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Merging phytochemistry and polymer science to develop a peel-off mask for its potential use in the treatment of hyperpigmentation

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Overproduction of melanin due to overexpression of tyrosinase stands responsible for various skin disorders/pigmentations. The use of kojic acid, mequinol, hydroquinone, or topical retinoids in hyperpigmentation can potentially cause irritation to the skin (dermal sensitization), DNA damage, and cancer at therapeutic doses. This study aims to highlight the phytochemicals that can multitarget 3 major dermatological targets, namely tyrosinase, elastase, and collagenase, and simultaneously optimize the polymer blend to develop a peel-off mask for the topical delivery of the bioactives. A total of 206 phytochemicals were chosen for the virtual screening to identify the hit compounds that can potentially act as skin-lightening agents. *In-vitro* tyrosinase inhibition assay for 4 hit molecules was performed. The cytotoxicity of the treatment solution and its effect on the H_2O_2-stressed environment were studied against the murine fibroblast cell line (L929). Subsequently, a bioactive-loaded peel-off mask formulation was developed for its cosmetic application. Considering hesperidin as a standard, our results listed 11 such bioactive compounds that can potentially act as multi-targeted inhibitors of all the 3 chosen receptors. The results indicate an inhibitory effect on oxidative stress in fibroblasts. The peel-off mask formulation exhibited the desired properties with short drying time and smooth surface morphology (SEM analysis). The bioactive compounds that are highlighted as hit molecules necessitate further investigations following *in-vivo* experiments to establish effective alternatives in the treatment of hyperpigmentation with minimal side effects.

Keywords: Hyperpigmentation, anti-aging, multi-target, polymer blend, drug-repurposing

Introduction

The growth in the cosmetic sector and its manufacturing divisions boomed in the recent past, encouraging research in developing novel formulations to meet the market demand for cosmeceuticals. As per reports from Fact.MR (www.factmr.com), the global market for skin-whitening cosmetics reached a valuation of approximately US$10 billion in 2021 and is predicted to increase to US$22 billion at a Compound Annual Growth Rate (CAGR) of 7.5 % by 2032. This accounts for a significant fraction of the global cosmetics market of US$800 billion in 2023[1].

Melanin has found its use in sclerotization of the cuticle, wound healing, protecting the skin from harmful ultraviolet (UV) rays, and scavenging reactive oxygen species. Its synthesis occurs in melanosomes and subsequently gets translocated to the surrounding epidermal keratinocytes. However, its overproduction due to overexpression of tyrosinase stands responsible for melasma, age spots, solar lentigo, and various skin disorders/pigmentations. Hence, tyrosinase is studied as the biological target to control the increased production of melanin because an initial inhibition of this enzyme's activity will essentially interrupt the melanin biosynthesis pathway. Hyperpigmentation seems to be an aesthetic problem that prevails in modern society. Advanced formulations are developed as skin-lightening topical cosmetics to counter hyperpigmentation complications. Such cosmeceuticals have gained significant limelight with their widespread market in the Middle East and Asia, including the countries China, India, Japan, Korea, and Indonesia[2]. Tyrosinase (molecular weight ranging from 13.4 kDa to 128 kDa) acts as the rate-limiting enzyme in the biosynthesis pathway of melanin and regulates melanogenesis within melanocytes. Melanogenesis starts with the oxidation of L-tyrosine and L-DOPA (i.e., levodopa or L-3,4-dihydroxyphenylalanine) in the presence of the catalyst tyrosinase to produce the pigment melanin. Tyrosinase directs the transformation of L-tyrosine to L-DOPA by hydroxylation (monophenolase activity), and

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subsequently L-DOPA to o-dopaquinone by oxidation (diphenolase activity). Finally, the conversion of o-dopaquinone to melanin readily occurs following a cascade of non-enzymatic reactions[3]. Other melanocyte-specific enzymes include tyrosinase-related protein 1 (TRP1) and tyrosinase-related protein 2 (TRP2)[4,5]. Normal functioning of melanocytes can be influenced by various intrinsic and extrinsic factors. Hyperpigmentation is triggered by several reasons: malfunctioning of melanocytes and keratinocytes, hormonal responses, inflammation, and injuries/surgeries. Chronic solar exposure promotes melanocyte production in the epidermis and forms melanocyte clusters, causing dark spots due to the imbalanced distribution of melanocytes[6]. The melanocyte signaling pathway is influenced by cytokines, such as basic fibroblast growth factor (bFGF) and endothelin-1 (ET-1). Tyrosinase inhibitors that are less expensive but possess high bioactivity and low toxicity are in huge demand in cosmetic industries to address hyperpigmentation and act as skin-lightening agents. Glabridin showed promising results in the inhibition of tyrosinase activity in B16 murine melanoma cells at concentrations ranging from 0.1 to 1.0 µg/mL; more specifically, it inhibited the T1 and T3 tyrosinase isozymes. Moreover, UVB-induced pigmentation and erythema were controlled in guinea pigs' skin upon topical applications of 0.5% glabridin. The main anti-tyrosinase activity of glabridin comes from the two hydroxyl groups present in its structure[7]. Apocarotenoids bixin and norbixin are reported to reversibly inhibit the enzymatic activity of tyrosinase in the B16-F0 melanoma cell line at higher concentrations[8]. Novel phytoconstituents are screened as skin-lightening agents based on their potential to inhibit tyrosinase, tyrosinase-related protein-1 (TRP1), and tyrosinase-related protein-2 (TRP-2)[9]. Floralginsenoside A isolated from *Panax ginseng* showed promising results in inhibiting tyrosinase and decreasing microphthalmia-associated transcription factor protein expression[10].

