

RESEARCH ARTICLE

Depigmenting topical therapy based on a synergistic combination of compounds targeting the key pathways involved in melasma pathophysiology

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Abstract

Melasma has a complex pathophysiology with different cell types and signalling pathways involved. Paracrine factors secreted by keratinocytes, fibroblasts and endothelial cells act on melanocytes and stimulate melanogenesis. These paracrine factors are involved in the oxidative stress, inflammatory, vascular and hormonal pathways, among others. Damage of the dermoepidermal barrier also occurs and facilitates melanin deposition in the dermis, also known as dermal or mixed melasma. We used artificial intelligence tools to define the best combination of compounds for skin pigmentation inhibition. Mathematical models suggested the combination of retinol, diosmin and ferulic acid to be the most effective one. In vitro cellular tyrosinase activity assay proved that this combination had a synergistic depigmenting effect. Further assays proved that the combination could inhibit key pathways involved in melasma by downregulating *ET-1* and *COX-2* gene expression and IBMX-induced dendricity in human melanocytes, and upregulated the gene expression of *IL-1b*, *TIMP3* and several endogenous antioxidant enzymes. The combination also reduced melanin levels in a phototype VI 3D epidermis model. These results indicate that the combination of retinol, diosmin and ferulic acid is an effective synergistic complex for the treatment of melasma by regulating the key molecular pathways involved in skin hyperpigmentation pathophysiology.

KEYWORDS

artificial intelligence, hormonal, hyperpigmentation, inflammation, melasma, synergistic effect, vascular

1 | INTRODUCTION

Melanogenesis is a highly regulated mechanism in which melanocytes synthesize melanin to protect the potential damage of DNA against solar radiation. It has been demonstrated by fluorescence microscopy that this pigment is located near the nucleus of keratinocytes

as a microparasol,¹ acting as a photoprotective structure that scatters ultraviolet radiation (UVA/UVB) and visible light (VL).²

Despite the physiological role of melanin, excessive accumulation of this pigment may occur in some conditions such as melasma, an acquired type of face hyperpigmentation characterized by symmetrical, irregular brown macules on sun-exposed areas.³ It possesses a

complex pathophysiology due to the different extrinsic and intrinsic triggering factors and the several cellular processes involved.⁴ The regulation of melanogenesis is key to control the clinical outcome of the different therapeutic approaches.

From a molecular point of view, two levels could be clearly differentiated in the pigmentation process: the intracellular regulation, including all the cellular processes within the melanocyte that are involved in the synthesis and transfer of melanin; and the intercellular regulation of melanogenesis, in which different signalling pathways are activated to modulate melanocyte behaviour.

Regarding intracellular regulation, Microphthalmia-associated Transcription Factor (MITF) is the main transcription factor of the melanogenic enzymes tyrosinase (TYR) and the tyrosinase-related proteins (TRPs) TRP1 and TRP2/DCT.^{5,6} The correct folding and function of these three melanin-producing enzymes located in the melanosomes is also a key step. The enzymatic inhibition of the oxidative reaction of L-tyrosine and/or L-dihydroxyphenylalanine (L-DOPA) to dopaquinone (DQ), which leads to the synthesis of eumelanin/pheomelanin, has been the main target of depigmenting methods.⁷ The regulation of cytoskeletal network is important for the control of the final steps: the four-stage vesicles of melanosome maturation, the increase of dendricity to reach keratinocytes and the transfer of melanosomes.

Concerning intercellular regulation, keratinocytes and fibroblasts secrete prostaglandins which directly induce melanogenesis and an increase of dendricity.⁸ These prostaglandins not only activate the melanocyte but also the mast cell, which in turn stimulates other cells such as macrophages, CD4⁺ T cells, basophils, dendritic cells, neutrophils, and inflammatory mediators such as histamine.⁹ The result is the triggering of an inflammatory cascade which is evidenced by the infiltration of mast cells in biopsies of skin with melasma.¹⁰

Another key signalling pathway especially important in the pathophysiology of melasma is the vascular route. Activated mast cells secrete vascular endothelial growth factor (VEGF) which induces angiogenesis.⁹ In fact, larger and elongated blood vessels and an increased expression of VEGF have been observed in skin with melasma, compared to perilesional skin.¹¹ The increase of blood vessels leads to a higher secretion of endothelial cells that secrete endothelin-1 (EDN1), which increases dendricity and stimulates melanogenesis.¹² Additionally, other surrounding cells like fibroblasts and keratinocytes produce EDN1 which will also induce melanogenesis.^{13,14}

