 0.1%. Controls were always treated with the same amount of DMSO (0.1%, v/v) as used in the corresponding experiments.

Cell viability measurement

Non-tumoral murine melanocytes were seeded at 60.000 cells on six plate wells and treated for 48 h with auraptene, isopentenyloxycoumarin, or umbelliprenin at the indicated concentrations or DMSO. Cells were detached by trypsinization, collected in phosphate buffer saline and centrifuged at 1500 rpm for 5 min. at 4 °C. Cells pellets were resuspended in the trypan blue solution (0.25%, w/v in PBS) and counted in a Malassez cell under a light microscope. The percentage of cell viability was calculated using the following formula: % cell viability= [1-(blue cells/total cells)] x100.

RNA isolation and qPCR analysis

Total RNA from cultured cells treated as indicated was isolated using TRIzol Reagent (Invitrogen). RNA was quantified (nanodrop; Thermofisher). Total RNA (1 μg) was reverse-transcribed using an iScript cDNA synthesis kit (Bio-Rad), according to the manufacturer's instructions. qRT-PCR was performed with an iCycler iQreal-time PCR detection system (Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad) and the indicated primers. The threshold cycle (Ct) values of the gene of interest were normalized with the Ct values of Cyclophiline A1.

Melanin content measurement

Non-tumoral murine melanocytes were seeded at 60,000 cells on six plate wells and treated for 48 hours with increasing doses of auraptene, isopentenyloxycoumarin, umbelliprenin, and kojic acid at the indicated concentration or carrier solvent (DMSO). $5x10^6$ cells were centrifuged at 1500 rpm for 5 min. at 4 °C. The cell pellet was washed twice with phosphate buffer saline, transferred in an

Eppendorf vial and centrifuged at 5000 g for 5 min. at 4 °C. The supernatant was discarded. 200 µL of water and 1 mL of EtOH/Ether (1/1) were added to remove opaque substances other than melanin. The mixture was incubated for 15 min. at room temperature, centrifuged at 5000 g for 5 min. and the supernatant was discarded. The precipitate containing melanin was solubilized by 300 µL of a mixture of aqueous sodium hydroxide (1 M)/DMSO 90/10 after heating at 80°C for 1 h. The absorbance was measured at 405 nm. The melanin content was expressed as a percentage of control (= 100%). UV experiments have been performed following the method reported by Liebermann and Hopkins in 2004 [20] and using a UVX radiometer (UVP, Inc., Upland, CA, USA).

Western blotting

Cells treated as indicated were washed with ice cold PBS, then scraped and centrifuged at 800 × g for 5 min at 4 °C. Pellets were resuspended in 100 µL of extraction buffer (50 mM Tris pH 7.4, 5mM NaCl, 1% Triton X-100, 10% glycerol) with 1% protease inhibitor mixture (Sigma-Aldrich), then vortexed and centrifuged at 10,000 × g for 10 min at 4 °C. Whole-cell extracts were fractionated by SDS/PAGE and transferred to a polyvinylidene difluoride membrane using a transfer apparatus according to the manufacturer's protocols (Life Technologies). After incubation with 5% nonfat milk in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 1% Tween 20) for 60 min., the membrane was incubated with antibodies against tyrosinase (1:1,000), TRP1 (1:500), TRP2 (1:500), MITF (1: 500), Rab27a (1: 200), or actin (1/20,000, C4; Merck Millipore) at 4 °C overnight. Membranes were then washed three times for 10 min. each and incubated either with a 1:10,000 dilution of horseradish peroxidase-conjugated anti-mouse (W402B; Promega) or antirabbit antibodies (W401B; Promega) for 1 h. Blots were then washed three times with TBST and developed with the ECL system (Amersham Biosciences), according to the manufacturer's protocol.

Reporter cell lines and luciferase assay

MELN cells expressed luciferase in an estrogen-dependent manner. Cells were routinely grown in DMEM, supplemented with 5% FBS. For experiments, cells were grown for 5 days in phenol red-free medium containing 5% dextran-coated charcoal-treated FBS. Luciferase assay were carried out exactly as described previously [21].