With aging and/or exposure to free radicals, the decrease in epidermal stem cells restricts the regeneration of the elastin and collagen fibers, thereby influencing the skin's elasticity. Hence, dermatologists and cosmetologists have shown keen interest in limiting the activity of the enzymes elastase and matrix metalloproteinases that are known to degrade collagen. The cleaving of proteins by elastase favorably occurs at the amino acid valine. Besides premature skin aging and wrinkle formation, overexpression of elastase is reported to trigger several pathological conditions, including rheumatoid arthritis, cystic fibrosis, chronic obstructive airway disease, and psoriasis, to name a few[11]. Numerous phytochemicals (such as agrimonilin, pedunculagin, and epigallocatechin gallate among polyphenols) have shown promising results in the inhibition of elastase activity. Among the hundred plant extracts that were tested to inhibit the activity of tyrosinase and elastase, 17 extracts exhibited an IC50 value of less than 100 µg/mL. A positive correlation was obtained between enzymatic inhibitory potential and the concentration of aromatic compounds in the plant extracts. Hydroalcoholic extract of *Hypericum scruglii* was found to exert an inhibitory effect selectively on the enzyme elastase[12]. In a study with 21 plant extracts, white tea is reported to be the most promising agent to possess anti-elastase and anti-collagenase activities[13]. Dermo-cosmetic formulations of the fruit extracts of *Ginkgo biloba*, *Punica granatum*, *Ficus carica*, and *Morus alba* were tested using an anti-collagenase assay (by monitoring the reduction of collagenase enzyme) and reviewed in human subjects. The treatment for 56 days showed a significant reduction in the depth, length, and area of wrinkles[14]. The ethanolic extract of cashew leaf extract exhibited significant tyrosinase and collagenase activities at 100 µg/mL and 40 µg/mL, respectively; hence, cosmeceutical formulations were developed using it as a whitening and anti-wrinkle skincare cream[15]. A cosmetic cream to treat melasma and facial wrinkles was prepared using a glycolic acid peel with a composition of 10% glycolic acid and 2% hydroquinone[9]. Studies revealed that lower doses of resveratrol, genistein, 11,13-dihydrohelenalin acetate, and parthenolide inhibited the release of elastase from neutrophil granulocytes and impaired NF-kappa-B activity. Based on our literature review, we have found 25 compounds that are reported as skin lightening agents (supported by *in-vitro* data and/or clinical trials) and can be listed as follows: arbutin (a glycosylated hydroquinone), niacinamide, N-acetylglucosamine, glabridin, kojic acid, N-Phenylthiourea, floralginsenoside A, aloin, betulinic acid, bixin, norbixin, crocin, 2-hydroxy-4-methoxybenzaldehyde, aleosin, alpha-bisabolol, ellagic acid, hesperidin, liquiritin, mequinol, procyanidin, silymarin, azelaic acid, glycolic acid, retinol, and salicylic acid[3,6,8,10,16-20].

Peel-off masks loaded with bioactive compounds are evenly applied to the skin surface and peeled off after complete drying. Due to its occlusive effect, it is widely used in the treatment of skin-related complications such as wrinkles, acne, and clogged pores due to the deposition of dust. After peeling the polymer film off the skin, dead cells/other deposited residues on the stratum corneum get removed[21]. The frequently encountered film-forming polymer in peel-off masks is PVA due to its biodegradability, swelling ability, and the ability to adjust the formulation viscosity. The awareness of the safety issues related to cosmetics draws attention to addressing the harmful outcomes of cheap chemical-based cosmetics. The use of kojic acid, mequinol, hydroquinone, or topical retinoids in hyperpigmentation can potentially cause irritation to the skin (dermal sensitization), DNA damage, and cancer at therapeutic doses[22,23]. Mequinol can induce erythema, desquamation, and hypopigmentation[9]. Hence, dermatological research prioritizes a quest to establish novel low-toxicity tyrosinase inhibitors with higher efficacy. This study on screening multi-targeted inhibitors of tyrosinase, elastase, and collagenase is a strategic shift from treating hyperpigmentation as a purely melanocytic disorder to treating it as a dermal-epidermal interaction disorder, highlighting the role of tyrosinase in melanin synthesis and matrix metalloproteinases (elastase and collagenase) in ECM degradation. Simultaneous inhibition of these three dermatological targets disrupts the inflammatory feedback loop, restores structural skin integrity, and reduces melanin output, thereby contributing to effective treatments for recalcitrant conditions like melasma, solar lentigines, and post-inflammatory hyperpigmentation in photoaged skin.

Materials and methods

Materials required

Trypsin-EDTA (0.25 %, Gibco), penicillin-streptomycin (10,000 U/mL, Gibco), 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide or MTT (Invitrogen), tyrosinase from mushroom (lyophilized powder, ≥1000 unit/mg solid from Sigma-Aldrich), L-DOPA (≥ 98% purity by TLC, Sigma-Aldrich), hesperidin (≥ 80%, Sigma-Aldrich), myricetin ((≥ 97% purity by HPLC, Tokyo Chemical Industry Co. Ltd.), luteolin supplement capsule (Double wood), rutin supplement capsule (Piping Rock), epigallocatechin gallate (EGCG) (98% purity by HPLC, Maple life sciences), Fetal Bovine Serum or FBS (Gibco), Dulbecco Modified Eagle Medium or DMEM (Gibco), polyvinyl alcohol or PVA (approximately 115 kDa, Loba Chemie), kappa-carrageenan (Sigma-Aldrich), carboxymethyl cellulose sodium salt (Loba Chemie), sodium alginate (Loba Chemie)

Molecular docking

The structures of three receptors (viz. tyrosinase (PDB ID: 5m8L), elastase (PDB ID: 1BRU), and collagenase (PDB ID: 1CGL) were downloaded from the website https://www.rcsb.org/ while phytochemicals/standard drugs (i.e., selected ligands) were obtained from the PubChem website (https://pubchem.ncbi.nlm.nih.gov). A total of 206 phytochemicals were chosen for the virtual screening to identify the hit compounds that can potentially act as skin-lightening agents. The database also includes 25 compounds that are marketed as skin-lightening agents and listed in the introduction section.

