

The anti-melanogenic properties of *Swietenia macrophylla* king

Camille Keisha Mahendra^a, Hooi-Leng Ser^b, Syafiq Asnawi Zainal Abidin^c, Shafi Ullah Khan^d, Priyia Pusparajah^e, Thet Thet Htar^a, Lay-Hong Chuah^a, Siah Ying Tang^{f,g,h}, Long Chiau Mingⁱ, Khang Wen Goh^j, Yatinesh Kumari^{k,**}, Bey Hing Goh^{a,l,*}

^a Biofunctional Molecule Exploratory Research Group, School of Pharmacy, Monash University Malaysia, 47500 Bandar Sunway, Selangor Darul Ehsan, Malaysia

^b Department of Biological Sciences, School of Medical and Life Sciences, Sunway University, 47500 Subang Jaya, Selangor, Malaysia

^c Liquid Chromatography Mass Spectrometry (LCMS) Platform, Jeffrey Cheah School of Medicine and Health Sciences, Monash University Malaysia, Jalan Lagoon Selatan, 47500 Bandar Sunway, Selangor Darul Ehsan, Malaysia

^d Product & Process Innovation Department, Qarshi Brands (Pvt) Ltd, Hattar Industrial Estate, 22610, Haripur, KPK, Pakistan

^e Medical Health and Translational Research Group, Jeffrey Cheah School of Medicine and Health Sciences, Monash University Malaysia, 47500 Bandar Sunway, Selangor Darul Ehsan, Malaysia

^f Advanced Engineering Platform, School of Engineering, Monash University Malaysia, Bandar Sunway 47500, Malaysia

^g Department of Chemical Engineering, School of Engineering, Monash University Malaysia, Jalan Lagoon Selatan, 47500 Bandar Sunway, Selangor, Malaysia

^h Tropical Medicine and Biology Platform, School of Science, Monash University Malaysia, Bandar Sunway 47500, Malaysia

ⁱ Faculty of Data Science and Information Technology, INTI International University, Nilai, Malaysia

^j PAPRSB Institute of Health Sciences, Universiti Brunei Darussalam, Gadong, Brunei Darussalam

^k Neurological Disorder and Aging Research Group (NDA), Microbiome and Bioresource Research Strength (MBRS), Jeffrey Cheah School of Medicine and Health Sciences, Monash University Malaysia, 47500, Selangor, Malaysia

^l College of Pharmaceutical Sciences, Zhejiang University, 866 Yuhangtang Road, Hangzhou 310058, China

ARTICLE INFO

Keywords:

Cosmeceutical
Anti-photoaging
Swietenia macrophylla
Skin whitening
Molecular docking
Zebrafish embryo
LC-MS/MS
Natural product
Anti-tyrosinase
Tyrosinase related protein-1

ABSTRACT

Fair flawless skin is the goal for some cultures and the development of irregular skin pigmentation is considered an indication of premature skin aging. Hence, there is a rising demand for skin whitening cosmetics. Thus, this research will be focusing on discovering the anti-pigmentation properties of *Swietenia macrophylla* seeds. Firstly, the seeds were extracted with ethanol and further fractionate based on their polarity before testing them on zebrafish embryos. The ethanolic extract of the seed demonstrated significant inhibition of both tyrosinase activity and melanin production in the embryos. However, after fractionation, the anti-melanogenic ability was observed to have decreased, signifying that the phytochemicals may be synergistic in nature. Still in the proteomic studies the ethanolic extract and its hexane fraction both induced the downregulation of cathepsin LB and cytoskeletal proteins that have connections to the melanogenic pathway, confirming that *S. macrophylla* seeds do indeed have anti-pigmentation properties that can be exploited for cosmetic use. Next, limonoids (tetraterpenoids found in the seed) were tested for their inhibitory effect against human tyrosinase related protein 1 (TYRP-1) via molecular docking. It was found that limonoids have a stronger binding affinity to TYRP-1 than kojic acid, suggesting that these phytochemicals may have the potential in inhibiting pigmentation. However, this still needs further confirmation before these phytochemicals can be developed into a skin whitening agent. Other assays like ex-vivo or 3D human skin culture can also be used to better study the seeds anti-pigmentation effect on humans.

1. Introduction

One's skin pigmentation can be classified from type I-VI according to

the Fitzpatrick skin type scale, where type I being the fairest and VI being the darkest skin tone [19]. These are all predetermined in our genes based on the types of short nucleotide polymorphisms (SNPs) in

* Corresponding author at: Biofunctional Molecule Exploratory Research Group, School of Pharmacy, Monash University Malaysia, 47500 Bandar Sunway, Selangor Darul Ehsan, Malaysia.

** Corresponding author.

E-mail addresses: yatinesh.kumari@monash.edu (Y. Kumari), goh.bey.hing@monash.edu (B.H. Goh).

<https://doi.org/10.1016/j.bioph.2023.114659>

Received 12 July 2022; Received in revised form 25 March 2023; Accepted 3 April 2023

Available online 15 April 2023

0753-3322/© 2023 The Author(s). Published by Elsevier Masson SAS. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

our melanogenic genes and how our melanin-containing melanosomes were distributed throughout the cells [75,77]. For example, African melanosomes are the largest and distributed throughout the cytoplasm and over the nuclear whereas skin type I Caucasians have smaller melanosomes and distributes exclusively at the supranuclear position. As for Asians, their melanosomes possess intermediary features of both races [75]. Besides that, the amount of eumelanin and pheomelanin in our skin also affects our skin tone and the colour of our hair and eyes. [65,69]. Both types of melanin are formed by melanocytes, and in humans, three enzymes are known as tyrosinase, TYRP-1, and tyrosinase-related protein 2 (TYRP-2), are responsible for the biosynthesis of melanin [37]. Based on the current biochemical viewpoint, tyrosinase firstly catalyzes L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) and from there L-DOPA is oxidized to dopaquinone. After dopaquinone comes cyclodopa which either goes on to form pheomelanin or dopachrome that becomes 5,6 -dihydroxyindole (DHI) or 5, 6-dihydroxy-indole-2-carboxylic acid (DHICA). The oxidation of DHI to indole 5,6-quinone (IQ) also occurs through tyrosinase. On the other hand, TYRP-2 is involved in the conversion of L-dopachrome to DHICA and TYRP-1 catalyzes the oxidation of DHICA to indole-5, 6-quinone-2-carboxylic acid (IQCA) as can be seen in Fig. 1. It is from these reactions that both eumelanin and pheomelanin are formed [17, 70]. Yet it must be known that the role of TYRP-1 is currently being challenged. Unlike mice, human TYRP-1 was reported to not be involved in the oxidation of DHICA to IQCA [5]. This is partly due to the difference in metal ions present in the active site. TYR has Cu^{2+} in its active site but the crystal structure of human TYRP-1 revealed that TYRP-1 had Zn^{2+} cofactors in its active site instead. This was confirmed when an exchange of the Zn^{2+} cofactors to Cu^{2+} in TYRP-1 displayed DHICA oxidase activity, while TYR with Zn^{2+} cofactors lost all activity [64].

Although the formation of melanin is an innate defense mechanism of our skin against the damaging effects of UV rays and is involved in our skin's circadian rhythm, irregular pigmentation is still undesired, especially in the Asian culture whereby fair, flawless, and ageless skin are prized [27,47,68]. Some of the characteristics of irregular pigmentations are the formation of age spots, freckles, solar lentigines, molted hyperpigmentation, and melasma, all of which are signs of premature skin aging [14,21,36,38]. There are many ways a depigmentation agent can reduce skin pigmentation; (1) is to inhibit the activity of the tyrosinase family, (2) is to interfere with the transport of melanosomes to the surrounding keratinocytes, (3) scavenge the formation of ROS during UV irradiation, (4) modulate the expression of MC1R, (5) accelerate the turnover of keratinocytes to disperse melanin [15]. Nevertheless, there is an upcoming trend where customers are looking for “natural”, “safe”, “green”, and “sustainable” cosmetics. This forces many companies to look toward natural products as a source of ingredients [16]. With billions of dollars on the line, many natural products are now being researched daily to find potential skin whitening bioproperties.

Thus, in the present study, we will be evaluating the bioactive properties of *Swietenia macrophylla* seeds for its potential application as a skin whitening agent. *S. macrophylla* is a timber tree from the Meliaceae family that can be found in the tropics of Central America and Mexico [13,79]. Other than its highly prized mahogany timber, the seed itself too had been used traditionally as a folk medicine for various illnesses like diabetes, malaria, hypertension, etc [18,53]. Subsequently, previous research had also reported that the extract from the seeds has wound healing, UV protective, anticancer, antioxidant, and antimicrobial properties [22,44,46,59]. These are traits that are desired and promising in the cosmetic business. For our *in vivo* study, we will be utilizing the zebrafish embryo as our model organism. Aside from that, molecular docking of reported limonoids, phytocompounds that were previously purified from the seeds, on human tyrosinase related protein-1 (TYRP-1) will be conducted. Limonoids are tetraterpenoids phytocompounds that are produced in the Meliaceae, Rutaceae, and Cneoraceae families. In the *Swietenia* genus itself, there are more than 160 limonoids that were previously isolated [72,73]. According to previous

findings, these compounds were found to be responsible for the many bioactivities exhibited by *S. macrophylla* as they were tested to have anti-microbial, anti-inflammatory, anti-viral, anti-cancer, antifeedant, anti-malarial, etc [43,53,72]. Hence, being secondary metabolisms that are greatly produced in the *S. macrophylla* and having a vast range of bioproperties, it was chosen as the candidate inhibitor against human TYRP-1. By conducting all these tests, the effect of *S. macrophylla* on the melanogenic pathway and its potential as an anti-pigmentation agent can be elucidated.

2. Materials and methods

2.1. Plant material and extraction of bioactive phytocompounds

The seeds of *S. macrophylla* (3 kg) were obtained from Temerloh, Malaysia and a voucher was deposited at the Herbarium of Institute of Biological Sciences, Faculty of Science, University of Malaya, Malaysia (Voucher: No. KLU46901). To extract the phytocompounds, the seeds were dried, finely ground, and soaked in ethanol for 72 h at room temperature. After that, the mixture was filtered and concentrated at 40°C with a rotary vacuum evaporator to obtain the crude ethanolic extract (SMCE). Some portions of the crude were then processed using solvent-solvent fractionation using hexane, ethyl acetate, and water. Firstly, hexane was applied to SMCE extracts and the liquid solution obtained were concentrated with the rotary vacuum evaporator at 40°C to produce the hexane fraction (SMHF). Then, the insoluble portions after the application of hexane were then filtered and subjected to a 1:1 portioning of ethyl acetate and water to form the ethyl acetate fraction (SMEAF) and aqueous fraction (SMWF), respectively. SMEAF were concentrated using the rotary evaporator at 40°C while SMWF was subjected to freeze drying. For analysis, the extract and fractions were dissolved in 100% (v/v) dimethyl sulfoxide (DMSO) solvent vehicle.

2.2. Pigment study utilizing zebrafish

2.2.1. Maintenance and breeding of zebrafish

Ethical approval for the use of zebrafishes in this experiment were obtained from Monash University Malaysia Animal Ethics Committee (ERM ID: 24394). Adult zebrafish (wild type strain) were kept and raised at 28 °C temperature under standard laboratory conditions. They were fed twice daily with live *Artemia salina* brine shrimps with supplementation of dry shrimp flakes. To breed them, a 1:1 ratio of male and female fishes were placed in hatching tanks the evening before spawning. The spawning was allowed to take place within two hours after the lights were turned on the next morning. Fertilized eggs were then collected and washed twice with Holt buffer (60 mmol/L NaCl (Merck, Darmstadt, Germany), 0.67 mmol/L KCl, 0.3 mmol/L NaHCO_3 (Merck, Darmstadt, Germany), and 0.9 mmol/L CaCl_2 (Merck, Darmstadt, Germany),) before being left to mature in the buffer that was treated with 0.1% methylene blue (Nacalai Tesque, Kyoto, Japan). After 24 h, 1 mg/mL pronase (Sigma, St. Louis, USA) was used to remove the chorion and the embryos were then used in subsequent treatments.

2.2.2. Toxicity analysis of zebrafish embryos

Healthy embryos ($n = 3$, 50 embryos per well) were treated with varying concentrations (12.5–100 $\mu\text{g/mL}$) of SMCE, SMHF, SMEAF, and SMWF at 24 h post fertilization (hpf) in 6 well plates. Besides that, 40 μM PTU (positive control) and 0.5% (v/v) DMSO (negative control) were also applied to 24hpf embryos to analyze their toxicity. To standardize, all treatments including the controls hereby contained 0.5% (v/v) DMSO in its final concentration. The mortality of the embryos was measured 24 h post-treatment.

2.2.3. Tyrosinase activity and melanin content determination analysis

The effect of *S. macrophylla* on zebrafish tyrosinase activity and melanin production was determined by treating the embryos ($n = 3$, 50

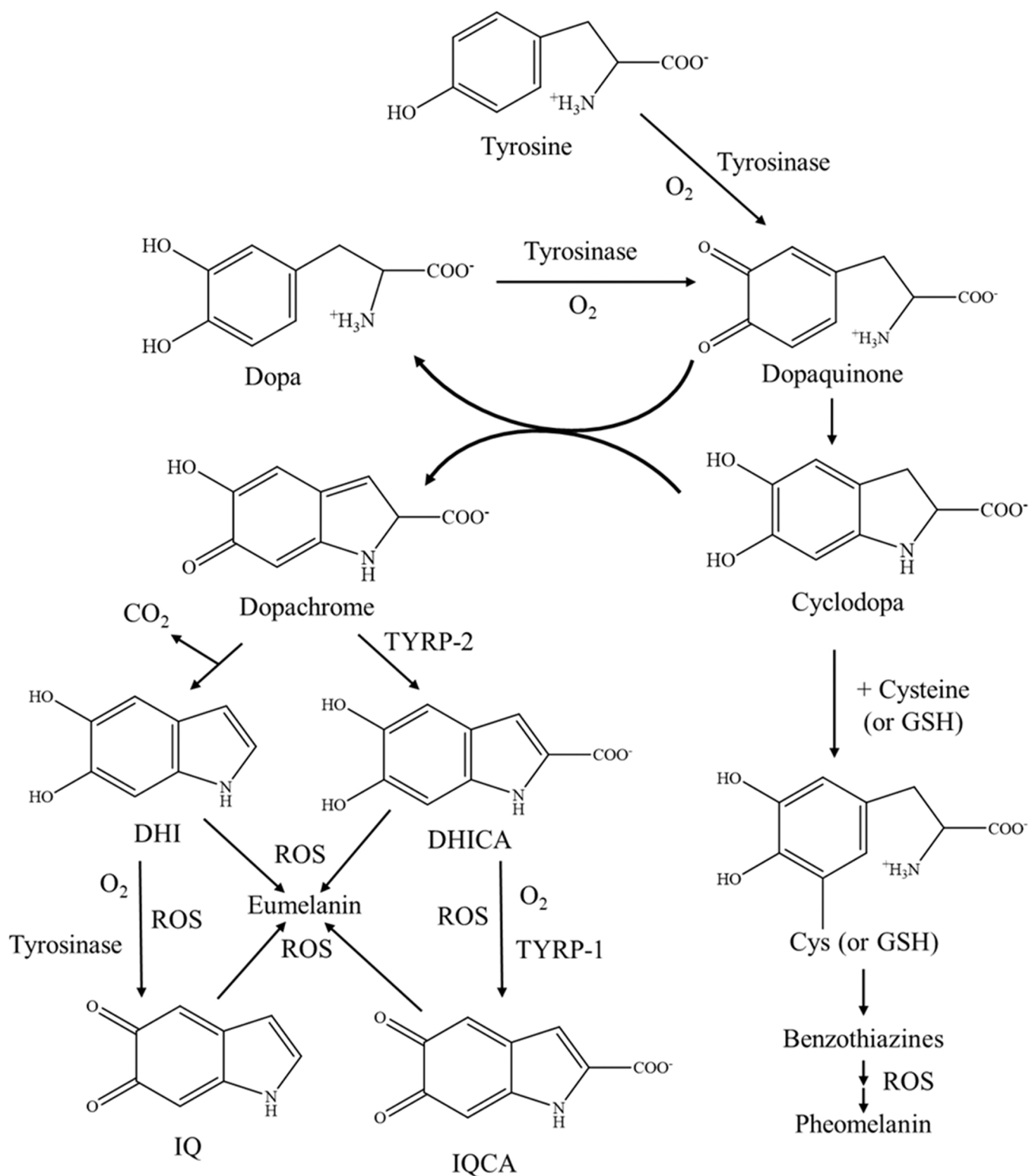


Fig. 1. The biosynthesis of eumelanin and pheomelanin in the melanogenesis process. Tyrosinase is the key enzyme involved in the oxidation of tyrosine to dopaquinone while TYRP-1 and TYRP-2 catalyze the final stages of eumelanin formation. The presence of ROS acts as an accelerator to the polymerization of indole units in eumelanogenesis. As for pheomelanogenesis, dopaquinone can react with thiol-containing products like glutathione (GSH) and cysteine to produce both benzothiazine and cysteinyl-dopa, leading to the formation of pheomelanin. The image was adapted from Solano [70].

embryos per well) with varying concentrations (12.5–100 µg/mL) of SMCE, SMHF, SMEAF, and SMWF in Holt buffer for 24 h. As a positive control, 40 µM of phenylthiourea was used, while 0.5% (v/v) DMSO in Holt buffer was designated as the negative control. Again, all treatments including the controls contained 0.5% (v/v) DMSO in its final concentration as DMSO is the solvent vehicle. The following day, 30 healthy embryos per well ($n = 3$) were picked to extract total protein content. After rinsing the embryos twice with ice cold PBS, the embryos were homogenized in ice cold lysis buffer (20 mM sodium phosphate (pH 6.8) (Merck, Darmstadt, Germany), 1% Triton X-100, 1 mM PMSF (Nacalai Tesque, Kyoto, Japan), 1 mM EDTA (1st Base, Singapore), and 1:200 protease inhibitor cocktail (Merck, Darmstadt, Germany)) using a homogenizing pestle. The solution was then centrifuged at 10,000×g, 4 °C and the total protein amount was measured using Bicinchoninic acid (BCA) assay with bovine serum albumin (BSA) as the standard. For the measurement of tyrosinase activity, 100 µg of protein in 15 µL was mixed with 100 µL of 5 mM L-DOPA in PBS in a 96 well plate. The mixture was incubated at 37 °C for 2 h before measuring the absorbance at 475 nm with a microplate reader. To measure the melanin content, the pellet obtained after centrifugation was resuspended and dissolved in 50 µL of 1 M NaOH for 30 min at 100 °C. For complete solubilization of the melanin pigment, the sample was vigorously vortexed. Finally, the absorbance of the samples was measured at 490 nm to determine the melanin content and the data was normalized against the control group.