Statistical analysis

Values are the mean ± S.E. of three independent experiments, each carried out in duplicate. Statistical analysis was carried out with GraphPad using a Student's *t* test for unpaired variables. *, **, and *** in the figures refer to *P* values of < 0.05, <0.01, p<0.001 respectively, compared with control cells that received the solvent vehicle alone.

Results and Discussion

The choice of Melan-a cell line, as an alternative to malignant melanocytes B16F10 or SK-MEL 28 strains, has been addressed by the fact that it represents a pharmacological model more suitable to investigate the effects of chemicals on melanogenesis. It has been in fact put in evidence how melanin biosynthesis is significantly up-regulated in cancer cells respect to Melan-a ones, so that the response of tumour lines to an elicitor or an inhibitor of melanin biosynthesis, like 7-isopentenyloxycoumarin or umbelliprenin respectively, can be more hardly tuned in B16F10 and SK-MEL 28 cells than in Melan-a line [22]. Melan-a cell line have been introduced for the first time by Bennet and coworkers in 1987 [23]. These Authors clearly outlined the differences between non-tumoral and tumorigenic B16 lines: all Melan-a cells contain the melanin pigment, even in the case of a rapid growth, they proliferate providing a confluent monolayer well visible under microscope examination, are round shape and dendritic, and do not grow in absence of a tumour promoter. On the other hand, B16F10 cells are irregularly pigmented, with few dendrites and proliferate randomly even in the absence of a tumour promoter. Similar considerations can be drawn for the comparison with SK-MEL 28 line [24]. Thus, in our opinion, the responsiveness of Melan-a cells is more sensitive and recordable. Oxyprenylated coumarins herein under investigation were synthesized

following a well validated and high yield method already reported in the literature[17]. Compounds **1-12** were preliminarily screened for their capacities in not affecting cell viability and subsequently in modulating melanin biosynthesis in Melan-a murine melanocytes. We first investigated the impact of oxyprenylated coumarins on proliferation and viability. Compounds **1-4** displayed no significant impact on cell viability (Figure 2A and 2B), whereas other coumarins under investigation exhibited either a marked cytotoxic effect at low doses (< 2 µM, e.g. psoralens **9-12**) or no appreciable tanning or whitening effect respect to controls at the same dose used for samples **2-4** (compounds **5-8**) in subsequent assays. Thus, these latters were not selected to perform further experiments. The concentration applied (40 µM) for umbelliferone **1** and its derivatives **2-4** corresponded to the highest solubility of such phytochemicals in the medium used to accomplish biological assays. Melanin content was measured on Melan-a cells exposed to increasing dose of oxyprenylated coumarins **2-4** and umbelliferone for 48 hours. Figures 2A and 2B showed that at the concentration of 40 µM cell viability remained virtually unaffected respect to untreated control cells. As reported in Figure 2C, all prenylated compounds showed appreciable differences on the extent of melanin biosynthesis compared to control. The unprenylated counterpart umbelliferone **1** did not virtually display any changes respect to the control untreated cells and was assayed merely to put in evidence the role of the *O*-side chain. A behaviour practically equal to compound **1** was recorded for hydroxylated coumarins, parent products of samples **5-12**. We observed that 7-isopentenyloxycoumarin **2** is a strong inducer of melanin production whereas umbelliprenine **4** exhibits whitening properties in a dose dependent manner. Results of auraptene **3** stand in the middle with a slight pro-pigmenting effect (1.3-fold induction) at the highest dose tested (40 µM). Tests have also been performed on Melan-a treated with 40 µM of oxyprenylated coumarins for 72 hours. The effect of these compounds on melanin content was higher at 72 hours of treatment compared to 48 hours underlying a time dependent effect. Compound **2** triggers a huge pigmenting effect with a 6-fold increase of melanin content whereas **3** increases melanin content by 2-fold compared to control (Figure 2D). On the other hand, compound **4** reduces melanin content by more

than 70% compared to control (Figure 2D). The photographs of cell pellets of Melan-a cells exposed to oxyprenylated coumarins highlight the strong impact of these natural compounds on pigmentation (Figure 2E).