Prior to molecular docking, the crystal structures of target receptors were virtually prepared (e.g., removing water molecules, hetero atoms, or any complexed ligands, adding hydrogen atoms, checking missing residues, adding charges, and performing other operations to refine the X-ray crystal structure) by using the software Biovia Discovery Studio 2020 and UCSF Chimera 1.15[24-26]. The ligand molecules were considered flexible and subjected to energy minimization using the universal force field (uff), and optimized to generate 3-D geometry and its tautomers prior to molecular docking. Molecular docking was performed using the AutoDock Vina wizard of the PyRx virtual screening tool[27]. The grid box parameters for each target receptor were listed in Supplementary Table 1. The Lamarckian Genetic Algorithm (LGA) was employed with a grid spacing value of 0.375 Å and exhaustiveness of 8. AutoDock Vina's empirical scoring function selected the top-scored conformations, and the final poses (i.e., ligand-receptor complexes) were ranked. The visualization of the results of molecular docking and its analysis (such as interacting residues, H-bonds, hydrophobic interactions, etc.) was done by the software Chimera X, Biovia Discovery Studio 2020[28]. Open Babel was used for any file conversion (e.g., converting .sdf to pdb file format)[29].

The inhibition constant (Ki) was calculated from the binding energy using Equation 1 as follows[30]:

Table 1 — Bioactive compounds multi-targeting tyrosinase, elastase, and collagenase with high binding affinity

Phytochemical	Binding affinity (in kcal/mol) Tyrosinase	Elastase	Collagenase
Hesperidin (standard skin-lightening agent)	-9.9	-8.4	-11.2
beta-Copaene	-10	-9.1	-8.6
Kaempferol-7-rhamnoside	-10.1	-8.8	-10.6
Abiesin	-10.2	-8.7	-11.5
Flavanomarein	-9.2	-8.2	-10.1
Quercitrin	-8.5	-8.2	-9.5
Taraxerol	-10.9	-8	-10.8
Friedelin	-9	-8	-8.6
Floralginsenoside A	-8.7	-8.3	-9.3
Liquiritin	-10.6	-8.2	-9.9
Silymarin	-8.3	-9.7	-10.8
Rutin	-8.4	-8.5	-10.5

$$K_i = e^{\frac{-\Delta G}{RT}} \quad \ldots(1)$$

where ΔG = binding affinity (in kcal/mol), R = Gas constant (1.987 × 10–3 kcal.K-1.mol-1) and T = 298.15 K

The analysis of the results is done by considering the binding affinity, interacting residues forming H-bonds and hydrophobic interactions, bond length, and inhibition constant.

In-vitro tyrosinase inhibition assay

In order to study the inhibition of tyrosinase activity by the selected phytochemicals, the protocol based on Mason and Peterson's method was followed as previously reported with slight modifications[31,32]. Briefly, stock solutions (at a concentration of 1 mg/ml) of phytochemicals were prepared using dimethyl sulfoxide (DMSO) and diluted accordingly for dose-dependent experiments. The reaction mixtures containing 20 units of mushroom tyrosinase (i.e., 20 µL of 1000 U/mL) and the test samples were diluted using 50 mM potassium phosphate buffer (pH 6.8) in a 96-well plate and pre-incubated on a rocking shaker for 10 min. Following, 10 mM L-Dopa (in phosphate buffer) was pipetted into each well to initiate the enzymatic reaction and allowed to further incubate for 20 min. The temperature was maintained at 298 K. In order to stop the enzyme-substrate reaction, 0.1N HCl was added to the assay solutions. The formation of dopachrome was measured by recording the optical density at 490 nm against a suitable blank (to eliminate the absorbance of the samples themselves) using a microplate reader (iMark™ Microplate Absorbance Reader). Hesperidin was chosen as the positive control (i.e., the standard).

The percentage inhibition of tyrosinase activity was calculated using Equation 2, and the 50% inhibitory concentration (IC_{50}) values were reported from the calibration curves of the respective test samples.

$$\% \text{ Inhibition } = \frac{(C_n - B_r) - (S_t - B_s)}{C_n} X\ 100\ \% \quad \ldots(2)$$

where C_n is negative control absorbance (i.e. DMSO in phosphate buffer with tyrosinase and L-Dopa), B_r is the absorbance of the prepared blank solution for the reaction mixture (i.e. solution of tyrosinase in phosphate buffer without test samples/positive control and substrate), S_t is the absorbance of the test sample (phytochemical dilutions) or positive control in presence of both tyrosinase and L-Dopa, B_s is the absorbance of prepared blank for the test sample (i.e. solution of the test sample in phosphate buffer with tyrosinase but without L-Dopa)

Cell viability using MTT assay

The murine fibroblast cell line (L929) was procured from the cell repository of the National Centre for Cell Sciences (NCCS), Pune, India. Cells were cultured in DMEM (pH 7.4) supplemented with 10% (v/v) FBS and antibiotics (penicillin and streptomycin at 100 U each per mL) and incubated at 37 °C in a humidified atmosphere with 5% CO_2. Media was replaced every alternate day, and the cells were subcultured once the flask was observed to be 75-80% confluent.