Another relevant pathway is the hormonal-related one. The melanocyte-stimulating hormone or melanotropin (α -MSH) is a peptide secreted by the pituitary gland, but it is also secreted in a paracrine manner by the keratinocyte, binding to the MC1R receptor and activating the cAMP pathway and downstream MITF.¹⁵ Immunolocalization with α -MSH antibody in ex vivo studies have shown greater expression of α -MSH in skin with melasma.¹⁶ In vitro studies have also demonstrated that there is an induction in dendricity of melanocytes when they are stimulated by α -MSH.¹²

The aim of this study was to use artificial intelligence (AI) to screen for the best combination of ingredients for a depigmenting topical formula and prove the efficacy on the main melanogenic pathways through in vitro and ex vivo models.

2 | METHODS

2.1 | Artificial intelligence

A set of 15 compounds with proven depigmenting efficacy, belonging to some of the main chemical families with melanogenesis inhibition activity (phenolic compounds, flavonoids, terpenoids, phenylpropanoids, stilbenes and isoprenoids),^{17,18} were analysed by AI using scientific data from trusted databases (DrugBank/FDA/Pubmed). Artificial Neural Networks (ANNs)-based approach was applied,¹⁹ which generates a model that simulates skin pigmentation. This model consists of a protein network that includes the main protein regulators of skin hyperpigmentation pathways according to the aforementioned databases, specifying the protein targets of each of the compounds selected for the screening (input). An algorithm then uses this information to predict synergistic combinations between the 15 compounds on the inhibition of skin hyperpigmentation, based on the regulation of the specified targets (output). Subsequently, sampling methods-based mathematical models²⁰ were used to elucidate the collaborative mechanism of action between the proposed compounds acting synergistically. These mathematical models predict the most probable path that leads from the stimulus (the regulation of the proteins by the different compounds) to the response (inhibition of hyperpigmentation).

2.2 | Cell culture

Human primary adult melanocytes (Cellsystems GmbH, Germany) (passage 4–5) were cultured in DermaLife Ma Medium supplemented with Zellshield (Minerva Biolabs GmbH, Germany). Human dermal adult fibroblasts (Promocell, Germany) were cultured in DMEM (Capricorn Scientific GmbH) supplemented with 10% fetal bovine serum (FBS) (Fisher Scientific) and Zellshield (Minerva Biolabs GmbH). Both cell types were incubated at 37°C in 5% CO₂. Unless specified, all reagents were obtained from Merck Life Science.

2.3 | Cell viability assay

To test the cell viability of the compounds, melanocytes were treated with the compounds at different concentrations for 72 h. SRB colorimetric assay was performed to assess cell viability of the compounds as previously described.²¹

2.4 | Cellular tyrosinase activity assay for synergy study

To quantify the possible synergy of the three compounds on the pigmentation process, melanocytes were treated in different conditions for 3 days:

- Control cells (non-treated).
- Stimulated cells: treated every 24 h with L-tyrosine 2 mM.

- Additive effect: treated every 24h with L-tyrosine 2mM+retinol 10 μ M, L-tyrosine-2mM+diosmin 5 μ M and L-tyrosine 2mM+ferulic acid 1mM, respectively.

- Synergy effect: treated every 24h with L-tyrosine 2mM+retinol 10 μ M+diosmin 5 μ M+ferulic acid 1mM.

After treatment, cells were trypsinized and lysed in PBS pH 7.0 with 1% Triton X-100. The cell lysate was incubated with L-DOPA and MBTH for 1 h at room temperature, and absorbance was measured at 492nm, as previously described.²² In parallel, BCA assay (Biovision, USA) was also performed in the cell lysates in order to normalize the cellular tyrosinase activity with the total protein content of each sample, according to manufacturer's protocol.

