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RESEARCH ARTICLE

Unveiling molecular melanogenesis inhibition of lime (*Citrus aurantifolia* (Christm.) Swingle) peel extract

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Abstract

The overproduction of melanin induces multiple types of hyperpigmentation, including post-inflammatory melanoderma, solar lentigo lesions, and melasma. Consequently, the design of melanogenesis inhibitors as skin-enlightening agents through various molecular mechanisms is urgently needed. *Citrus aurantifolia* (Christm.) Swingle, or lime, has garnered significant interest as a natural product that contains secondary metabolites to inhibit melanogenesis. Nevertheless, the molecular mechanism of its effect on melanogenesis is not yet fully understood. The lime peel was extracted using 70% ethanol, and the major compounds of the extract were detected by thin-layer chromatography (TLC). The gene target and the top ten target proteins associated with molecular pathways were further investigated based on bioinformatics analysis. Additionally, molecular docking studies were carried out on two specific protein targets to assist in further study. Finally, *in vitro* study of tyrosinase (TYR) and carbonic anhydrase 2 (CA2) enzyme inhibition was carried out to confirm the extract's anti-melanogenic effect. There are 10 target genes, including *CA2, CYP1B1, CA1, CYP19A1, CA9, ABCB1, CA3, ABCG2, TYR,* and *ADORA1*. An *in silico* study of hesperidin (HD) and tangeretin (TN) to bind with TYR and CA2 found that the binding affinity was binding energy of -10.01 and -6.85 kcal/mol. The *in vitro* study demonstrated that the extract inhibited TYR with an IC_{50} value of 59.71 ppm, which is *in vitro* more effective in inhibiting it than kojic acid. Based on these findings, *Citrus aurantifolia* peel is effective in impeding melanogenesis and can be explored as a plant-based cosmetic.

Keywords: bioinformatics, citrus, cosmetics, melanin, skin lightening, tyrosinase

INTRODUCTION

Skin is a vital organ in the human body where various cells reside to serve its functions to provide a barrier to water loss and pathogens, as well as to protect against diverse physical trauma, including chemical, thermal, and ultraviolet (UV) radiation (Abdo et al., 2020). Human skin, a crucial part of the innate immune system, possesses multiple molecular mechanisms to protect this organ from UV exposure (Mohania et al., 2017; Orazio et al., 2013). The outermost layer of the epidermis functions as the primary barrier against external harmful effects, which later respond to other immune cells such as Langerhans cells and T-lymphocyte cells. Melanin in keratinocytes plays a crucial role in protecting against UV radiation in the epidermis layer (Brenner & Hearing, 2008; Fu et al., 2020). Melanin is a pigment produced by melanocytes in the basal layer of the epidermis, hindering UV penetration by blocking and hindering it as a harmless heat (Gromkowska et al., 2021; Mohania et al., 2017). UV radiation escaping melanin blocking later leads to DNA damage by either directly causing DNA breaks or reactive oxygen species (ROS) (Cabaco et al., 2022; Mohania et al., 2017).

Melanin is produced by melanocytes as a product of the physiological process called melanogenesis, which is accelerated by UV radiation and heat (Gillbro & Olsson, 2011; Li et al., 2022; Zhang et al., 2023). It

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requires several enzymes, including tyrosinase, tyrosinase-related protein (TRP1)1, and TRP2 in the melanosome (Serre et al., 2018). Among them, tyrosinase (TYR) is the rate-limiting step of melanogenesis, and its transcription is regulated by a microphthalmia-associated transcription factor (MITF) (Li et al., 2022). In particular, the binding of α-melanocyte-stimulating hormone (α-MSH) to melanocortin receptor (MC1R) in melanocytes activates cAMP-dependent protein kinase A (PKA), which regulates the cAMP response element-binding protein (CREB), triggering MITF induction (Alam et al., 2018). In the nucleus, MITF acts as the transcription factor for TYR, TRP-1, and TRP2 (Lee et al., 2022; Yoon & Youn, 2022). The melanin-synthesizing enzymes convert L-tyrosine to melanin through a multi-stage biological process, which is interfered with by pH inside the melanosome (Fuller et al., 2001; Serre et al., 2018; You et al., 2022). Due to the lower pH of melanocytes, Caucasian skin has a slower melanogenesis rate than Black skin (Fuller et al., 2001; You et al., 2022). Subsequently, melanin granule is transported to keratinocytes and accumulate in the outer part of the epidermis layer, resulting in skin pigmentation (Masum et al., 2019; Serre et al., 2018). The overproduction of melanin induces multiple types of hyperpigmentation, including post-inflammatory melanoderma, solar lentigines, and melasma. Thus, melanin is further believed to be responsible for sunburn and mottle formation (Merecz-Sadowska et al., 2022; Mohania et al., 2017). Therefore, the development of melanogenesis inhibitors as skin-lightening agents through various molecular mechanisms is urgently needed.