2.2.4. BCA protein assay

The concentration of proteins harvested was measured using the BCA Protein Assay Kit (Thermo Fisher, Waltham, MA, USA), following the manufacturer's instructions. Briefly, reagents A and B were added together at a 50:1 ratio to form the working reagent. Next, 10 µL of the protein sample was added to a 200 µL working reagent in a 96 well plate before incubating at 37 °C for 30 min and then measuring at 562 nm. Before calculating the concentration from the standard curve, the absorbance of each sample was subtracted with the blank absorbance. The standard curve was built using BSA (Sigma, St. Louis, USA) as the sample at increasing concentrations (0–1500 µg/mL).

2.2.5. Image acquisition

Images of pigmentation patterns on the body of the zebrafish embryos (dorsal and lateral) were taken utilizing Zeiss Axio Zoom V16 microscope (Zeiss, Oberkochen, Germany). Before that, the zebrafish embryo was anaesthetized using 1 g/mL tricaine (Nacalai Tesque, Kyoto, Japan) and was partially immobilized in 3% methylcellulose (Sigma, St. Louis, USA).

2.2.6. Statistical analysis

The results obtained were expressed as mean $\pm$ standard deviation of means (SD) and were statistically analyzed using Statistical Package for Social Science version 27.0 (SPSS). To compare the means of multiple groups, one-way analysis of variance (ANOVA) and post hoc Tukey test were used. When $p \leq 0.05$, the difference was considered as statistically significant.

2.3. LC-MS/MS analysis of pigment-related pathways in zebrafish

2.3.1. In solution tryptic digestion

Denaturation of 100 µg zebrafish protein samples were conducted using by mixing protein samples with 1 µL of 200 mM 1,4-dithiothreitol (DTT), 25 µL of 100 mM ammonium bicarbonate, and 25 µL tri-fluoroethanol (TFE). After vortexing the mixture, the samples were heated for 1 h at 60 °C. Next, 4 µL of 200 mM iodoacetamide (IAM) stock was added to the mixture for 1 h in the dark at room temperature to alkylate the proteins before adding 1 µL of 200 mM DTT to quench excess IAM. The mixture was once again incubated for 1 h at room temperature in the dark. Following that, 100 µL of 25 mM ammonium bicarbonate and 300 µL of ultrapure water were added to the samples to

raise sample pH before the addition of 1 µL of 20 µg/mL MS grade trypsin (reconstituted in 50 mM acetic acid). To complete the protein digestion, samples were incubated at 37 °C. The digestion was stopped after 16 h of incubation by adding 1 µL of formic acid. Complete drying of the samples at 60 °C was then conducted using a MiniVac speed vacuum concentrator (Lobogene, Denmark). Dried samples were stored at -20 °C.

2.3.2. Desalting and cleanup of protein samples

Protein samples were cleaned and desalted using Pierce® C18 Spin column (Thermoscientific, USA) according to the manufacturer's instructions. Before proceeding with LCMS/MS analysis, the samples were dried again with MiniVac speed vacuum concentrator Lobogene, Denmark) at 60 °C.

2.3.3. Protein analysis with nanoflow-ultra high-performance chromatography-tandem mass spectrometry (LC-MS/MS)

The operation of LC-MS/MS was conducted according to the method that was previously established [44]. Briefly, protein samples were reconstituted using 30 µL 0.1% formic acid before centrifugation at 14,000 rpm for 10 min. One microliter of the sample was then loaded into Agilent C18, 300 Å large capacity chip (Agilent Technologies, USA), and then mounted onto the Agilent 1200HPLC-Chip/MS interface coupled with Agilent 6500 iFunnel quadrupole-time of flight (Q-TOF) LC/MS system. The flow rate was adjusted to 0.5 µL/min from Agilent 1200 Series Nano Pump with solution A (0.1% formic acid in water) and solution B (90% acetonitrile and 0.1% formic acid in water) and 4 µL/min from Agilent 1200 Series Capillary pump. Elution of samples was then conducted using a multi-step gradient of 5–75% of solution B (30 min of 5–75% solution B, 9 min of 75% solution, and 8 min of 75–5% solution B. Further adjustment of ion polarity of Q-TOF to positive with capillary voltage of 2050 V, gas temperature at 325 °C, fragmentor voltage at 360 V, and drying gas flow rate at 5 L/min were done. Acquired spectra were in auto MS/MS mode with an MS scan range of 110–3000 m/z and an MS/MS scan range of 50–3000 m/z . Finally, the precursor charge state selection and preference were fixed as doubly, triply, or more than triply charged state, with the exclusion of precursor 299/294457 m/z ($Z = 1$) and 1221.990637 m/z ($Z = 1$) (reference ions).

2.3.4. Identification and differential expression of proteins

Utilizing PEAKS studio 7.5 (Bioinformatics Solution Inc., Canada), protein samples were identified and their expressions were compared with the negative control. *Danio rerio* (Uniprot database) was used in the homology search and the identification of the proteins. Carbamidomethylation with maximum missed cleavages of 3 was preset as a fixed modification. Parent and fragment mass error tolerance were chosen to be at 0.1 Da with monoisotopic as the precursor mass search type. For the digestion enzyme, Trypsin was selected. The data filtering parameters that were used are unique peptides ≥ 1 and 1% false discover rate (FDR). The LFQ parameters were: mass error tolerance of 20 ppm, retention time shift tolerance of 6 min, and FDR threshold of 1%. Protein expressions of PTU, SMCE, and SMHF were compared against the negative control (untreated embryos) using hierarchical clustering. Heatmaps were generated after setting the parameters to a protein significance of ≥ 13 , which is the p -value of 0.05. Fold change was set to be ≥ 1 with at least one unique peptide. The significance was calculated with PEAKSQ and automatic normalization of protein ratios was done in accordance with the total ion chromatogram [44].

2.4. Real time PCR analysis of pigment related gene expression

Thirty random zebrafish embryos (48 hpf) that were treated with either PTU, SMCE or SMHF for 24 h were homogenized in 100 µL of Trizol (Thermo Fisher Scientific, USA) to obtain its total mRNA. The mRNA of embryos that were not treated with anything except 0.5% DMSO were the negative control in this experiment. To purify the

mRNA, 1:5 ratio of chloroform (Nacalai Tesque, Kyoto, Japan) was added to the trizol before vortexing and incubating the solution for 3 min in room temperature. Next, the solution was centrifuged at 13,500 rpm for 15 min at 4 °C to obtain the upper aqueous phase. After separating the colourless liquid from the main solution, 100% isopropyl alcohol was added at a 1:2 ratio, mixed gently and incubated for another 10 min at room temperature. After that, the mRNA was pelleted through centrifugation at 13,500 rpm at 4 °C for 15 min and the supernatant was discarded. The pellet collected was rinsed and centrifuged twice with 100 μ L 75% ethanol at 10,500 rpm at 4 °C for 5 min before air drying for 5 min. The mRNA pellet was finally dissolved in 10 μ L DEPC ultrapure water and its concentration was measured at A260/A280. To standardize, 500 ng of mRNA was converted to cDNA with High Capacity cDNA Reverse Transcription kit (Applied Biosystem, USA) before analyzing its gene expression using Power SYBR Green OCR Master Mix (Applied Biosystem, USA) and QuantStudio™ 5 Real-Time PCR System (Thermo Fisher Scientific, USA). The primers used in this analysis can be seen in Table 1 below with *Cyrr1* as the housekeeping gene. All primers were designed using NCBI Primer Blast.

2.5. Molecular docking of limonoids on TYRP-1

2.5.1. Protein structure preparation

Human TRYP-1 crystal protein structure (PDB ID: 5M8M) was obtained from the RCSB protein data bank. The protein structure had a resolution of 2.65 Å and cocrystallized with an inhibitor, kojic acid [37]. To prepare the protein for docking, chains B, C, and D were removed in Biovia Discovery Studio Visualizer. Besides that, all water molecules were removed, except the water molecule (HOH601) which was binded to the Zn²⁺ cofactors, and kojic acid. The cleaned protein was then saved as a new PDB file and its structure was assessed by Ramachandran plot and its z-score analysis. The Ramachandran plot was obtained through Biovia Discovery Studio Visualizer, while the z-score was calculated using Yasara WHAT IF program.

2.5.2. Preparation of ligand structure

Limonoid compounds that were previously reported to be found in the seed of *S. macrophylla* and had available 3D structure in Pubchem were selected to be tested as an inhibiting ligand on TYRP-1. The list of limonoids can be seen in Table 2. A few limonoid compounds, although not having available 3D structure but having an almost similar structure to other existing compounds, were also chosen and had optimized in the Chemdraw 3D. Stereoisomers of the compounds were also taken into account as well for docking. Meanwhile, kojic acid was also retrieved from Pubchem (CID: 3840) to validate the docking method. Finally, all compounds were screened carefully to ensure the correct formation of bonds before being energy minimized in Chemdraw 3D and saved as PDB files.

2.5.3. Molecular docking analysis

Molecular docking was done using Autodock 4.0 software with binary files of Autodock4 and Autogrid4. The docking procedure of ligands to 5M8M protein was performed according to the protocol published by Khan, et al. [31] with slight changes. In the 5M8M PDBQT file, both zinc atoms were adjusted manually to possess the charge of

+ 2, while the grid dimensions of 60 × 60 × 60 were centroid around the co-crystal ligand, Kojic acid having XYZ coordinates of (−31.389, −3.555, −24.998) with a spacing of 0.375 Å. To validate the docking methods, kojic acid was redocked and its RMSD value was calculated using the DockRMSD website (<https://zhanglab.ccmb.med.umich.edu/DockRMSD/>). After validation, all compounds were docking using the optimized protocol to generate 100 docked poses for each ligand. Docked poses of the ligands were then screened by cluster analysis and visualized using Biovia Discovery Studio Visualizer.

3. Results

3.1. Toxicity study of *S. macrophylla* extract on zebrafish embryos

To determine the toxicity of *S. macrophylla* on zebrafish embryos, 24 hpf embryos were treated with 0–100 μ g/mL of SMCE, SMHF, SMEAF, and SMWF. After 24 h, the mortality of each treatment was recorded and the percentage of survival was calculated by normalizing against the non-treated control. As shown in Fig. 2, none of the samples induces death, even at the 100 μ g/mL concentration. There were also no morphological defects seen in the zebrafish embryos at 48 hpf. The same could be seen for the PTU at 40 μ M. Hence, these concentrations will be used to measure the tyrosinase activity and melanin content in the embryos.

3.2. Anti-pigmentation properties of *S. macrophylla*

The effect of SMCE, SMHF, SMEAF, and SMWF on zebrafish melanogenesis was tested by analyzing the tyrosinase activity and melanin content of zebrafish embryo 24 h after treatment. Total protein and melanin were extracted from 48 hpf embryos via homogenization and in both assays, PTU was chosen as the positive control due to its ability to inhibit tyrosinase [32]. Subsequently, Fig. 3 demonstrates that SMCE was able to significantly inhibit tyrosinase activity by 40.88 ± 4.6% and 65.66 ± 2.96% at 100 and 50 μ g/mL, respectively. This then contributed to a substantial decrease in melanin production as well. Melanin content in SMCE treated embryos were recorded to be 69.44 ± 5.95% at 50 μ g/mL treatment and 48.58 ± 2.5% at 100 μ g/mL treatment (Fig. 4). The evident whitening effect of SMCE on the embryos at 100 μ g/mL is also supported by the images shown in Fig. 5. In fact, SMCE is comparable with PTU which successfully suppressed tyrosinase activity down to 76 ± 1.28% and decreased melanin content to 54.13 ± 3.36%. On the other hand, SMHF too demonstrated significant inhibition in tyrosinase activity at 100 μ g/mL concentration down to 71.45 ± 0.21%. Despite that, this inhibition did not translate to a substantial decrease in melanin content, whereby 100 μ g/mL SMHF only decreased the melanin content by 14.67 ± 4.37%. As for SMEAF, although the data obtained were not significant, there was still a slight dose-dependent decrease in both melanin and tyrosinase activity induced after treatment. Conversely, SMWF treated embryos experienced the opposite where it induced dose-dependent elevation in tyrosinase activity and melanin content as concentration increased.

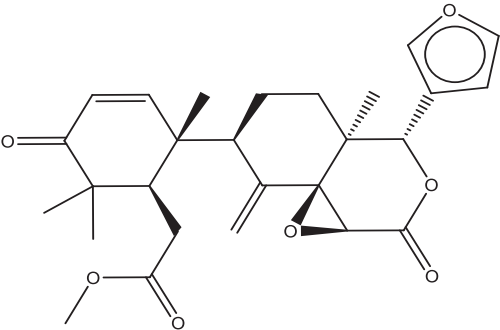
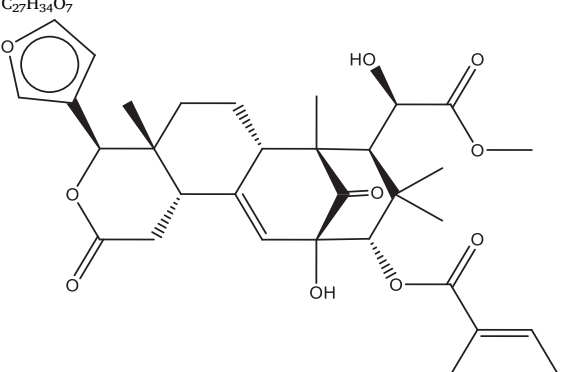
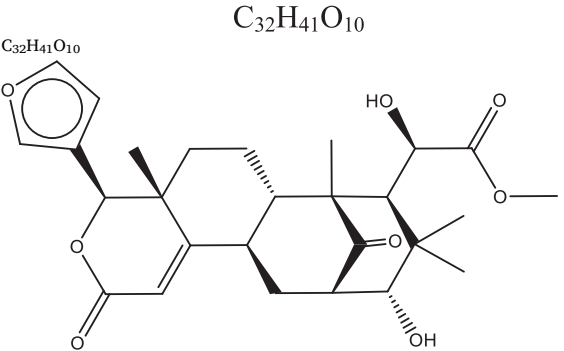
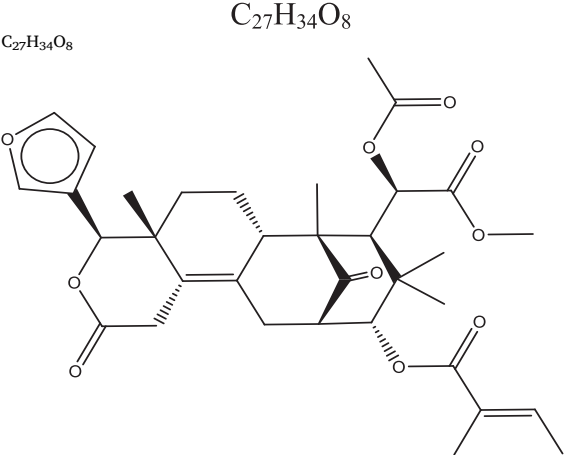
3.3. Proteomic study on SMCE and SMHF treated zebrafish embryos using LC-MS/MS

To investigate SMCE and SMHF-induced changes in protein expression, zebrafish embryos (24 hpf) were subjected to treatment with either 100 μ g/mL of SMCE or SMHF for 24 h. As a comparison for pigmentation-related proteomic changes, 40 μ M PTU (positive control) too was used to treat the embryos separately. After homogenizing the embryos at 48 hpf, the proteins were extracted and analyzed using LC/MS-MS. Using PEAKS studio 7.5, a ratio comparing the negative control and each treatment (PTU, SMCE, or SMHF) was generated. Additionally, heatmaps demonstrating the protein changes were produced as well by the PEAKS software and they can be seen in Fig. 6. However, due to the

Table 1
Sequences of the Primers.