2.5 | Gene expression quantification by qPCR

To quantify the effect of the compounds combination on genes involved in pigmentation, fibroblasts were treated with retinol 10 μ M+diosmin 5 μ M+ferulic acid 1mM or with IL-1 α 10 ng/mL+retinol 10 μ M+diosmin 5 μ M+ferulic acid 1mM for 24h, and harvested for qPCR analysis. Total RNA was extracted using the Total RNA Purification Kit (Norgen, Canada). Retrotranscription of 200ng of RNA was performed using the PrimeScript RT reagent kit (Takara Bio). Finally, the cDNA obtained was amplified using the TB Green Premix Ex Taq (Takara Bio, Japan). The primers used are detailed in Suppl table 1. The qPCR conditions were 30s at 95°C followed by 40 cycles of 5 s at 95°C and 30s at 60°C.

2.6 | Melanocyte dendricity assay

To quantify the effect of the compounds combination on dendricity, melanocytes were treated for 24h with IBMX 1mM+retinol 10 μ M+diosmin 5 μ M+ferulic acid 1mM. Pictures of melanocytes were taken using the Olympus CKX41 (10x magnification) microscope. Cell counting using a haemocytometer was performed based on the number of dendrites per cell. Ten pictures for each condition were analysed.

2.7 | Live melanin quantification

To quantify the amount of melanin produced in live melanocytes, cells were treated for 24h with L-tyrosine 2mM+IBMX 1mM with or without retinol 10 μ M+diosmin 5 μ M+ferulic acid 1mM, and recorded using the manual CF-X 3D Cell Explorer microscope (Nanolive, Switzerland). A picture from the last hour of the treatment was taken and melanin granules (which appear in white colour) were stained in red using the Steve software from Nanolive to better appreciate the difference between conditions.

2.8 | Ex vivo assay

To test the efficacy of the combination in a skin 3D model, SkinEthicTM RHPE/Reconstructed Human Pigmented Epidermis Phototype VI (Episkin, France) was treated topically with retinol 50 μ M+diosmin 25 μ M+ferulic acid 5mM once a day for 6 days and harvested on day 8 for MTT and histology analysis. For the MTT assay, tissues were washed with PBS and incubated with MTT 0.5 mg/mL for 3 h at 37°C. Then, tissues were incubated in isopropanol 100% for 2 h at room temperature. Absorbance of the solution was quantified at 570nm. For the histology analysis, tissues were washed with PBS, incubated in 4% paraformaldehyde for 12h and embedded in paraffin. 10 μ m sections were performed using the microtome and Fontana Masson staining (Abcam) was used to detect melanin in the samples. All images were obtained by optical microscopy (Olympus Iberia).

2.9 | Statistical analysis

The experiments were performed three times under the same conditions and the average and standard deviation calculated for each condition was compared using Student's *T*-test (**p* < 0.05, ***p* < 0.01, ****p* < 0.001).

3 | RESULTS

3.1 | Artificial intelligence analysis of the best combination of compounds

The ANNs mathematical model proposed several potential combinations of compounds for skin hyperpigmentation treatment. From these, the combination of three compounds, retinol, diosmin and ferulic acid was chosen based on their synergistic action on pigmentation regulating, involving the key cellular processes that regulate melanogenesis: oxidative stress, inflammation, vascular and hormonal pathway. The sampling methods-based mathematical models show the proposed mechanism of action of this combination of compounds (Figure 1A).

3.2 | Cell viability of the compounds

Melanocytes were treated for 72h with the three compounds at different concentrations (Appendix S1). Maximum non-cytotoxic dose for retinol, diosmin and ferulic acid were 10 μ M, 5 μ M and 1mM, respectively. The combination of the compounds showed no cytotoxicity at these concentrations. Regarding fibroblasts treatment, the combination was used at the same concentrations as in melanocytes.

