



RESEARCH ARTICLE

Mechanisms of Selected Cassipourea Metabolites for Melasma Treatment: Network Pharmacology and Molecular Dynamics Study

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Abstract

Background

Melasma is a common dyschromia, mainly found in women with darker skin types. Although asymptomatic, melasma significantly impacts patients' quality of life. Due to this complex pathogenesis, melasma is difficult to treat. Plant and plant-derived products have been explored as alternatives for the treatment of melasma.

Methods

This study utilized network pharmacology coupled with molecular docking and molecular dynamics simulations to investigate the molecular mechanisms of three selected *Cassipourea* metabolites in the treatment of melasma.

Results

Of the 202 genes obtained from the 14 profiled metabolites, only PTGS2, TYR, ESR2, and ESR1 were common among metabolites and targets implicated in melasma. From this, The gene ontology highlighted the intracellular steroid hormone receptor, signalling

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1. **Jared Jagdeo**, State University of New York, Downstate Health Sciences University, Brooklyn, USA

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pathway, macromolecular complex, and estrogen receptor activity as the top enriched functional annotations, while the KEGG pathway analysis identified five signalling pathways, from which the prolactin signalling pathway, endocrine resistance, and estrogen signalling pathway were implicated in the pathogenesis of melasma. These pathways were further connected by their linkage to ESR2 and ESR1. Of all *Cassipourea* metabolites and standards, with afzelechin having the highest docking score for both gens. Further binding interaction analysis showed that ESR2-bound tamoxifen had the highest binding free energy of -47.68 kcal/mol, however, among the interacting *Cassipourea* metabolites, sitosterol-glycoside exhibited the highest negative binding affinity for both ESR2 (-40.50 kcal/mol) and ESR1 (-78.97 kcal/mol) over 150 ns simulation, suggesting its potential as a dual modulator. Altogether, the metabolites presented remarkable binding stability and thermodynamic compactness with the apo-genes.

Conclusion

The finding that the selected *Cassipourea* metabolites are associated with the genes and enzymes implicated in melasma pathogenesis, together with their significant binding effects on the enriched genes, suggests their regulatory potential on the profiled targets and, consequently, in the treatment of melasma.

Keywords

melasma, *Cassipourea* species, Network pharmacology, Molecular dynamics simulation, Signalling pathways

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1. Introduction

The skin is the largest organ in the human body. It forms a significant anatomical barrier between internal and external environments.¹ The body is continuously exposed to various physical and chemical exogenous polluting substances.² Ultraviolet radiation (UVR) from excessive sun exposure is the primary exogenous factor that harms the skin. This process has various harmful effects on the skin as it alters the composition of the skin, causing elastic fiber accumulation, collagen reduction, and degeneration leading to wrinkles, sagging, and glycosaminoglycan deposition, resulting in premature aging, known as photoaging.³ Moreover, overexposure to ultraviolet (UV) rays stimulates melanin synthesis owing to the rapid proliferation of melanocytes. Furthermore; to stimulated melanin synthesis, excessive exposure to sunlight, especially UVA and UVB, causes overexpression of reactive oxygen species (ROS) that harm lipids, proteins, and deoxyribonucleic acids.⁴

Melanin is produced in the epidermis of the skin via a pathway known as melanogenesis, with tyrosinase playing a key role as the rate-limiting enzyme.⁵ The enzyme catalyzes three steps in melanin biosynthesis: hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA), oxidation of DOPA to DOPA quinone, and conversion of 5,6-dihydroxyindole to indolequinone.^{3,6} Thus, tyrosinase is the primary target for determining skin-lightening agents for cosmetic applications or skin lightening.⁵

While melanin shields the skin from UV radiation, excessive production can result in dermatological hyperpigmentation of the skin in clinical conditions such as dark spots, freckles, melasma, solar lentigo, linea nigra, and post-inflammatory hyperpigmentation (PIH), which can affect the appearance of the skin.⁷ Melasma is a common dermatological condition characterized by hyperpigmentation (light brown or dark brown), flaky or reticular patches, and macules that appear on the facial skin, and much less often on the neck and forearms. This condition primarily affects adult females, especially those with darker skin phototypes (Fitzpatrick skin phototypes III-VI).^{8,9} Although common to females, men are also affected; the incidence of melasma is estimated at 1% worldwide; however, it varies between 8.8-50% of at-risk populations.^{10,11} The pathogenesis of melasma is complex, as it is linked to melanocytes, keratin-forming cells, endothelial cells, fibroblasts, and alterations in the basement membrane.^{12,13} Visible light and UV exposure, hormonal changes, and genetic predisposition have all been linked to the onset of melasma.¹²⁻¹⁴ Melasma can also be caused by various factors such as the use of photosensitizing medication, thyroid diseases, ovarian tumors, hepatopathies, parasitic infestation, certain foods, and stress.⁹ Thyroid dysfunction, menstrual cycle irregularities, and insulin resistance which may also be caused by hormonal imbalance, have been linked to the diagnosis of melasma.^{9,14}

Although not life-threatening, being a facial disorder, melasma is disfiguring, and as such, impacts patients' lives and psychological well-being, potentially leading to anxiety, depression, and other disorders.¹²⁻¹⁵ Owing to this complex pathogenesis, melasma is difficult to treat. Currently, there is no cure or standardized treatment for melasma. Hydroquinone is a popular skin-whitening agent that inhibits tyrosinase. However, it is not suitable for all skin types, and prolonged and unsupervised use can cause undesirable side effects such as dermatitis, edema, allergic reactions, and ochronosis.^{15,16} Common treatments include sun protection, topical creams, such as niacinamide, vitamins A and C, oral medications, chemical peels, laser and light therapy, tranexamic acid, and microneedling. However, these treatments are prone to chemical irritation, inflammatory reactions, and hyperpigmentation. Other disadvantages include the possibility of recurrence and unpredictable efficacy.¹²⁻¹⁵

Patients often prefer the use of complementary and alternative medicines to supplement or replace traditional treatments, licorice extract, arbutin, and kojic acid are among the many tyrosinase inhibitors used in skin-lightening treatments.^{6,15} Additionally, tyrosinase inhibitors from phyto-molecules are equally valuable for scavenging ROS, which cause skin damage from excessive sun exposure.⁵ Since ancient times, medicinal plants have been used as therapeutic agents, dating back to 4000-5000 BC.¹⁷ The biological activities of plants are unique to specific species or groups, which supports the idea that a plant's secondary metabolites are taxonomically distinct.¹⁸ Screening of such secondary metabolites as active compounds from plants has led to the invention of a drug discovery process in pharmaceutical science for isolating new natural drugs with efficient protection and treatment roles against various diseases.¹⁸

Studies have explored natural products that inhibit UV-induced ROS, suppress enzymes, and reduce melanin formation as potential alternatives to current treatments have been conducted. This strategic shift is due to the adverse effects of synthetic agents.¹⁹ Phytocompounds such as aloesin, arbutin, licorice, hesperidin, gentisic acid, flavonoids, niacinamide, polyphenols, and yeast derivatives have demonstrated great potential to inhibit melanogenesis without harming melanocytes.^{4,18} Topical botanicals are becoming increasingly popular for skin care because of their perceived safety, low side effects, formulation stability, efficacy, cost-effectiveness, and quick metabolism when applied to the skin compared with conventional treatments.^{12,17,20} According to ethnobotanical literature, topical application is the most commonly used mode of application because it guarantees direct and immediate interaction of specific botanical compounds with the site of action.²¹⁻²³

Recently, three *Cassipourea* species (*Cassipourea flanaganii*, *Cassipourea malosana*, and *Cassipourea gummiflua* Tul. *Verticillata*) have been reported to be used for skin lightening by women in rural areas in the Eastern Cape and Kwa Zulu-Natal provinces of South Africa.²⁰ Although common names of the species are often used interchangeably by the rural community, the phytochemical comparison showed that each species is distinct, but they share skin-lightening characteristics. All three plants have been demonstrated to be effective and safe for use as topical skin lighteners, with no side effects.^{17,22–24}

In this context, the biological activities of the three *Cassipourea* species were systematically analyzed for their potential molecular mechanisms in the treatment of melasma using network pharmacology. Network pharmacology is an emerging science that examines the “compound-protein/gene-disease” system, thus, it is an effective measure for describing the intricacy of biological systems, drugs, and diseases from a network-based context.^{19,25} However, to gain further insight into the binding interaction and stability of the metabolites and hub targets, molecular docking and dynamics simulations were conducted.

2. Methods

2.1 Acquisition of *Cassipourea* metabolites

Active compounds from the three *Cassipourea* species screened through Chromatography-Mass Spectrometry (LC-MS/MS) analysis in the negative mode were used to generate a library of compounds for *Cassipourea*. The LCMS/MS analysis detected twenty-four compounds from various chemical classes, including fatty acids, steroids, di- and triterpenoids, flavonoids, and phenolic acids (<https://doi.org/10.6084/m9.figshare.26418361.v1>) (accessed on 01 August 2024). Eighteen compounds were tentatively identified.²⁰ The compounds were identified based on their structure and molecular mass, which shared similarities with known substances (<https://doi.org/10.6084/m9.figshare.26418361.v1>) (accessed on 01 August 2024). This was validated further with previous reports, characteristic fragmentation patterns, and data from a large bank and the SciFinder database.