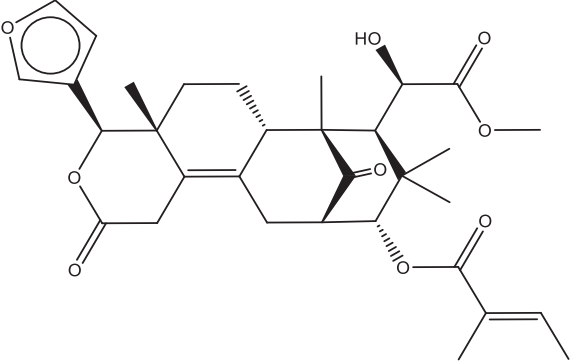
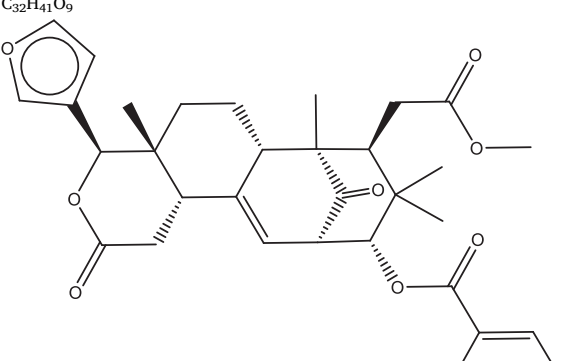
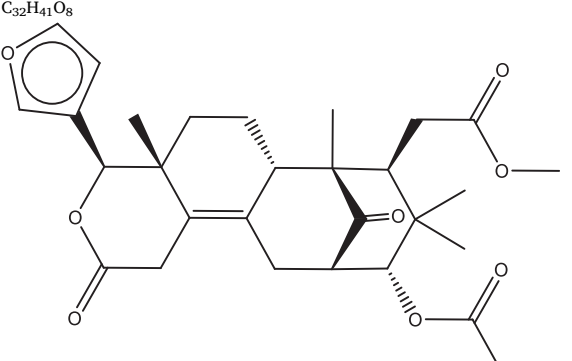
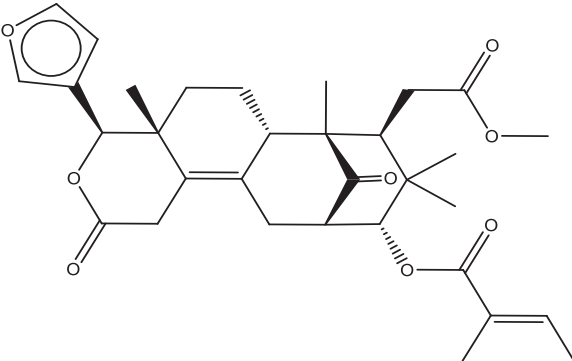
Target gene	Sequence
<i>Cyrr1</i>	Forward: 5' AAGAGGAAGAAGTAGCGCGG 3' Reverse: 5' ACTCGCACITCTGGGATTCT 3'
<i>PSEN1</i>	Forward: 5' TGGCCGTCCATCAGTTCTAC 3' Reverse: 5' GAGGAAAGGTGTGCTCGTCT 3'
<i>PSEN2</i>	Forward: 5' CTGCCATGGTCTGGATGGTT 3' Reverse: 5' TTCTGTGTCTTACTCCGC 3'

Table 2List of previously reported Limonoids in *S. macrophylla* seeds used in docking against Tryp-1 protein.

No.	Type	Pubchem CID	Limonoid Compounds	2D structure	References
1.	Andirobin	266444	Andirobin		[54]
2.	Mexicanolide	76326957	2-hydroxyswietenine	 C ₂₇ H ₃₄ O ₇	[54]
3.	Mexicanolide	NA	3-β,6-dihydroxydihydrocarapin	 C ₃₂ H ₄₁ O ₁₀	[54]
4.	Mexicanolide	23258996	3-O-tigloyl-6-O-acetylswietenolide	 C ₂₇ H ₃₄ O ₈ C ₃₄ H ₄₃ O ₁₀	[42]

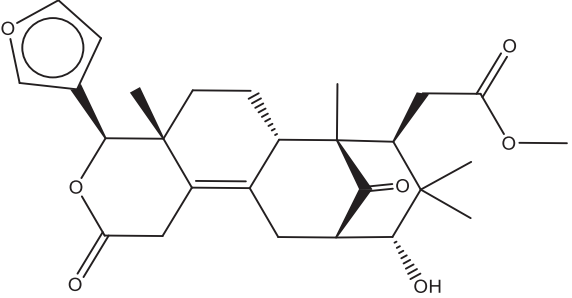
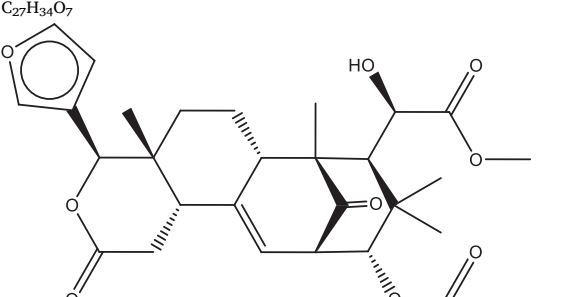
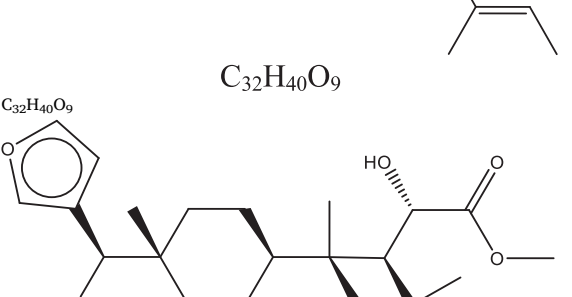
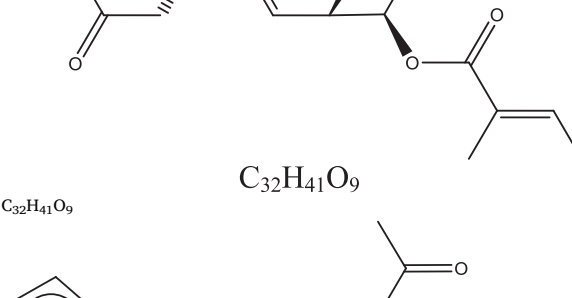
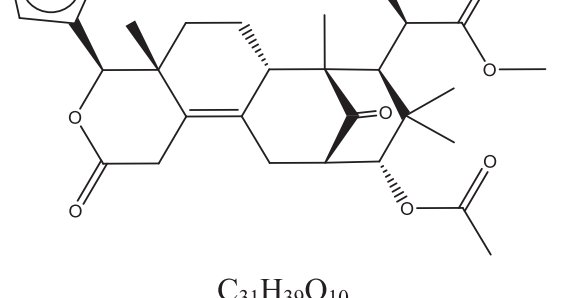
(continued on next page)

Table 2 (continued)

No.	Type	Pubchem CID	Limonoid Compounds	2D structure	References
5.	Mexicanolide	101396725	3-O-tigloylswietenolide	 $C_{32}H_{41}O_9$	[42]
6.	Mexicanolide	NA	Febrifugin (6-desoxyswietenine)	 $C_{32}H_{41}O_9$	[42]
7.	Mexicanolide	6451333	Fissinolide	 $C_{32}H_{41}O_8$	[42]
8.	Mexicanolide	NA	Khayasin T	 $C_{32}H_{41}O_8$	[42]

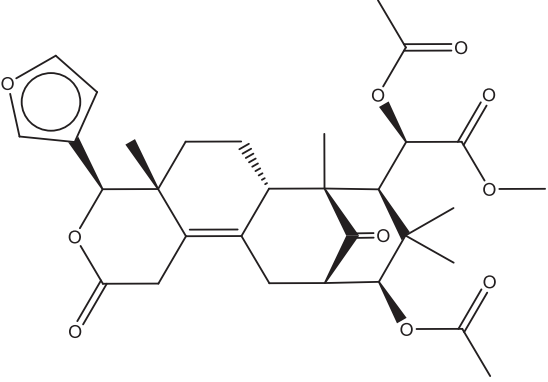
(continued on next page)

Table 2 (continued)

No.	Type	Pubchem CID	Limonoid Compounds	2D structure	References
9.	Mexicanolide	23258999	Proceranolide	 $C_{27}H_{34}O_7$	[39]
10.	Mexicanolide	14262276	Swietenine	 $C_{27}H_{34}O_7$	[39]
11.	Mexicanolide	23891055	Swietenine	 $C_{32}H_{40}O_9$	[39]
12.	Mexicanolide	23258997 (44575317)	Swietenolide diacetate (3,6-O,O-diacetylswietenolide)	 $C_{32}H_{41}O_9$	[23]
				 $C_{31}H_{39}O_{10}$	

(continued on next page)

Table 2 (continued)

No.	Type	Pubchem CID	Limonoid Compounds	2D structure	References
13	Mexicanolide	98042567	Swietenolide diacetate (3,6-O,O-diacetylswietenolide)		[23]

C₃₁H₃₈O₁₀

*NA indicates “not available”

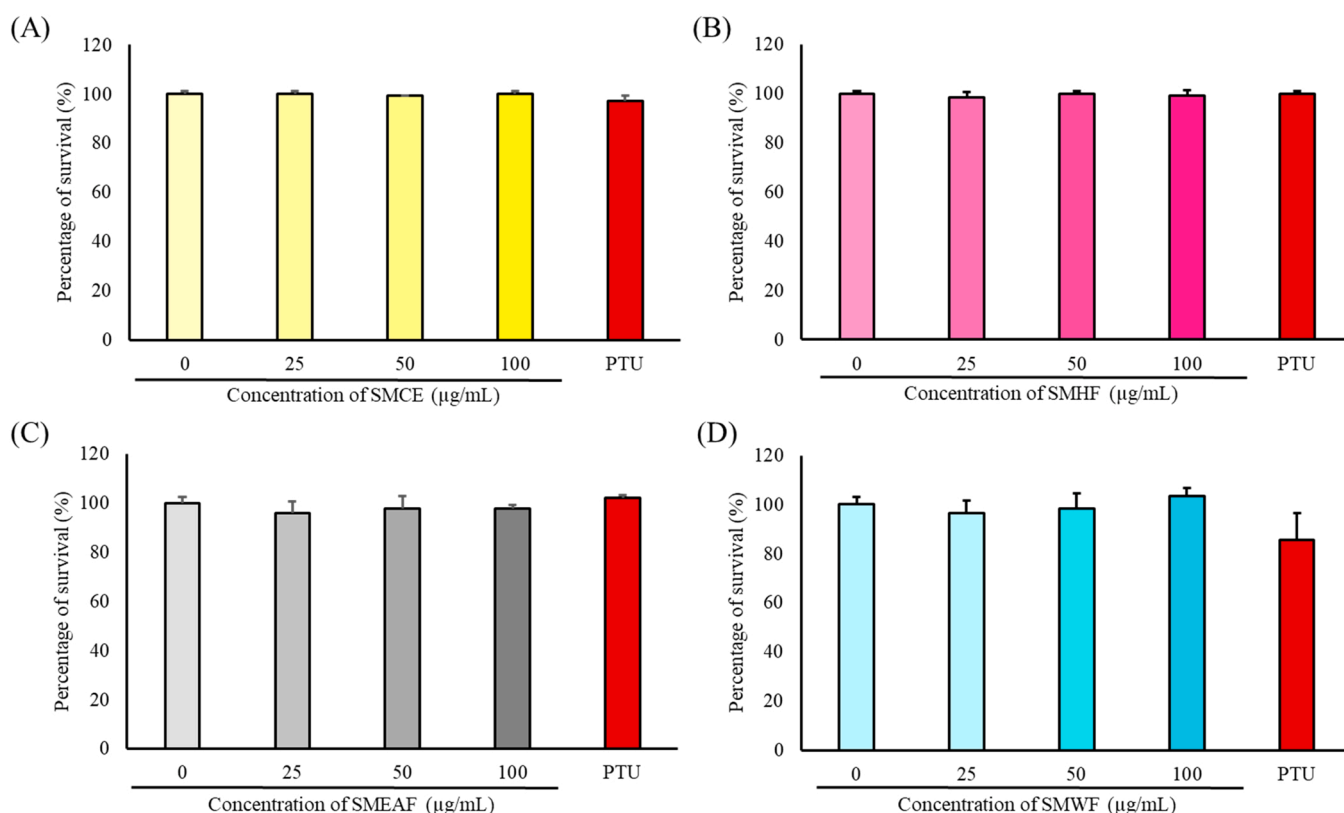


Fig. 2. Analysis of *S. macrophylla* extract and fractions toxicity on zebrafish embryos. The embryos were treated with various concentrations of (A) SMCE, (B) SMHF, (C) SMEAF, and (D) SMWF, with concentrations ranging from 0 to 100 µg/mL for 24 h. The toxicity of 40 µM PTU (positive control) was also analyzed (n = 3; * *p*-value ≤ 0.05, ** *p*-value ≤ 0.01; *** *p*-value ≤ 0.001).

limited number of zebrafish embryos obtained per breeding, SMCE and SMHF treatment had to be done in separate batches (Tables 3 and 4), each with its own negative and positive control. Nevertheless, despite having slightly different proteins detected per batch, the protein expression changes induced by the positive control in the same/repeated proteins were similar across both batches except for the protein parvalbumin. This indicates that although being separate batches, there is not many discrepancies between both batches and the data is consistent enough for comparison across the batches.

From the data obtained, SMCE and SMHF were observed to induce a

downregulation of almost all the listed proteins. Among the proteins tabulated, the common proteins that are downregulated across the group are cathepsin LB, embryonic 1 beta-globin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Whereas, individually in Table 4, both SMHF and PTU further suppressed 8 more proteins; which are apolipoprotein A-I, embryonic alpha globin e1, hbae1 protein, hemoglobin (alpha embryonic 1.3), novel alpha-globin, parvalbumin-2, vitellogenin, and vitellogenin 2. SMCE alone was observed to reduce the expression of elongation factor 1-alpha (EF-1a), keratin 4, and vitellogenin 1, whereas SMHF alone affected actin beta 1. PTU in Table 4 too

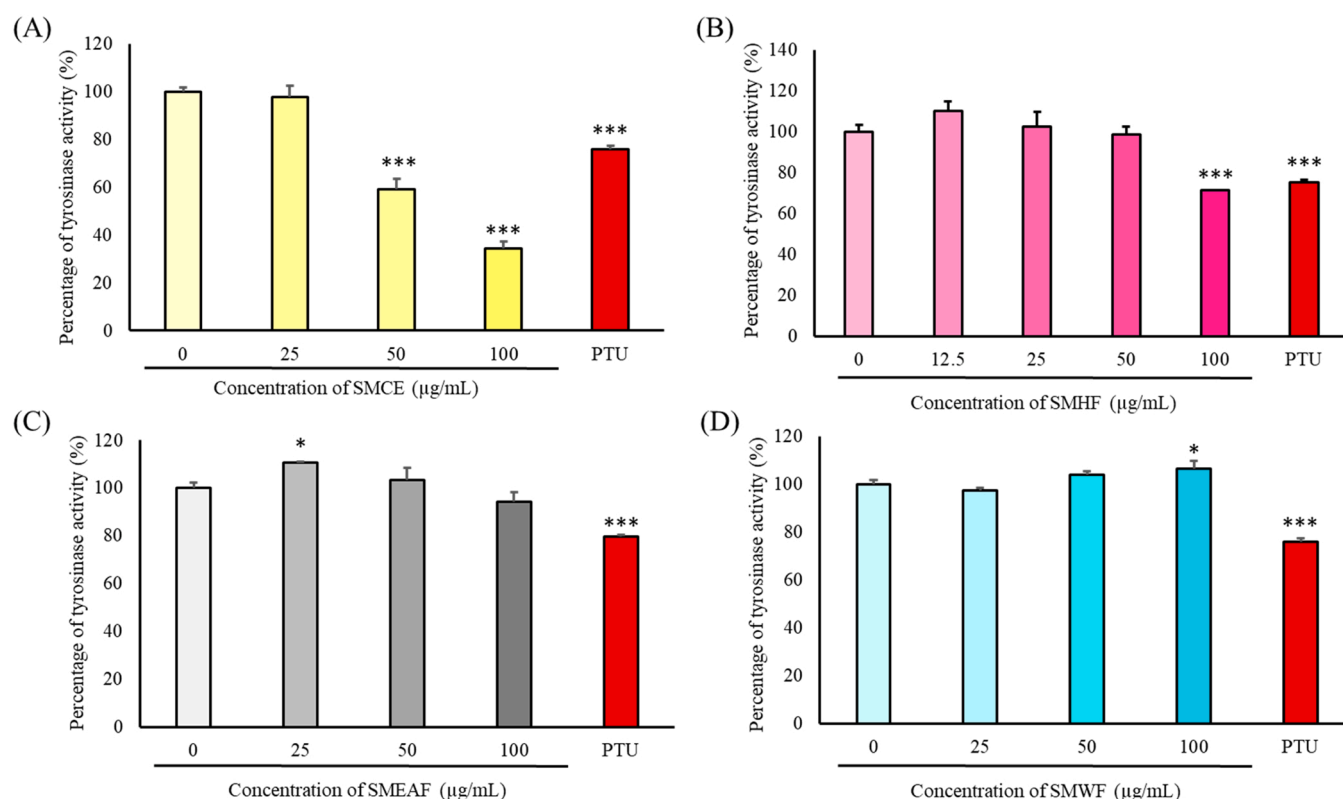


Fig. 3. Measurement of tyrosinase activity after treatment with *S. macrophylla* seed extract and fractions. Zebrafish embryos were treated with 0–100 $\mu\text{g/mL}$ of (A) SMCE, (B) SMHF, (C) SMEAF, and (D) SMWF for 24 h. The harvested protein was then added with L-DOPA and the absorbance was measured using a microplate reader at 475 nm. For the positive control, 40 μM of PTU was utilized. (n = 3; * p -value ≤ 0.05 , ** p -value ≤ 0.01 ; *** p -value ≤ 0.001).