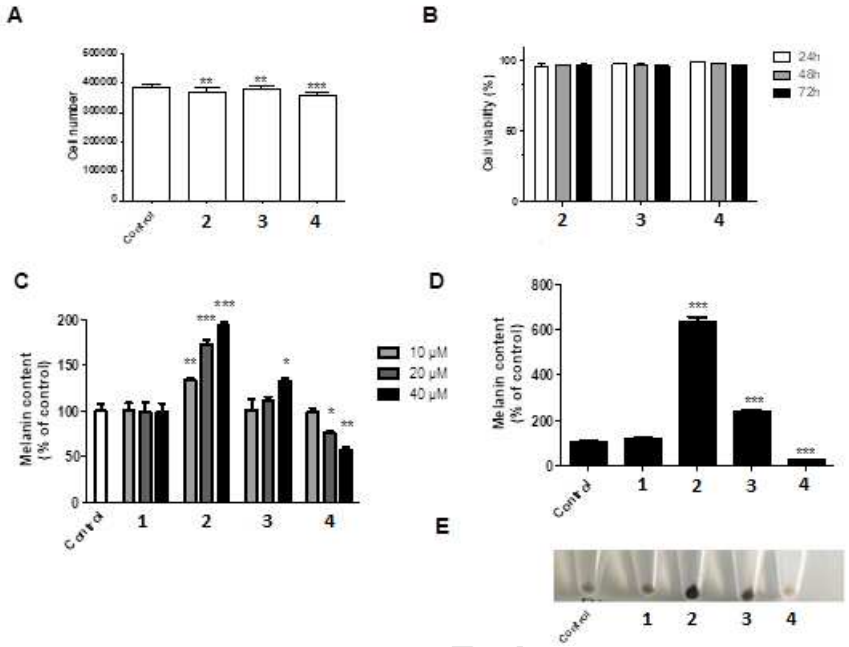


Figure 2. Effects of prenyoxycoumarins on cell proliferation, cell viability, and melanin content in Melan-a cells. Cell number analysis of Melan-a cells treated with 7- isopentenyloxycoumarin **2** auraptene **3**, and umbelliprenin **4** at 40 µM for 3 days (A). Cell viability analysis was performed by trypan blue exclusion test on Melan-a cells treated with **1**, **2**, **3**, and **4** at 40 µM for the indicated time (B). Melanin content measurement on Melan-a cells treated with **1**, **2**, **3**, and **4** at the indicated concentrations for 2 days (C). Melanin content measurement on Melan-a cells treated with **1**, **2**, **3**, and **4** at 40 µM for 3 days (D). Photographs of Melan-a cells treated with solvent vehicle (control), **1**, **2**, **3**, and **4** at 40 µM for 3 days at 40 µM for 3 days (E) Data are shown as mean ± SEM; *p < 0.05, **p<0.01, ***p<0.001 compared with solvent vehicle treated cells (control), t test.

The main consideration that can be drawn from these experiments is that: a) prenyloxycoumarins impact melanin production without inducing cytotoxicity and b) the type of prenylation (e.g. length of the side chain) has a deep influence on the effects exerted by coumarins on melanogenesis: the shorter is the chain, the greater is the pigmentation, while the longer is the chain the more evident is