Skin-lightening agents work through wide molecular mechanisms: tyrosinase transcription inhibition (vitamin A derivative; tretinoin, retinoid acid, retinol), tyrosinase inhibition, epidermal turnover acceleration (alpha/beta hydroxy acid), melanosome transfer inhibition, anti-inflammation, and radical scavenger (Burger et al., 2016; Couteau & Coiffard, 2016). Most melanogenesis inhibitors in the market target tyrosinase (Orazio et al., 2013), such as hydroquinone (HQ) (Arndt & Thomas, 2015; Kumari et al., 2018), kojic acid (Kolbe et al., 2013), arbutin (Chandorkar et al., 2021), and tranexamic acid (Maeda, 2022; Molina et al., 2022). Many effective hyperpigmentation agents, such as hydroquinone and retinoic acid, must be delivered under a dermatologist's supervision, while others are allowed in cosmetic products (Maeda, 2022). Discontinuation of tyrosinase inhibitor) The regimen includes recurrent effects of hyperpigmentation (Maeda, 2022), skin irritation, erythema, permanent skin depigmentation, and contact dermatitis (Kanthraj, 2010; Masub & Khachemoune, 2022). Due to the unfavorable side effects of current hyper-pigmentation treatments, safe plant-derived melano-genesis inhibitors to treat skin hyperpigmentation disorders are urgently needed.

Recently, *Citrus aurantifolia* (Christm.) Swingle), or lime, has received great interest as a natural product that contains secondary metabolites to impede melanogenesis. Lime peel is rich in polymethoxylflavone (PMF), such as tangeretin and nobiletin, which previously revealed anti-melano-genesis activity (Kim et al., 2015; Yoshizaki et al., 2017), through tyrosinase inhibition activity (Obaid et al., 2021). PMF mixture extracted from orange peel performed anti-inflammatory activity by suppressing prostaglandin E2 after UV radiation (Yoshizaki et al., 2014). Instead of flavonoid, essential oil compounds in Citrus, such as β-elemene, farnesene, limonene, sabinene, terpinene-4-ol, revealed inhibition of tyrosinase activity and gene expression (Yang et al., 2023). Furthermore, another Citrus flavonoid, hesperidin, showed anti-melanogenesis activity through inhibition of tyrosinase and MITF degradation (Lee et al., 2015; Liu-Smith & Meyskens, 2016). Therefore, this study emphasized the activity of *Citrus aurantifolia* investigation from a molecular mechanism's perspective of melanogenesis.

To support the ultimate goal of the United Nations Sustainable Development Goal (SDG) to reduce waste production (UN SDGs, 2015), this study repurposes *Citrus aurantifolia* peel extract to inhibit melanogenesis. Due to its various Citrus secondary metabolites as potential active compounds, we investigate the whole extract of *Citrus aurantifolia* peel on melanogenesis. After identifying its predominant compounds by Thin Layer Chromatography (TLC), bioinformatics analysis was conducted to search for responsible genes and proteins targeted by the compounds. Furthermore, an *in silico* experiment revealed the molecular interaction between the compounds and protein targets. Finally, this study quantitatively investigated an *in vitro* inhibition study of the extract against one of the selected protein targets.

MATERIALS AND METHODS

Materials

Citrus aurantifolia fruit was harvested from Klaten, Central Java, Indonesia, with authentication number 01/LKTO/Far-USD/XI/2023. The fruit peel of *Citrus aurantifolia* was extracted using ethanol (Smart-Lab A-1035) at pro-analyst grade. Thin-layer chromatography system used silica gel GF254 (Sigma-Aldrich) as the stationary phase, and the

mobile phase was butanol (Smart-Lab 71-36-3) and acetic acid (Smart-Lab 64-19-7). Sigma-Aldrich (MAK257-1KT), a tyrosinase inhibition kit, is used to confirm tyrosinase inhibition activity. The e-tubes were Axygen, all tips were Biologix, and the 96-well plate was Corning**.**

Citrus peels extraction

The fruit peels were collected, dried at 50°C, pulverized, and sieved through a 60-mesh screen. The resulting powder was subjected to maceration with ethanol 90% (1:30 w/v ratio) for 20 hours under continuous shaking. The macerated extract underwent filtration through Whatman no.1 filter and was concentrated with a rotary evaporator. Subsequently, the concentrate was further dried in an oven at 50°C to constant weight. The extraction yield was documented based on the final mass oof the dried extract.

Phytochemical screening

The ethanol extract of *Citrus aurantifolia* underwent phytochemical screening via TLC to identify tangeretin and hesperidin. Thus, a mobile phase composed of butanol-acetic acid-water (3:1:1 v/v%) was utilized on silica gel GF254 plates that had been pre-activated at 110°C for 30 minutes. In the testing process, 5 µL of the sample and reference solutions were dropped onto the TLC plate using a micropipette and allowed to develop. To confirm the presence of tangeretin and hesperidin, the retention factors of the sample and reference solutions were calculated and compared under UV light at 254 nm.