Considering the binding affinities and cost of the compounds, luteolin, rutin, myricetin, and EGCG were selected as cheaper alternatives to hesperidin (as tyrosinase inhibitors) to develop cost-effective formulations in the treatment of hyperpigmentation. Each stock solutions of rutin, luteolin, and EGCG were prepared in incomplete DMEM at a concentration of 100 µg/mL. Myricetin is sparingly soluble in aqueous buffers; hence, its stock solution was prepared in DMSO at a concentration of 5 mg/mL. The poly-drug solution (having a concentration of 100 µg bioactives/mL) was finally developed by homogeneous mixing of the stock solutions of rutin, luteolin, EGCC, and myricetin in the ratio 1:1:1:0.02 with the final DMSO content of 0.66 %. This solution was serially diluted (2X, 4X, and 8X dilutions), and the cytotoxicity was determined against the L929 cell line using the MTT solution (prepared at a concentration of 2 mg/mL). The trypsinized cells were resuspended, and a 96-well

tissue culture plate was seeded with $5x10^4$ cells/well, following incubation at 37°C in a humidified 5% CO_2 incubator. After 24 h, the growth medium was replaced with fresh medium, and the treatment was done, followed by incubation for a further 24 h. Upon addition of MTT solution, the plate was incubated for 4 h. Subsequently, the supernatant (MTT solution with the growth medium) was aspirated, and DMSO was added to dissolve the formed formazan salt. Absorbance was recorded using a microplate reader (iMark™ Microplate Absorbance Reader) at 595 nm. The percentage of relative cell viability was calculated using Equation 3, and the data were compared to that of untreated control cells. 10% DMSO was used as the positive control in our study.

$$\% \text{ Cell viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells } (Negative\ control)} X\ 100\ \% \quad \ldots(3)$$

Any changes in the morphology of cells, such as membrane shrinkage, presence of apoptotic bodies, membrane blebbing, and presence of condensed nuclei were observed by a phase-contrast microscope (Olympus CX21). Further, hydrogen peroxide (H_2O_2) was used to induce oxidative stress, and the response of the poly-drug solution on L929 under such a stressed environment was assessed. After 24 h incubation of the cell-seeded well plate, fibroblasts were treated with different dilutions of the poly-drug solution and preincubated for 1 h. Subsequently, co-incubation was done with 300 µM H_2O_2 for 24 h prior to the MTT assay, following the above-mentioned protocol.

Preparation of peel-off mask

Polymer solutions (aqueous) prepared for optimizing the blend composition are as follows: 8 % (w/v) PVA, 6 % (w/v) sodium alginate, and 3 % (w/v) CMC. The blend was prepared by mixing the solutions in a ratio of 5:1:2. The final composition was adjusted by adding kappa-carrageenan (1% (w/v)), glycerol (2.5% (w/v)), and propylene glycol (4% (w/v)) to get the desired consistency. 2.5 mg of each bioactives (luteolin, rutin, EGCG, myricetin) was dissolved in 1 mL of DMSO so that the final concentration of bioactives reaches 10 mg/mL in DMSO. This polydrug solution acts as the active ingredient in the product. 1 mL of polydrug solution is then added to 99 mL of base polymer solution to prepare the drug-loaded peel-off mask with a concentration of 0.1 mg/mL.

The peel-off gel was evenly spread on a surface, and a strip of film was removed after its complete drying. Repeated folding at the same place was done to manually determine the folding endurance of the film. Subsequently, 1 g of gel was dissolved in 100 mL distilled water, and the pH of the formulation was recorded. In order to study the thermodynamic stability of the prepared formulation, 10 accelerated freeze-thawing cycles (from 4 °C to 40 °C) were performed.

Statistical analyses

All experiments were performed in triplicate, and data are represented as mean±SD. Statistical analyses were carried out using GraphPad Prism version 9.0, and a p-value of less than 0.05 was considered statistically significant. Comparisons between multiple treatment groups and the control were performed using a one-way analysis of variance (ANOVA), followed by Dunnett's post-hoc test for comparisons against the single untreated control group, or Tukey's honestly significant difference (HSD) test for pairwise comparisons among all experimental groups, where applicable.

Results and discussions

Molecular docking

A dataset of 618 binding affinities (206 ligands docked with 3 receptors) was obtained after performing molecular docking. A higher magnitude of binding affinity indicates a stronger interaction in the ligand-receptor complex, and hence the ligand can act as a potent inhibitor of the receptor with lower values of inhibition constant (Ki). Among the 25 chosen skin-lightening phytochemicals (established and currently marketed), hesperidin exhibited strong interaction with all the 3 receptor molecules; hence, it is considered as the standard compound in our study. Any phytochemical with binding affinity close to that of hesperidin is highlighted as the hit molecule. Apart from hesperidin, other marketized skin-lightening agents, namely glabridin, betulinic acid, procyanidin, floralginsenoside A, liquiritin, and silymarin reported to be high-affinity ligands of tyrosinase. However, the remaining 18 tyrosinase inhibitors reflected poor *in-silico* data. Considering hesperidin as a standard, our results listed 11 such bioactive compounds that can potentially act as multi-targeted inhibitors of all the 3 chosen receptors (presented in Table 1). Besides, 36 phytochemicals formed stable complexes with both tyrosinase and collagenase, hence listed as

the second-line of hit molecules (listed from Sl. No. 2-37 in Supplementary Table 1).