the depigmentation. We then investigated whether oxyprenylated coumarins that display potent impact on melanogenesis (i.e compounds **2** and **4**) modulate the expression of major players in melanin production (tyrosinase, TRP-1, TRP-2, and MITF). For this purpose, qPCR analysis was performed on Melan-a cells treated for 24 hours with **2** and **4**. As shown in Figure 3A, we revealed that **2** induces the expression of the melanogenic enzymes tyrosinase, TRP-1 and TRP-2 as well as MITF. Consistently with its whitening property, **4** inhibits by more than 2-fold the expression of proteins belonging to the melanogenic machinery (Figure 3A). Quite surprisingly however Western Blotting analysis revealed that **4** slightly increases tyrosinase protein level when assayed at 40 µM (Figure 3B). It can be hypothesized, stating that Real-time PCR and Western Blotting analysis have been assayed at different times, 24 h and 48 h respectively, the inhibitory effect of umbelliprenin on the genomic expression of tyrosinase can be transient and after a prolonged time a slight increase in the concentration of this enzyme could be observed as outlined in Figure 2B. On the contrary it virtually abolished the synthesis of TRP-1, TRP-2, MITF, and Rab27a, this latter being a melanosome transporter protein the lack of which is the main cause of the Griscelli syndrome, and such inhibition may the effective mechanism underlying the depigmenting effects of umbelliprenine. Western Blotting analysis on the same proteins was also carried out in the case of compound **2**, providing a pattern of results completely overlapping results reported in Figure 2A and thus was not reported herein. Altogether, these data showed that prenyloxycoumarins impact the expression of enzymes (tyrosinase, TRP-1 and TRP-2), transcription factor (MITF) and transporter (Rab27a) that are key players of the melanogenic machinery.

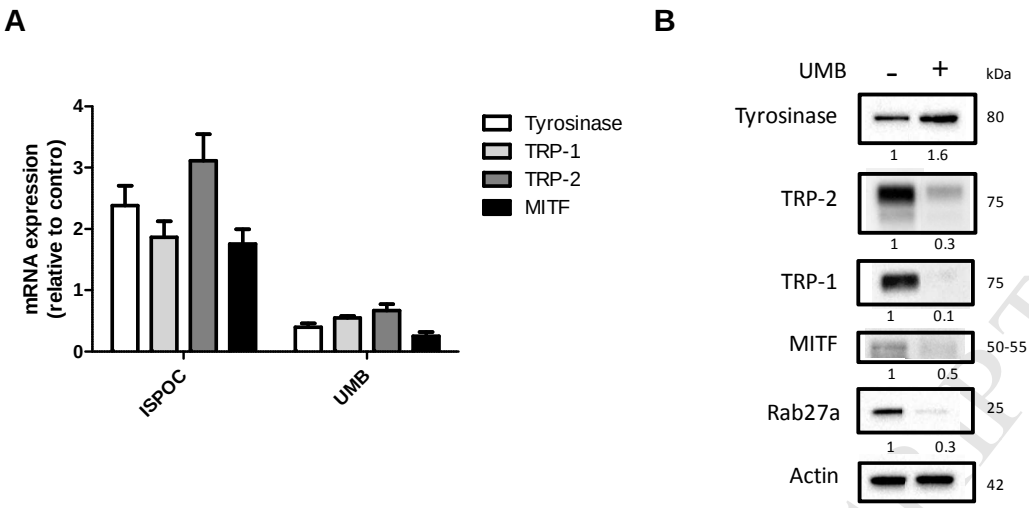


Figure 3. Effects of prenyoxycoumarins on the expression of proteins of the melanogenic machinery. Real-time PCR of tyrosinase, TRP-1, TRP-2, and MITF expression in Melan-a cells treated with 40 µM of **2** and **4** for 24 hours (A). Western blot analysis of tyrosinase, TRP-1, TRP-2, MITF and Rab27a levels in Melan-a cells treated with **4** at 40 µM for 2 days (B).

Considering that compound **4** is a whitening agent, we evaluate its ability to inhibit the pigmenting effect of physiological regulator of melanin production (UV irradiation and α-MSH). Melan-a cells were exposed for 48 hours to α-MSH, UV irradiation (254 nm, 30 mJ/cm^2) or **4** alone or to the combination α-MSH / **4** or UV irradiation / **4**. Melanin content measurement showed that **4** fully inhibits the stimulation of melanin production induced by UV irradiation and α-MSH (Figure 4). Our results demonstrate that umbelliprenin is effective to reduce melanin content both in basal condition or after exposure to a physiological pro-pigmenting stimulus. Globally considered the data collected herein indicated that all the three oxyprenylated coumarins **2-4** are able to interfere with melanogenesis and thus can be claimed as active principles of plant origin with a great potential for therapeutic and cosmetic purposes.