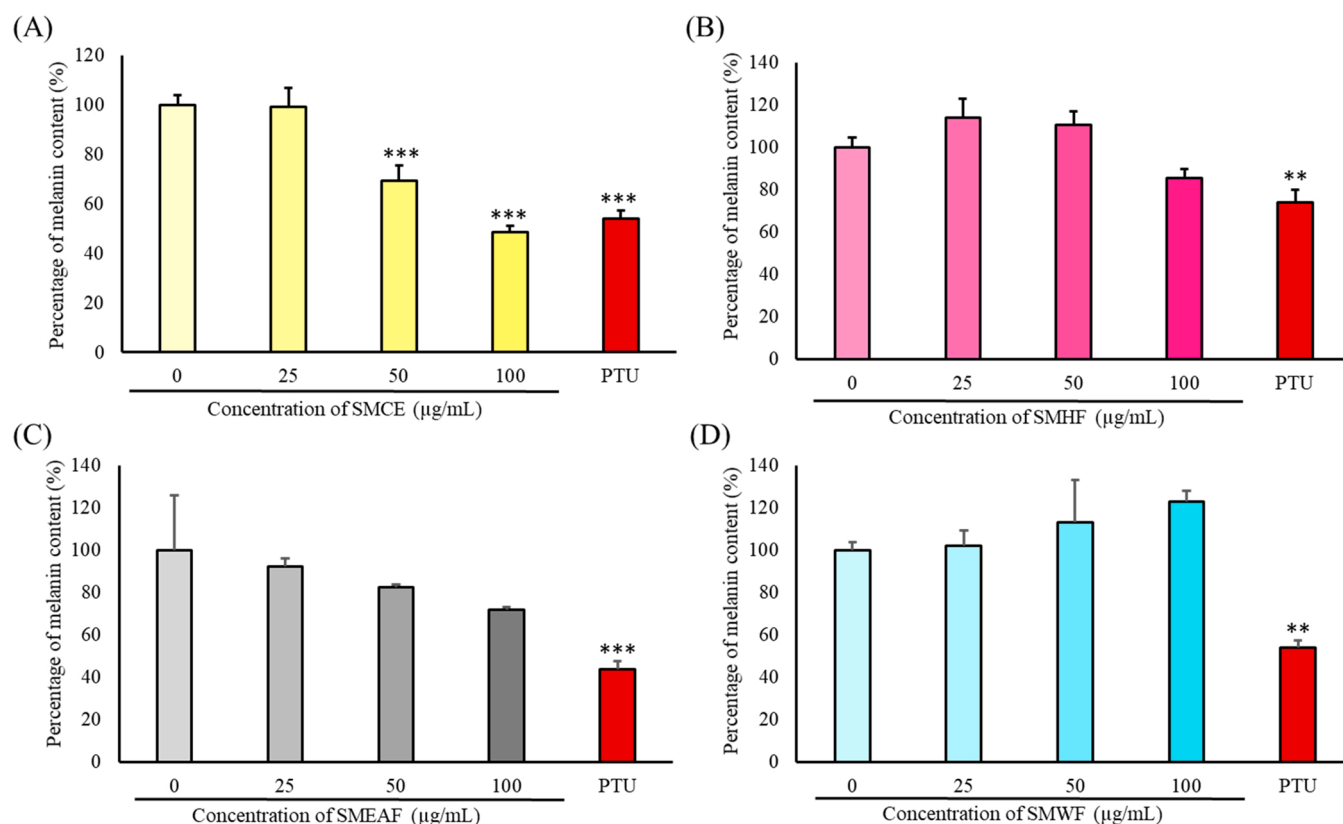


Fig. 4. The percentage of melanin content after treatment 0–100 $\mu\text{g/mL}$ of (A) SMCE, (B) SMHF, (C) SMEAF and (D) SMWF for 24 h. As a positive control, 40 μM of PTU was used to induce a decrease in melanin content in the zebrafish embryos (n = 3 * p -value ≤ 0.05 , ** p -value ≤ 0.01 ; *** p -value ≤ 0.001).

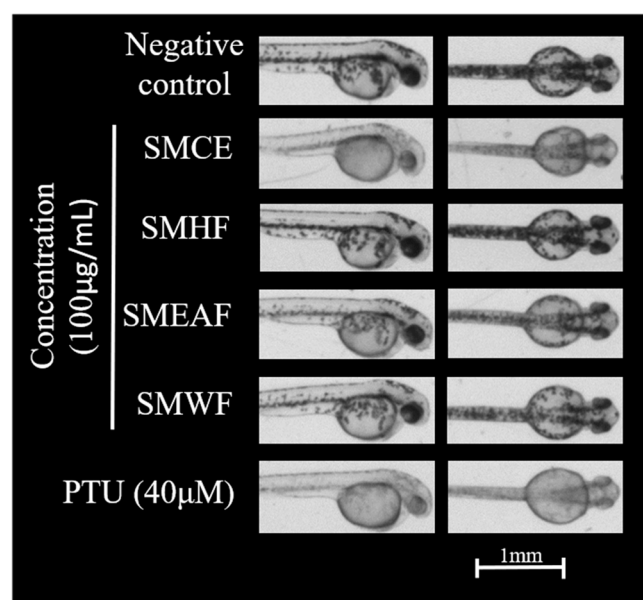


Fig. 5. Representative images of zebrafish embryos that were treated with 100 µg/mL of SMCE, SMHF, SMEAF, and SMWF for 24 h. PTU is the positive control in this assay (n = 50).

additionally decreased the expression of several actin cytoplasmic proteins, apolipoproteins, bactin, and heat shock proteins. The expression of heat shock protein in Table 4 was probably triggered during the process of euthanizing with ice, as Kulkeaw, et al. [35] had proven that heat shock proteins too can be expressed in zebrafishes under cold conditions. Nonetheless, after many literature searches, a few proteins such as cathepsin LB, keratin 4, actin, and EF-1a were previously discovered to have some involvement in skin pigmentation and these proteins will be discussed in depth in the discussion section. Additional detailed data on each identified protein can be found in the [supplementary materials](#) (Table A.1 and A.2).

3.4. qPCR analysis of zebrafish embryos treated with SMCE and SMHF

The total mRNA of the embryos were collected and analyzed after 24 h treatment of PTU, SMCE or SMHF for changes in *PSEN1* and *PSEN2* expression. The expression of *PSEN1* and *PSEN2* were chosen as subject of investigation because it was previously suggested that both these genes were linked with the formation of melanin in zebrafish embryos. When silenced, zebrafish embryos were observed to experience a loss in pigment formation [61]. As can be seen in Fig. 7, there were no significant changes in the expression of *PSEN1* while SMCE and SMHF significantly increased *PSEN2*. However, when treated with SMCE and SMHF, *PSEN2* was significantly increased which contradicts with the data where melanin production was observed to be decreased (Fig. 4). This suggests that although there is an increase in *PSEN2* expression, SMCE and SMHF might be inhibiting other pigment-related proteins downstream of *PSEN1* and 2 instead of *PSEN1* and 2 themselves,

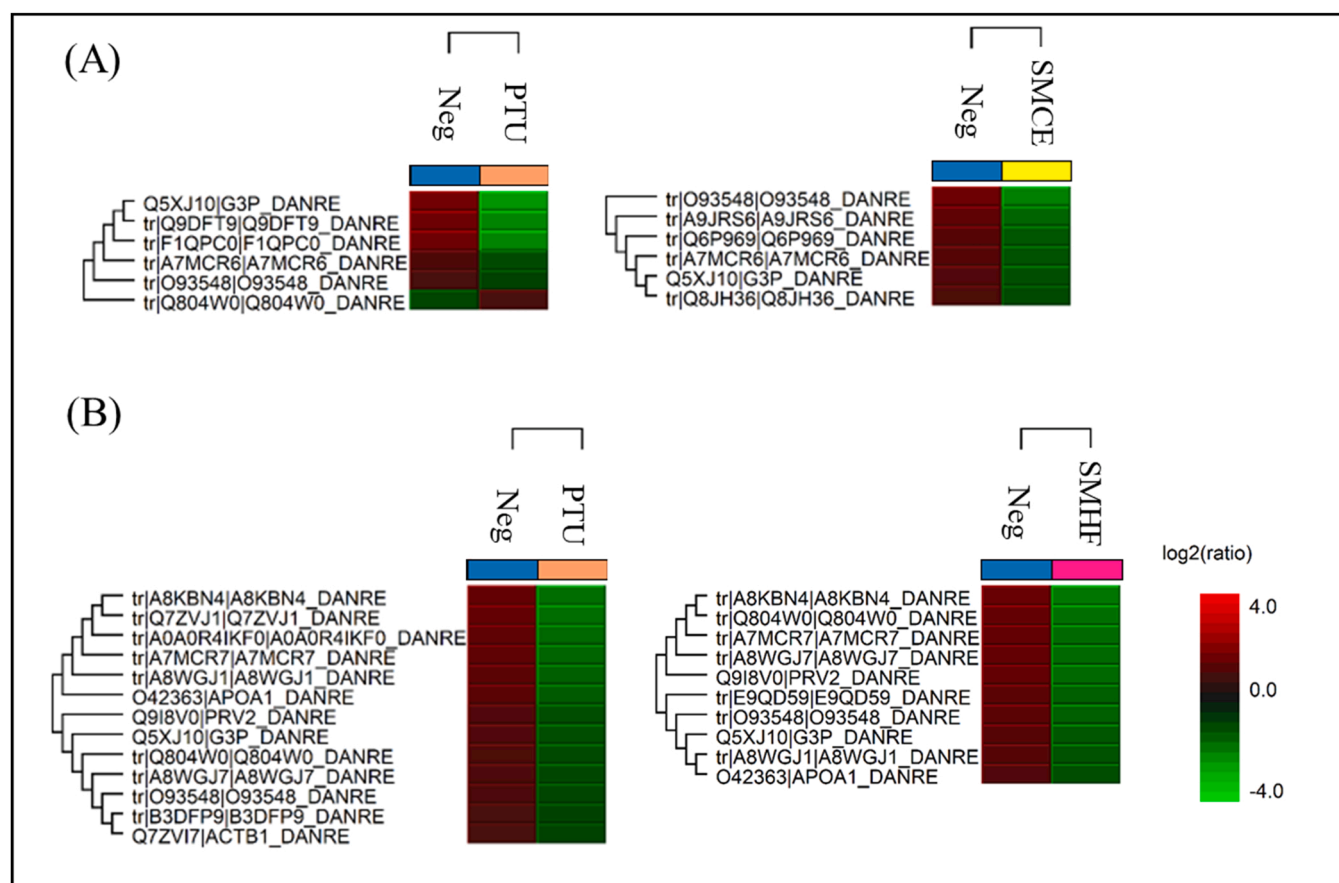


Fig. 6. Heatmaps on the proteins that were upregulated (red) and downregulated (green) in the zebrafish embryo by PTU, SMCE, and SMHF after 24 h treatment. (A) Comparison of negative control with 40 µM PTU or 100 µg/mL SMCE, (B) Comparison of negative control with 40 µM PTU or 100 µg/mL SMHF; (Neg: negative control, whereby zebrafish embryos were treated with only the vehicle solvent 0.5% (v/v) DMSO)(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

Table 3
Proteomic changes in zebrafish embryos after 24 h of PTU or SMCE treatment.

No	Name of protein (Uniprot ID)	Comparison ratio of negative control with PTU or SMCE (negative control: treatment)	
		40 μ M PTU (Positive control)	100 μ g/mL SMCE
1	Cathepsin LB (A7MCR6, A7MCM3, A7MCR8, A7MCR7, A0A2R8Q790, E9QE79 or A8E587)	1.00:0.63 (↓)	1.00:0.57 (↓)
2	EF-1a (Q6P969, A0A2R8QSR4, C5IG39, A0A2R8Q469, Q92005, A8WKG7, Q568F0, A2VCX2, Q4KMK1, A7YYA5, Q4JIY5, A0A0R4IE10, Q6DHT2 or A0A2R8RKC7)	N/S	1.00:0.54 (↓)
3	Embryonic 1 beta-globin (O93548)	1.00:0.72 (↓)	1.00:0.26 (↓)
4	GAPDH (Q5XJ10)	1.00:0.18 (↓)	1.00:0.59 (↓)
5	Keratin 4 (A9JRS6)	N/S	1.00:0.43 (↓)
6	Parvalbumin (Q804W0)	1.00:1.46 (↑)	N/S
7	Vitellogenin 1 (Q8JH36)	N/S	1.00:0.64 (↓)
8	Vitellogenin 3, phosphovitellogenin (F1QPCC03, F1QQQ3, or Q9DFT9)	1.00:0.25 or 0.24 (↓)	N/S

* N/S represents data that was not significant, while protein upregulation and downregulation were represented as (↑) and (↓), respectively. Both PTU and SMCE were compared against the negative control. (n = 3, whereby each tube contained 30 embryos; the p-value is set at 0.05 significant level).

ultimately inhibiting the production of melanin.

3.5. Molecular docking of *S. macrophylla* limonoids against TYRP-1

3.5.1. Validation of docking protocol

The use of molecular docking comes with its benefits. As a computational scheme, it can be used to predict the structure of intermolecular complexes formed through interaction between two or more molecules [52,71]. It is also comparatively a much effective tool in screening enzymatic substrate and pure compounds on a large scale, which aids chemists in making rational decisions during drug design and synthesis [78]. In our study, the X-ray crystallographic structure of human TYRP-1 (PDB ID: 5M8M), with a resolution of 2.65 Å and kojic acid bound within its active site, was downloaded from the protein databank and used as the protein model for molecular docking (Fig. 8). Next, to determine the structure quality of the protein, a Ramachandran plot of the protein was plotted as shown in Fig. 9. A Z-score of the plot was also obtained from Yasara WhatIF and the score was −1.854, which is acceptable as protein structures with a resolution less than 2.8 Å commonly have an average Z-score of -0.7 ± 1.3 standard deviation (Hooft et al., 1997). Besides that, there were very few protein residues in the outlier region, but those were located at the outer loops of the protein itself and were nowhere near the active site. Kojic acid that was also already bounded within the active site was removed and redocked to validate our docking protocol. The root mean square deviation (RMSD) of the redocked kojic acid ligand was found to be 1.13 Å when compared with the co-crystal bounded kojic acid. Upon comparison of co-crystallized and redocked Kojic acid poses, as depicted in Figure A.1, minor differences in position and bond formation were observed but were within an acceptable range (RMSD < 2 Å) for further docking of limonoids in TYRP-1.

3.5.2. Docking of limonoids on TYRP-1

Limonoids are tetranotriterpenoids and secondary metabolites from *S. macrophylla* exhibiting anti-microbial, anti-inflammatory,

Table 4
Modulation of protein expression in zebrafish embryos by PTU and SMHF after 24 h of treatment (n = 30 embryos per biological replicate).