Considering the best-scored pose of hesperidin with tyrosinase, it is found to interact with the amino acid residues of chain D with a binding affinity of -9.9 kcal/mol. At the same binding pocket of the hesperidin-tyrosinase complex, the phytochemicals luteolin, myricetin, and rutin exhibit high affinity. Detailed analysis of the interaction site using Biovia Discovery Studio reveals the interacting residues that coexist in the complexes of hesperidin and other ligand molecules with tyrosinase. Both luteolin and hesperidin are seen to interact with the Lys-233, Pro-115, Arg-114 and Glu-237 of chain D. Among hesperidin and myricetin the common interacting residues are found to be Leu-229, Pro-445, Thr-112, Ser-106, Pro115, Lys233, and Arg114, while rutin-tyrosinase complex shares bonds with Lys-233, Thr-112, and Glu-237 of chain D. The 2D interaction diagram (figure 1) compares the binding pocket of the standard compound with that of the identified hit molecules. These findings indicate that a similarity in the mechanism of action of hesperidin, luteolin, rutin, and myricetin on tyrosinase can exist due to their closely related binding pockets in the ligand-receptor complexes. The bioactive compounds that are highlighted as hit molecules necessitate further investigations following *in-vitro* and *in-vivo* experiments to establish effective alternatives in the treatment of hyperpigmentation with minimal side effects.

Tyrosinase inhibitory activity

The IC_{50} values (calculated by regression analysis) reveal the extent of inhibition of tyrosinase enzyme activity by the selected bioactives (presented in Figure 2). The positive control hesperidin exhibits an IC_{50} value of 171.17 ± 3.59 µg/mL. Among the hit molecules, luteolin possesses the highest IC_{50} value of 338.527 ± 7.61 µg/mL; hence, it does not seem to

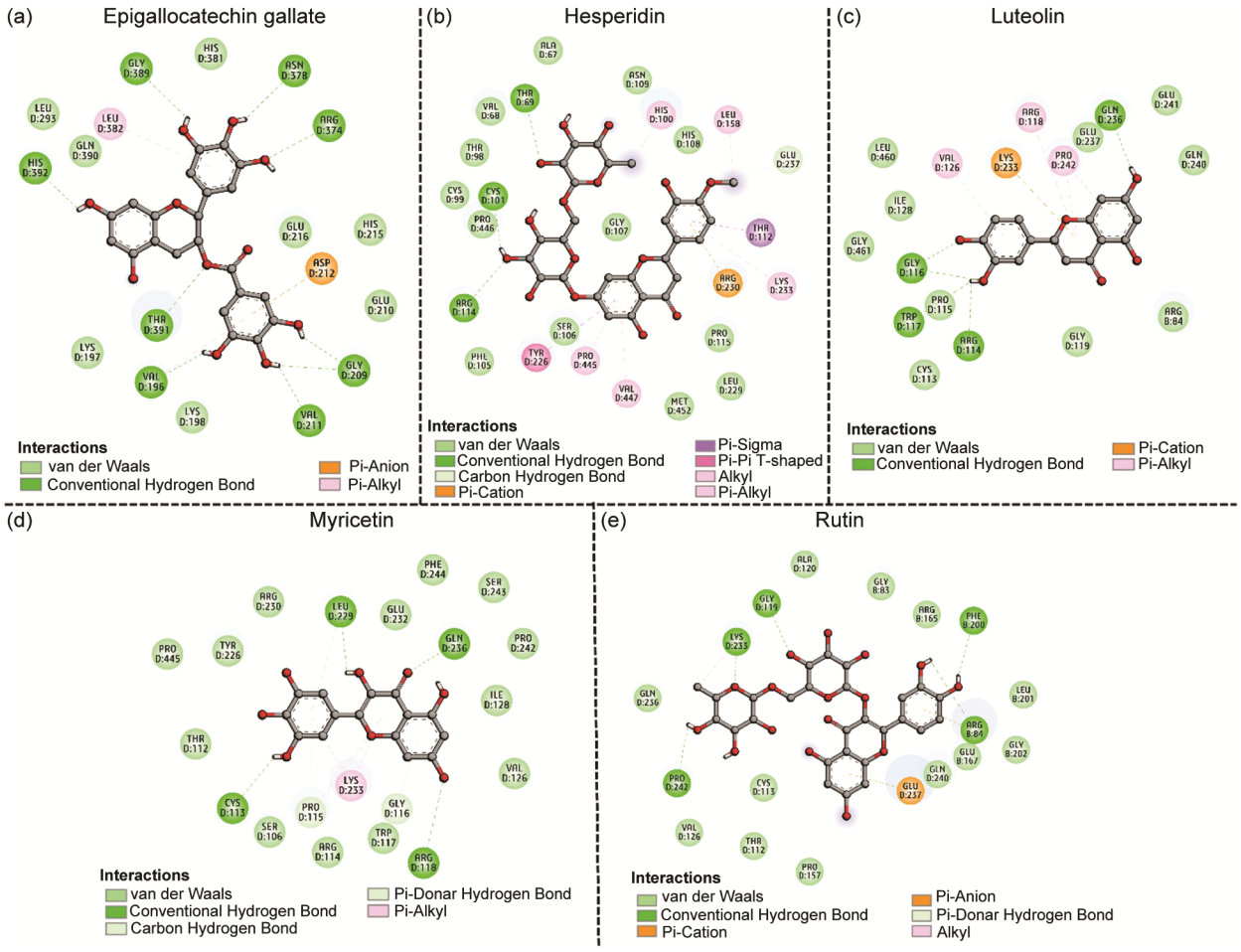

Fig. 1 — The 2D interaction diagram analyzing the binding pocket and the interacting residues of the receptor tyrosinase with the identified hit molecules (i.e., the potential multi-targeting inhibitors).