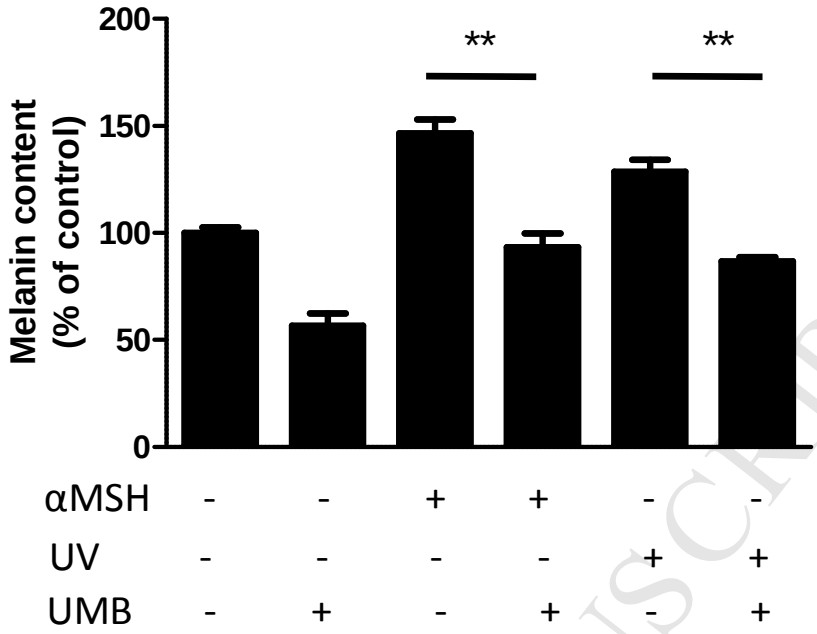


Figure 4. Effects of compound **4** on the pigmenting effect of physiological pro-pigmenting stimuli. Melanin content measurement on Melan-a cells treated for 2 days with **4** (40 µM), α-MSH (100 nM), UV irradiation (254 nm) or the indicated combination. Data are shown as mean ± SEM; **p<0.01, **p< 0.001, t-test.

To this concern we subsequently decided to investigate their chemical stability in view of their potential use as ingredients of preparations for skin topical applications, in the same way we recently did with umbelliprenin [24]. Indeed, we previously reported the formation of products deriving from inter- and intramolecular [2+2] cycloadditions for umbelliprenin exposed to UV (254 nm) and sunlight. The stability of both 7-isopentenyloxycoumarin and auraptene (as 0.5 % w/w solution in ethanol) was assayed over a period of 6 days under light exposure by TLC analysis. Light exposure of umbelliprenin was also performed and used as reference. As shown in Figure 5A, coumarins **2** and **3** were largely degraded after a 6 days exposure with formation of products (compound P1) with lower R_f. However, the degradation profile of both auraptene and isopentenyloxycoumarin differ from those of umbelliprenin since no compounds with higher Rf (compound P2, Figure 5A) resulting from an intramolecular interaction between double bonds of

the side chain and the α,β-unsaturated carbon-carbon double bond have been observed. The addition of UV-A filters commonly found in commercially available sunscreens like Uvinul A (diethylaminohydroxybenzoylhexyl benzoate) (Figure 5B and 5C) prevented to a large extent the degradation of both coumarins. Preparative TLC allowed to isolate the main product P1 from the degradation of both coumarins. As expected, these were identified as the coumarin-dimers by LC-MS analysis (data not shown). Our data showed that compounds **2** and **3** undergo under light exposure an intermolecular [2+2] cycloaddition that is prevented by UV-A filters.