Bioinformatics analysis

Before commencing the analysis, information regarding proteins and genes associated with melanogenesis and hyperpigmentation was extracted from credible databases, including PubMed (www.ncbi.nlm.nih.gov), OMIM (www.omim.org), and GeneCard (www.genecard.org). Subsequently, an exploration of proteins and genes influenced by Tangeretin (TN) and Hesperidin (HD), both directly and indirectly, was conducted using the Swiss target prediction database (http://www.swisstargetprediction.ch/). Overlapping genes associated with TN and HD within the scope of pigmentation and melanogenesis were investigated using a Venn diagram (www.interactivenn.net). Protein-protein interaction (PPI) networks and gene clustering patterns were revealed using the www.string-db.org platform. The resulting datasets were further processed and visualized using Cytoscape 3.9.1 and STRING-DB v11.5 software (Shannon et al., 2003; Szklarczyk et al., 2021). To identify the top 10 genes, this study utilized the MCC and Degree algorithms from the Cyto-Hubba plugin, classifying them as hub genes (Chin et al., 2014).

In vitro tyrosinase inhibition test

The quantification of the extract's tyrosinase inhibitory activity was evaluated *in vitro* using a colorimetric assay with L-tyrosine as the substrate. In this study, kojic acid (0.75 mM) was utilized for comparison as a positive control (Setiawati et al., 2024). The tyrosinase substrate solution was diluted in the tyrosinase assay buffer with an enhancer in a 2:23:5 v/v ratio. Then, the extract was introduced at concentrations of 0.1, 1, 10, 100, and 1000 µg/mL in the tyrosinase buffer. Additionally, 20 µL of kojic acid was added to a 96-well plate. The tyrosinase enzyme solution was then dropped to each well, followed by a 10-minute incubation at 25°C. Afterward, L-tyrosinase as much as 30 µL was dropped into each well. A multi-mode reader (Allsheng FlexA-200) was used to observe the absorbance within a 30 to 60-minute range. The following formula was applied to compute the relative inhibition percentage:

$$\frac{(Slope\ (EC) - Slope\ (S))}{Slope\ (EC)} \times 100\%$$

Molecular docking

CA2 (1A42) and TYR (5M8O) were retrieved from the protein data bank www.rcsb.org. The native ligand was separated from macromolecules and protonated using BIOVIA Discovery Studio 2021 as a docking control. Ligand was protonated by applying Gaisteiger charge, while protein was applied Kollman charge using AutodockTools 1.5.7. For grid box settings, the ligand was caged at coordinates x = -4.138, y = 4.641, z = 14.520. The size of the grid box is 40 x 40 x 40 grid points with a spacing point 0.375 Å. Molecular docking was performed using Genetic algorithm parameters with GA runs = 100. The docking method is considered valid by evaluating the control RMSD value between the previous and after re-docking using LigRMSD (https://ligrmsd.appsbio.utalca.cl/), where the RMSD value must be less than or equal to 2.0 Å. Complex data is stored and projected in 3D and surface model superimposition using BIOVIA Discovery Studio 2021 before and after re-docking. For cross-docking, the used ligand (tangeretin & hesperidin) was retrieved from PubChem (https://pubchem.ncbi.nlm.nih.gov). The ligand was then prepared and re-docked using the same method and parameters as the control docking. Docking data are employed to observe the free-binding energy that occurs. Docking results are also visualized to see the protein-ligand molecular interactions that occur in 2D and 3D.

RESULTS

This study extracted the dried peels with ethanol, and then qualitatively analyzed the components of this extract using thin-layer chromatography (TLC) (Figure 1a). Based on TLC analysis, our lime peel extract contained Citrus flavonoids: tangeretin (TN) and hesperidin (HD). The Rf value of 0.71 in the extract was identified as tangeretin, while the Rf value of 0.76 was attributed to hesperidin (Figure 1b). Furthermore, our study used tangeretin and hesperidin to conduct bioinformatics analysis on melanogenesis-related genes affected by the extract.

Molecular pathway inhibition of those compounds (Figure 2a) in melanogenesis was explored with bioinformatics analysis. There were 12,567 genes involved in melanogenesis and pigmentation, while 100 genes were interfered with by tangeretin and hesperidin, respectively. Based on Venn diagram analysis, 19 pigmentation and melanogenesis-related genes were affected by tangeretin and hesperidin (Figure 2b). These genes were then analyzed for their interactions using a protein-protein interaction (PPI) network from STRING to see the interactions of the obtained genes. All the proteins are connected in the network, except F2 and MMP-13 (Figure 2c). Further analysis was conducted using Cytoscape with the plug-in cytohubba. Cytohubba is used to identify important nodes or relationships in a data network using several algorithms. In this study, the algorithm used was maximum clique centrality (MCC). Based on the analysis conducted, 10 genes were found to have the strongest connections, namely *CA2* (carbonic anhydrase 2), *CYP1B1* (cytochrome P450 1B1), *CA1* (carbonic anhydrase 1), *CYP19A1* (cytochrome P450 19A1), *CA9* (carbonic anhydrase 9), *ABCB1* (ATP binding Cassette subfamily B member 1), *CA3* (carbonic anhydrase 3), *ABCG2* (ATP binding Cassette subfamily G member 2), *TYR* (tyrosinase) and *ADORA1* (Adenosine A1 receptor) (Figure 2d). All of those genes play important roles in producing melanin or are related to melanin transport directly or indirectly (Table 1).