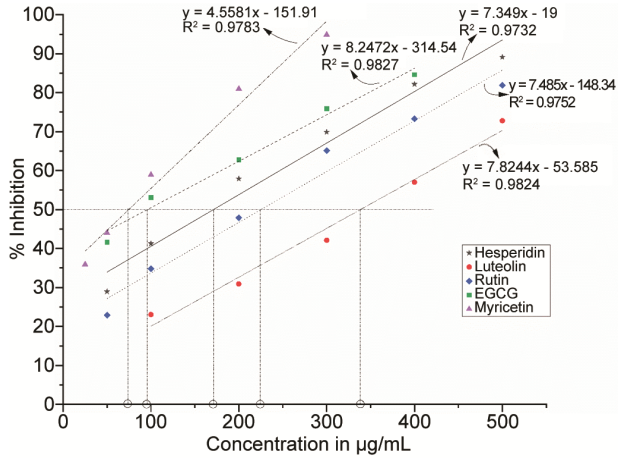

Fig. 2 — 2 Regression analysis indicating the IC 50 values of the respective compounds against tyrosinase.

directly interact with mushroom tyrosinase. However, as previously reported, the skin whitening effect of luteolin may occur due to the inhibition of adenyl cyclase involved in the signal transduction pathway of α-MSH[33]. In α-MSH-stimulated B16 melanoma cells, luteolin is found to inhibit both tyrosinase activity and melanin production at a concentration of 10 µM. Rutin, with an IC_{50} of 224.47 ± 3.67 µg/mL suggested to moderately inhibit the enzyme activity. In a published work, rutin is found to restrict ROS-induced skin aging, with increased mRNA expression of collagen, type I, alpha 1 (COL1A1) with a simultaneous decrease in the mRNA expression of matrix metallopeptidase 1 (MMP1)[34]. Hence, rutin is frequently used in cosmetic formulations to improve skin elasticity and lessen the length, area, and number of wrinkles. Compared to hesperidin, the phytochemicals myricetin and EGCG proved to be potent tyrosinase inhibitors with much lower IC_{50} values of 74.07 ± 6.06 µg/mL and 95.97 ± 6.25 µg/mL, respectively.

Cytotoxicity assessment

The prepared poly-drug solution (i.e., the stock solution containing 100 µg of bioactives per mL) exhibited a slight decrease in the cell viability of L929 cells after 24 h of incubation. 2-fold, 4-fold, and 8-fold serial dilutions, however, exhibited no noticeable toxicity against L929 fibroblasts (as represented in Figure 3). This is in good accordance with the previous studies, which indicated the non-toxic behavior of rutin-rich extract on fibroblasts and monocyte/macrophages at a high concentration of 1000 µg/mL[35]. Similar articles on wound healing and cell migration revealed that a dose of 3 µM of

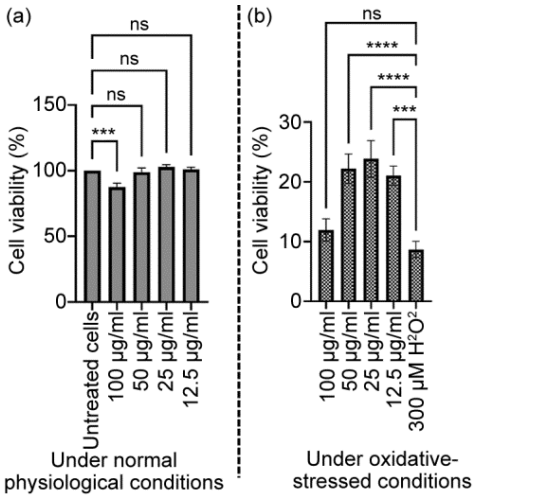

Fig. 3 — Cytotoxic effect of different dilutions of poly-drug solution on L929 cells cultured in (a) normal physiological conditions and (b) under H_2O_2 treated induced oxidative-stressed environment.

myricetin exhibited approximately 87 % cell migration rate, while its high concentration of 100 µM reported more than 90 % cell viability in human dermal fibroblasts[36]. Figure 4 portrays the morphology and growth of L929 cells upon treatment with 100 µg of bioactives per mL and compares with the untreated cells. No significant morphological changes were observed in the treated fibroblasts, and the absence of apoptotic bodies, cell shrinkage, and condensed nuclei was reported. Subsequently, the cytoprotective activity of the poly-drug solution on L929 was assessed against H_2O_2. H_2O_2 permeates through the cellular membrane and directs remodeling, inflammation, apoptotic fate, and several vascular processes of cells by acting as an intracellular second messenger. The results (presented in Figure 3) indicate an inhibitory effect on oxidative stress, establishing the antioxidant potential of the bioactives.

The primary objective of our study was to evaluate their cytotoxicity and biocompatibility as a prerequisite for potential future applications. The L929 fibroblasts were selected specifically for this purpose because it represent a standard, well-characterized cell line recommended by ISO 10993-5 for initial cytotoxicity screening of biomaterials and compounds, thereby establishing the safe concentration range of the poly-drug solution (before proceeding to more specialized cell models) and encouraging *in-vivo* validation using appropriate animal models to confirm efficacy and safety in a complex biological system.

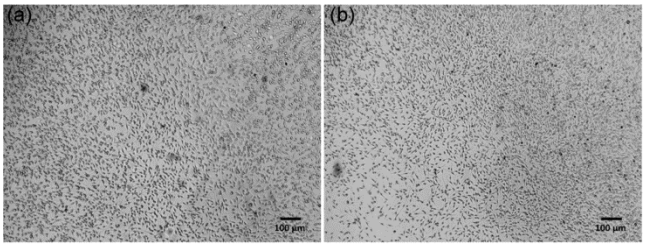

Fig. 4 — Morphology and growth of L929 cells cultured (a) without any treatment, (b) with a treatment of 100 µg bioactives/mL, exhibiting its non-toxicity at the administered dose.