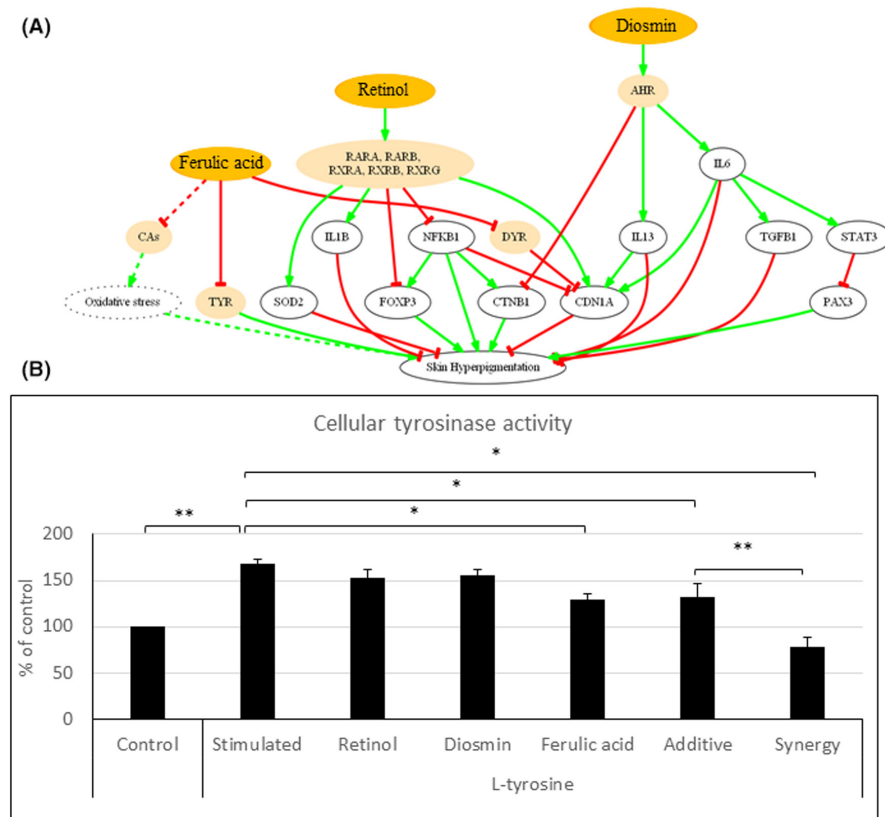


FIGURE 1 (A) Graphical representation of the most probable collaborative mechanism of action between retinol, diosmin and ferulic acid for skin depigmentation effects. These paths have been predicted by sampling methods-based mathematical modelling and biologically contextualized. (B) Percentage of cellular tyrosinase activity in non-treated cells (Control), cells stimulated with L-tyrosine 2 mM (Stimulated), cells stimulated with L-tyrosinase 2 mM and treated with retinol 10 μ M, diosmin 5 μ M or ferulic acid 1 mM alone (Retinol, Diosmin, Ferulic acid), cells stimulated with L-tyrosine 2 mM and treated with retinol 10 μ M the first 24 h, diosmin 5 μ M the next 24 h and ferulic acid 1 mM the final 24 h (Additive), and cells stimulated with L-tyrosine 2 mM and treated with retinol 10 μ M, diosmin 5 μ M and ferulic acid 1 mM simultaneously for 72 h (Synergy).

3.3 | Synergistic depigmenting effect of the combination

To test whether the combination of compounds have a synergic effect on skin depigmentation, cells were treated with L-tyrosine (stimulated), treated with L-tyrosine plus each of the compounds for 24 h alone, for a total of 72 h (additive) or treated with L-tyrosine plus the combination of compounds for the full 72 h (synergy), and cellular tyrosinase activity was measured. The results showed that retinol or diosmin alone slightly decreased tyrosinase activity (although this difference was not significant), while ferulic acid significantly decreased tyrosinase activity by 40%. The additive treatment with the compounds significantly decreased cellular tyrosinase activity by 36%, while the combination of compounds significantly decreased the cellular tyrosinase activity by 90% (Figure 1B). These results indicate that the combination of compounds has a synergic effect on skin pigmentation inhibition, as the depigmenting activity is higher with the compounds combined compared to applying each compound separated but sequentially in the same cells.

3.4 | Regulation of key hyperpigmentation pathways

Several papers have recently reported the key role of inflammation, oxidative stress, vascularization, and hormonal pathways in the regulation of hyperpigmentation disorders such as melasma.^{1,23,24} Thus, we investigated whether this combination of compounds could

regulate some of these key pathways, as suggested by the artificial intelligence study.