The molecular docking study was conducted to predict theoretically potential inhibitors through the binding energy related to interactions between the predicted compound and the amino acid residues within the active site. TYR and CA2 were selected as the target proteins for docking with hesperidin and tangeretin. TYR and hesperidin show a lower binding energy of -10.01 kcal/mol with seven H-bonds to His224, Arg321, Arg374, Arg377, Gln390, Thr391, and His404. In addition, there are hydrophobic bonds with Asp212, His215, and Thr391 (Table 2, Figure 3a). Meanwhile, tangerine showed a binding energy of -6.85 kcal/mol with seven H-bonds with a hydrophobic bond that His244, Arg321, Arg374, His377, Gln390, Thr391, and His377 (Table 2, Figure 3b). There is a similarity of residues that form hydrogen bonds with hesperidin and tangeretin. His192, His215, His381, Leu382, and Phe400 are residues that show hydrophobic bonds with tangeretin. Uniqueness occurs where eight residues bind van der Waals with hesperidin, that is His192, His215, His381, Leu382, and Phe400, but none bind to tangeretin. The results of molecular docking are considered valid with an RMSD control value of 1.23 Å for CA2 and 1.96 Å for TYR.

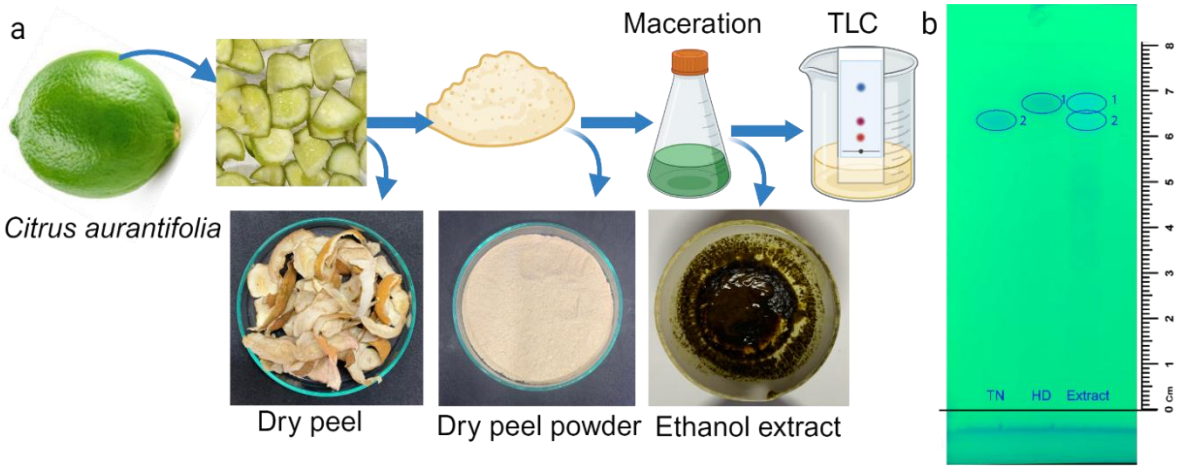

Figure 1. Extraction and Identification of Lime (*Citrus aurantifolia* (Christm.) Swingle) peel. a. Extraction scheme, b. Identification in the Extract, TN: tangeretin, HD: hesperidin, and Extract: ethanolic extract of lime peel.