2.2 Drug-likeness and pharmacokinetic screening of *Cassipourea* metabolites

The metabolites of the *Cassipourea* species were evaluated using Lipinski's rule of five (Ro5) for drug-likeness properties of the metabolites.²⁶ The SwissADME server (<http://www.swissadme.ch/>; accessed April 01, 2024) was used to predict the absorption, distribution, metabolism, and excretion properties of metabolites.²⁷

2.3 Acquisition of target genes related to *Cassipourea* metabolites and melasma

The metabolite target genes were mined from two independent databases. Genes related to *Cassipourea* metabolites, whose SMILES were available on PubChem (Afzelechin, azelaic acid, cassipourol, chlorogenic acid, chrysin 8-C glucoside, decahydroretinol, emodin 6,8 dimethyl ether, hexose, isorhamnetin-3-O-rhamnoside, lupeol, lyoniside, methyl linoleate, sitosterol-glycoside and triclin) were identified from the Swiss Target Prediction (STP) database (<http://www.swisstargetprediction.ch/>) (accessed April 01, 2024) using the Simplified Molecular Input Line Entry System (SMILES) retrieved from PubChem (<https://pubchem.ncbi.nlm.nih.gov/>) (accessed April 01, 2024). Genes related to melasma were acquired from the Online Mendelian Inheritance in Man (OMIM) database (<https://www.omim.org/>) (accessed on April 01, 2024) and the GeneCards database (<https://www.genecards.org/>) (accessed April 01, 2024). The Venny 2.1.0 (<https://bioinfogp.cnb.csic.es/tools/venny/>) tool was used to identify and characterize intersecting target genes²⁸ between *Cassipourea* metabolites and melasma.²⁹

2.4 Generation of protein-protein interaction (PPI) network

The Search Tool for the Retrieval of Interacting Genes (STRING) database (<https://string-db.org/>) (accessed April 01, 2024) was used to construct a PPI network.³⁰ The parameters for the analysis were set to *Homo sapiens* with a confidence level of < 0.4, followed by the input of common target genes between metabolites and melasma. The PPI network was then classified using Cytoscape v3.8.2.²⁸ A degree algorithm was used to identify key genes in the network (Equation 1).³¹

$$\text{Deg}(v) = |N(v)| \quad (1)$$

where $N(v)$ = a node neighbor, and v = each node's neighbors.

2.5 Analysis of the gene ontology (GO) and Kyoto Encyclopedia of Genes and Genome (KEGG) enrichment parameters

To illustrate the roles of the identified common targets in biological processes (BP), cellular components (CC), and molecular function (MF), gene ontology was conducted using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) tool (<https://david.ncifcrf.gov/tools.jsp>),³² with search parameters fixed to *Homo sapiens*. The GO and KEGG enrichment pathway analysis plots were generated using SRPlot (<http://www.bioinformatics.com.cn/en>), and

from the KEGG pathway enrichment, the most significant pathway was selected based on the lowest false discovery rate (FDR).^{33,34}

2.6 Construction of the pathway compound target network (PCT)

The PCT comprising melasma-related signaling pathways, their interacting genes, and metabolites were constructed using the Cytomerge plugin in Cytoscape software v3.9.1.³⁵ Thereafter, network topology analysis was conducted with edges depicting node interactions and their degrees of significance.³⁵

2.7 Molecular docking

Crystal X-ray structures of the target genes ESR2 [PDB: 2GIU] and ESR1 [PDB: 1QKU] were downloaded from the RCSB Protein Data Bank (PDB) (<https://www.rcsb.org/>) (accessed on April 01, 2024) and optimized by the elimination of all non-standard residues, co-crystallized ligands, and water molecules. Thereafter, ESR2 and ESR1 interacting metabolites and reference standards for melasma and the gene agonists tranexamic acid and tamoxifen, respectively, were developed. 3D conformers (ligands) were obtained from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>) (accessed April 01, 2024). Ligands were optimized using the Open Babel program plug-in on Python Prescription v 0.9.5 (PyRx)³⁶ through the addition of Gasteiger charges. Binding at the gene active site was confirmed by the identification and selection of ESR2 and ESR1 active site amino acid residues. Docking studies were conducted on PyRx, from which the grid box coordinates, in correlation with the x-y-z coordinates, were ascertained using the BIOVIA Discovery Studio v21.1.0.³⁷ Following molecular docking, the top five ligands with the highest negative docking score relative to their reference standards were visualized using Discovery Studio for their docking interactions before the simulation process. To avoid pseudo-positive binding conformations, the docking protocol was validated by measuring the root mean square deviation (RMSD) of docked ligands from the reference pocket containing native ligands (estradiol) in the experimental co-crystal. Where an RMSD of 0.5 Å was obtained for both docking validations (Figure 1).³⁸

2.8 Molecular Dynamic (MD) simulations

MD simulations were conducted at 150 ns using the graphical processing unit (GPU) version of the AMBER 18 package (force field with FF18SB variant) of the Center for High Performance Computing (CHPC), Cape Town, South Africa.³⁹ Ligand atomic partial charges were harnessed through ANTECHAMBER using a general amber force field (GAFF) and restrained electrostatic potential (RESP). The systems were neutralized using hydrogen atoms and Na⁺ and Cl⁻ counter ions from the Leap module. The amino acid residues of ESR2 and ESR1 were subsequently numbered and encased in an orthorhombic box of TIP3P water molecules, such that all atoms were at most 8 Å away from any box edge. The SHAKE algorithm was used to constrain hydrogen atom bonds within simulated systems, with each simulation comprising a 2 fs step-size, concurrent with the isobaric-isothermal ensemble (NPT) randomized seeding, a Langevin thermostat with a collision frequency of 1.0 ps, 1 bar, 2 ps pressure-coupling constant, and 300 K temperature. Thereafter, along with the binding free

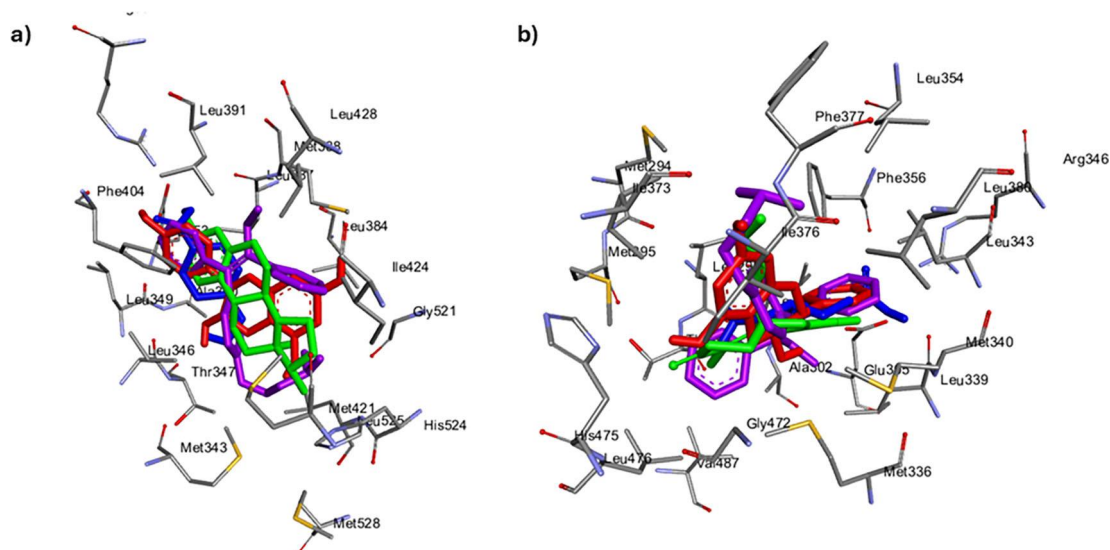


Figure 1. (a) Superimposition of ESR1 native ligand estradiol (green), gene modulator: tamoxifen (blue), reference standard: tranexamic acid (purple) and top performing plant metabolite afzelechin (red), with an RMSD of 0.5 Å. (b) Superimposition of ESR2 native ligand estradiol (green), gene modulator: tamoxifen (blue), reference standard: tranexamic acid (purple) and top performing plant metabolite afzelechin (red), with an RMSD of 0.5 Å.

energy, the post-dynamic parameters, namely the root mean square deviation (RMSD), root mean square fluctuation (RMSF), radius of gyration (RoG), and solvent-accessible surface area of each protein-ligand system were assessed.⁴⁰

3. Results

3.1 *Cassipourea* metabolites library

A total number of 18 common metabolites were identified from the previously characterized *Cassipourea* species. They are comprised of different chemical classes, including fatty acids, steroids, di- and tri-terpenoids, flavonoids, and phenolic acids (Table 1).

3.2 Drug-likeness, Pharmacokinetic screening of *Cassipourea* metabolites

Of the 14 common metabolites of *Cassipourea* species whose SMILES were available in PubChem, only lyoniside had three violations of Lipinski's Ro5 (molecular weight ≤ 500 g/mol, hydrogen bond donor and acceptor ≤ 5 and 10, respectively, bioavailability score ≤ 0.55 , and lipophilicity (MLOGP) ≤ 4.5), as it had a molecular weight of 552.57 g/mol, hydrogen bond acceptor and donor of 12 and 6, respectively; other metabolites had ≤ 2 violations (Table 2). Pharmacokinetic analysis revealed that, except for sitosterol-glycosides, the metabolites were soluble in water to varying degrees. The metabolites also possess relatively high gastrointestinal absorption, are mostly non-substrate for glycoproteins, and are impermeable to the blood-brain barrier (BBB). However, azelaic acid, decahydroretinol, and emodin 6,8 dimethyl showed BBB permeability (Table 2). The metabolites demonstrated significant non-inhibition of the cytochrome P (cytochrome P450) isoenzymes (Table 2).

3.3 Acquisition of target genes related to *Cassipourea* metabolites and melasma

In total, 644 and 545 *Cassipourea* metabolite genes were collected from the STP and SEA databases, respectively. A total of 202 genes were common in both databases. Of the 34 melasma genes acquired from the GeneCards database and 24282 genes acquired from OMIM, only 31 genes were common to both. A further probe of the gene interactions identified four genes directly linked to *Cassipourea* and melasma (Figure 2).

3.4 Generation of protein-protein interaction (PPI) network

The PPI network constructed from common genes from melasma and *Cassipourea* metabolites comprised 4 nodes and 12 edges, an average node degree of 2, and an enrichment p -value of 0.0107 (Figure 3). The identified estrogen receptors 1 and 2 (ESR1 and 2), prostaglandin-endoperoxide synthase 2 (PTGS2), and tyrosinase (TYR) genes interact with each other.

Table 1. Common metabolites identified from the three investigated *Cassipourea* species.