3.4.1 | Vascular pathway

First, we proved that the combination of compounds reduced the expression of *ET-1* and *COX-2* in fibroblasts incubated with IL-1 α (Figure 2A). These results confirm that the combination of the three compounds can regulate the inflammatory and the vascular component in hyperpigmentation. Regarding the inflammatory component, we also observed that the combination of compounds induced the expression of IL-1 β (Figure 2B), a cytokine which can inhibit melanogenesis according to previous research.²⁵

3.4.2 | Oxidative stress pathway

The combination of compounds also increased the expression of genes involved in antioxidant defense (*SOD1*, *TXNRD1*, *GPX3*, *GSTT1*, *CAT*, *HMOX1*) (Figure 2B), which is another key mechanism to counteract skin hyperpigmentation.²⁶ Another process occurring in the pathophysiology of hyperpigmentation is the alteration of the dermoepidermal barrier, mainly formed by collagen IV.²⁴ We observed that the combination did not increase the expression of *COL4A1*, but it did increase the gene expression of *TIMP3* (Figure 2B), which inhibits matrix metalloproteinases and thus protects collagen degradation.²⁷

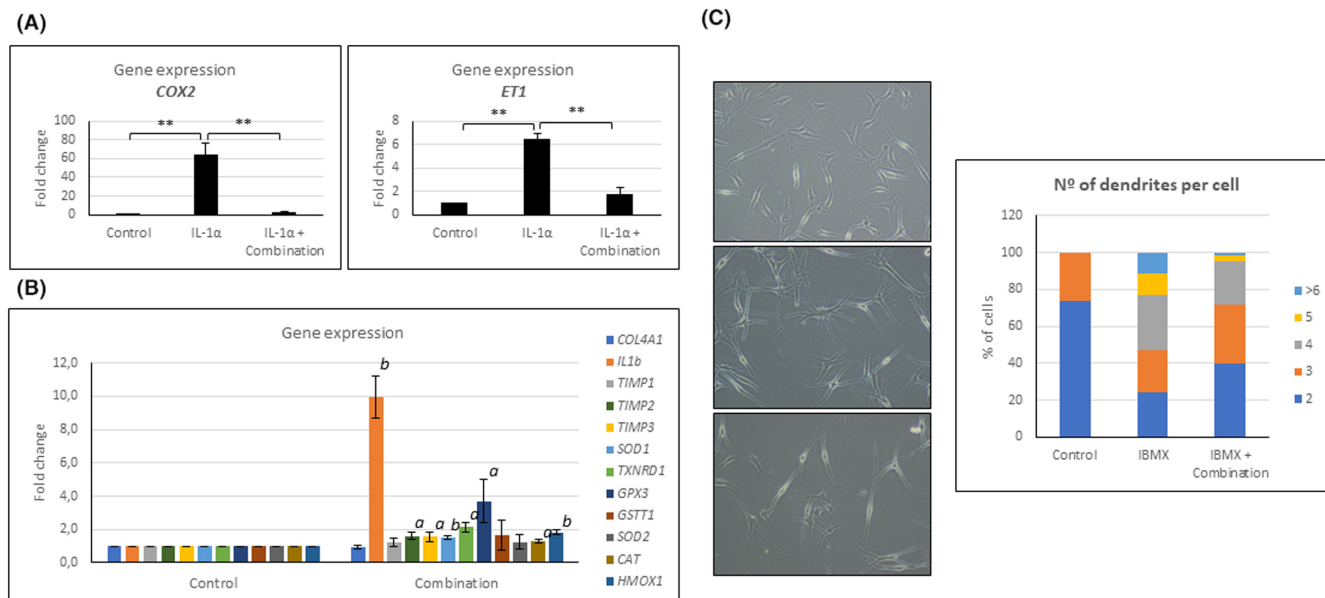


FIGURE 2 (A) mRNA levels of *COX2* and *ET1* in control fibroblasts, IL-1 α -stimulated fibroblasts and IL-1 α -stimulated fibroblasts treated with retinol 10 μ M, diosmin 5 μ M and ferulic acid 1 mM simultaneously during 24 h. (B) mRNA levels of *COL4A1*, *IL1b*, *TIMP1*, *TIMP3*, *SOD1*, *TXNRD1*, *GPX3*, *GSTT1*, *SOD2*, *CAT* and *HMOX1* in control fibroblasts and fibroblasts treated with retinol, diosmin and ferulic acid for 24 h. Statistical analysis: *a* where Student's *T*-test between control and combination is $p < 0.05$, *b* where Student's *T*-test between control and combination is $p < 0.01$, *c* where Student's *T*-test between control and combination is $p < 0.001$. (C) Representative optical microscope images, 10x magnification, of control melanocytes (1), IBMX-stimulated melanocytes (2) and IBMX-stimulated melanocytes treated with retinol, diosmin and ferulic acid (3), plus the cell count based on n° of dendrites per cell for each condition.