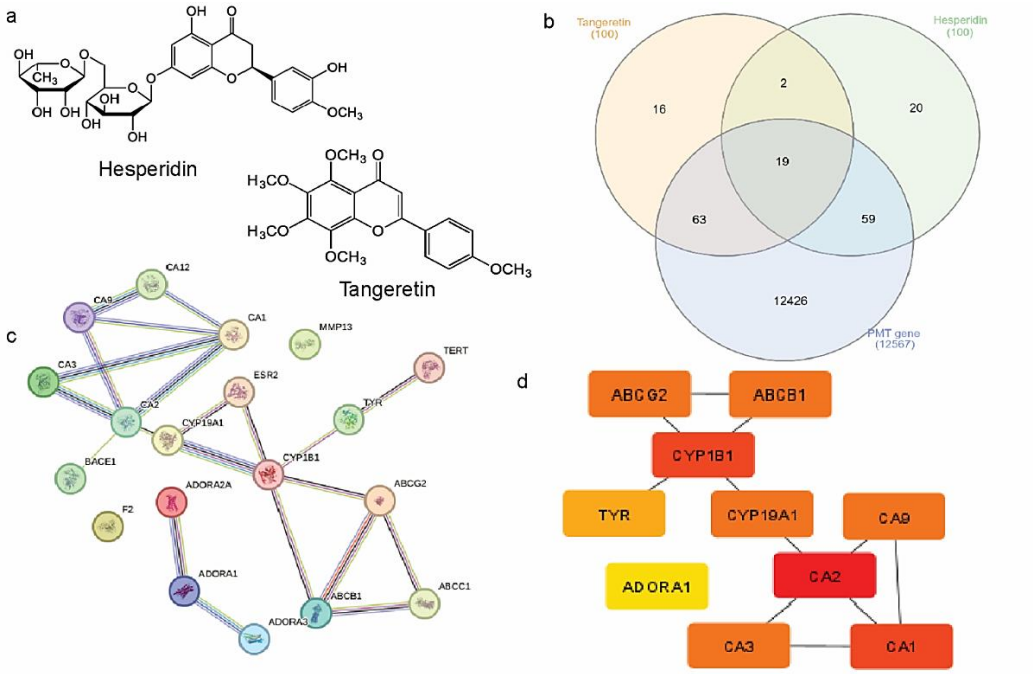

Figure 2. The top gene targets and proteins of melanogenesis and hyperpigmentation targeted by Citrus flavonoids. a. HD's and TN's structure, b. Venn diagram of the compounds and hyperpigmentation-interfered genes, c. Protein-protein interaction (PPI) network of the overlapping genes, d. The clustering of the top 10 genes associated to melanogenesis and hyperpigmentation was interfered with by the compounds according to MCC.

Furthermore, hesperidin showed a binding energy of -6.44 kcal/mol with three H-bonds with Gln92, Thr199, and Pro202 residues on CA2. In addition, there are hydrophobic interactions with Ile91, His94, His96, His119, Val121, Phe131, Val135, Leu198, Pro202, Leu204, Trp209. There are several van der Waals bonds with Gly132, Val143, and Thr200. Hence, tangeretin shows a binding energy of -6.21 kcal/mol in three H-bonds with residues Gln92, Thr199, and Pro202. In addition, there are hydrophobic interactions with Ile91, His94, His96, His119, Val121, Phe131, Val135, Leu198, Pro202, Leu204, Trp209. And there are some van der Waals bonds with Gly132, Val143, and Thr200 (Table 3, Figure 3c). While the molecular docking results between CA2 and tangeretin showed five H bonds with Gln92, His94, Thr199, Thr200, Pro201. and Ile91, His94, Phe131, Val135, Leu198 as hydrophobic bond residues. There are also some van der Waals bonds with Trp5, Asn62, His64, His67, Glu69, His 96, His119, Val121, Val143, Ser197, Pro202, Leu203, Leu204, Val207 (Table 3, Figure 3d). It is concluded that hesperidin and tangeretin have the potential to inhibit TYR and CA2, so *in vitro* studies need to be carried out to prove the activity of tangeretin and hesperidin against TYR and CA2.

Subsequently, the anti-melanogenesis activity of the ethanol extract was validated by an *in vitro* cell-free tyrosinase inhibition assay based on Wang and colleagues (2022) (Figure 4a). This study prefers to conduct an assay on TYR, rather than CA2, due to its activity in the rate-limiting step in melanogenesis. For the test inhibitor, we used kojic acid in this study. Kojic acid inhibited tyrosinase activity with sufficient stability, results which is not easily affected by different testing conditions, such as differences in substrate concentration, incubation time, and tyrosinase substrate source (Wang et al., 2022). The concentration of kojic acid used is 0.75 mM, which is equivalent to 106.58 ppm (Figure 4b). The extract demonstrated a concentration-dependent inhibition activity, which was further analyzed to obtain the IC_{50} value based on an equation of y= 17x + 19.806 with an R-value of 0.997 (Figure S1). In this study, the IC_{50} value of lime peel extract was found to be 59.71 ppm, which indicated a strong tyrosinase enzyme inhibitor. The tyrosinase enzyme inhibition activity of natural substances was categorized into three categories based on their IC_{50} value, namely strong (IC_{50}< 100 ppm), weak (IC_{50} value of 100-450 ppm), and very weak (IC_{50} value of 451-700 ppm) (Tahir et al., 2021). Thus, the IC_{50} value of lime fruit peel extract (IC_{50} 59.71 ppm), smaller than the IC_{50} value of kojic acid (106.58 ppm) in our previous study (Setiawati et al., 2024). To date, our extract potently impedes the melanogenesis process with tyrosinase enzyme as the target.

Table 1. Top 10 melanogenesis genes interfered by hesperidin and tangeretin, and their biological function pH is regulated by the concerted interplay between acid/base transporters and carbonic anhydrase

No	Gene symbol	Protein name	Biological Function	References
1	*CA2*	Carbonic anhydrase 2	Cytoplasm pH regulation in melanocytes.	(Becker, 2020)
2	*CYP1B1*	Cytochrome P450 1B1	In keratinocytes, it converts estrogen (17β-estradiol) to 4-hydroxy-estradiol. Estrogen activates G-coupled protein receptor (GPCR), which stimulates CREB, a transcription factor of MITF.	(Natale et al., 2016)(Dika et al., 2019)
3	*CA1*	Carbonic anhydrase 1	Cytoplasm pH regulation in melanocytes.	(Bernardino et al., 2019)
4	*CYP19A1*	Cytochrome P450 19A1	In keratinocytes, it converts testosterone to estradiol, which regulates melanin synthesis by activating GPCR.	(Yamamoto et al.,2021)
5	*CA9*	Carbonic anhydrase 9	Regulating extracellular pH.	(You et al.,2022)
6	*ABCB1*	ATP-binding cassette subfamily B member 1	An active transporter in melanocytes, to maintains melanin melanin-containing melanosome structure to be transferred to keratinocytes.	(You et al.,2022)
7	*CA3*	Carbonic anhydrase 3	Maintaining cytoplasm pH in melanocytes.	(Bernardino et al., 2019)
8	*ABCG2*	ATP-binding cassette subfamily G member 2	An active transporter in keratinocytes that regulates the uric acid transporter in melanocytes.	(Cheong et al.,2021)
9	*TYR*	Tyrosinase	Rate rate-limiting step enzyme converting tyrosine to melanin in the melanosome	(Yuan et al.,2019)
10	*ADORA1*	Adenosine A1 receptor	Adrenergic receptor linked to GPCR type Gi, which inhibits protein kinase A (PKA), leading to suppression of CREB formation, a MITF transcription factor.	(Bang & Zippin 2021; Kim et al., 2014)