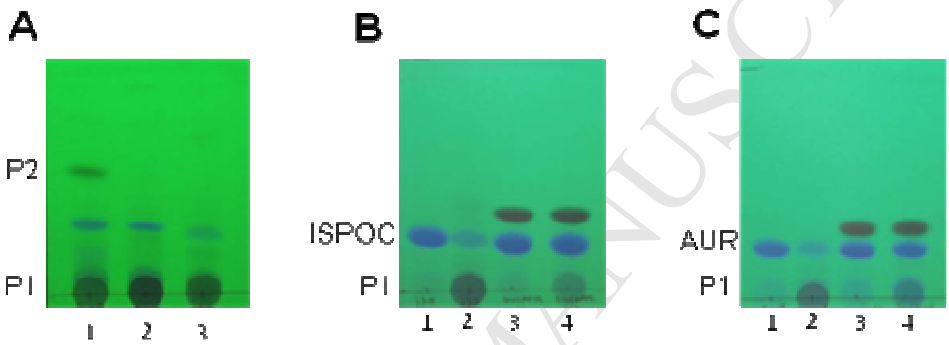
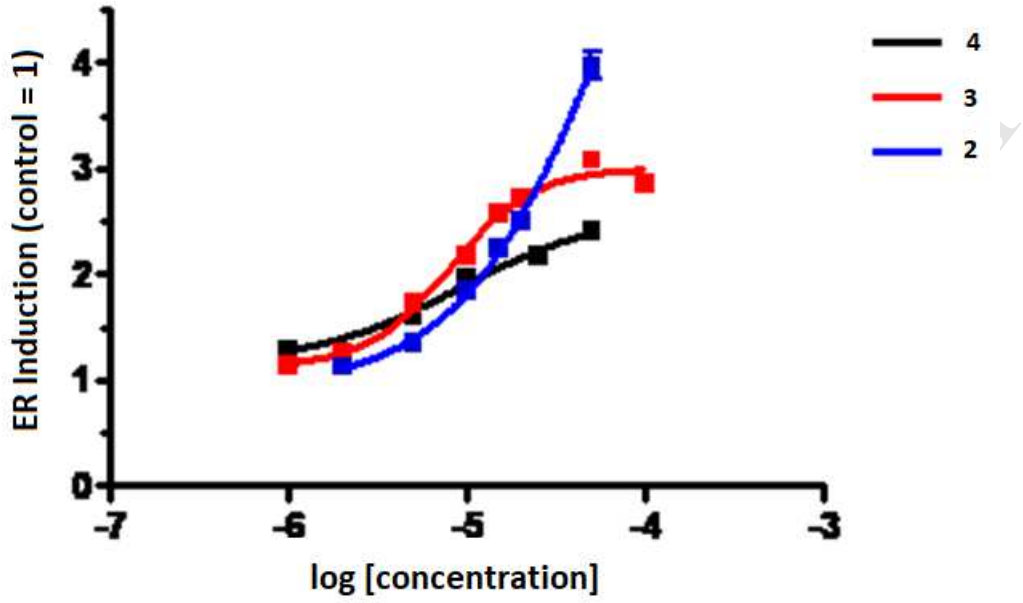