3.4.3 | Hormonal pathway

Another key pathway regulating hyperpigmentation is the one controlled by the hormone α -melanocyte-stimulating hormone (α -MSH).²³ We observed that the combination inhibited melanocyte dendricity stimulated by IBMX (Figure 2C,D), a cAMP phosphodiesterase inhibitor that activates α -MSH pathway.²⁸

3.5 | Depigmenting effect of the combination in cultured melanocytes and 3D epidermis model

A live cell assay stimulating melanocytes with L-tyrosine 2 mM + IBMX 1 mM with or without retinol 10 μ M + diosmin 5 μ M + ferulic acid 1 mM proved that this combination inhibits the accumulation of melanin granules in cultured melanocytes (Figure 3A). Moreover, Fontana Masson staining of tissues indicated that the combination was also able to reduce the accumulation of melanin in a 3D epidermis model (Figure 3B) without affecting the viability of the tissues according to the MTT assay.

4 | DISCUSSION

Melasma is a skin condition with a complex pathophysiology. At the cellular level, skin hyperpigmentation is regulated by two different processes. On one hand, at the intracellular level, in the melanocyte, where different pathways converge in the activation of MITF,

subsequently activating the expression of the key genes involved in melanogenesis, such as *TYR*, *TYRP1* or *TYRP2*.^{5,6} On the other hand, at the intercellular level, in the neighbouring cells (fibroblasts, keratinocytes, endothelial cells and immune system cells) which produce different paracrine factors (such as α -MSH, ET-1, PGE2, SCF or WNT) that can bind to melanocyte receptors and activate melanogenesis.^{1,24} Thus, these pathways, involved in oxidative stress, inflammation, vascularization and hormone regulation, are dysregulated in melasma pathogenesis and control excessive melanin accumulation.

In our research we used computational Artificial Neural Networks-based approach to select the best combination of compounds to treat skin hyperpigmentation. The mathematical model suggested the combination of retinol, diosmin and ferulic acid to synergistically inhibit the pigmentation process. The most probable mechanism of action suggested by this model included proteins involved in some of the key regulatory pathways previously mentioned: *SOD2* in oxidative stress or *IL1B*, *NFKB*, *IL13*, *IL6* in inflammation.^{25,26} Previous research has shown that retinol, diosmin and ferulic acid can individually act on these pathways.

Retinol has antioxidant and inflammation regulatory properties, being a good choice for skin photoaging treatment.²⁹ In fact, as proposed by the mathematical model used, the activation of retinoic acid receptors (RAR) may lead to a wide range of depigmentation mechanisms such as the downmodulation of inflammation through NF- κ B, the increase of interleukin-1 β or the increase of endogenous antioxidant enzymes such as *SOD2*.^{30–32} Diosmin has been widely described as regulator of inflammation.³³ According to