No	Name of protein (Uniprot ID)	Comparison ratio of negative control with PTU or SMHF	
		40 μ M PTU (positive control)	100 μ g/mL SMHF
1	Actin, beta 1 (E9QD59)	N/S	1.00:0.47 (↓)
2	Actin, cytoplasmic 1 (Q7ZV17)	1.00:0.71 (↓)	N/S
3	Actin, cytoplasmic 2 (Q7ZVF9)	1.00:0.71 (↓)	N/S
4	Apolipoprotein A-I (O42363)	1.00:0.49 (↓)	1.00:0.60 (↓)
5	Apolipoprotein A-II (A0A0R4IKF0)	1.00:0.40 (↓)	N/S
6	Apolipoprotein A-III (B3DFP9)	1.00:0.69 (↓)	N/S
7	Bactin1 (B2GS08)	1.00:0.71 (↓)	N/S
8	Bactin2 (A8WG05)	1.00:0.71 (↓)	N/S
9	Cathepsin LB (A7MCR6, A7MCM3, A7MCR8, A7MCR7, A0A2R8Q790, E9QE79, or A8E587)	1.00:0.42 (↓)	1.00:0.38 (↓)
10	Embryonic 1 beta-globin (O93548)	1.00:0.68 (↓)	1.00:0.47 (↓)
11	Embryonic alpha globin e1 (Q7ZT21)	1.00:0.36 (↓)	1.00:0.31 (↓)
12	GAPDH (Q5XJ10)	1.00:0.61 (↓)	1.00:0.52 (↓)
13	Hbae1 protein, haemoglobin alpha embryonic 1 (A8KBN4)	1.00:0.36 (↓)	1.00:0.31 (↓)
14	Heat shock 70 kDa protein 8 (Q6TEQ5)	1.00:0.36 (↓)	N/S
15	Heat shock cognate 71 kDa protein (A0A2R8Q860, A0A2R8QEN9, Q1LY29, A0A0R4IMF8, or Q6NRY4)	1.00:0.36 (↓)	N/S
16	Heat shock protein 8 (Q7ZVJ1)	1.00:0.36 (↓)	N/S
17	Heat shock protein family A (Hsp70) member 8b (F1R9V3 or R4GEU2)	1.00:0.36 (↓)	N/S
18	Hemoglobin, alpha embryonic 1.3 (F6P731)	1.00:0.36 (↓)	1.00:0.31 (↓)
19	Novel alpha-globin (Q7SZV8)	1.00:0.36 (↓)	1.00:0.31 (↓)
20	Parvalbumin (Q804W0)	1.00:0.64 (↓)	1.00:0.35 (↓)
21	Parvalbumin-2 (Q9I8V0)	1.00:0.60 (↓)	1.00:0.39 (↓)
22	Vitellogenin [gene: vtg4] (A8WGG7)	1.00:0.65 (↓)	1.00:0.39 (↓)
23	Vitellogenin 2 (A8WGG1)	1.00:0.45 (↓)	1.00:0.57 (↓)

* N/S represents data that was not significant, while protein upregulation and downregulation were represented as (↑) and (↓), respectively. Both PTU and SMHF were compared against the negative control. (n = 3, whereby each tube contained 30 embryos; the p-value is set at a 0.05 significant level).

antifeedant, anti-malarial, anti-viral, anti-cancer activities, etc [43,53,72]. Hence, being secondary metabolites that are extensively produced in the *S. macrophylla* and having a vast range of bioproperties, it was chosen as the potential inhibitor against human TYRP-1. 3D structures of various limonoids were downloaded from Pubchem, followed by energy minimization and docking within TYRP-1 employing the validated docking parameters. Table 5 shows the binding energy, binding interactions, and interacting residues of each ligand with 5M8M TYRP-1. The interactions between ligand and targeted protein are illustrated in Fig. 10.

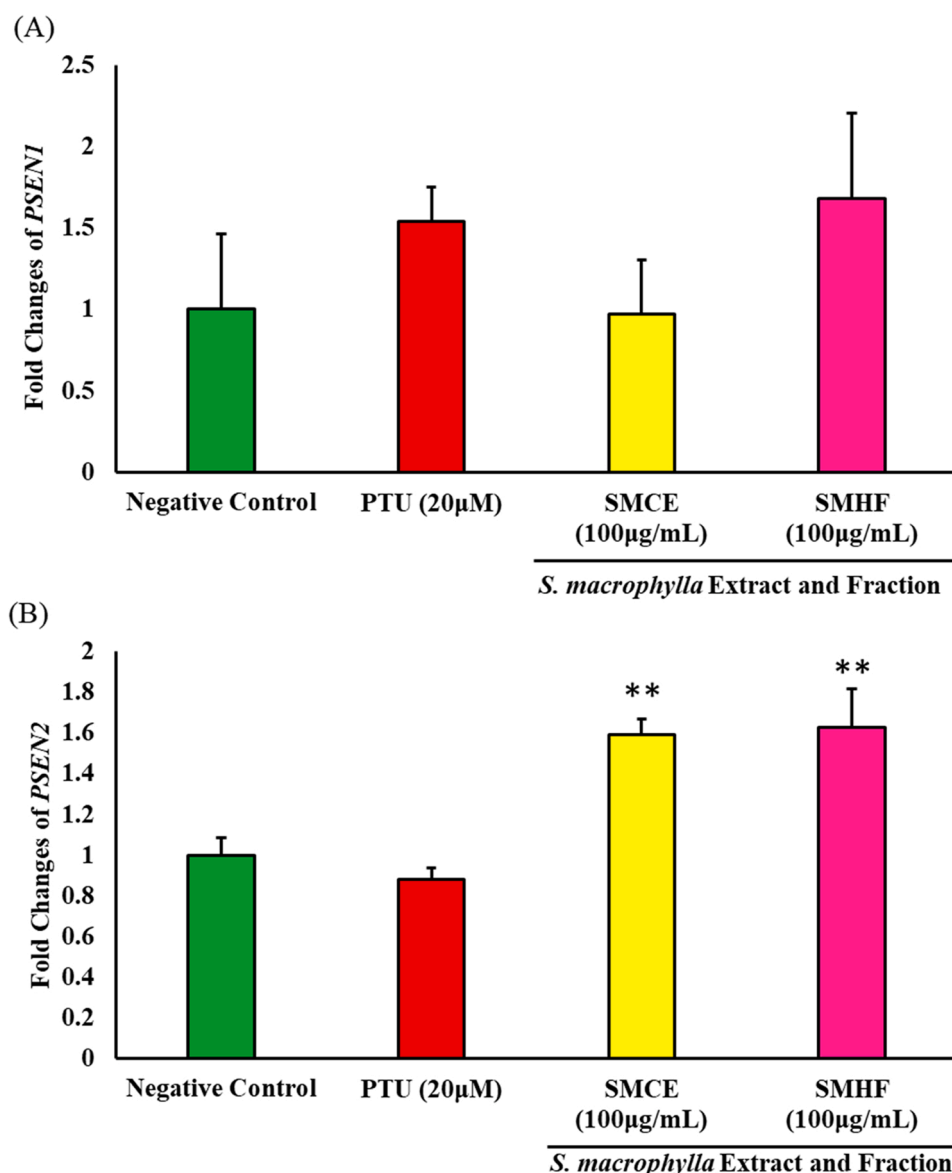


Fig. 7. The gene expression changes of *PSEN1* and *PSEN2* in zebrafish embryos that were treated with PTU, SMCE, and SMHF. The negative control are embryos that were treated with only the vehicle solvent (0.5% v/v DMSO). (n = 3; * p-value ≤ 0.05 , ** p-value ≤ 0.01 ; *** p-value ≤ 0.001).

Comparing kojic acid and limonoids, it was evident that the limonoids have a stronger binding affinity than kojic acid. This is due to it having more side chains as compared to kojic acid. Many of the bindings are hydrogen bonds as well which indicates strong binding and almost all of them, except 2-hydroxyswietenine, have bonds with the water molecule located in between the binuclear zinc-binding site. According to Lai, Wichers, Soler-Lopez and Dijkstra [37], L-tyrosine, L-DOPA, L-mimosine, and kojic acid all showed interaction with the water molecule that bridges the two zinc ions. Therefore, it is important that the limonoids have interaction with the water molecule. Besides that, other interactions like Thr391, Arg374, Ser394, and His 381 were also considered as Lai and his team reported these amino acids as interacting amino acids in TYRP-1 [37]. Overall, these phytochemicals may act as potential inhibitors of TYRP-1 and possess anti-pigmentation properties.

4. Discussion

The utilization of zebrafish embryo as a model to study pigmentation had been on a rise. Some of the benefits of using these embryos are (1) the mechanism of melanocyte development and melanin biosynthesis

between humans and fish is highly conserved, (2) the transparency of the fish makes it easy to observe pigment change, (3) the short time required for the formation of melanocytes and pigment in the embryos, (4) easy evaluation of drug toxicity, (5) high number of offspring, (6) ease of maintenance and handling due to a small size, and finally (7) increased efficiency of drug penetration via the gills and skin [1,50]. However, there is a fundamental difference between human and fish is that zebrafish do not have a melanosome transportation process like humans. Hence, the changes in pigmentation by the drugs tested are solely due to the changes in melanocytes or the process of melanin synthesis and/or metabolism [50].

Following the treatment of zebrafish with *S. macrophylla* extract and fractions, SMCE as the crude extract demonstrated the lowest tyrosinase activity and melanin content. The value obtained was even lower than PTU, signifying its potency as an anti-pigmentation agent. Then after fractionation of SMCE, 100 µg/mL SMHF too significantly suppressed tyrosinase. SMEAF instead had just a slight decrease in tyrosinase activity, while SMWF produced the opposite effect of increasing tyrosinase activity. Yet when the melanin content was measured, SMHF was observed to decrease only a little of the melanin content in the embryos

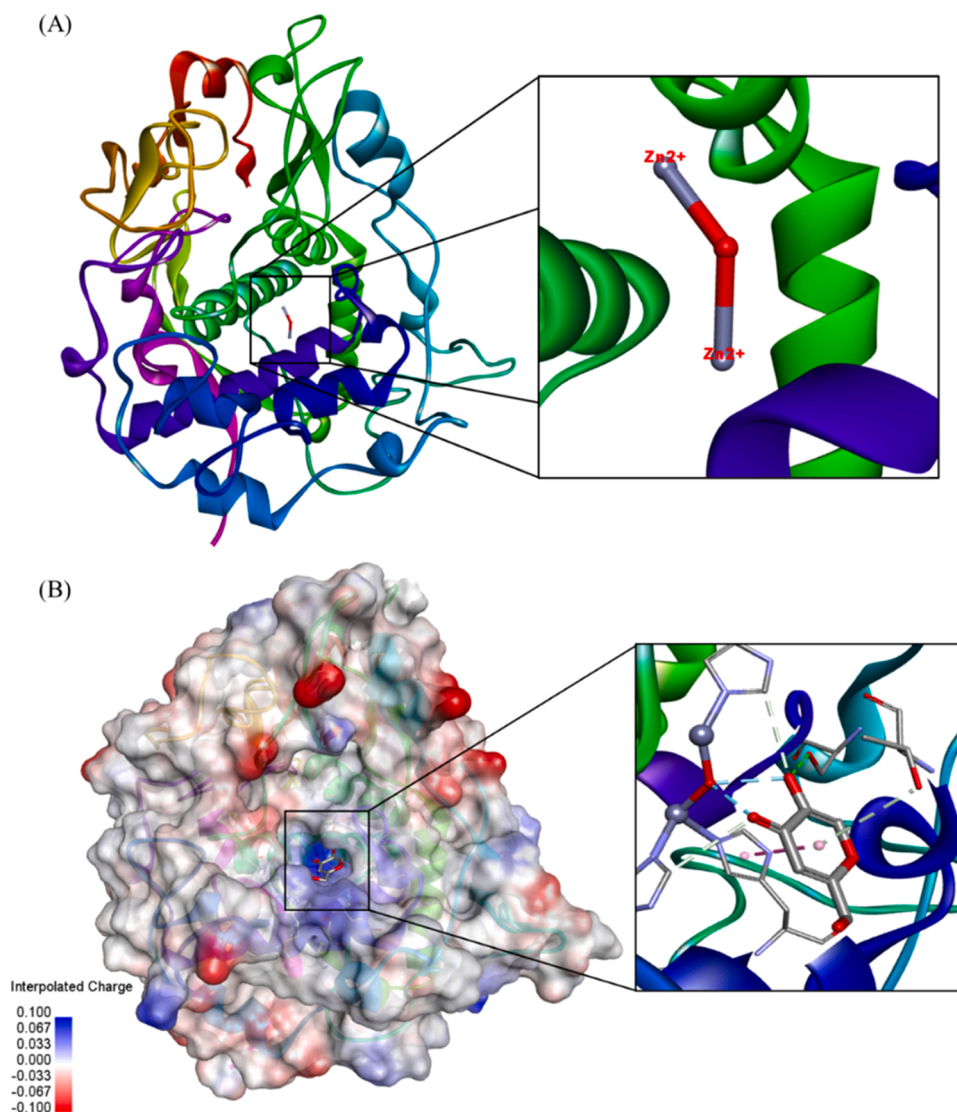


Fig. 8. Protein structure of TYRP-1 (5M8M) obtained from protein databank. (A) Image of the binuclear zinc active site for protein binding. (B) The binding pocket of ligands is colour coded with an interpolated charged surface (blue/red = positive/ negative). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

at the highest concentration. The same was seen for SMEAF as well whereby a dose-dependent decrease in melanin content was obtained. Whereas, SMWF increased the melanin content as it increases the tyrosinase activity of the embryos. Based on the data, *S. macrophylla* does have anti-pigmentation properties and it can be found in SMCE.

Further research using LC-MS/MS revealed that SMCE and SMHF similarly downregulated several proteins like PTU. Among the proteins that were detected to experience a significant change in the embryos after the treatment, there are a few that stand out and had connections to the melanogenesis process. The first one is cathepsin LB, which was downregulated by SMCE and SMHF. Cathepsin LB is a lysosomal cysteine protease produced during embryogenesis and is potentially involved in hatching and tissue remodeling [58]. In zebrafish, the gene expression of cathepsin LB was reported to be affected when there are mutations in *presenilin (PSEN) 1* and *2* genes [58]. PSENs are transmembrane proteins that are the catalytic core of γ -secretase complex, regulating the proteolysis of type I membrane proteins [58]. Although these genes are interrelated with Alzheimer's disease, mutations in zebrafish PSEN1 and 2 were reported to reduce melanotic pigmentation. Zebrafish embryos that were silenced in both *PSEN1* and *2* were observed to have impaired pigment formation [61]. According to Jiang,

et al. [29], T141_L142delinsMISLISV homozygous mutated *PSEN2* zebrafish have fainter melanotic cells on somewhat normal stripes, while N140fs homozygous mutated *PSEN2* fishes lack or had very faint surface melanotic stripes. Thus, this confirms that modification to *PSEN2* causes a loss of pigmentation and as cathepsin LB are also affected by *PSEN2*, it may be possible that SMCE and SMHF might affect the expression of *PSEN2*, cathepsin LB, and in turn the pigmentary system in zebrafish. Thus, the gene expression of both *PSEN1* and *PSEN2* in SMCE and SMHF embryos were further investigated. Unexpectedly, it was discovered that *PSEN2* expression was instead significantly upregulated by both SMCE and SMHF, while *PSEN1* remained unchanged in its expression. This data suggests that, alternatively, the inhibition of pigmentation by SMCE and SMHF could be occurring downstream of these two genes (like cathepsin LB) and not on the gene itself. In hindsight, this discovery is a good outcome as *PSEN1* and *2* are essential genes in the development of the brain and spine of zebrafish larvae [57, 61]. In another research, mice with null *PSEN1* were born with skeletal defects, impaired brains, and die shortly after birth [67]. Whereas, there had been cases whereby mutated *PSEN2* had been associated with early onset Alzhiemers and dementia in patients [20,41]. Therefore, the fact that SMCE and SMHF did not downregulate both genes indicate that