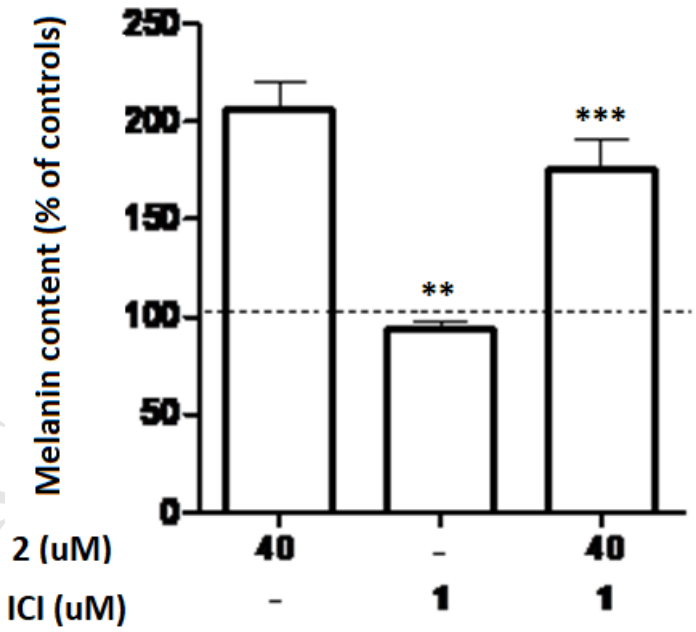
Figure 5. Evaluation of isopentenyloxycoumarin and auraptene stability under light exposure. Representative TLC of umbelliprenin, auraptene, and isopentenyloxycoumarin photodegradation. Ethanolic solutions of coumarins (0.5% w/w) were exposed to sunlight for 6 days and analyzed by TLC. Lane 1: umbelliprenin; lane 2: auraptene; lane 3: isopentenyloxycoumarin (A). TLC showing the impact of Uvinul A (0.5% w/w) on the photodegradation of isopentenyloxycoumarin (B) and auraptene (C) after 6 days of light exposure using the conditions described in the Experimental Section. Lane 1: prenyloxycoumarin control; lane 2: prenyloxycoumarin exposed to light; lane 3: prenyloxycoumarin + Uvinul A control; lane 4: prenyloxycoumarine + Uvinul A exposed to light. Prenyloxycoumarins are respectively isopentenyloxycoumarin and auraptene for Fig 3B and 3C.

Trying to rationalize the mechanism of action underlying the herein observed effects, we took in consideration our previous acquired data about the interaction of auraptene with ERβ [21]. As a result of this investigation, we showed that compound **3** behaves as a partial agonist. Furthermore, it

is well known that ERβ play a crucial role in melanogenesis [26]. So, we assessed the extent of the induction capacities of the three oxyprenylated coumarins **2-4** towards ERβ using the same experimental model used by us in 2010 [20]. Results are reported in Figure 6A.



(A)



(B)

Figure 6. Interaction of compounds **2-4** with ERβ (A) and effects of 7-isopentenyloxycoumarin on melanin content in Melan-a cells in the presence of the steroidal estrogen antagonist ICI 182780 (B).

What is evident at a glance is that opposite results have been obtained again for 7-isopentenyloxycoumarin and umbelliprenin. Thus, a parallelism with results recorded in Melan-a cells can be traced. So, it may be hypothesized that the modulation in both senses of melanogenesis by oxyprenylated coumarins may be due to their interaction with ERβ. To further enforce our hypothesis, we estimated also the effect of compound **2** on melanin biosynthesis in the same cell line pre-treated with ICI 182780 1 μM (Faslodex®), a steroidal estrogen antagonist, over a period of 48 h. Results are reported in Figure 6B. As expected, ICI 182780 was able to maintain the melanin biosynthesis ate the same level of controls, while 7-isopentenyloxycoumarin was effectively able to antagonize its effect. Indeed, in cells pretreated with the ER antagonist for 1 h and then with compound **2**, biosynthesis of melanin was almost double respect to that recorded for cells treated only with ICI 182780. For what concerns the pigmenting activity displayed by 7-isopentenyloxycoumarin, its effect can be also mediated by the aryl hydrocarbon receptor, towards which we have recently demonstrated that compound **2** is an effective agonist. The involvement of this receptor as an efficient activator of melanogenesis has been also demonstrated [27].