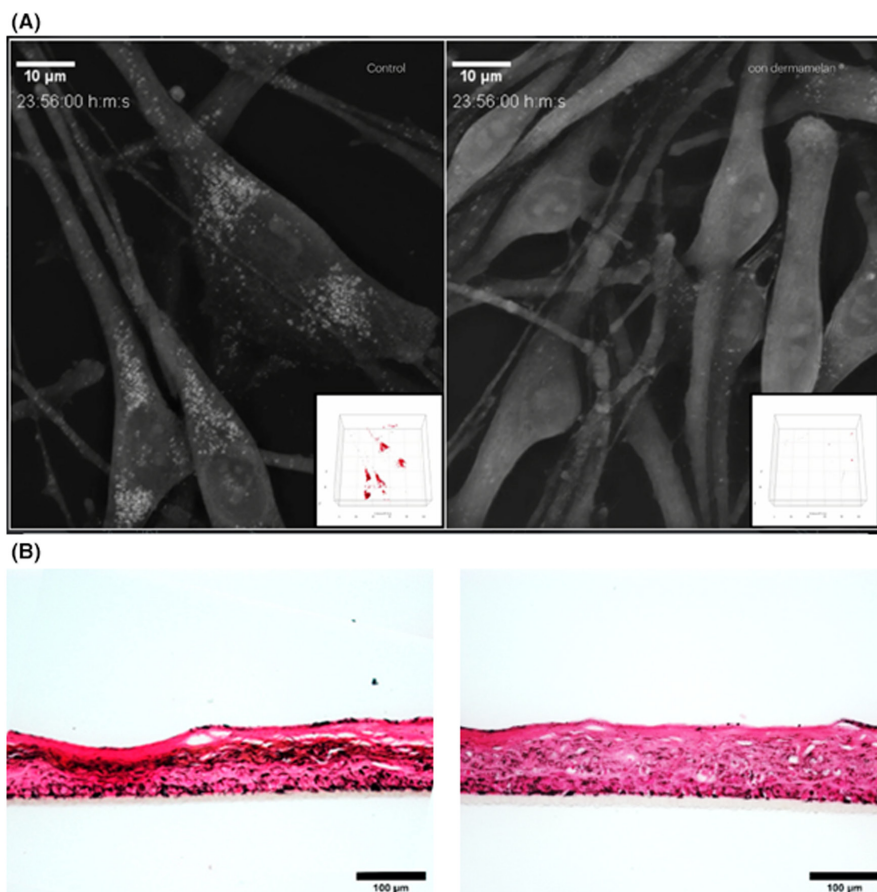


FIGURE 3 (A) Live cell images of melanocytes stimulated with L-tyrosine + IBMX (left) and melanocytes stimulated with L-tyrosine + IBMX + retinol + diosmin + ferulic acid (right). 3D representations with melanin stained in red from the same pictures are shown in the bottom-right part of each picture. (B) Representative optical microscopy images, 20x magnification, at day 7 from non-treated human reconstructed epidermis (left) and human reconstructed epidermis treated with retinol, diosmin and ferulic acid daily for 5 days. Fontana Masson staining was performed to evidence melanin granules.

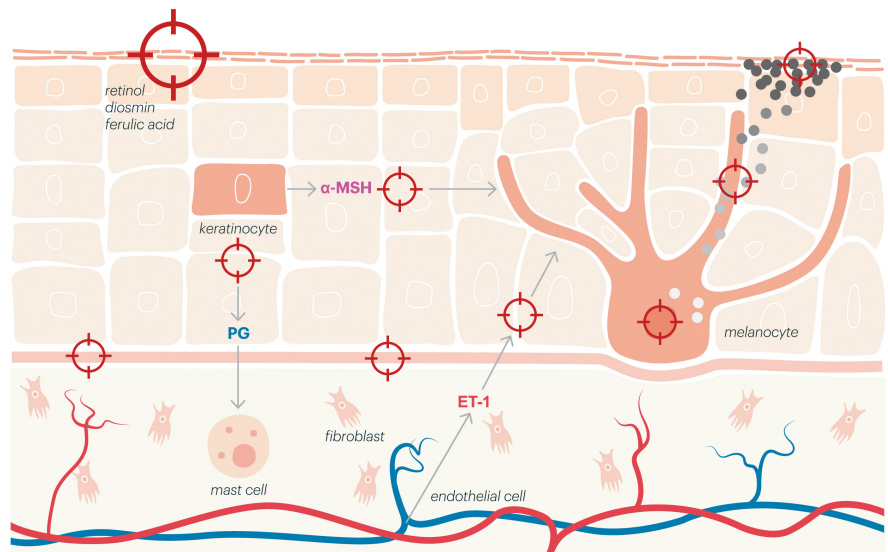
our results, the most probable way that diosmin contributes to the depigmenting effects is through the activation of aryl hydrocarbon receptor (Ahr),³⁴ which at the same time induces anti-melanogenic cytokines such as IL-6, IL-13 and TGF- β .^{35–37} Both retinol and diosmin converge in the regulation of *CTNB1*, which codifies for β -catenin, an intracellular mediator of the Wnt pathway. Further research could reveal if this combination of compounds also targets this key pathway involved in melasma pathogenesis. Ferulic acid is another natural compound with proven antioxidant and anti-inflammatory properties, among others,³⁸ and can also directly inhibit tyrosinase, the key enzyme in melanogenesis.³⁹

The proposed mechanism of action by the artificial intelligence study also highlights other potential targets for hyperpigmentation regulation. For example, the transcription factor PAX3 is regulated by the inflammation pathway and can directly regulate MITF levels.⁴⁰ In fact, other authors have described inhibitors of the PAX3-MITF axis.^{41,42}