Table 2. Molecular docking results of hesperidin and tangeretin against tyrosinase (TYR)

Ligand	Binding energy kcal/mol	H-Bond Residue	Hidrophobic Residue	van der Waals residue
Hesperidin	-10.01	His224, Arg321, Arg374, Arg377, Gln390, Thr391, His404	Asp212, His215, Thr391	His192, Val196, Val211, Arg321, His377, Asn378, Gln388, Gly389, Gln390, Phe400.
Tangeretin	-6.85	His244, Arg321, Arg374, His377, Gln390, Thr391, His377	His192, His215, His381, Leu382, Phe400	-

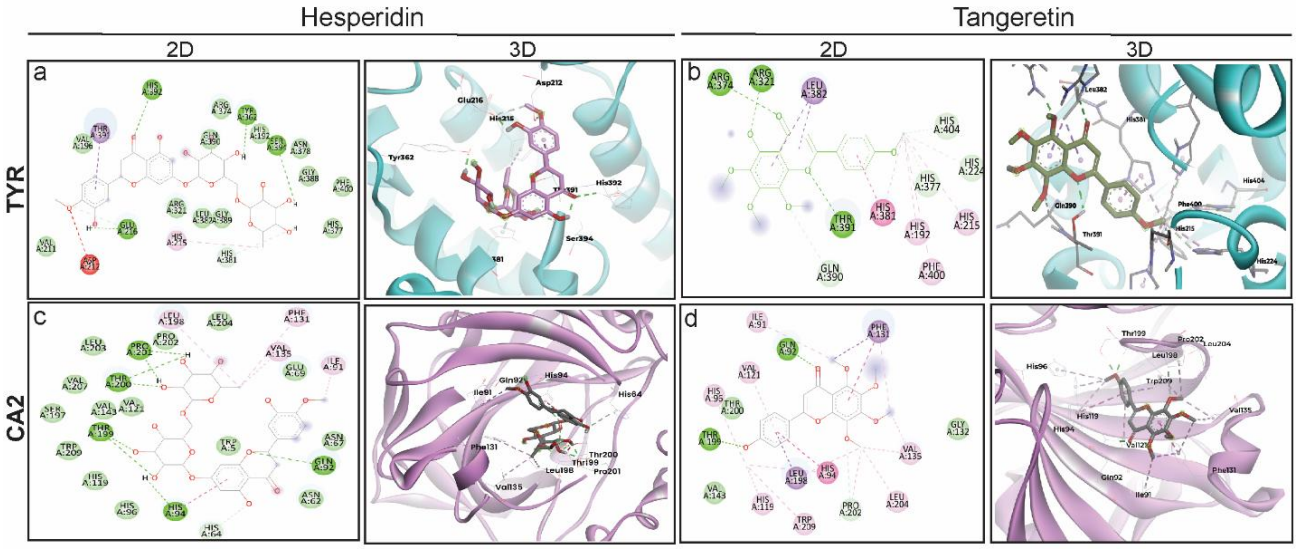

Figure 3. The best binding poses of tyrosinase (TYR) and carbonic anhydrase 2 (CA2) binding pocket in 2D view and 3D view. Interaction between hesperidin and tangeretin to TYR (a, b) and CA2 (c, d). Yellow, red, and white indicated carbon, oxygen, and hydrogen atoms. The green, pink, orange, and purple represent van der Waals, alkyl/pi-alkyl, pi-sulfur, and pi-sigma.