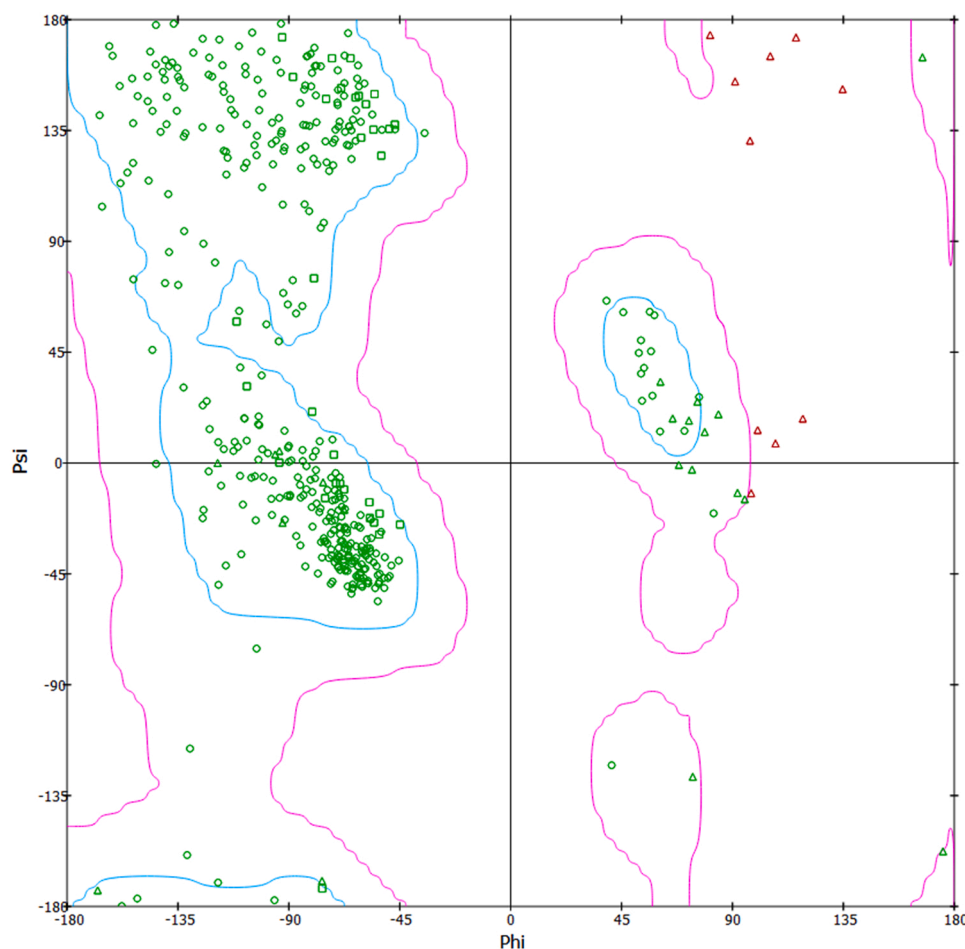


Fig. 9. Ramachandran plot of the protein (5M8M) obtained from Biovia Discovery Studio. The blue region represents the favoured region, while the pink region is the allowed region. Green circles and triangles are protein residues that are within the favoured and allowed region, while the red triangles represent protein residues that are in the outlier regions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

SMCE and SMHF are solely suppressing the production of melanin and not essential genes involved in the development of the embryos.

Next, cytoskeletal proteins (keratin and actin) and actin binding protein EF-1a have a role to play in the formation of pigmentation in the eye and its downregulation by either SMCE or SMHF probably affected the melanogenic process. According to literature, diseases such as epidermal bullosa simplex with mottled pigmentation, Dowling-Degos disease, and Naegeli-Franceschetti-Jadassohn syndrome all had hyperpigmentation problems that are interconnected to mutations in various keratins [26]. In regards to keratin 4, it was discovered that the missense mutation in keratin 4 produced a breed of mice with brighter and lighter coat colors (*Bcc1* mutant) [51]. Subsequently, it was previously reported that a double mutant (*yap*^{-/-} *taz*^{-/-}) zebrafish embryo demonstrated not only defective retinal pigment epithelial (RPE) differentiation but also a significant decrease of keratin 4, keratin 8, and pigmentation genes *tyr* and *tyrp1b* in the dissected head at 18 hpf [8]. RPE are pigmented cells that can be found in the retina and are essential in their support of the photoreceptors [45]. Due to the nature of its role, RPEs are highly polarized and have an extensive cytoskeleton, which comprises three filament types; actin, microtubules, and intermediate filaments. The intermediate filaments instead are composed of keratins, vimentin, desmin, and neurofilaments [28,74]. The function of these cytoskeletons is to maintain outer retinal homeostasis and in zebrafishes, these cytoskeletons restructure themselves as RPE in the fish flattens and stretch themselves to match the retinal curvature and promote the folding of the optic vesicle [55,74]. Not forgetting, mutations in EF-1a protein was also reported to alter the organization of the cytoskeleton [25]. Hence,

the decrease of actin protein by SMHF or the suppression of keratin 4 and EF-1a expression by SMCE could be interconnected with defective RPE differentiation and pigmentation, decreasing the colouration in zebrafish eyes as observed in Fig. 5. In support of that, the protein parvalbumin, which is also expressed in the retina was also affected in its expression by SMHF [80]. This further confirms that the lightening of eye pigmentation induced by SMCE and SMHF was by interfering with normal retinal development. For better understanding, a summary of the proteins affected by *S. macrophylla* in the anti-pigmentation process is depicted in Fig. 11.

Despite that, there are still many proteins listed that had yet to be studied in depth for their connection with zebrafish pigmentation. Some of these proteins, like EF-1a, GAPDH, and actin beta 1 too are used as housekeeping proteins [62,63,9]. However, if samples, especially PTU, are affecting the expression of these proteins, these genes or proteins should not be used for housekeeping as it causes the expression of controls and samples to no longer be comparable. It should be noted that it is very common to see PTU being used as a pigment inhibitor to maintain fish transparency for imaging whilst doing gene or protein studies [3,33,56]. As for the other proteins like vitellogenin, apolipoprotein, bactin, and more, their downregulation during the treatment of PTU potentially signifies that these proteins may have a connection to the melanogenesis process of zebrafish in one way or another. These are research gaps that have yet to be undertaken and this is the first time these proteins had been brought to attention in the study of zebrafish pigmentation.

Finally, molecular docking of previously reported isolated limonoids

Table 5

Binding energy and interactions of limonoids with human TRYP-1.

Ligand	Pubchem CID	Binding energy (ΔG) (kcal/mol)	Binding interactions	Binding residues
Reported kojic acid	NA*	NA	Conventional H bond Water H bond Carbon H bond Pi-Pi stacked Pi-donor H bond	Ser394 HOH601 HIS192, His377 His381 Thr391
Redocked kojic acid	3840	-4.2	Conventional H bond Water H bond	Ser394, Gly389 HOH601
2-hydroxyswietenine	76326957	-6.6	Conventional H bond Carbon H bond Pi-Cation Pi-Pi stacked	Tyr362, Ser394, Glu216 Thr391, Gly388 Zn513 His381
3- β ,6-dihydroxydihydrocarapin	NA	-6.36	Conventional H bond Water H bond Pi-Sigma	Arg374, Thr391 HOH601 His215
3-O-tigloyl-6-O-acetylswietenolide	23258996	-6.08	Conventional H bond Water H bond Carbon H bond Pi-Pi stacked Pi-pi T-shaped Pi-alkyl	Tyr362, Arg374 HOH601 Thr391 His377 His381 TYR348
3-O-tigloylswietenolide	101396725	-6.62	Water H bond Conventional H bond Carbon H bond Alkyl	HOH601 Arg374, Ser394, Thr391 His377, Gly388 Val196
Adirobin	266444	-7.15	Conventional H bond Water H bond Pi-Lone pair Pi-Anion Pi-Alkyl	TYR348, Arg374 HOH601 His381 Glu216 His215, Tyr362
Febrifugin	NA	-7.48	water-carbon H bond Conventional H bond Carbon H bond Alkyl	HOH601 His392 Thr391, Glu216 Lys197, Lys198, Leu293
Fissinolide	6451333	-7.36	water-carbon H bond Conventional H bond Carbon H bond Pi-Cation Amide-Pi stacked	HOH601 Arg321, Arg374, Ser394 Asp212 Zn513 Gln390
Khayasin T	NA	-7.32	water-carbon H bond Conventional H bond Pi-Sigma Pi-pi T-shaped	HOH601 Arg374, Thr391, His381 His392
Proceranolide	23258999	-7.66	Water H bond Conventional H bond Pi-Sigma Pi-Pi stacked	HOH601 Arg374, Thr391 Thr391 His381
Swietenine	14262276	-7.22	water-carbon H bond Conventional H bond Carbon H bond Pi-pi T-shaped	HOH601 Thr391, His392, Asp212, Glu216, Thr391, Gly209, Val211 His381
Swietenine (stereoisomer)	23891055	-6.85	Water H bond Conventional H bond Carbon H bond Pi-Pi stacked Alkyl	HOH601 Arg321, Ser349, Arg374 Asn378 His381 Thr362, Tyr369
Swietenolide diacetate (3,6-O,O diacetylswietenolide)	44575317	-7.64	water-carbon H bond Conventional H bond Carbon H bond Pi-sigma Pi-Pi stacked Amide-Pi stacked	HOH601 Arg321, Thr391 Gly389 Thr391 His381 Gln390
Swietenolide diacetate (stereoisomer)	98042567	-7.15	water-carbon H bond Conventional H bond Carbon H bond Pi-Pi T-shaped	HOH601 Thr391 His215, Asp212, Glu360 His215

*NA indicates “Not available”; H-bond: hydrogen bond.

from the seeds of human TYRP-1 was conducted. Although the function of TYRP-1 in catalyzing the oxidation of DHICA to IQCA in the melanogenesis process had been challenged, its role in the formation of pigment is undeniable [37]. The loss of TYRP-1 had been related to a form of skin albinism known as oculocutaneous albinism type III (OCA3). Trademarks of OCA3 are light brick red skin tones accompanied

with gold hair which darkens to ginger or red-brown colour with age. They also have decreased visual acuity and some have photophobia as well [34]. In some cases, patients with OCA3 also have blue/gray irides [6]. In *in vitro* cultures, mouse melan-b melanocytes that were mutant for TYRP-1 and had a brown pigmentation, begin to form black to dark coloration after being transfected with TYRP-1 cDNA. This suggests that

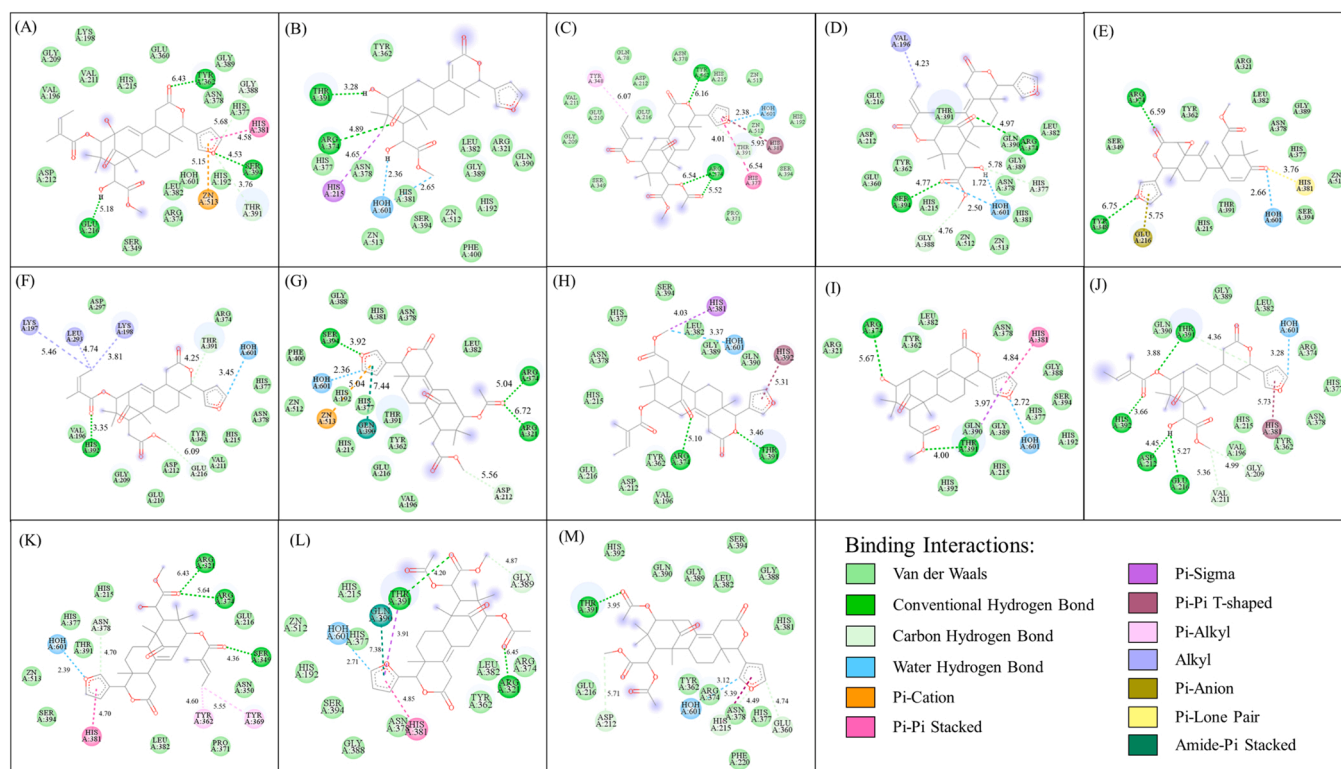


Fig. 10. Illustration of 2D binding interactions between limonoids and TYRP-1. (A–M) shows limonoid 2-hydroxyswietenine, 3- β ,6-dihydroxydihydrocarapin, 3-*O*-tigloyl-6-*O*-acetylswietenolide, 3-*O*-tigloylswietenolide, andirobin, febrifugin, fassinolide, khayasin T, proceranolide, swietenine (14262276), swietenine (23891055), swietenolide diacetate (44575317), and swietenolide diacetate (98042567), respectively. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

TYRP-1 is essential to the formation of black or darker pigmentation [4]. On another note, TYRP-1 had also been suggested to be involved in the stabilization of TYR protein itself. In a study done by Manga, et al. [49], COS-7 cells had been transfected with both murine TYR and TYRP-1. The co-transfection revealed that not only was tyrosinase activity significantly increased but the number of tyrosinase protein was elevated as well. This increase was conferred to the ability of TYRP-1 in stabilizing tyrosinase as further analysis demonstrated that the protein extract from cells transfected with both tyrosinase and TYRP-1 had the greatest stability in terms of tyrosinase activity in comparison to cells transfected with TYR alone under 50 °C incubation [49]. On the other hand, it is noted that mutated TYRP-1 produced in a melan-b (murine melanocyte cell line with mutated TYRP-1 gene) could not (1) leave the endoplasmic reticulum (ER), (2) is sensitive to the enzyme endoH, and (3) was degraded quickly because they were unable to mature. Not only that, but the mutations of TYRP-1 also retarded the maturation of tyrosinase. Tyrosinase produced in these cells was found to have difficulty leaving the ER, stayed sensitive to endoH, and was degraded at an accelerated pace [76].

In this study, all the limonoids tested demonstrated to higher binding affinity than kojic acid (−4.2 kcal/mol), with the highest being proceranolide (−7.66 kcal/mol), followed by swietenolide diacetate (−7.64 kcal/mol), febrifugin (−7.48 kcal/mol) and so on. This is because of the additional bonds the limonoids have with TYRP-1 like Pi-sigma, Pi-cation, Pi-lone pair, Pi-anion, Pi-Pi T-shaped, Pi-alkyl, alkyl, and amide-Pi stacked with the surrounding amino acids, which contributed to a stronger binding affinity than kojic acid. Those with low binding energies also have the same binding to important residues like Thr391, Arg374, His381, and HOH601. These binding interactions are significant ligand-receptor binding interactions as observed with kojic acid in Figure A.1 and as reported by Lai and his team [37]. Thus having similar binding interactions and higher binding affinity to known

TYRP-1 inhibitors, these limonoids can be potential TYRP-1 inhibitors. Additionally, the strong binding of proceranolide towards TYRP-1 is a positive sign as previously, proceranolide demonstrated considerable anti-inflammatory properties against the production of nitric oxide (NO) in RAW264.7 macrophage cells [60]. Other limonoids that had a high binding affinity with TYRP-1 such as 3-*O*-tigloylswietenolide, swietenine, 3-*O*-tigloyl-6-*O*-acetylswietenolide, and swietenolide diacetate (3, 6-*O*,*O*-diacetylswietenolide) too were tested to be potent inhibitors of inflammation [11,12,48]. Skin inflammation and pigmentation are interconnected in the skin photodamage process. Under UVR exposure, excessive reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated [2]. These then lead to oxidative and nitrosative stress which triggers the inflammatory system. The production of pro-inflammatory markers instigates the infiltration of neutrophils which continue to produce more NO, upregulating the tyrosinase protein level and activity [10,30,40,66]. NO also stimulates the α -melanocyte stimulating hormone (α -MSH) in melanocytes inducing an increase in melanin production [24]. Now adding on to their anti-inflammatory properties, it could be possible that these limonoids may also have anti-pigmentation properties. Nonetheless, further verification of their TYRP-1 inhibitory activity still needs to be conducted to confirm their skin whitening ability. In short, Fig. 12 showcases the overview of the project and the potential *S. macrophylla* has as an anti-pigmentation agent.