Through these diverse mechanisms of action, this mathematical model indicated that the combination of active ingredients could have a synergistic effect on skin depigmentation. In order to prove the synergistic effect of the combination, tyrosinase activity from melanocytes treated with the compounds in an additive way or in combination was analysed. The positive results in this assay were a solid indicator of the synergistic effect of this combination of depigmenting agents. Further analysis of the key pathways involved in melasma pathology was another point in favour of the use of this complex. Endothelin-1 is secreted by fibroblasts, keratinocytes and

endothelial cells and can directly active melanogenesis. In fact, skin hyperpigmentation is normally accompanied by increased vascularity.⁴³ Inflammation is another key pathway involved in melasma.²⁵ By downregulating the levels of COX-2, an enzyme involved in the synthesis of prostaglandin E2 and initiator of the inflammatory cascade, the combination also inhibits the increased inflammation that occurs in skin hyperpigmentation. Another protein previously described to regulate melanogenesis is IL-1b, which has a depigmenting effect.^{25,44} As previously mentioned, oxidative stress can contribute to increased skin pigmentation.^{45,46} The increase of endogenous antioxidant enzymes such as SOD1, TXNRD1, GPX3, GSTT1, CAT, HMOX1 can reduce reactive oxygen species and prevent oxidative stress-activated pathways. Dermal accumulation of melanin may also occur in melasma, caused by the damage of matrix metalloproteinases (mainly MMP-2 and MMP-9) on the dermoepidermal barrier.²⁴ The combination of compounds can prevent this process by inducing the expression of TIMP2 and TIMP3, two of the endogenous proteins produced to inhibit the activity of matrix metalloproteinases.²⁷ Besides, previous research reported the extracellular matrix-enhancing and antiaging properties of these compounds, which potentiates the structural features of the skin.^{47,48} As human dermal fibroblasts are involved in all these processes and are key regulation of hyperpigmentation, we have used these cells to study the effect of the combination of compounds on the previously mentioned markers. However, further research on the effect of these compounds on other skin cells such as keratinocytes or endothelial cells is warranted to better characterize

FIGURE 4 Schematic view of the intercellular pathways regulating melanogenesis and levels of action regulated by the combination of retinol, diosmin and ferulic acid. Several cell types secrete paracrine factors which modulate melanocyte behaviour via specific signalling pathways, such as ET-1, prostaglandins (PG) or α -MSH. The combination of retinol, ferulic acid and diosmin can target some of these key pathways, protect the dermoepidermal barrier and inhibit of melanin production in the melanocyte.



the mechanism of action of this combination of compounds. On the other hand, proteomic studies that quantify the protein levels of these key regulators of the melanogenesis-regulating pathways (ET-1 and COX-2) are also needed to confirm the influence of the three compounds on the vascular and inflammatory pathways.

The depigmenting efficacy of the combination was finally proven in an ex vivo model of phototype VI reconstructed human epidermis. The positive results of the combination in this model suggests that these agents can penetrate the skin to reach their target cells and be effective also in the clinical treatment of hyperpigmentation conditions.

Thus, these results prove that this synergistic combination of compounds can target some of the main pathways involved in melasma pathogenesis, such as oxidative stress, inflammation, vascular pathway and hormonal pathway. Further research is warranted to study if this combination can target other key pigmentation regulation pathways. For instance, Wnt pathway, which has been described to be hyperactivated in melasma.²³ Our artificial intelligence study suggests that this pathway is also targeted both through the AhR and NF- κ B pathway on their action on β -catenin.^{49,50} Another factor involved in pigmentation regulation is stem cell factor (SCF), which is produced mainly by keratinocytes and fibroblasts.²³ Future studies could prove if this combination of compounds can also regulate this pathway.

Figure 4 summarizes the mechanism of action of this novel combination. On one hand, at the intercellular level, targeting melanin synthesis directly in the melanocyte. On the other hand, at the intercellular level, targeting the main factors and pathways in the neighbouring cells, which can directly activate the melanocyte and damage the dermoepidermal barrier.

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AUTHOR CONTRIBUTIONS

AM has designed the experiments, analysed results, written the manuscript. MP and EP have performed the experimental work and analysed results. SG has written the manuscript. AM, SG, MG and LS have proposed the initial research idea and supervised the work.

CONFLICT OF INTEREST STATEMENT

All authors work for the company mesoestetic Pharma Group. s.l. The company has applied for a patent, WO2022064083A1, for the use of retinol, diosmin and ferulic acid combination as a depigmenting dermopharmaceutical composition.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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