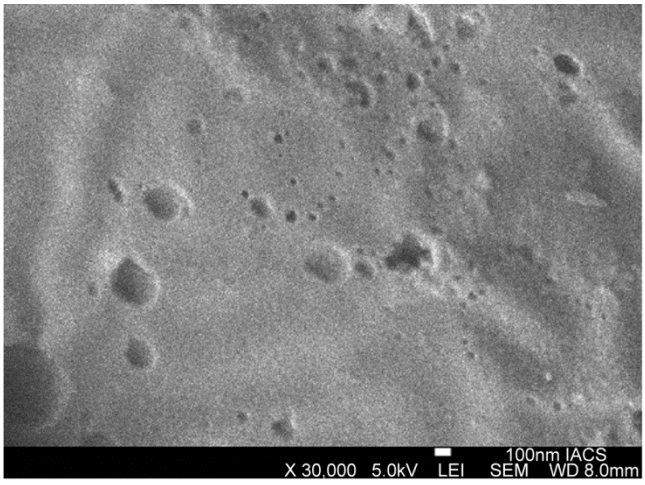

Fig. 5 —The SEM image of the peel-off mask reveals its smooth surface morphology with no pore formation upon its drying.

Peel-off mask

The base formulation (i.e., without the active ingredient) is observed to be off-white in color, homogeneous, odorless, and easily spreadable without dripping. It exhibits good thin-film-forming capability and a low drying time of around 15 min. While the mask slowly dehydrates, moisture gets collected in the layers beneath and releases the bioactives, enabling better penetration into the skin within a short period of time. The folding endurance test on the films allowed them to be folded 100 times at the same position without breaking. This ensures that it can be easily removed from the applied surface without any breaks. The prepared base formulation (i.e., without the active ingredient) of the peel-off mask can also be used to load other bioactive molecules depending on its intended purpose. The average pH of the formulation is found to be around 7.5. The formulation is found to be stable under freeze-thaw stress. The SEM image of the peel-off mask reveals its smooth surface morphology with no pore formation upon drying (Figure 5).

Conclusion

A dataset of 618 binding affinities (206 ligands docked with 3 receptors) was obtained after performing molecular docking. Among the 25 chosen skin-lightening phytochemicals (established and currently marketed), hesperidin exhibited strong interactions with all the 3 receptor molecules; hence, it is considered the standard compound in our study. Our results listed 11 such bioactive compounds that can potentially act as multi-targeted inhibitors of all the 3 chosen receptors. Besides, 36 phytochemicals formed stable complexes with both tyrosinase and collagenase, hence listed as the second-line of hit molecules. *In-vitro* assay revealed myricetin and epigallocatechin gallate as potent inhibitors of tyrosinase. The prepared poly-drug solution at a concentration of 100 µg of bioactives per mL exhibited a slight decrease in cell viability after 24 h of incubation, simultaneously established an inhibitory effect on oxidative stress in fibroblasts. The peel-off mask formulation exhibited the desired properties of short drying time and smooth surface morphology with no pore formation. Summarily, this work on predicting the multi-target activity is based entirely on molecular docking predictions and binding free energy calculations. Although these *in-silico* findings provide structural insights to prioritize lead compounds, the bioactive compounds that are highlighted as hit molecules necessitate further investigations following *in-vitro* and *in-vivo* experiments to establish effective alternatives in the treatment of hyperpigmentation with minimal side effects.

Conflict of Interest

The authors declare that they have no relevant financial or non-financial conflict of interest to disclose.

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References

1 Liu JK, Natural products in cosmetics. *Nat Prod Bioprospecting*, 12 (2022) 1. https://doi.org/10.1007/s13659-022-00363-y

2 Masum MN, Yamauchi K, & Mitsunaga T, Tyrosinase Inhibitors from Natural and Synthetic Sources as Skin-lightening Agents. *Rev Agric Sci*, 7 (2019) 41.

3 Kundu A, & Mitra A, Evaluating tyrosinase (monophenolase) inhibitory activity from fragrant roots of Hemidesmus indicus for potent use in herbal products. *Ind Crops Prod*, 52 (2014) 394.

4 Zaidi KU, Ali SA, Ali A, & Naaz I, Natural Tyrosinase Inhibitors: Role of Herbals in the Treatment of Hyperpigmentary Disorders. *Mini Rev Med Chem*, 19 (2019)796.

5 Bonesi M, Xiao J, Tundis R, Aiello F, Sicari V, & Loizzo MR, Advances in the Tyrosinase Inhibitors from Plant Source. *Curr Med Chem*, 26 (2019) 3279.

6 El Khoury R, Michael Jubeli R, El Beyrouthy M, Baillet Guffroy A, Rizk T, Tfayli A, & Lteif R, Phytochemical screening and antityrosinase activity of carvacrol, thymoquinone, and four essential oils of Lebanese plants. *J Cosmet Dermatol*, 18 (2019) 944.

7 Yokota T, Nishio H, Kubota Y, & Mizoguchi M, The inhibitory effect of glabridin from licorice extracts on melanogenesis and inflammation. *Pigment Cell Res*, 11 (1998) 355.

8 Anantharaman A, Hemachandran H, Priya RR, Sankari M, Gopalakrishnan M, Palanisami N, & Siva R, Inhibitory effect of apocarotenoids on the activity of tyrosinase: Multi-spectroscopic and docking studies. *J Biosci Bioeng*, 121 (2016) 13.

9 Kaur H, Afsana, Aggarwal G, & Nagpal M, Potential benefits of phytochemicals for treatment of hyperpigmentation. *J Drug Deliv Ther*. 9 (2019) 420.

10 Lee DY, Lee J, Jeong YT, Byun GH, & Kim JH, Melanogenesis inhibition activity of floralginsenoside A from Panax ginseng berry. *J Ginseng Res*, 41 (2017) 602.