Metabolites	Molecular formula	Metabolite class
2 α ,3 α -Epoxyflavan-5,7,4'-triol-(4 β $\rightarrow$ 8)-afzelechin	C ₃₀ H ₂₄ O ₁₀	Flavonoids
Afzelechin	C ₁₅ H ₁₄ O ₅	Flavonoids
Azelaic acid	C ₉ H ₁₆ O ₄	Fatty acids
Cassipourol	C ₂₀ H ₃₈ O	Triterpenoids
Chlorogenic acid	C ₁₆ H ₁₈ O ₉	Phenolics
Chrysin 8-C-glucoside	C ₂₇ H ₄₄ O ₃	Flavonoids
Decahydroretinol	C ₂₀ H ₄₀ O	Tocopherol
Ellisinin A	C ₁₈ H ₁₈ O ₂	Sesquiterpenes
Emodin 6,8-dimethyl ether	C ₁₇ H ₁₄ O ₅	Anthraquinone derivative
Ent-atis-16-en-19-oic acid	C ₃₀ H ₂₄ O ₁₀	Di-terpenoids
Hexose (glucose)	C ₆ H ₁₂ O	Monosaccharide
Isorhamnetin-3-O-rhamnoside	C ₂₂ H ₂₂ O ₁₁	Flavonoids
Lupeol	C ₃₀ H ₅₀ O	Triterpenoids
Lupeol stearate	C ₄₈ H ₈₄ O ₂	Triterpene ester
Lyoniside	C ₂₇ H ₃₆ O ₁₂	Iridoid glycoside
Methyl linoleate	C ₁₉ H ₃₄ O ₂	Fatty acid methyl ester
Sitosterol glycoside	C ₃₅ H ₆₀ O ₆	Sterol glycoside derivate
Tricin	C ₁₇ H ₁₄ O ₇	Methylated flavonoids

Table 2. Pharmacokinetic and Lipinski's Ro5 screening of *Cassipourea* metabolites.

Melasma metabolites	MW (g/mol)	HBA	HBD	LOGP	BS	LV	Sol.	GIA	BBB-P	Pgp-S	CYP1A2	CYP2C19	CYP2C9	CYP2D6	CYP3A4
M1: Afzelechin	274.27	5	4	1.38	0.55	0	Soluble	High	No	Yes	No	No	No	No	No
M2: Azelaic acid	188.22	4	2	1.44	0.85	0	Very soluble	High	Yes	No	No	No	No	No	No
M3: Cassipourol	294.52	1	1	3.90	0.55	1	Mod. Soluble	High	No	No	No	No	Yes	Yes	No
M4: Chlorogenic acid	354.31	9	6	0.96	0.11	1	Very soluble	Low	No	No	No	No	No	No	No
M5: Chrysin 8-C-glucoside	416.38	9	6	2.18	0.55	1	Soluble	Low	No	No	No	No	No	No	No
M6: Decahydroretinol	286.45	1	1	4.05	0.55	1	Mod. soluble	High	Yes	No	Yes	No	Yes	No	No
M7: Emodin 6,8 dimethyl ether	298.29	5	1	2.66	0.55	0	Mod. soluble	High	Yes	No	Yes	Yes	Yes	No	Yes
M8: Hexose (glucose)	180.16	6	5	0.24	0.55	0	Highly soluble	Low	No	Yes	No	No	No	No	No
M9: Isorhamnetin-3-O-rhamnoside	462.40	11	6	2.26	0.17	2	Soluble	Low	No	Yes	No	No	No	No	Yes
M10: Lupeol	426.72	1	1	4.68	0.55	1	Poorly soluble	Low	No	No	No	No	No	No	No
M11: Lyoniside	552.57	12	6	4.07	0.17	3	Soluble	Low	No	Yes	No	No	No	No	No
M12: Methyl linoleate	294.47	2	0	4.61	0.55	1	Mod. soluble	High	No	No	Yes	No	Yes	No	No
M13: Sitosterol-glycoside	576.85	6	4	4.98	0.55	1	Poorly soluble	Low	No	No	No	No	No	No	No
M14: Tricin	330.29	7	3	2.58	0.55	0	Mod. soluble	High	No	No	Yes	No	Yes	Yes	Yes

MW: Molecular weight; HBA: Hydrogen bond acceptor; HBD: Hydrogen bond donor; LOGP: Hydrophobicity; BS: Bioavailability score; LV: Lipinski's violation Sol.: Solubility, Mod.: Moderately, GIA: Gastrointestinal absorption; BBB-P: Blood brain barrier permeant; Pgp-S: phosphoglycoprotein permeant; CYP: cytochrome P450 isoenzyme inhibition.

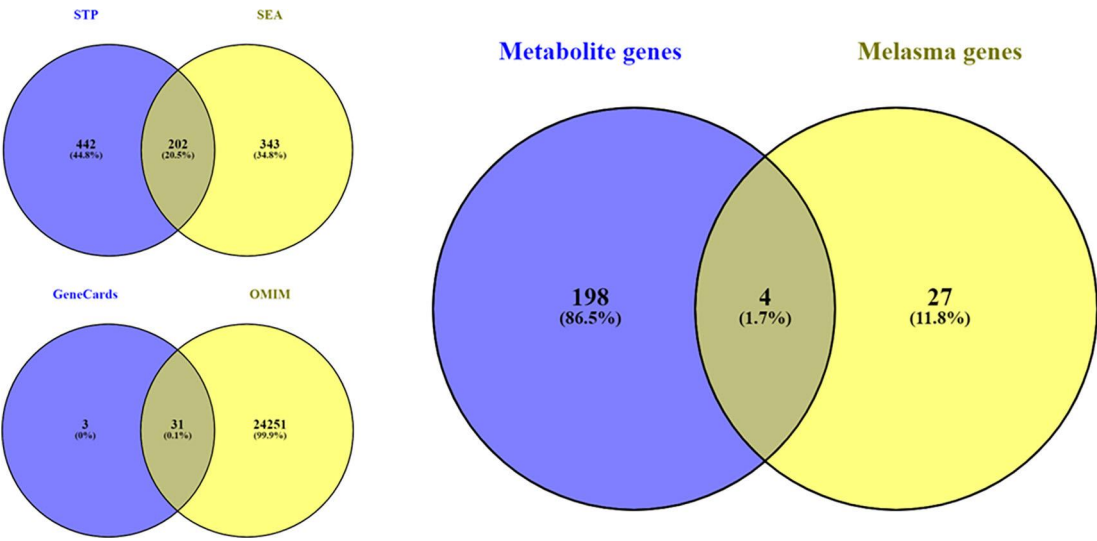


Figure 2. Common genes between *cassipourea* metabolites and melasma, extrapolated from the relevant databases.

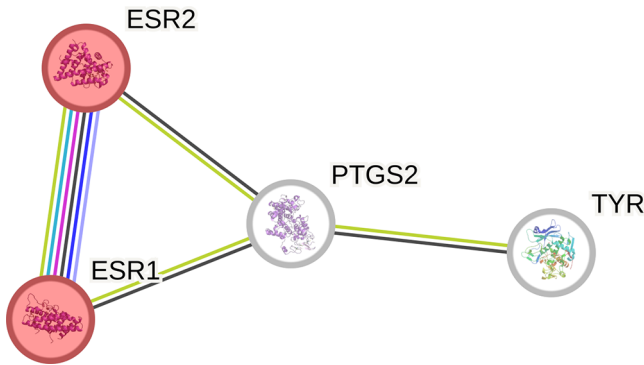


Figure 3. PPI network analysis of common genes between *Cassipourea* metabolites and melasma.

3.5 Analysis of the Gene Ontology (GO) and Kyoto Encyclopaedia of Genes and Genome (KEGG) enrichment parameters

Gene ontology analysis indicated the potential effect of *Cassipourea* metabolites on BP, CC, and MF relative to melasma. Amongst the BP, the intracellular steroid hormone receptor signalling pathway (2.0×10^{-3}) was identified as the most enriched, followed by response to vitamin D (2.9×10^{-3}) and cellular response to estrogen stimulus (3.4×10^{-3}). Of the CCs, only the macromolecular complex (9.8×10^{-2}) was marginally enriched. Estrogen receptor activity (6.3×10^{-4}) was both the most enriched MF within the GO, followed closely by enzyme binding (1.2×10^{-3}) and estrogen response element binding (1.7×10^{-3}) (Figure 4).

KEGG pathway enrichment analysis generated five pathways, three of which were related to melasma (Figure 5). All three pathways, namely the prolactin signaling pathway (0.0238), estrogen signaling pathway (0.0238), and endocrine resistance (0.0238), shared the same FDR. To account for this, pathway strength was used to screen for the most enriched pathways. The prolactin signaling pathway was subsequently identified as the most prominent pathway (Table 2).

3.6 Construction of the pathway compound target network

The resultant PCT comprised the prolactin signaling pathway, estrogen signaling pathway, and endocrine resistance (Figure 7) encompassing 13 nodes, 20 edges, and an average node degree of 3.07. From the top three pathways identified, the prolactin signalling pathway was highlighted as the most significant for its greater pathway strength (Figure 6). Network topology analysis identified seven metabolites that interact with the significant genes ESR1 and ESR2 (Figures 8 and 9).

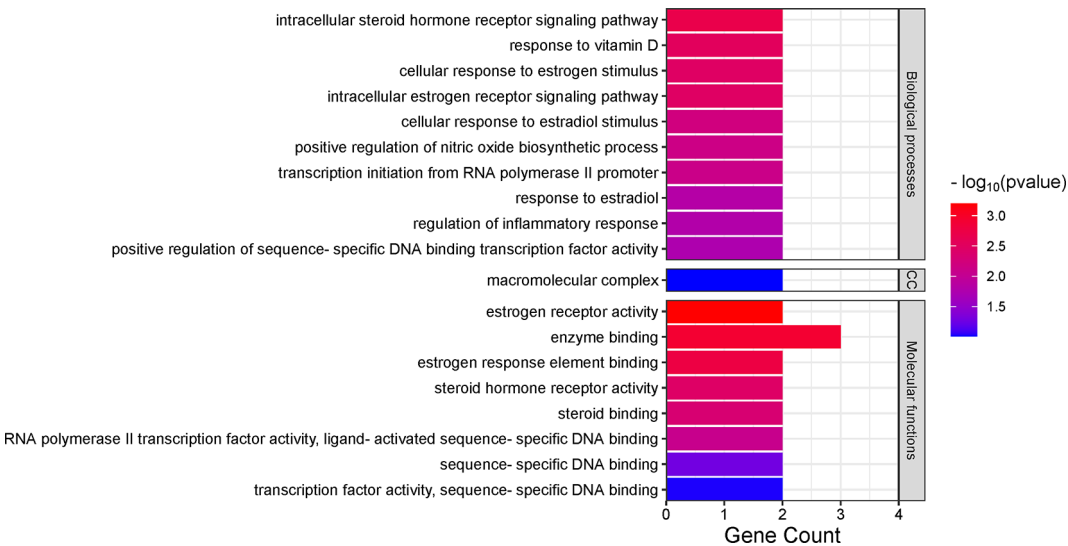


Figure 4. GO enrichment analysis of interacting *Cassipourea* metabolites and melasma genes.