5. Conclusion

In conclusion, *S. macrophylla* seeds do possess anti-pigmentation properties that can potentially be developed as a skin whitening cosmetic agent. The treatment with SMCE displayed a significant decrease in pigment formation in 48 hpf zebrafish embryo. The same was later observed when SMCE was fractionated to SMHF and SMEAF,

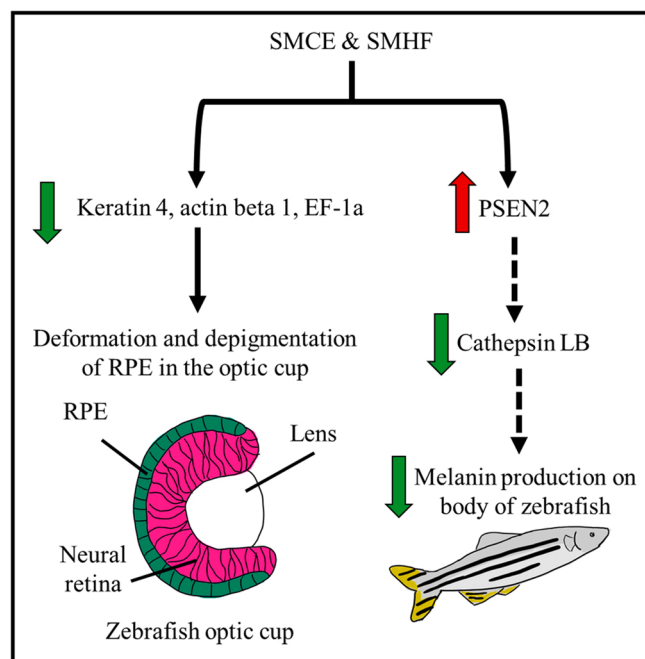


Fig. 11. The protein changes induced by SMCE and SMHF during the melanogenesis process of zebrafish. The decrease in keratin, actin, and EF-1a might have caused deformation and depigmentation of RPE in zebrafish embryos, giving rise to pale eye colour. In the meantime, the downregulation of cathepsin LB by SMCE and SMHF could be a factor in the decrease of melanin production in the body of the zebrafishes. The green arrows represent the downregulation of expression by either SMCE or SMHF, whereas the red arrows represent genes that were upregulated. The dashed lines are proposed interconnected pathways that might have been activated during treatment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

albeit having a much lower effect than SMCE. Nevertheless, it is seen that SMHF like SMCE induces the downregulation of cathepsin LB and cytoskeletal proteins that affected the pigmentation process. This signifies that not only are there phytochemicals in SMCE and SMHF that can affect the melanogenic process of zebrafish but the LC-MS/MS data also had revealed unforeseen and unconventional novel protein functions that can be explored further. Finally, molecular docking against human TYRP-1 was conducted using limonoids that were previously reported to be found in the seeds. Based on the data, limonoids were observed to be binding stronger to human TYRP-1 as compared to kojic acid. These are good indicators as many studies had showed that limonoids have many bioproperties and are most likely the main contributor to the bioactivity detected in the seed. Even so, more studies still need to be conducted in the future on these compounds before confirming their anti-melanogenic properties. Other than that, for future work, the extract and fractions can be tested on different models like ex vivo or 3D human skin cultures to better study the whitening effect of *S. macrophylla* on humans.

Funding

Not Applicable.

CRediT authorship contribution statement

Conceptualization, C.K.M., H.-L.S. and B.H.G.; Methodology, C.K.M., S.H.-L. and S.U.K.; Validation, S.U.K., T.T.H., H.-L.S. and B.H.G.; Formal analysis, C.K.M., H.-L.S. and S.A.Z.A.; investigation, C.K.M.; Resources, S.A.Z.A., and K.Y.; Writing—original draft preparation, C.K.M.; Writing—review and editing, S.U.K., T.T.H. and B.H.G.; Funding acquisition, B.H.G., L.H.C, P.P., K.W.G., L.C.M. and S.Y.T. All authors have read and agreed to the published version of the manuscript.

Conflict of interest statement

The authors declare no conflict of interest.

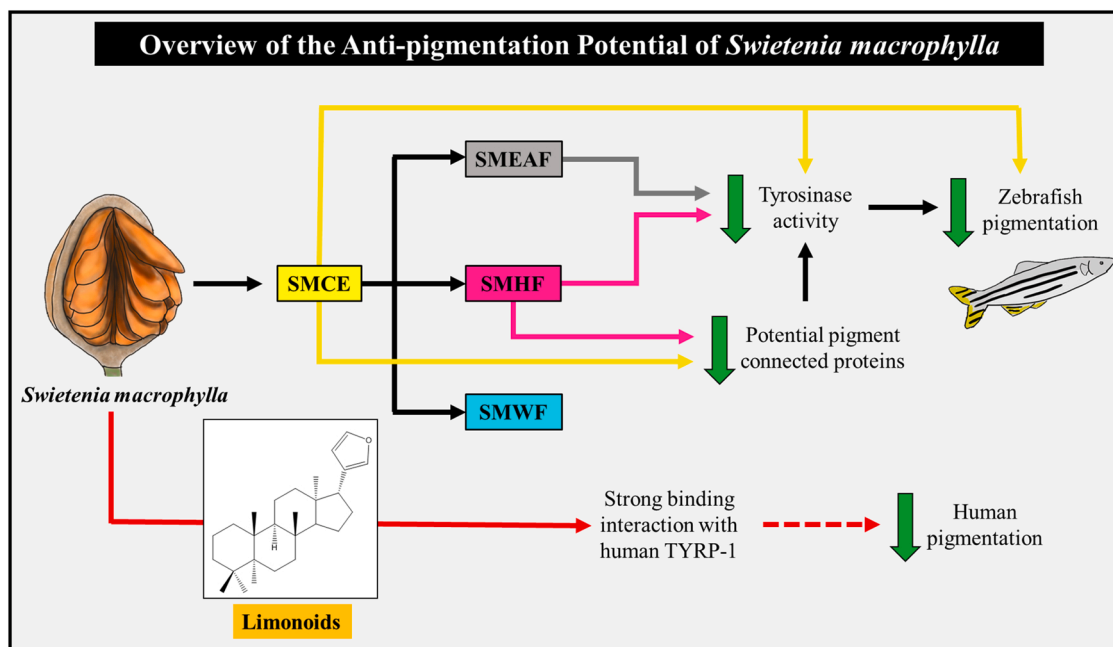


Fig. 12. An overview of the anti-pigmentation potential of *S. macrophylla*. The potential pathways mediated by *S. macrophylla* seeds in decreasing zebrafish tyrosinase activity and therefore reducing the melanin formed in the embryos. Pure limonoids from the seed too may potentially be a good inhibitor against human TYRP-1, decreasing human pigmentation. (Coloured lines indicate the influence of each extract, fraction, or compound against pigment formation. Yellow: SMCE; grey: SMEAF; pink: SMHF; blue: SMWF; red: limonoids. The dashed lines represent possible effect on skin pigmentation. The green arrows represent downregulation.) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Data availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.biopha.2023.114659](https://doi.org/10.1016/j.biopha.2023.114659).

References

- [1] A. Agalou, M. Thrapsianiotis, A. Angelis, A. Papakyriakou, A.-L. Skaltsounis, N. Aliagannis, D. Beis, Identification of novel melanin synthesis inhibitors from *Crataegus pycnoloba* using an *in vivo* zebrafish phenotypic assay, *Front. Pharmacol.* (2018) 9, <https://doi.org/10.3389/fphar.2018.00265>.
- [2] G.R. Aitken, J.R. Henderson, S.C. Chang, C.J. McNeil, M.A. Birch-Machin, Direct monitoring of UV-induced free radical generation in HaCaT keratinocytes, *Clin. Exp. Dermatol.* 32 (2007) 722–727, <https://doi.org/10.1111/j.1365-2230.2007.02474.x>.
- [3] A. Al-Samadi, K. Tuomai, A. Kivimäki, A. Salem, S. Al-Kubati, A. Hyytiäinen, M. Parikka, K. Mesimäki, T. Wilkman, A. Mäkitie, et al., PCR-based zebrafish model for personalised medicine in head and neck cancer, *J. Transl. Med.* 17 (2019) 235, <https://doi.org/10.1186/s12967-019-1985-1>.
- [4] D.C. Bennett, D. Huszar, P.J. Laipis, R. Jaenisch, L.J. Jackson, Phenotypic rescue of mutant brown melanocytes by a retrovirus carrying a wild-type tyrosinase-related protein gene, *Development* 110 (1990) 471–475, <https://doi.org/10.1242/dev.110.2.471>.
- [5] R.E. Boissy, C. Sakai, H. Zhao, T. Kobayashi, V.J. Hearing, Human tyrosinase related protein-1 (TRP-1) does not function as a DHICA oxidase activity in contrast to murine TRP-1, *Exp. Dermatol.* 7 (1998) 198–204, <https://doi.org/10.1111/j.1600-0625.1998.tb00324.x>.
- [6] R.E. Boissy, H. Zhao, W.S. Oetting, L.M. Austin, S.C. Wildenberg, Y.L. Boissy, Y. Zhao, R.A. Sturm, V.J. Hearing, R.A. King, et al., Mutation in and lack of expression of tyrosinase-related protein-1 (TRP-1) in melanocytes from an individual with brown oculocutaneous albinism: A new subtype of albinism classified as "OCA3", *Am. J. Hum. Genet.* 58 (1996) 1145–1156.
- [7] M. Brenner, V.J. Hearing, The protective role of melanin against UV damage in human skin, *Photochem. Photobiol.* 84 (2008) 539–549.
- [8] L. Buono, J. Corbacho, S. Naranjo, M. Almuendo-Castillo, T. Moreno-Marmol, B. de la Cerda, E. Sanabria-Reinos, R. Polvillo, F.-J. Díaz-Corrales, O. Bogdanovic, et al., Analysis of gene network bifurcation during optic cup morphogenesis in zebrafish, *Nat. Commun.* 12 (2021) 3866, <https://doi.org/10.1038/s41467-021-24169-7>.
- [9] R. Casadei, M.C. Pelleri, L. Vitale, F. Facchini, L. Lenzi, S. Canaider, P. Strippoli, F. Frabetti, Identification of housekeeping genes suitable for gene expression analysis in the zebrafish, *Gene Expr. Patterns* 11 (2011) 271–276, <https://doi.org/10.1016/j.gexp.2011.01.003>.
- [10] H.-R. Chang, D.-A. Tsao, S.-R. Wang, H.-S. Yu, Expression of nitric oxide synthases in keratinocytes after UVB irradiation, *Arch. Dermatol. Res.* 295 (2003) 293–296, <https://doi.org/10.1007/s00403-003-0433-4>.
- [11] J.-J. Chen, S.-S. Huang, C.-H. Liao, D.-C. Wei, P.-J. Sung, T.-C. Wang, M.-J. Cheng, A new phragmalin-type limonoid and anti-inflammatory constituents from the fruits of *Swietenia macrophylla*, *Food Chem.* 120 (2010) 379–384, <https://doi.org/10.1016/j.foodchem.2009.09.093>.
- [12] L.-C. Chen, H.-R. Liao, P.-Y. Chen, W.-L. Kuo, T.-H. Chang, P.-J. Sung, Z.-H. Wen, J.-J. Chen, Limonoids from the seeds of *Swietenia macrophylla* and their anti-inflammatory activities, *Molecules* 20 (2015) 18551–18564, <https://doi.org/10.3390/molecules201018551>.
- [13] Y.-B. Cheng, Y.-T. Chien, J.-C. Lee, C.-K. Tseng, H.-C. Wang, I.W. Lo, Y.-H. Wu, S.-Y. Wang, Y.-C. Wu, F.-R. Chang, Limonoids from the seeds of *Swietenia macrophylla* with inhibitory activity against dengue virus 2, *J. Nat. Prod.* 77 (2014) 2367–2374, <https://doi.org/10.1021/np5002829>.
- [14] J.H. Chung, Photoaging in Asians, *Photodermatol. Photoimmunol. Photomed.* 19 (2003) 109–121, <https://doi.org/10.1034/j.1600-0781.2003.00027.x>.
- [15] B. Desmedt, P. Courselle, J.O. De Beer, V. Rogiers, M. Grosber, E. Deconinck, K. De Paepe, Overview of skin whitening agents with an insight into the illegal cosmetic market in Europe, *J. Eur. Acad. Dermatol. Venereol.* 30 (2016) 943–950, <https://doi.org/10.1111/jdv.13595>.
- [16] I. Dini, S. Laneri, The new challenge of green cosmetics: natural food ingredients for cosmetic formulations, *Molecules* 26 (2021) 3921, <https://doi.org/10.3390/molecules26133921>.
- [17] M.B. Dolinska, P.T. Wingfield, K.L. Young, Y.V. Sergeev, The TYRPI-mediated protection of human tyrosinase activity does not involve stable interactions of tyrosinase domains, *Pigment Cell Melanoma Res* 32 (2019) 753–765, <https://doi.org/10.1111/pcmr.12791>.
- [18] A. Eid, N. Elmarzugi, H. El Enshasy, A review on the phytopharmacological effect of *Swietenia macrophylla*, *Int. J. Pharm. Pharm. Sci.* 5 (2013) 47–53.
- [19] S. Eilers, D.Q. Bach, R. Gaber, H. Blatt, Y. Guevara, K. Nitsche, R.V. Kundu, J. K. Robinson, Accuracy of self-report in assessing Fitzpatrick skin phototypes I through VI, *JAMA Dermatol.* 149 (2013) 1289–1294, <https://doi.org/10.1001/jamadermatol.2013.6101>.
- [20] M. Ezquerro, A. Lleó, M. Castellví, R. Queralt, P. Santacruz, J. Pastor, J. L. Molinero, R. Blesa, R. Oliva, A Novel Mutation in the PSEN2 Gene (T430M) Associated With Variable Expression in a Family With Early-Onset Alzheimer Disease, *Arch. Neurol.* 60 (2003) 1149–1151, <https://doi.org/10.1001/archneur.60.8.1149>.
- [21] K. Ezzedine, E. Mauger, J. Latreille, R. Jdid, D. Malvy, F. Gruber, P. Galan, S. Herberg, E. Tschachler, C. Guinot, Freckles and solar lentigines have different risk factors in Caucasian women, *J. Eur. Acad. Dermatol. Venereol.* 27 (2013) e345–e356, <https://doi.org/10.1111/j.1468-3083.2012.04685.x>.
- [22] B.H. Goh, H.A. Kadir, *In vitro* cytotoxic potential of *Swietenia macrophylla* King seeds against human carcinoma cell lines, *J. Med. Plant Res.* 5 (2011) 1395–1404.
- [23] B.H. Goh, H. Abdul Kadir, S.N. Abdul Malek, S.W. Ng, Swietenolide diacetate from the seeds of *Swietenia macrophylla*, *Acta Crystallogr. Sect. E Struct. Rep. Online* 66 (2010) o1396, <https://doi.org/10.1107/S1600536810017733>.
- [24] A. Graham, P. Manning, C.J. McNeil, D. Szatkowski, M. Tsatmali, J. Ancans, A. M. Graham, A.J. Thody, α -Melanocyte-stimulating hormone modulates nitric oxide production in melanocytes, *J. Invest. Dermatol.* 114 (2000) 520–526, <https://doi.org/10.1046/j.1523-1747.2000.00879.x>.
- [25] S.R. Gross, T.G. Kinzy, Translation elongation factor 1A is essential for regulation of the actin cytoskeleton and cell morphology, *Nat. Struct. Mol. Biol.* 12 (2005) 772–778, <https://doi.org/10.1038/nsmb979>.
- [26] L.-H. Gu, P.A. Coulombe, Keratin function in skin epithelia: a broadening palette with surprising shades, *Curr. Opin. Cell Biol.* 19 (2007) 13–23, <https://doi.org/10.1016/j.cceb.2006.12.007>.
- [27] Y. Hu, H. Zeng, J. Huang, L. Jiang, J. Chen, Q. Zeng, Traditional asian herbs in skin whitening: The current development and limitations, *Front. Pharmacol.* 11 (2020) 982, <https://doi.org/10.3389/fphar.2020.00982>.
- [28] R.C. Hunt, A.A. Davis, Altered expression of keratin and vimentin in human retinal pigment epithelial cells *in vivo* and *in vitro*, *J. Cell Physiol.* 145 (1990) 187–199, <https://doi.org/10.1002/jcp.1041450202>.
- [29] H. Jiang, M. Newman, M. Lardelli, The zebrafish orthologue of familial Alzheimer's disease gene PRESENILIN 2 is required for normal adult melanotic skin pigmentation, *PLoS One* 13 (2018), e0206155, <https://doi.org/10.1371/journal.pone.0206155>.
- [30] S.K. Katiyar, H. Mukhtar, Green tea polyphenol (–)–epigallocatechin-3-gallate treatment to mouse skin prevents UVB-induced infiltration of leukocytes, depletion of antigen-presenting cells, and oxidative stress, *J. Leukoc. Biol.* 69 (2001) 719–726, <https://doi.org/10.1189/jlb.69.5.719>.
- [31] S.U. Khan, N. Ahemad, L.-H. Chuah, R. Naidu, T.T. Htar, Illustrated step by step protocol to perform molecular docking: Human estrogen receptor complex with 4-hydroxytamoxifen as a case study, *Prog. Drug Disco Biomed. Sci.* 3 (2020) 1–21, <https://doi.org/10.36877/pdbs.a0000054>.
- [32] D.-C. Kim, S. Kim, K.-S. Hwang, C.-H. Kim, p-Coumaric acid potentially down-regulates zebrafish embryo pigmentation: comparison of *in vivo* assay and computational molecular modeling with phenylthiourea, *Biomed. Sci. Lett.* 23 (2017) 8–16, <https://doi.org/10.15616/BSL.2017.23.1.8>.
- [33] J.H. Kim, D.H. Kim, J.H. Kim, S.G. Lee, H.S. Kim, H.C. Park, I.-H. Kim, Recovery of pigmentation following selective photothermolysis in adult zebrafish skin: clinical implications for laser toning treatment of melasma, *J. Cosmet. Laser Ther.* 14 (2012) 277–285, <https://doi.org/10.3109/14764172.2012.738908>.
- [34] J.G.R. Kromberg, Chapter 2 - Clinical features, types of albinism, and natural history, in: J. Kromberg, P. Manga (Eds.), *Albinism in Africa*, Academic Press, 2018, pp. 27–55.
- [35] K. Kulkeaw, T. Ishitani, T. Kanemaru, O. Ivanovski, M. Nakagawa, C. Mizuochi, Y. Horio, D. Sugiyama, Cold exposure down-regulates zebrafish pigmentation, *Genes Cells* 16 (2011) 358–367, <https://doi.org/10.1111/j.1365-2443.2011.01498.x>.
- [36] S.-H. Kwon, J.-I. Na, J.-Y. Choi, K.-C. Park, Melasma: updates and perspectives, *Exp. Dermatol.* 28 (2019) 704–708, <https://doi.org/10.1111/exd.13844>.
- [37] X. Lai, H.J. Wichers, M. Soler-Lopez, B.W. Dijkstra, Structure of human tyrosinase related protein 1 reveals a binuclear zinc active site important for melanogenesis, *Angew. Chem. Int. Ed.* 56 (2017) 9812–9815, <https://doi.org/10.1002/anie.201704616>.
- [38] X. Lai, H.J. Wichers, M. Soler-Lopez, B.W. Dijkstra, Structure and function of human tyrosinase and tyrosinase-related proteins, *Chemistry* 24 (2018) 47–55, <https://doi.org/10.1002/chem.201704410>.
- [39] W.K. Lau, B.H. Goh, H.A. Kadir, A.C. Shu-Chien, T.S. Muhammad, Potent PPAR γ ligands from *Swietenia macrophylla* are capable of stimulating glucose uptake in muscle cells, *Molecules* 20 (2015) 22301–22314, <https://doi.org/10.3390/molecules201219847>.
- [40] P.L. Lee, H. Van Weelden, P.L.B. Bruijnzeel, Neutrophil infiltration in normal human skin after exposure to different ultraviolet radiation sources, *Photochem. Photobiol.* 84 (2008) 1528–1534, <https://doi.org/10.1111/j.1751-1097.2008.00380.x>.
- [41] A. Lleó, R. Blesa, J. Gendre, M. Castellví, P. Pastor, R. Queralt, R. Oliva, A novel presenilin 2 gene mutation (D439A) in a patient with early-onset Alzheimer's disease, *Neurology* 57 (2001) 1926, <https://doi.org/10.1212/WNL.57.10.1926>.
- [42] Y.-Q. Ma, K. Jiang, Y. Deng, L. Guo, Y.-Q. Wan, C.-H. Tan, Mexicanolide-type limonoids from the seeds of *Swietenia macrophylla*, *J. Asian Nat. Prod. Res.* 20 (2018) 299–305, <https://doi.org/10.1080/10286020.2017.1335715>.
- [43] C.K. Mahendra, K.W. Goh, L.C. Ming, G. Zengin, L.E. Low, H.-L. Ser, B.H. Goh, The prospects of *Swietenia macrophylla* King in skin care, *Antioxidants* 11 (2022) 913, <https://doi.org/10.3390/antiox11050913>.
- [44] C.K. Mahendra, S.A.Z. Abidin, T.T. Htar, L.H. Chuah, S.U. Khan, L.C. Ming, S. Y. Tang, P. Pusparajah, B.H. Goh, Counteracting the ramifications of UVB irradiation and photoaging with *Swietenia macrophylla* King seed, *Molecules* 26 (2021) 2000, <https://doi.org/10.3390/molecules26072000>.
- [45] C.K. Mahendra, L.T.H. Tan, P. Pusparajah, T.T. Htar, L.-H. Chuah, V.S. Lee, L. E. Low, S.Y. Tang, K.-G. Chan, B.H. Goh, Detrimental effects of UVB on retinal