In this paper we disclosed for the first time the effect of naturally occurring prenyloxycoumarins biosynthetically related to umbelliferone on melanogenesis using a non tumoral cell line. The research on skin tanning and lightening agents has a great importance not only for therapeutic aspects, but also for economic ones, when considered in the context of cosmetic products market. The profits for companies commercializing tanning activators and skin whitening lotions and creams are in rapid increase over the last decade. We have clearly demonstrated how a minor structural modification (e.g. increase from 5 carbon atoms to 15 carbon atoms) has a deep impact on the modulatory properties on melanogenesis. Furthermore, other determinants of capacities of the interaction of prenyloxycoumarins with the melanogenic machinery seem to be the presence of only hydrogen atoms in positions of the benzochromone ring other than 7. Indeed, as stated above, coumarins substituted in positions 8, like collinin **5** and lacinartin **6**, 6, like 7-geranyloxy-6-methoxycoumarin **7**, and 5, like, 5-geranyloxy-7-methoxycoumarin **8**, were totally ineffective in

boosting or decreasing melanin biosynthesis in Melan-a cells. Psoralens cannot be taken into consideration for the elaboration of a structure-activity relationship due to their great toxicity towards the same cell line at very low doses. The observed effects seem not to be mediated by a direct triggering of one or more components of the melanin biosynthetic machinery but instead it may rely on an interaction with ERβ. All the three coumarins herein under investigation have been found as components of the phytochemical pool of several plant species, the most of which belonging to the Rutaceae and Apiaceae families. This is the case of *Angelica sinensis* [Oliv. (Diels)] for which a marked depigmenting effect by apolar extracts from roots has been recorded [28], of *Citrus* spp. for which extracts from fruits enriched in flavonoids exhibited a skin whitening effects of more than 60 % and half of that recorded for kojic acid [29], and of several other species exhibiting both or tanning activities. Apart from individual natural products or phytopreparations, other natural remedies like bee products, in which we have recently demonstrated the presence of 7-isopentenyloxycoumarin, auraptene, and umbelliprenin [18], have shown effects on melanogenesis. In 2015 Jantakee and Tragoolpua reported that honey has an anti-tyrosinase activity [28], caffeic acid derivatives from propolis inhibit melanogenesis at different levels [29]. Tanning and/or skin lightening effects have been ascribed in all reported cases to selected classes of secondary metabolites like flavonoids, monoterpenes, tannins, and chromenes. The one reported herein is the first example in the literature, to the best of our knowledge, showing the modulatory properties of naturally occurring oxyprenylated coumarins on melanogenesis in a non-cancer cell line. Results described herein may largely contribute to consider such phenylpropanoids and phytopreparations containing them as effective therapeutic remedies for skin diseases featured by hyper- or hypopigmentation of dermal tissues as well as a novel class of ingredients for cosmetics and cosmeceuticals. Effects have been recorded for applied doses up to 40 µM. Such a range of concentration values are similar to those of ingredients, like arbutin and kojic acid, commonly employed in commercial products and thus can be considered safe for a potential use in humans, also considering that up to 40 µM practically no effects on cell viability were recorded. Our recent

studies clearly indicate that oxyprenylated coumarins can be regarded as additional components of the phytochemical pool of Apiaceae and Rutaceae plant species as well as of bee products like honey and propolis of different geographical origin. Coumarins investigated herein may act in synergy with already described chemicals from such plants or food preparations sharing the same activity on melanogesis (e.g. flavonoids, caffeic acid derivatives, quinones). Such synergies surely deserve to be studied in more details in the next future as well as the search for alternative oxyprenylated secondary metabolites and their effects on the melanin biosynthetic machinery.

Conclusions

In conclusion we have demonstrated herein that 7-isopentenyloxycoumarin, auraptene, and umbelliprenin are able to modulate the biosynthesis of melanin in murine Melan-a cells through the interactions with selected biological targets like ERβ and AhR, the effectiveness of which we have investigated recently with both receptors [20, 25]. Such a modulation is strongly dependant on the structure of the coumarin being the presence of an emiterpenyl chain a structural determinant for tanning activation whereas a farnesyl one leads to the opposite effect. The compound without such moiety, like umbelliferone, or placed in different position of the benzopyrone ring, did not provide any interaction. Once more it is confirmed how prenylation largely ameliorate the pharmacological effectiveness of a natural and/or a semisynthetic product [30].

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1) Oxyprenylated coumarins as skin tanning or whitening agents

2) The observed effect strictly depends on the length of the *O*- side chain

3) Oxyprenylated coumarins show a great potential in the cosmetic field