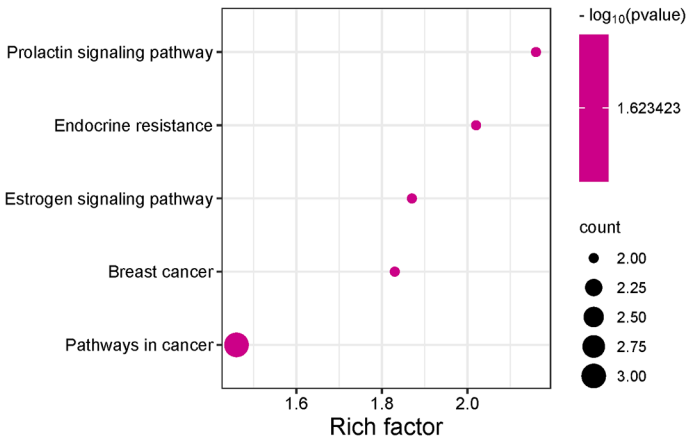


Figure 5. Enriched signalling pathways from the KEGG analysis of the common *Cassipourea* metabolites and melasma genes.

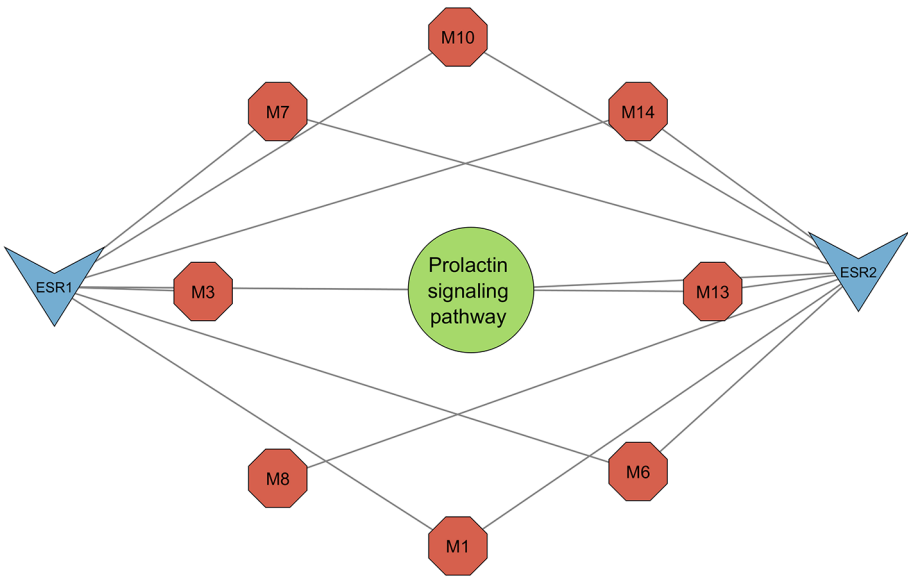


Figure 6. PCT network depicting the interactions of *Cassipourea* metabolites with the most enriched pathway.

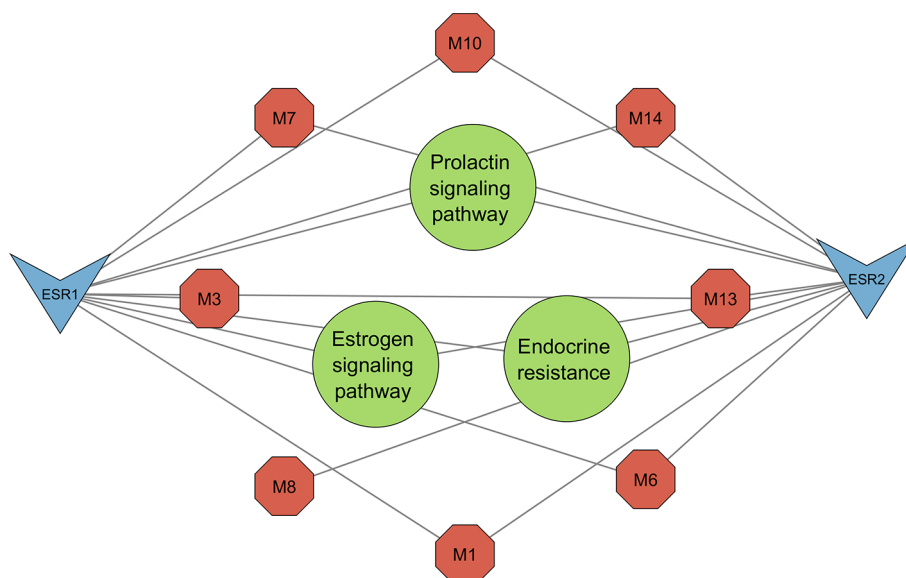


Figure 7. PCT network depicting the interactions of *Cassipourea* metabolites with the three most enriched signalling pathways.

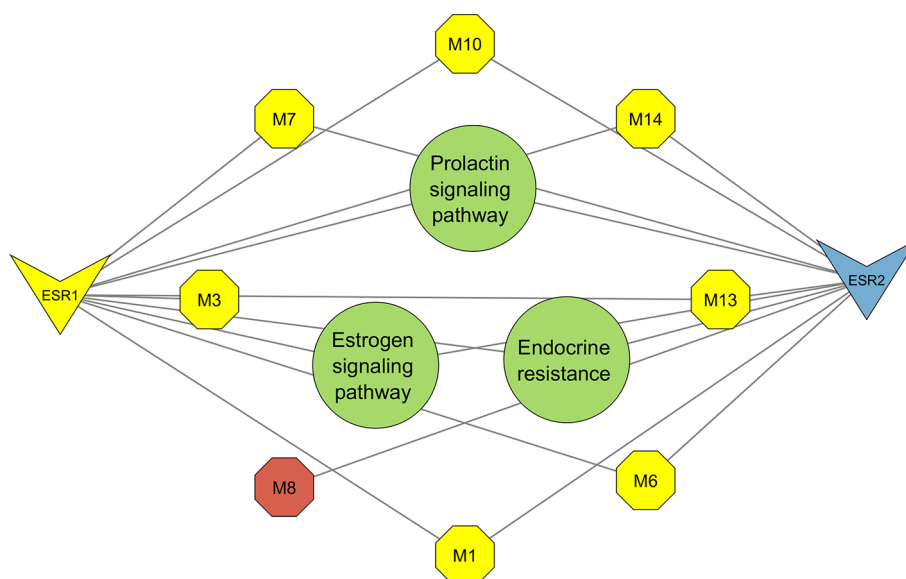


Figure 8. Interaction of ESR1 gene with 7 *Cassipourea* metabolites (yellow) and the most significant pathways (green) common to melasma and *Cassipourea* metabolites.

3.7 Molecular docking

From the ESR2 bound *Cassipourea* metabolites, hexose (-5.4 kcal/mol), lupeol (-5.4 kcal/mol) and sitosterol-glycoside (-4.9 kcal/mol) had lower negative docking scores than tamoxifen (-6.2 kcal/mol) and tranexamic acid (-5.5 kcal/mol) (Table 3). Except for decahydroretinol (-6.0 kcal/mol), all ESR1-bound metabolites exhibited higher negative docking scores than both tamoxifen (-6.1 kcal/mol) and tranexamic acid (-5.9 kcal/mol) (Table 4). Observably, when bound to both ESR2 and ESR1, afzelechin exhibited the highest negative docking scores of -7.3 kcal/mol and -8.8 kcal/mol, respectively. Regarding ESR2, afzelechin formed 27 intermolecular interactions comprising two hydrogen bonds (Ile373, Arg346), 12 van der Waals interactions (Met340, Ile376, Leu362, Met294, Phe377, Leu380, Leu354, Val487, Thr299, Trp335, Leu301, Arg346), and nine other important interactions, viz. hydrophobic interactions (Glu305, Leu343, Phe356, Ala302, Leu339, Leu476, Met336, Met295, Leu298). Twenty intermolecular interactions and bonds were observed in ESR1 bound afzelechin namely, two hydrogen bonds (Phe404, Met517), 13 Van der Waals

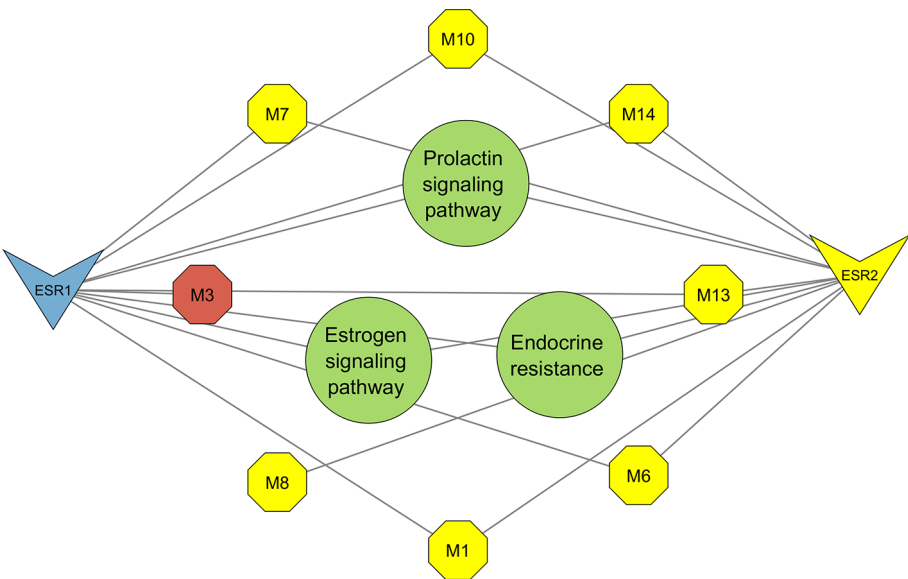


Figure 9. Interaction of ESR2 gene with 7 *Cassipourea* metabolites (yellow) and the most significant pathway (green) common to melasma and *Cassipourea* metabolites.

Table 3. Profiles of the 5 signalling pathways enriched in KEGG analysis of the common *Cassipourea* metabolites and melasma genes.

#term ID	Pathways	Strength	FDR	Target genes
hsa01522	Endocrine resistance	2.02	0.0238	ESR2, ESR1
hsa04915	Estrogen signalling pathway	1.87	0.0238	ESR2, ESR1
hsa04917	Prolactin signaling pathway	2.16	0.0238	ESR2, ESR1
hsa05200	Pathways in cancer	1.46	0.0238	ESR2, PTGS2, ESR1
hsa05224	Breast cancer	1.83	0.0238	ESR2, ESR1

interactions (Arg394, Glu353, Thr347, Leu346, Met343, His524, Leu525, Met421, Gly521, Ser518, Ile424, Leu428, Leu387), and five other important interactions (Met388, Leu384, Leu391, Ala350, Leu349) (Tables 4 and 5).