- pigment epithelial cells and its role in age-related macular degeneration, *Oxid. Med. Cell Longev.* 2020 (2020) 1904178, <https://doi.org/10.1155/2020/1904178>.
- [46] C.K. Mahendra, L.T. Tan, C.K. Mahendra, H.-L. Ser, P. Pusparajah, T.T. Htar, L.-H. Chuah, W.H. Yap, S.Y. Tang, L.C. Ming, et al., The potential of sky fruit as an anti-aging and wound healing cosmetic agent, *Cosmetics* (2021) 8, <https://doi.org/10.3390/cosmetics8030079>.
- [47] C.K. Mahendra, H.-L. Ser, P. Pusparajah, T.T. Htar, L.-H. Chuah, W.H. Yap, Y.-Q. Tang, G. Zengin, S.Y. Tang, W.L. Lee, et al., Cosmeceutical therapy: Engaging the repercussions of UVR photoaging on the skin's circadian rhythm, *Int. J. Mol. Sci.* 23 (2022) 2884, <https://doi.org/10.3390/ijms23052884>.
- [48] K.-K. Mak, Z. Shiming, M.K. Balijepalli, A.T. Dinkova-Kostova, O. Epemolu, Z. Mohd, M.R. Pichika, Studies on the mechanism of anti-inflammatory action of swietenine, a tetranortriterpenoid isolated from *Swietenia macrophylla* seeds, *Phytomed* 1 (2021), 100018, <https://doi.org/10.1016/j.phyplu.2020.100018>.
- [49] P. Manga, K. Sato, L. Ye, F. Beermann, M.L. Lamoreux, S.J. Orlow, Mutational analysis of the modulation of tyrosinase by tyrosinase-related proteins 1 and 2 *in vitro*, *Pigment Cell Res* 13 (2000) 364–374, <https://doi.org/10.1034/j.1600-0749.2000.130510.x>.
- [50] K. Martinson, N. Stueven, A. Monte, C.-C. Huang, A novel stilbene-like compound that reduces melanin through inhibiting melanocyte differentiation and proliferation without inhibiting tyrosinase, *Cosmetics* 5 (2018) 45, <https://doi.org/10.3390/cosmetics5030045>.
- [51] K.A. McGowan, H. Fuchs, M.H. de Angelis, G.S. Barsh, Identification of a keratin 4 mutation in a chemically induced mouse mutant that models white sponge nevus, *J. Invest. Dermatol.* 127 (2007) 60–64, <https://doi.org/10.1038/sj.jid.5700498>.
- [52] M. Mihāsan, What *in silico* molecular docking can do for the 'bench-working biologists', *J. Biosci.* 37 (2012) 1089–1095, <https://doi.org/10.1007/s12038-012-9273-8>.
- [53] S.Z. Moghadamtousi, B.H. Goh, C.K. Chan, T. Shabab, H.A. Kadir, Biological activities and phytochemicals of *Swietenia macrophylla* King, *Molecules* 18 (2013) 10465–10483, <https://doi.org/10.3390/molecules180910465>.
- [54] B.S. Mootoo, A. Ali, R. Motilal, R. Pingal, A. Ramlal, A. Khan, W.F. Reynolds, S. McLean, Limonoids from *Swietenia macrophylla* and *S. aubrevilleana*, *J. Nat. Prod.* 62 (1999) 1514–1517, <https://doi.org/10.1021/np990199x>.
- [55] T. Moreno-Mármol, M. Ledesma-Terrón, N. Tabanera, M.J. Martín-Bermejo, M. J. Cardozo, F. Cavodeassi, P. Bovolenta, Stretching of the retinal pigment epithelium contributes to zebrafish optic cup morphogenesis, *eLife* 10 (2021), e63396, <https://doi.org/10.7554/eLife.63396>.
- [56] G.K. Nasrallah, R. Salem, S. Da'as, O.L.A. Al-Jamal, M. Scott, I. Mustafa, Biocompatibility and toxicity of novel iron chelator Starch-Deferoxamine (S-DFO) compared to zinc oxide nanoparticles to zebrafish embryo: An oxidative stress based apoptosis, physicochemical and neurological study profile, *Neurotoxicol. Teratol.* 72 (2019) 29–38, <https://doi.org/10.1016/j.ntt.2019.01.004>.
- [57] M. Newman, E. Ebrahimie, M. Lardelli, Using the zebrafish model for Alzheimer's disease research, *Front. Genet.* (2014) 5, <https://doi.org/10.3389/fgene.2014.00189>.
- [58] M. Newman, B. Tucker, S. Nornes, A. Ward, M. Lardelli, Altering presenilin gene activity in zebrafish embryos causes changes in expression of genes with potential involvement in Alzheimer's disease pathogenesis, *J. Alzheimer's Dis.: JAD* 16 (2009) 133–147, <https://doi.org/10.3233/jad-2009-0945>.
- [59] K. Nilugal, S. Fattepur, M.F. Asmani, I. Abdullah, S. Vijendren, R.E. Ugandar, Evaluation of wound healing activity of *Swietenia macrophylla* (Meliaceae) seed extract in albino rats, *Am. J. PharmTech Res.* 7 (2017) 113–124.
- [60] T.S.R. Nogueira, Md.S. Passos, S.D. Calixto, T.L.B. Ventura, E. Lassounskaia, M. F. Muzitano, R. Braz-Filho, I.J.C. Vieira, Anti-mycobacterium tuberculosis activity of compounds from *Cedrela fissilis* Vell. seeds (Meliaceae), *Rev. Virtual Quim.* 13 (2021) 1116–1121, <https://doi.org/10.21577/1984-6835.20210056>.
- [61] S. Nornes, M. Newman, S. Wells, G. Verdile, R.N. Martins, M. Lardelli, Independent and cooperative action of Psen2 with Psen1 in zebrafish embryos, *Exp. Cell Res.* 315 (2009) 2791–2801, <https://doi.org/10.1016/j.yexcr.2009.06.023>.
- [62] D.J. Page, R. Thuret, L. Venkatraman, T. Takahashi, K. Bentley, S.P. Herbert, Positive feedback defines the timing, magnitude, and robustness of angiogenesis, *e3135, Cell Rep.* 27 (2019) 3139–3151, <https://doi.org/10.1016/j.celrep.2019.05.052>.
- [63] D.S. Pei, Y.H. Sun, S.P. Chen, Y.P. Wang, W. Hu, Z.Y. Zhu, Zebrafish GAPDH can be used as a reference gene for expression analysis in cross-subfamily cloned embryos, *Anal. Biochem.* 363 (2007) 291–293, <https://doi.org/10.1016/j.ab.2006.12.005>.
- [64] T. Pillaiyar, M. Manickam, V. Namasivayam, Skin whitening agents: Medicinal chemistry perspective of tyrosinase inhibitors, *J. Enzym. Inhib. Med. Chem.* 32 (2017) 403–425, <https://doi.org/10.1080/14756366.2016.1256882>.
- [65] T. Pillaiyar, M. Manickam, S.-H. Jung, Recent development of signaling pathways inhibitors of melanogenesis, *Cell Signal* 40 (2017) 99–115, <https://doi.org/10.1016/j.cellsig.2017.09.004>.
- [66] M. Sasaki, T. Horikoshi, H. Uchiwa, Y. Miyachi, Up-regulation of tyrosinase gene by nitric oxide in human melanocytes, *Pigment Cell Res* 13 (2000) 248–252, <https://doi.org/10.1034/j.1600-0749.2000.130406.x>.
- [67] J. Shen, R.T. Bronson, D.F. Chen, W. Xia, D.J. Selkoe, S. Tonegawa, Skeletal and CNS Defects in Presenilin-1-Deficient Mice, *Cell* 89 (1997) 629–639, [https://doi.org/10.1016/S0092-8674\(00\)80244-5](https://doi.org/10.1016/S0092-8674(00)80244-5).
- [68] A. Slominski, Neuroendocrine activity of the melanocyte, *Exp. Dermatol.* 18 (2009) 760–763, <https://doi.org/10.1111/j.1600-0625.2009.00892.x>.
- [69] F. Solano, Photoprotection versus photodamage: Updating an old but still unsolved controversy about melanin, *Polym. Int.* 65 (2016) 1276–1287, <https://doi.org/10.1002/pi.5117>.
- [70] F. Solano, Photoprotection and skin pigmentation: Melanin-related molecules and some other new agents obtained from natural sources, *Molecules* (2020) 25, <https://doi.org/10.3390/molecules25071537>.
- [71] S.F. Sousa, P.A. Fernandes, M.J. Ramos, Protein–ligand docking: Current status and future challenges, *Protein.: Struct. Funct. Genet.* 65 (2006) 15–26, <https://doi.org/10.1002/prot.21082>.
- [72] Y.-P. Sun, W.-F. Jin, Y.-Y. Wang, G. Wang, S.L. Morris-Natschke, J.-S. Liu, G.-K. Wang, K.-H. Lee, Chemical structures and biological activities of limonoids from the genus *Swietenia* (Meliaceae), *Molecules* 23 (2018) 1588, <https://doi.org/10.3390/molecules23071588>.
- [73] Q.-G. Tan, X.-D. Luo, Meliaceous limonoids: Chemistry and biological activities, *Chem. Rev.* 111 (2011) 7437–7522, <https://doi.org/10.1021/cr9004023>.
- [74] I.-S. Tarau, A. Berlin, C.A. Curcio, T. Ach, The cytoskeleton of the retinal pigment epithelium: From normal aging to age-related macular degeneration, *Int. J. Mol. Sci.* 20 (2019) 3578, <https://doi.org/10.3390/ijms20143578>.
- [75] H.Y. Thong, S.H. Jee, C.C. Sun, R.E. Boissy, The patterns of melanosome distribution in keratinocytes of human skin as one determining factor of skin colour, *Br. J. Dermatol.* 149 (2003) 498–505, <https://doi.org/10.1046/j.1365-2133.2003.05473.x>.
- [76] K. Toyofuku, I. Wada, J.C. Valencia, T. Kushimoto, V.J. Ferrans, V.J. Hearing, Oculocutaneous albinism types 1 and 3 are ER retention diseases: mutation of tyrosinase or Tyrp1 can affect the processing of both mutant and wild-type proteins, *FASEB J.* 15 (2001) 2149–2161, <https://doi.org/10.1096/fj.01-0216com>.
- [77] S. Walsh, L. Chaitanya, K. Breslin, C. Muralidharan, A. Bronikowska, E. Pospiech, J. Koller, L. Kovatsi, A. Wollstein, W. Branicki, et al., Global skin colour prediction from DNA, *Hum. Genet.* 136 (2017) 847–863, <https://doi.org/10.1007/s00439-017-1808-5>.
- [78] Z. Wang, Y. Li, C. Ai, Y. Wang, *In silico* prediction of estrogen receptor subtype binding affinity and selectivity using statistical methods and molecular docking with 2-arylnaphthalenes and 2-arylquinolines, *Int. J. Mol. Sci.* 11 (2010) 3434–3458, <https://doi.org/10.3390/ijms11093434>.
- [79] S.F. Wu, C.K. Lin, Y.S. Chuang, F.R. Chang, C.K. Tseng, Y.C. Wu, J.C. Lee, Anti-hepatitis C virus activity of 3-hydroxy caruilignan C from *Swietenia macrophylla* stems, *J. Viral Hepat.* 19 (2012) 364–370, <https://doi.org/10.1111/j.1365-2893.2011.01558.x>.
- [80] J.-Y. Yeo, E.-S. Lee, C.-J. Jeon, Parvalbumin-immunoreactive neurons in the inner nuclear layer of zebrafish retina, *Exp. Eye Res.* 88 (2009) 553–560, <https://doi.org/10.1016/j.yexcr.2008.11.014>.