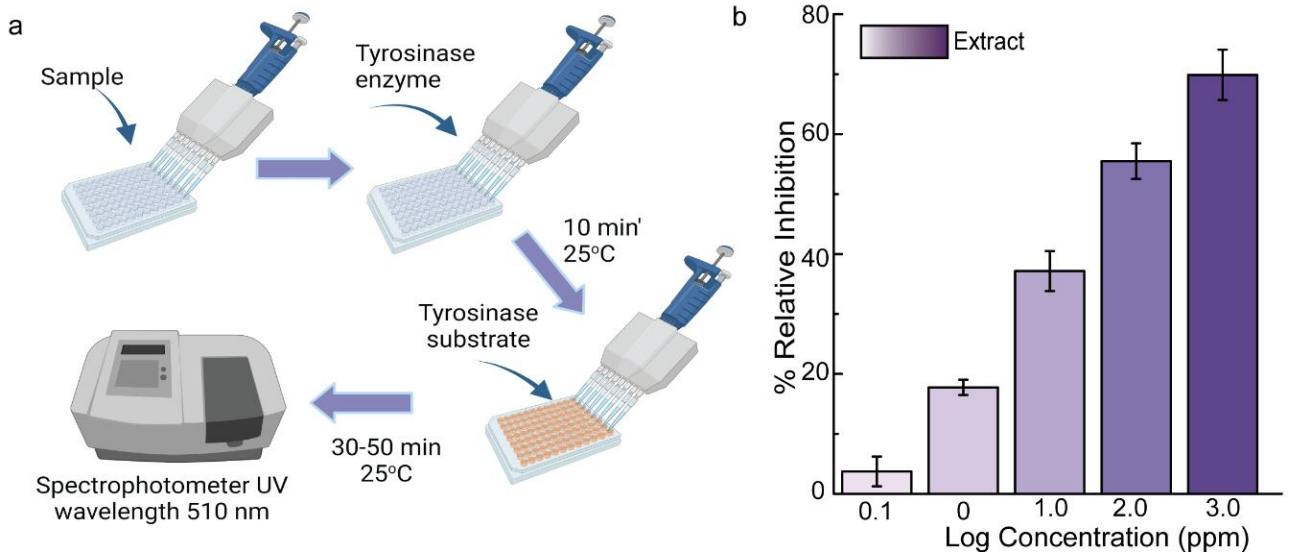

Figure 4. *In vitro* tyrosinase inhibition assay of CF-containing extract. a. Experiment scheme, b. Relative TYR inhibition of the extract t various concentration.

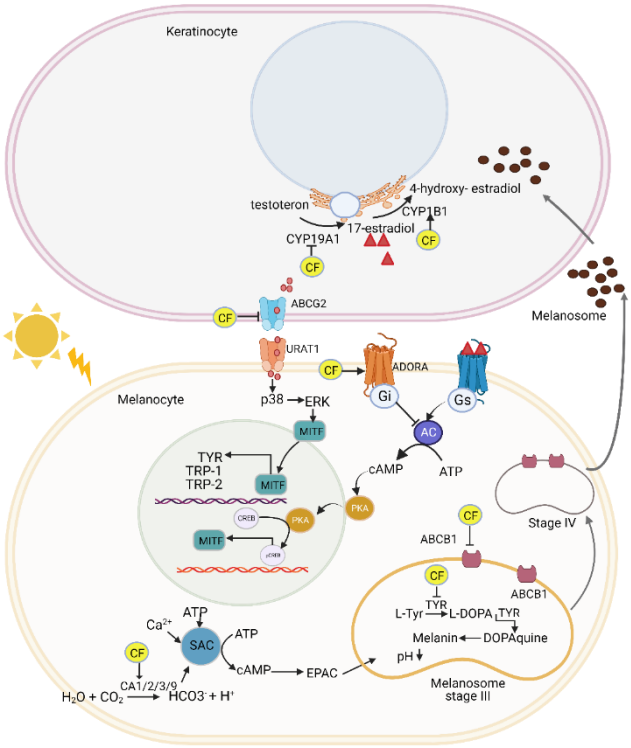

Figure 5. A predicted molecular mechanism of melanogenesis and pigmentation of the skin. CF: Citrus flavonoid, CYP1B1:cytochrome P450 1B1, CYP19A1: cytochrome P450 19A1, ABCB1: ATP binding Cassette subfamily B member 1, CA: carbonic anhydrase, ABCG2: ATP binding Cassette subfamily G member 2, TYR: tyrosinase, ADORA1: adenosine A1 receptor, URAT1: uric acid transporter 1, ERK: extracellular signal-regulated kinase, MITF: microphthalmia-associated transcription factor, CREB: cAMP response element-binding protein, PKA: protein kinase A, cAMP: cyclic adenosine monophosphate, SAC: soluble adenylyl cyclase, AC: adenylyl cyclase, TRP-1: tyrosinase related protein-2, TRP-2: tyrosinase related protein-2, EPAC: exchange proteins directly activated by cAMP proteins, ATP: adenosine triphosphate, L-tyr: levotyrosine, L-DOPA: levodopa. The figure was prepared in Biorender.

DISCUSSION

This study investigated the anti-melanogenesis effect of lime (*Citrus aurantifolia*) peel. We extracted the dried peels with ethanol, and then qualitatively analyzed the components of this extract using thin-layer chromatography (TLC) (Figure 1a). Our study used tangeretin and hesperidin to conduct a bioinformatics analysis and molecular docking study on melanogenesis-related genes affected by the extract. In bioinformatics analysis, the MCC method shows better results in determining important proteins from PPI network data (Chin et al., 2014). The molecular docking study was conducted to predict theoretically potential inhibitors through the binding energy related to interactions between the predicted compound and the amino acid residues in the active site (Pinzi & Rastelli, 2019).