3.8 Molecular dynamic simulations of *Cassipourea* metabolites with the enriched genes

Regarding ESR2 bound systems, all *Cassipourea* metabolites displayed lower negative binding affinities (ranging from -18.71 kcal/mol to -40.50 kcal/mol) than tamoxifen (-47.68 kcal/mol) and higher negative binding affinities than tranexamic acid (17, 60 kcal/mol). From the top performing *Cassipourea* metabolites bound to ESR1, sitosterol-glycoside (-78.97 kcal/mol), decahydroretinol (-63,34 kcal/mol), lupeol (-61.23 kcal/mol), emodin 6,8 dimethyl ether (-56.45 kcal/mol), and tricin (-48.22 kcal/mol), had higher negative binding affinities than tamoxifen (-47.61 kcal/mol) and tranexamic acid (-16.68 kcal/mol), with emodin 6,8 dimethyl ether (-36.26 kcal/mol) exhibiting the lowest negative binding affinity (Table 6).

With the exception of ESR2-hexose and ESR1-tricin bound complexes at 90 ns and 120 ns, respectively, all trajectories of ESR2 and ESR1 bound and unbound systems were relatively stable and experienced minimal fluctuations (Figure 10a and 10b). Among ESR2-bound metabolites, tranexamic acid (1.44 Å) had the lowest RMSD value. Along with tranexamic acid, afzelechin (1.60 Å), emodin 6,8 dimethyl ether (1.58 Å) and sitosterol glycoside (1.59 Å) exhibited RMSD values comparable to or lower than apo-ESR2 (1.60 Å). Excluding hexose (1.98 Å), all ESR2-bound metabolites had lower RMSD values than tamoxifen (1.98 Å) (Table 7). In ESR1 bound systems, tamoxifen (1.51 Å) had the lowest RMSD value. Conversely, tranexamic acid (1.91 Å) and sitosterol-glycoside (1.91 Å) had the highest RMSD values among the ESR1-bound metabolites. Whereas tamoxifen (1.51 Å), afzelechin (1.61 Å), cassipourol (1.70 Å), decahydroretinol (1.55 Å), emodin 6,8 dimethyl ether (1.55 Å) and lupeol (1.78 Å) had similar or lower RMSD values than apo-ESR1 (1.78) (Table 7).

Table 4. Docking scores and structural interactions of the plant metabolites and their reference standards bound to ESR2.

Metabolite name	Docking scores (kcal/mol)	No. of interactions	No. of hydrogen bonds and interacting residues	No. of Van der Waal forces and interacting residues	Other important interactions and residues
Tranexamic acid	-5.5	10	2 (Arg346, Leu339)	5 (Met340, Glu305, Leu301, Met336, Met295)	3 (Phe356, Leu343, Leu298)
Tamoxifen	-6.2	21	1 (Ile373)	12 (Met340, Ile376, Leu362, Met294, Phe377, Leu380, Leu354, Val487, Thr299, Trp335, Leu301, Arg346)	9 (Glu305, Leu343, Phe356, Ala302, Leu339, Leu476, Met336, Met295, Leu298)
Afzelechin	-7.3	17	2 (Ile373, Arg346)	11 (Met295, Gly472, Met336, Ala302, Leu339, Leu301, Glu305, Met340, Leu298, Leu380, Phe377)	4 (His475, Ile376, Phe356, Leu343)
Tricin	-6.6	13	4 (Pro277, Glu305, Val338, Lys401)	6 (Pro278, Val280, Leu339, Met341, Gly342, Tyr397)	3 (His279, Arg346, Trp345)
Decahydroretinol	-6.4	15	-	11 (Trp345, Gly342, Lys401, Trp312, Met309, Leu339, Pro277, His308, Glu305, Pro278, Val280)	4 (Ala357, Pro358, His279, Arg346)
Emodin 6,8 dimethyl ether	-6.4	19	1 (Glu305)	9 (Arg346, Leu380, Met340, Gly472, Thr299, Val487, Ala302, Leu301, Leu339)	9 (Leu343, Phe356, Met336, His475, Ile376, Ile373, Leu476, Met295, Leu298)
Hexose (glucose)	-5.4	13	5 (Glu305, Arg346, Gly342, Lys402, Val338)	8 (Pro278, Val280, His308, Pro277, Met309, Trp312, Leu339, Tyr397)	-
Lupeol	-5.4	8	1 (Pro277)	7 (Glu276, Pro278, Arg346, His279, Pro358, Ile355, His350)	
Sitosterol-glycoside	-4.9	14	3 (Asn470, Ser333, Glu332)	10 (Arg466, Glu337, Cys334, Ser469, Met336, Trp335, Met473, Tyr488, Glu474, Asn478)	1 (Leu477)

Table 5. Docking scores and structural interactions of the plant metabolites and their reference standards bound to ESR1.

Metabolite name	Docking scores (kcal/mol)	No. of interactions	No. of hydrogen bonds and interaction residues	No. of Van der Waal forces and interactions residues	Other important interactions and residues
Tranexamic acid	-5.9	12	2 (Arg394, Glu353)	7 (Leu384, Leu391, Leu349, Leu346, Leu525, Leu540, Thr347)	3 (Phe404, Leu387, Ala350)
Tamoxifen	-6.1	18	-	8 (Gly521, Met421, Leu525, Met343, Thr347, Phe404, Glu353, Arg394)	10 (Ile424, Leu384, Met388, Leu391, Leu428, Leu349, Ala350, Leu387, Leu346, His524)
Afzelechin	-8.8	20	2 (Phe404, Met517)	13 (Arg394, Glu353, Thr347, Leu346, Met343, His524, Leu525, Met421, Gly521, Ser518, Ile424, Leu428, Leu387)	5 (Met388, Leu384, Leu391, Ala350, Leu349)
Cassipourol	-8.1	20	-	13 (Arg394, Glu353, Leu349, Thr347, Leu540, Leu346, Leu540, Gly521, Ile424, Leu428, Phe425, Met421, Met522, Met388)	7 (Leu387, Phe404, Ala350, Leu391, Leu384, Leu525, Trp383)
Sitosterol-glycoside	-7.7	28	-	21 (Pro324, Lys449, Glu323, Ile326, Gly390, Met388, Leu346, Gly521, Ile424, Leu384, Met522, Trp383, His524, Leu540, Met421, Phe425, Met343, Thr347, Leu349, Met357, Ile386)	7 (Leu525, Ala350, Arg394, Glu353, Leu387, Phe404, Leu391)
Emodin 6,8 dimethyl ether	-7.7	19	1 (Leu387)	5 (Met517, Gly521, Leu428, Met421, Gly353, Thr347)	12 (Met388, Ile424, Leu384, Met528, Met343, His524, Leu525, Leu346, Ala350, Phe404, Leu349, Leu391)
Lupeol	-6.9	10	1 (Glu397)	8 (Met396, Gly442, Leu320, Glu323, Ile326, Pro325, Pro324, Arg394)	1 (Trp393)
Tricin	-6.5	19	2 (Met517, Met388)	8 (Leu428, Leu384, Gly521, Met421, His524, Thr347, Glu353, Arg394)	9 (Phe404, Ala350, Leu391, Met343, Leu346, Leu525, Ile424, Leu387, Leu349)
Decahydroretinol	-6.0	11	1 (Met438)	8 (Asn439, Arg503, Leu495, Met490, Glu444, Gln441, Glu443, Lys492)	2 (Leu489, Ala493)

Table 6. Thermodynamic analysis of the *Cassipourea* metabolites bound to ESR2 and ESR1 systems over 150 ns.

Energy framework (kcal/mol)					
Complex	ΔE_{vdw}	ΔE_{elec}	ΔG_{gas}	ΔG_{solv}	ΔG_{bind}
ESR2					
Ref. STD: Tranexamic acid	-20.31±2.46	-146.65±9.22	-166.95±8.96	149.35±8.50	-17.60±2.58
GM: Tamoxifen	-50.70±3.05	-139.49±17.17	-190.19±16.79	142.51±15.29	-47.68±3.63
M1: Afzelechin	-41.15±2.65	-16.99±6.36	-58.15±5.81	21.58±3.29	-36.57±3.40
M6: Decahydroretinol	-35.04±3.36	-16.80±4.44	-56.40±4.99	21.20±2.96	-35.21±4.09
M7: Emodin 6,8 dimethyl ether	-46.45±2.38	-9.66±2.00	-56.12±3.04	16.23±1.62	-39.88±2.67
M8: Hexose	-17.64±4.87	-61.88±11.61	-79.52±8.85	50.82±7.96	-28.70±4.19
M10: Lupeol	-23.83±5.42	-4.27±6.14	-28.10±9.91	9.39±4.38	-18.71±6.53
M13: Sitosterol-glycoside	-34.53±4.07	-40.70±11.07	-75.62±10.67	35.12±7.55	-40.50±4.96
M14: Tricin	-36.85±4.41	-50.67±6.99	-87.52±8.29	50.57±5.23	-36.95±5.49
ESR1					
Ref. STD: Tranexamic acid	-22.01±2.32	-148.81±15.75	-170.82±15.63	154.14±13.56	-16.68±3.77
GM: Tamoxifen	-50.46±2.78	-152.53±13.11	-202.99±13.36	155.37±12.28	-47.61±3.11
M1: Afzelechin	-41.19±2.96	-33.01±4.32	-74.20±4.05	28.67±2.09	-45.52±2.99
M3: Cassipourol	-49.70±2.57	-6.75±2.52	-67.54±4.05	4.20±1.74	-63.34±3.89
M6: Decahydroretinol	-48.43±2.59	-13.27±4.41	-66.42±4.99	9.97±2.06	-56.45±4.42
M7: Emodin 6,8 dimethyl ether	-43.32±2.43	-5.96±2.88	-49.28±4.16	13.02±3.13	-36.26±2.43
M10: Lupeol	-58.12±2.66	-1.76±2.87	-59.88±3.43	-1.34±2.35	-61.23±3.02
M13: Sitosterol-glycoside	-75.58±4.11	-38.12±9.66	-114.16±8.96	35.19±9.12	-78.97±5.52
M14: Tricin	-47.40±3.38	-20.37±8.58	-67.77±6.97	19.55±5.40	-48.22±3.09