11 Siedle B, Hrenn A, & Merfort I, Natural compounds as inhibitors of human neutrophil elastase. *Planta Med*, 73 (2007) 401.

12 Chiocchio I, Mandrone M, Sanna C, Maxia A, Tacchini M, & Poli F, Screening of a hundred plant extracts as tyrosinase and elastase inhibitors, two enzymatic targets of cosmetic interest. *Ind Crops Prod*, 122 (2018) 498.

13 Thring TS, Hili P, & Naughton DP, Anti-collagenase anti-elastase and anti-oxidant activities of extracts from 21 plants. *BMC Complement Altern Med*, 9 (2009) 27.

14 Ghimeray AK, Jung US, Lee HY, Kim YH, Ryu EK, & Chang MS, In vitro antioxidant collagenase inhibition and in vivo anti-wrinkle effects of combined formulation containing Punica granatum, Ginkgo biloba, Ficus carica, and Morus alba fruits extract. *Clin Cosmet Investig Dermatol*, 8 (2015) 389.

15 Srisuksomwong P, Kaenhin L, & Mungmai L, Collagenase and Tyrosinase Inhibitory Activities and Stability of Facial Cream Formulation Containing Cashew Leaf Extract. *Cosmetics*, 10 (2023) 17.

16 Leyden JJ, Shergill B, Micali G, Downie J, & Wallo W, Natural options for the management of hyperpigmentation. *J Eur Acad Dermatol Venereol*, 25 (2011) 1140.

17 Chen J, Yu X, & Huang Y, Inhibitory mechanisms of glabridin on tyrosinase. *Spectrochim Acta A Mol Biomol Spectrosc*, 168 (2016) 111.

18 Ali SA, Galgut JM, & Choudhary RK, On the novel action of melanolysis by a leaf extract of Aloe vera and its active ingredient aloin, potent skin depigmenting agents. *Planta Med*, 78 (2012) 767.

19 Biswas R, Chanda J, Kar A, & Mukherjee PK, Tyrosinase inhibitory mechanism of betulinic acid from Dillenia indica. *Food Chem*, 232 (2017) 689.

20 Clark AK, & Sivamani RK, Phytochemicals in the treatment of hyperpigmentation. *Bot Targets Ther*, 6 (2016) 89.

21 Kulkarni SV, Gupta DAK, & Bhawsar S, Formulation and evaluation of activated charcoal peel off mask. *Int J Pharm Res Technol*, 9 (2019) 44.

22 Burnett CL, Bergfeld WF, Belsito DV, Hill RA, Klaassen CD, Liebler DC, Marks JG, Shank RC, Slaga TJ, Snyder PW, & Andersen FA, Final report of the safety assessment of Kojic acid as used in cosmetics. *Int J Toxicol*, 29 (2010) 244.

23 Masub N, & Khachemoune A, Cosmetic skin lightening use and side effects. *J Dermatol Treat*, 33 (2022) 1287.

24 Biovia DS, Discovery studio modeling environment. (2017).

25 Design L, Pharmacophore and ligand-based design with Biovia Discovery Studio. (2014).

26 Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, & Ferrin TE, UCSF Chimera-a visualization system for exploratory research and analysis. *J Comput Chem*, 25 (2004) 1605.

27 Dallakyan S, & Olson AJ, Small-Molecule Library Screening by Docking with PyRx. *Chemical Biology: Methods and Protocols*, (2015) 243.

28 Pettersen EF, Goddard TD, Huang CC, Meng EC, Couch GS, Croll TI, Morrin JH, & Ferrin TE, UCSF ChimeraX: Structure visualization for researchers, educators, and developers. *Protein Sci Publ Protein Soc*, 30 (2021) 70.

29 O'Boyle NM, Banck M, James CA, Morley C, Vandermeersch T, & Hutchison GR, Open Babel: An open chemical toolbox. *J Cheminformatics*. 3 (2011) 33.

30 Cournia Z, Allen B, & Sherman W, Relative Binding Free Energy Calculations in Drug Discovery: Recent Advances and Practical Considerations. *J Chem Inf Model*, 57 (2017) 2911.

31 Mason HS, & Peterson EW, Melanoproteins I Reactions between enzyme-generated quinones and amino acids. *Biochim Biophys Acta*, 111 (1965) 134.

32 Zhang C, Lu Y, Tao L, Tao X, Su X, & Wei D, Tyrosinase inhibitory effects and inhibition mechanisms of nobiletin and hesperidin from citrus peel crude extracts. *J Enzyme Inhib Med Chem*, 22 (2007) 91.

33 Choi MY, Song HS, Hur HS, & Sim SS, Whitening activity of luteolin related to the inhibition of cAMP pathway in α-MSH-stimulated B16 melanoma cells. *Arch Pharm Res*, 31 (2008) 1166.

34 Choi SJ, Lee SN, Kim K, Joo DH, Shin S, Lee J, Lee HK, Kim J, Kwon SB, Kim MJ, Ahn KJ, An IS, An S, & Cha HJ, Biological effects of rutin on skin aging. *Int J Mol Med*, 38 (2016) 357.

35 Sinsuebpol C, Nakpheng T, Srichana T, Sawatdee S, Pipatrattanaseree W, Burapapadh K, & Changsan N, Assessing the Anti-Aging and Wound Healing Capabilities of Etlingera elatior Inflorescence Extract: A Comparison of Three Inflorescence Color Varieties. *Molecules*, 28 (2023) 7370.

36 Chaniad P, Tewtrakul S, Sudsai T, Langyanai S, & Kaewdana K, Anti-inflammatory, wound healing and antioxidant potential of compounds from Dioscorea bulbifera L. bulbils. *Plos One*, 15 (2020) e0243632.