Subsequently, the anti-melanogenesis properties of the ethanol extract were validated through *an in vitro* cell-free tyrosinase inhibition assay based on Wang and colleagues (2022) (Figure 4a). The tyrosinase enzyme inhibition activity of natural substances was categorized into three categories based on their IC_{50} value, namely strong (IC_{50}< 100 ppm), weak (IC_{50} value of 100-450 ppm), and very weak (IC_{50} value of 451-700 ppm) (Tahir et al., 2021). So, the IC_{50} value of lime peel extract, which was found to be 59.71 ppm, indicated a strong tyrosinase enzyme inhibitor

Figure 5 shows the molecular cascade of Citrus flavonoids (HD and TN) in inhibiting melanogenesis. Citrus flavonoids directly inhibit the tyrosinase enzyme, the main enzyme in the melanogenesis process, as well as indirectly inhibit the melanogenesis process by affecting the molecular process in melanogenesis. Since the function of the tyrosinase enzyme in melanin synthesis is greatly influenced by the pH, its activity was interfered with by the carbonic anhydrase (CA) enzyme, which regulates the pH level in the cytoplasm (You et al., 2022). When the cytoplasmic pH drops, it activates soluble adenyl cyclase (sAC) to lower the pH of the melanosome, thereby inhibiting the activity of the tyrosinase enzyme in melanin synthesis (You et al., 2022). Citrus flavonoids also target ADORA, which is a Gi-type G-coupled protein receptor (GPCR) that inhibits the action of adenylate cyclase that synthesizes cAMP, which in turn activates protein kinase A (PKA) or exchange protein activated by cAMP 1 (EPAC1). Active PKA induces MITF to move to the nucleus and acts as a transcription factor for TYR, TRP1, and TRP2 (Bang & Zippin, 2021; Kim et al., 2014).

Another molecular mechanism of melanogenesis involves estradiol, an estrogen hormone, through a tyrosinase transcription factor, Microphthalmia-associated transcription factor (MITF) (Lee et al., 2022; Natale et al., 2016). Bioinformatics study also revealed that Citrus flavonoids induce the CYP1B1 enzyme and inhibit the CYP19A1 enzyme to reduce the amount of estradiol. CYP19A1 is aromatase,

cleaving the C10−C19 carbon−carbon bond of androgens to form estrogen hormone in a three-step process (Yamamoto et al., 2021). CFs are predicted to inhibit CYP19A1 to decrease estrogen levels in the cytoplasm. Thus, they stimulate CYP1B, a cytochrome enzyme that converts estrogen to 4-hydroxy estrogen, which subsequently reduces estrogen levels.

Furthermore, molecular pathway inhibitions of CFs also involved protein transporters, ABCG2 and ABCB1. ABCG2 is the main transporter of uric acid from keratinocytes, which then transports it to melanocytes through uric acid transporter 1 (URAT1) to activate the p38 pathway through Mitogen-Activated Protein Kinase (MAPK), including extracellular signal-regulated kinase (ERK) (Basu et al., 2019). Phosphorylated ERK is responsible for regulating MITF stability, increasing tyrosinase (TYR), tyrosinase-related protein-1 (TRP-1), and tyrosinase-related protein-2 (TRP2) expression levels (Chen et al., 2010). CF inhibition of ABCG2 affected uric acid depletion in the cytoplasm, leading to melanogenesis inhibition in melanocytes. On the other hand, ABCB1 is the transporter of melanosomes in melanin synthesis (Cheong et al., 2021). ABCB1 transporter is found in melanocyte cells. This transporter ensures that melanosomes that already contain melanin can be released into keratinocytes. Inhibition of Citrus flavonoids on the ABCB1 transporter can inhibit this melanosome transfer process (Chen et al., 2010).

To sum up, lime peel ethanolic extract mainly contained Citrus flavonoids: hesperidin and tangeretin. Based on bioinformatics and molecular docking studies, Citrus flavonoids in our extract interfere with proteins related to melanogenesis, including enzymes and receptors. The main enzymes affected by Citrus flavonoid, such as rate-limiting step enzymes, are tyrosinase, estrogen metabolic enzyme; CYP19A1, CYP1B1, as well as carbonic anhydrase 2 (CA2), which is responsible for maintaining pH in the melanosome. Furthermore, upcoming studies are urgently needed to unveil mechanisms and the effectiveness of skin lightening effect in in-vivo animal tests; in parallel, clinical trials are needed, followed by topical cosmetics formulations that have not yet been applied.

CONCLUSION

Based on bioinformatics analysis, this study indicates that compounds in lime (*Citrus aurantifolia* (Christm.) Swingle) peel extract modulate melanogenesis. The identified Citrus flavonoid compounds: HD and TN inhibited protein related to melanogenesis through an enzyme (CA1/2/3/9, CYP1B, and TYR), protein transporter (ABCB1, and ABCG2), and protein receptor (ADORA1). Thus, an in-silico study was evaluated using HD and TN to bind with TYR and CA2, with binding energy values of -10.01 and -6.85 kcal/mol. The extract was revealed to be more effective in *in vitro* inhibiting TYR than kojic acid, a commercially available skin-lightening agent.

CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

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