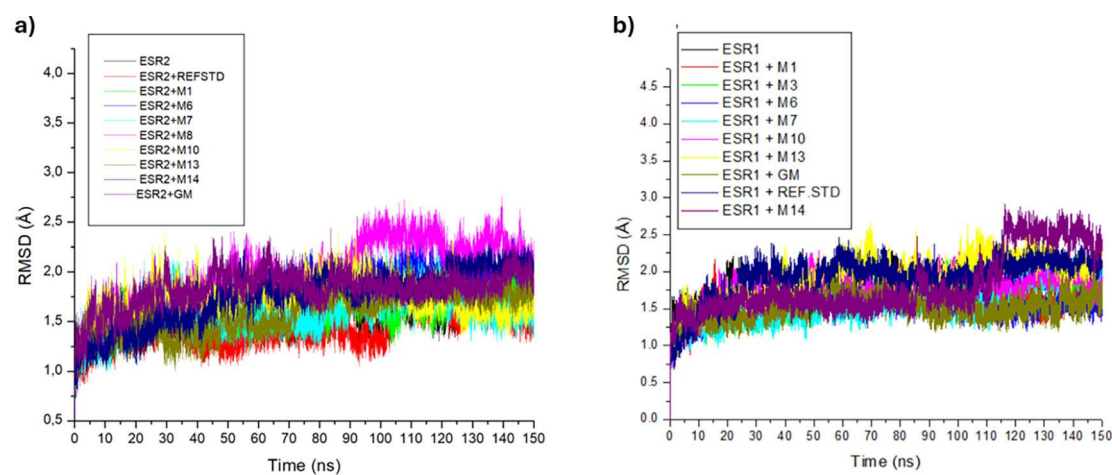


Figure 10. Comparative RMSD of a) ESR2 and b) ESR1 bound systems with *Cassipourea* metabolites over 150 ns.

Table 7. Post-dynamic analysis of the *Cassipourea* metabolites bound to ESR2 and ESR1 systems over 150 ns.

Dynamics				
Complex	RMSD (Å)	ROG (Å)	RMSF (Å)	SASA (Å)
ESR2				
Apo-ESR2	1.60±0.17	1.20±0.60	17.70±0.09	12411.51±304.13
Ref STD: Tranexamic acid	1.44±0.17	1.15±0.51	17.76±0.06	12291.71±258.62
GM: Tamoxifen	1.83±0.18	1.21±0.59	17.64±0.06	12227.35±290.42
M1: Afzelechin	1.60±0.17	1.20±0.59	17.70±0.07	12103.98±265.74
M6: Decahydroretinol	1.80±0.25	1.33±0.65	17.59±0.08	12216.00±291.91
M7: Emodin 6,8 dimethyl ether	1.58±0.19	1.17±0.59	17.62±0.07	121116.71±256.02
M8: Hexose	1.98±0.31	1.50±0.72	17.60±0.11	12123.38±321.02
M10: Lupeol	1.72±0.17	1.34±0.68	17.71±0.08	12475.06±292.96
M13: Sitosterol-glycoside	1.59±0.21	1.27±0.60	17.74±0.07	12397.75±347.87
M14: Tricin	1.75±0.27	1.34±0.66	17.63±0.07	11892.74±337.47
ESR1				
Apo-ESR1	1.78± 0.2	18.67 ±0.81	1.175± 0.55	13584.25 ± 234
Ref. STD: Tranexamic acid	1.91±0.32	18.81 ±0.62	1.19± 0.80	13706.67 ± 276
GM: Tamoxifen	1.51±0.23	18.71 ±0.53	1.07± 0.57	13588.03 ± 321
M1: Afzelechin	1.61± 0.16	18.54 ±0.75	1.05± 0.45	12995.41 ± 248
M3: Cassipourol	1.70±0.21	18.66 ±0.63	1.28± 0.53	13672.21 ± 312
M6: Decahydroretinol	1.55±0.19	18.62 ±0.42	1.11± 0.63	13132.37 ± 278
M7: Emodin 6,8 dimethyl ether	1.55±0.32	18.69 ±0.86	1.21± 0.46	13615.75 ± 265
M10: Lupeol	1.78±0.23	18.64 ±0.75	1.17± 0.74	13602.97 ± 213
M13: Sitosterol-glycoside	1.91±0.24	18.62 ±0.44	1.30± 0.66	13451.55 ± 314
M14: Tricin	1.81±0.21	18.52 ±0.60	1.18± 0.30	13287.54 ± 254

Trajectories of ESR2 bound systems experienced a multitude of high-rising fluctuations on amino acid residues 290, 215-218, and 402-412 and again from residues to 469-482 (Figure 11a). All ESR2 bound and unbound systems had comparable RMSF values, with hexose (1.50 Å) and tranexamic acid (1.15 Å) exhibiting the highest and lowest RMSF values, respectively. Against apo-ESR2 (1.20 Å), tranexamic acid, afzelechin (1.20 Å), and emodin 6,8 dimethyl ether (1.17 Å) had lower RMSF values (Table 7). In the ESR1 bound and unbound systems, fluctuations in residues

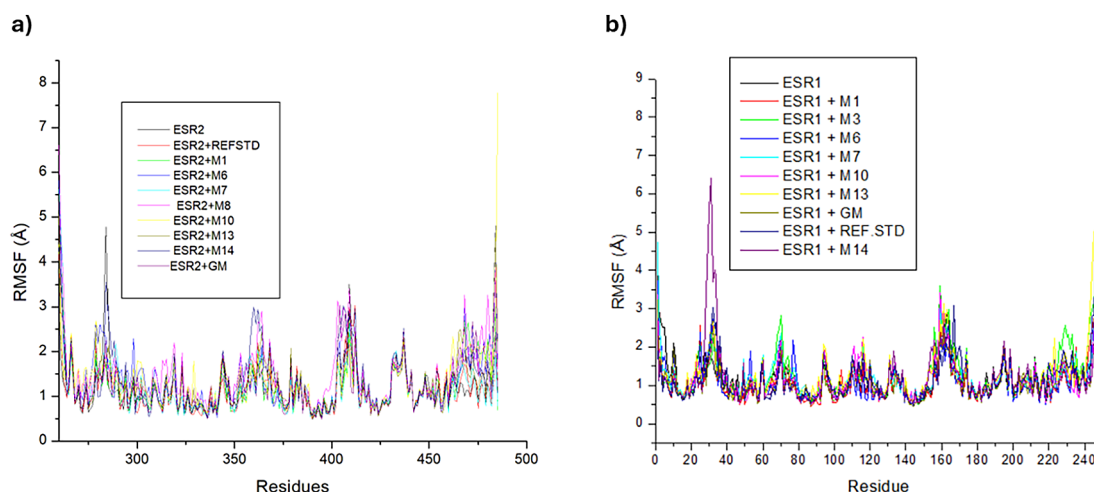


Figure 11. Comparative RMSF of a) ESR2 and b) ESR1 bound systems with *Cassipourea* metabolites over 150 ns.

30, 70, 230, and 247, and from residues 155-165 were prominent (Figure 11b). Besides sitosterol-glycoside (1.30 Å), cassipourol (1.28 Å), and emodin 6,8 dimethyl ether (1.21 Å), all other ESR1 bound metabolites had comparable or lower RMSF values than apo-ESR1 (1.18 Å) and tranexamic acid (1.19 Å). From the ESR1-bound metabolites, only afzelechin (1.05 Å) had a lower RMSF value than apo-ESR1, tranexamic acid, and tamoxifen (1.07 Å) (Table 7).

In the ESR2 and ESR1 bound and unbound systems, no complex fluctuations were observed (Figure 12a and 12b). The ESR2 bound systems exhibited non-significant differences in their RoG values, with decahydroretinol (17.59 Å), emodin 6,8 dimethyl ether (17.62 Å) and hexose (17.60 Å) displaying lower RoG values than tranexamic acid (17.76 Å), apo-ESR2 (17.70) and tamoxifen (17.64 Å) (Table 7). There was a marginal variance in ESR1 bound the RoG values. Excluding emodin 6,8 dimethyl ether (18.69 Å), all ESR1 bound metabolites complexes had lower RoG values than apo-ESR1 (18.67 Å), tranexamic acid (18.81 Å) and tamoxifen (18.71 Å), with triclin (18.52 Å) displaying the lowest RoG value (Table 7).

Excluding ESR2 bound lupeol and ESR1 bound triclin, the trajectories of ESR2 and ESR1 bound and unbound systems experienced minor disruptions (Figure 13a and 13b). Except for sitosterol-glycoside (12397.75 Å) and lupeol (12475.06 Å), ESR2-bound metabolites exhibit lower mean SASA values than tranexamic acid (12291.71 Å), tamoxifen (12227.35 Å) and apo-ESR2 (12411.51 Å), with triclin (11892.74 Å) exerting the most impact on the SASA (Table 7). In ESR1 bound and unbound systems, tranexamic acid (13706.67 Å) had the highest SASA value. ESR1 bound cassipourol

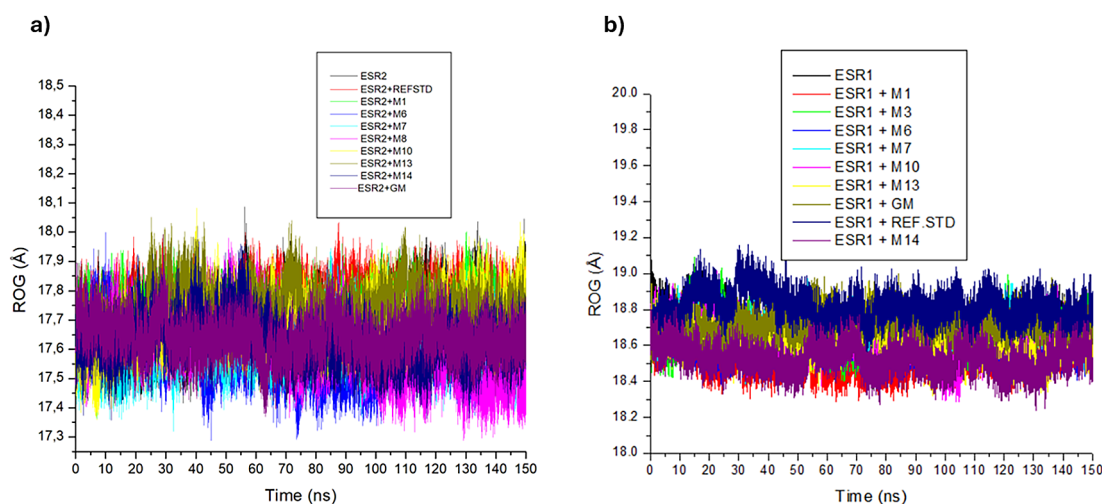


Figure 12. Comparative RoG of a) ESR2 and b) ESR1 bound systems with *Cassipourea* metabolites over 150 ns.

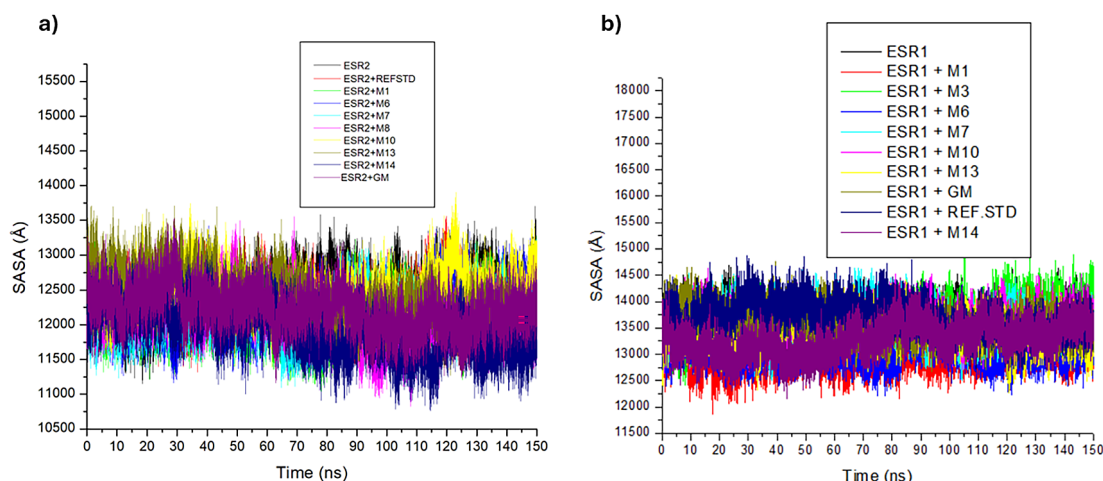


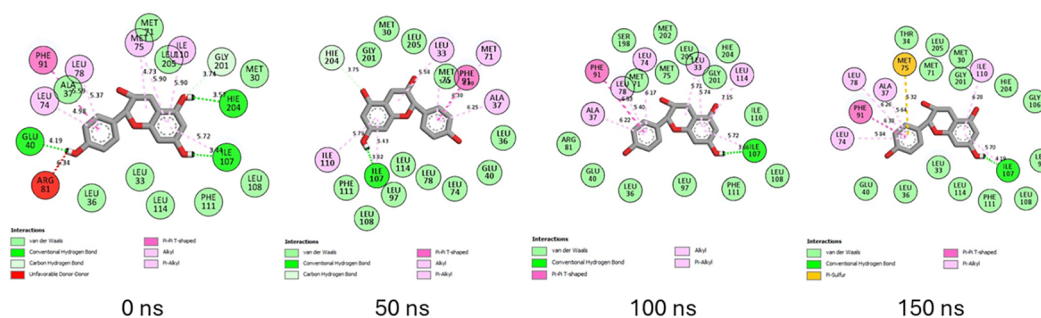
Figure 13. Comparative SASA plots of a) ESR2 and b) ESR1 bound systems with *Cassipourea* metabolites over 150 ns.

(13672.21 Å), Emodin 6,8 dimethyl ether (13615.75 Å) and lupeol (13602.97 Å) exhibited higher SASA values than tamoxifen (13558.03 Å) and apo-ESR1 (13584.25 Å), with afzelechin (12995.41 Å) exhibiting the lowest SASA value (Table 7).

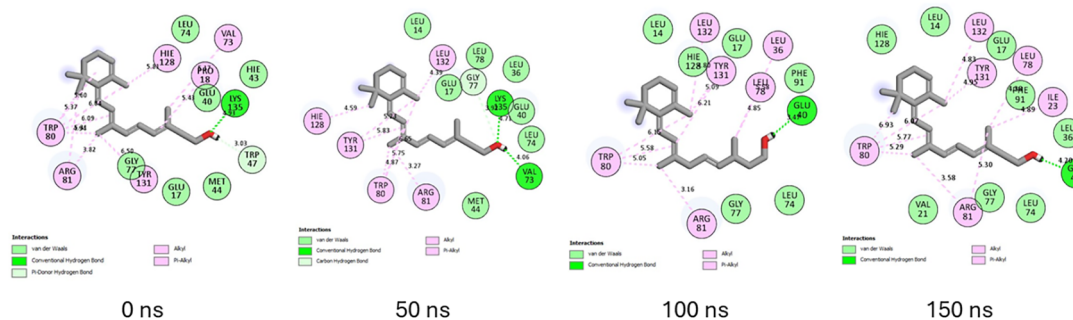
The 2D interaction plots of ESR2 bound systems displayed the formation of hydrogen bonds (carbon-hydrogen, pi-hydrogen donor, and conventional), hydrophobic interactions (pi-pi-T-shaped, alkyl, pi-alkyl, pi-sulfur, pi-cation, amide-pi-stacked, attractive charge, salt bridge, and unfavorable donor-donor bonds), and van der Waals interactions (Figure 14). From 0 ns to 150 ns, afzelechin is unable to maintain hydrogen bond formation (4 → 1) and forgoes its unfavorable donor-donor bond to form a π -sulfur bond, thus maintaining six important interactions and bonds (Figure 14a). Decahydroretinol retains six hydrophobic interactions at 0 ns and 150 ns but is unable to maintain its hydrogen bonds (2 → 1) (Figure 14b). Emodin 6,8 dimethyl ether formed nine hydrophobic interactions and three hydrogen bonds at 0 ns, which were reduced to seven hydrophobic interactions and two hydrogen bonds at 150 ns (Figure 14c). However, at 0 and 150 ns, hexose formed four and seven hydrogen bonds, respectively, and experienced a decrease in the total intramolecular interactions from 15 to 13 (Figure 14d). At 150 ns, lupeol was unable to maintain hydrogen bond formation on Arg81 and retained only four hydrophobic interactions (Figure 14e). Sitosterol-glycoside retained four hydrogen bonds at 0 ns and 150 ns and formed a hydrophobic alkyl interaction on Met292 at 150 ns (Figure 14f). Regarding triclin, at 0 ns and 150, the number of hydrogen bonds decreased from five to four, and the number of hydrophobic interactions increased from three to four (Figure 14g). Based on its interactions at 0 ns, tranexamic acid was unable to retain its hydrogen bond (one), hydrophobic interactions, and intramolecular interactions (12) at 150 ns (Figure 14h). Although tamoxifen was able to maintain its hydrophobic interactions (seven) at 0 and 150 ns, it did not retain its hydrogen bond formation (one) or intramolecular interactions (22) (Figure 14i).

A probe into the binding interactions of ESR1 and its metabolites revealed the presence of hydrogen bonds, van der Waals interactions, and hydrophobic interactions such as pi-sulfur, pi-alkyl, alkyl, pi-pi-T-shaped, amide-pi-stacked, pi-sigma, and unfavorable donor-donor interactions (Figure 15). At 0 and 150 ns, afzelechin retained its five hydrophobic interactions and formed four hydrogen bonds at 150 ns (Figure 15a). From 0 to 150 ns, cassipourol forms nine hydrophobic interactions and two hydrogen bonds (Figure 15b). Decahydroretinol maintains one hydrogen bond and, in total, forms 11 hydrophobic interactions from 0 to 150 ns (Figure 15c). Against emodin 6,8 dimethyl ether's intramolecular interactions at 0 ns, hydrogen bond formation increased to two, and hydrophobic and total intramolecular interactions decreased to nine and 18, respectively (Figure 15d). Lupeol exhibited hydrogen bond formation on Phe101 and an increase in hydrophobic (10) and intramolecular interactions (23) (Figure 15e). Sitosterol-glycoside forms three hydrogen bonds and replaces the three unfavorable bumps with eight hydrophobic alkyl interactions (Figure 15f). At 0 ns and 150 ns, triclin forms two and five hydrogen bonds, respectively, and forgoes the unfavorable acceptor bond at 0 ns for eight hydrophobic interactions (Figure 15g). In tranexamic acid, from 0 ns to 150 ns, hydrogen bond formation increased to three, intramolecular interactions were altered to 18, and hydrophobic interactions decreased to two (Figure 15h). Similarly, in tamoxifen, hydrogen bonds and intramolecular interactions are increased to two and 22, respectively, and hydrophobic interactions are reduced to six (Figure 15i).

a) ESR2 + Afzelechin



b) ESR2 + Decahydroretinol



c) ESR2 + Emodin 6,8 dimethyl ether

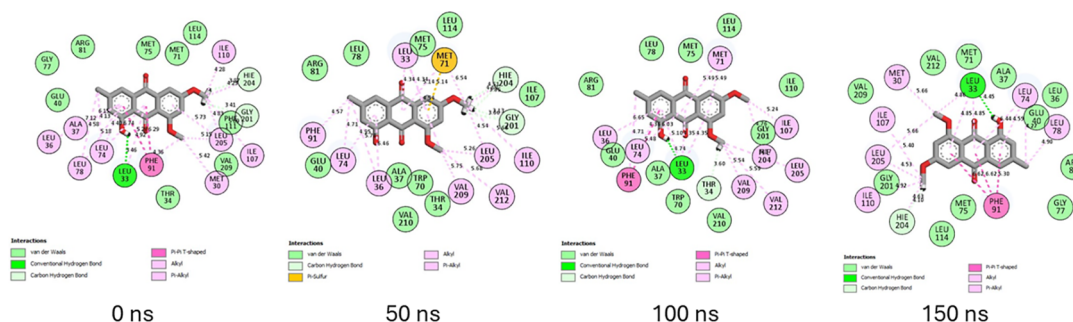


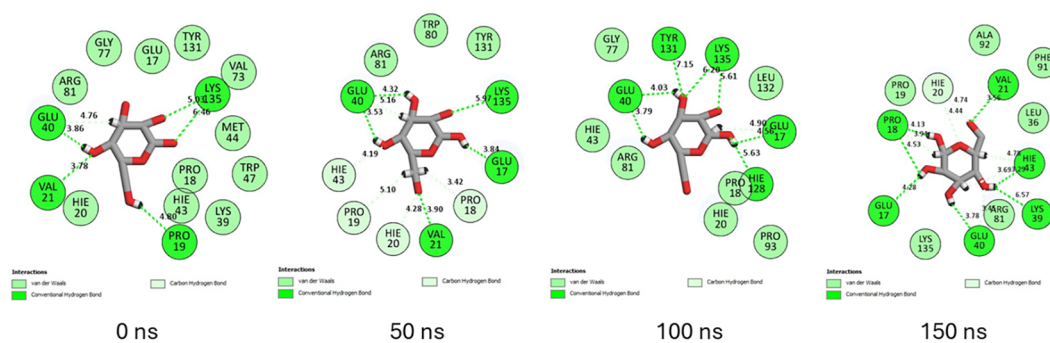
Figure 14. a: 2D interaction plot of ESR2 and afzelechin over 150 ns simulation. b: 2D interaction plot of ESR2 and decahydroretinol over 150 ns simulation. c: 2D interaction plot of ESR2 and emodin 6,8 dimethyl ether over 150 ns simulation. d: 2D interaction plot of ESR2 and hexose over 150 ns simulation. e: 2D interaction plot of ESR2 and lupeol over 150 ns simulation. f: 2D interaction plot of ESR2 and sitosterol glycoside over 150 ns simulation. g: 2D interaction plot of ESR2 and tricin over 150 ns simulation. h: 2D interaction plot of ESR2 and tranexamic acid over 150 ns simulation. i: 2D interaction plot of ESR2 and tamoxifen over 150 ns simulation.

4. Discussion

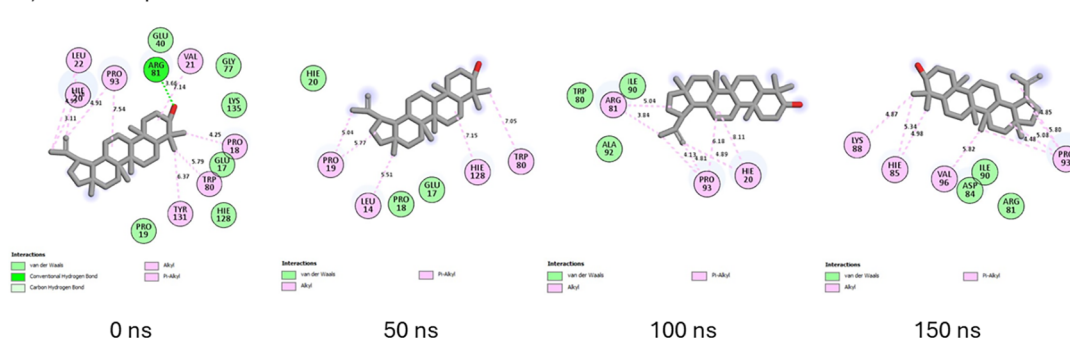
Several studies have established the benefits of natural products, including plants, for various biological purposes.⁴¹ Metabolites identified in the *Cassipourea* species are reportedly implicated in skin pigmentation, treatment of skin diseases, and inhibition of tyrosinase activity.^{20,24} Specifically, azelaic acid, a dicarboxylic acid found in many grains, has been reported to possess anti-tyrosinase and anti-inflammatory effects,⁴² whereas lupeol and its derivatives are implicated in wound healing.⁴³ These studies corroborate the therapeutic potential and identify putative leads of *Cassipourea* metabolites in melasma.

Interestingly, *Cassipourea* metabolites demonstrated remarkable drug-likeness and pharmacokinetic properties, indicating their use not only as potential topical agents but also as oral drug candidates for melasma treatment. This is evident by the finding that most metabolites are in conformity with Lipinski's Ro5 for oral drug candidates and show appreciable bioavailability for therapeutic applications. In addition, the metabolites were relatively non-inhibitors of CYP450

d) ESR2 + Hexose



e) ESR2 + Lupeol



f) ESR2 + Sitosterol glycoside

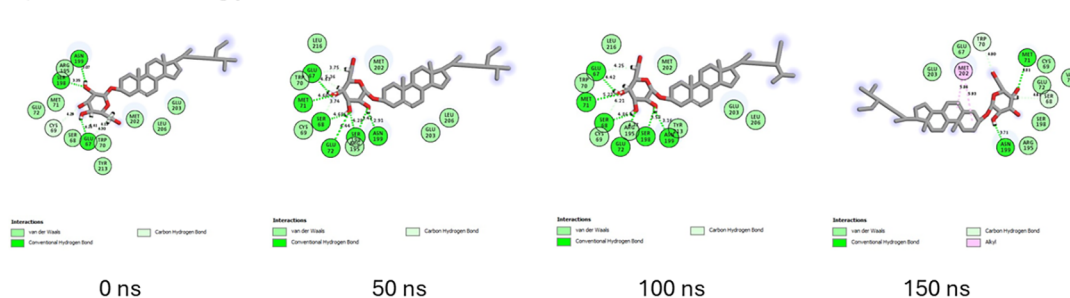


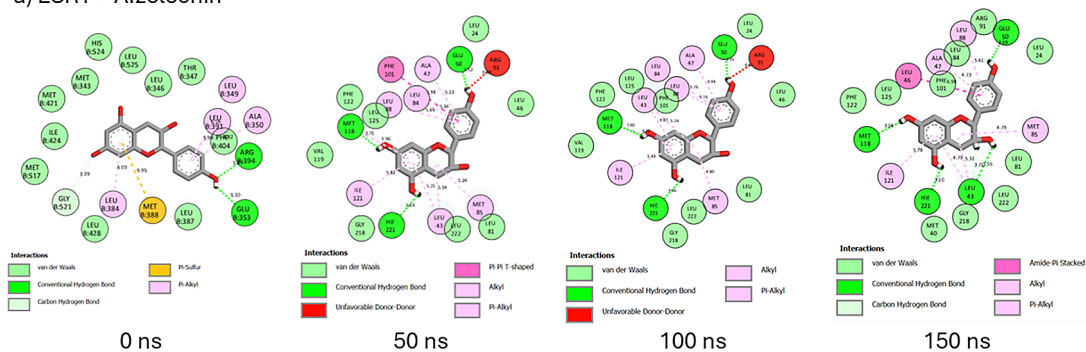
Figure 14. (continued)

enzymes; hence, they can be easily biotransformed into more polar and excretable by-products in biological systems. The GIA and Pgp-S profiles also potentiated *Cassipourea* for both applications. For example, sitosterol-glycoside and lupeol are hydrophobic compounds, and their low GIA and poor solubilities are suggestive of their biological effect being more applicable topically with potentially longer-lasting and better effects on the skin due to their hydrophobicity. To gain further insight into the mechanism of action of *Cassipourea* metabolites in melasma treatment, NP and MD simulations were conducted.

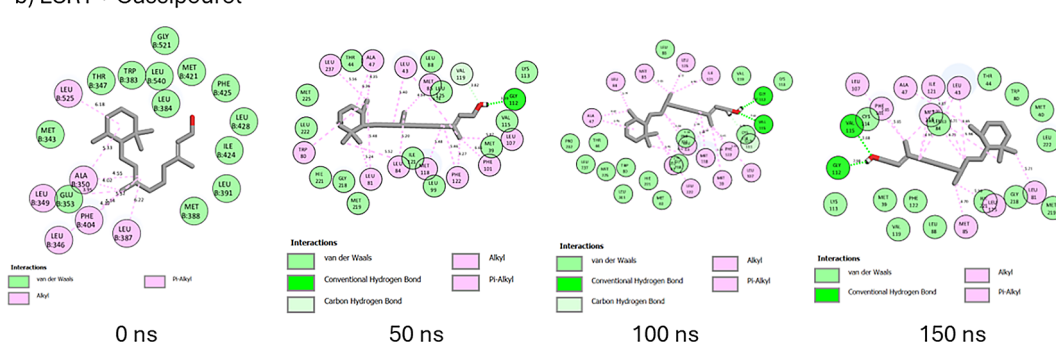
Although only four genes were common between *Cassipourea* metabolites and melasma, they have been reported to exert significant effects on skin pigmentation and melasma. Factors that reportedly affect the pathogenesis of melasma include genetics and hormonal activity.⁴⁴ Estrogen is a female sex hormone that influences skin pigmentation by stimulating melanin synthesis in melanocytes, improving skin tonicity, thickness, healing, elasticity, and preventing age-related diseases when produced in adequate amounts.⁴⁵ Estrogen levels differ with stages in women; for example, estrogen levels gradually rise from the pre-menopausal to childbearing stages, followed by a decline during post-menopause. This could be attributed to the reduction in melanin synthesis with increasing age.⁴⁵

Estrogen exerts its effects through signalling pathways and genes that are important in skin physiology and pathology. Dysregulation of estrogen gene activity in the skin might result in abnormal skin pigmentation, including melasma.

a) ESR1 + Afzelechin



b) ESR1 + Cassipourol



c) ESR1 + Decahydroretinol

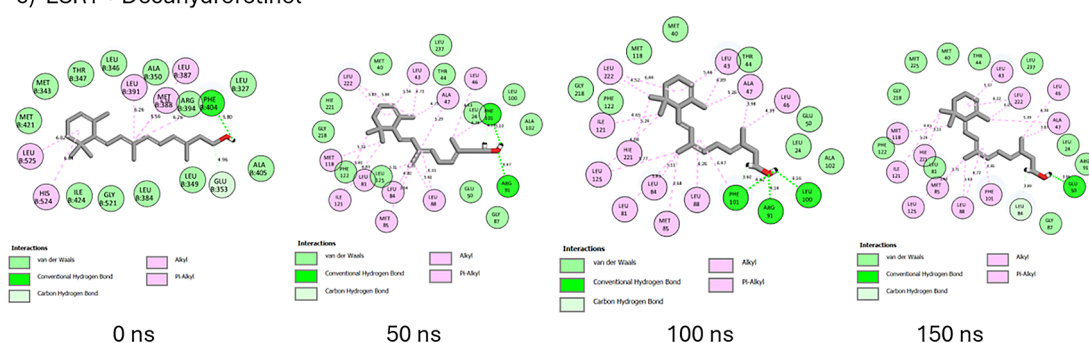
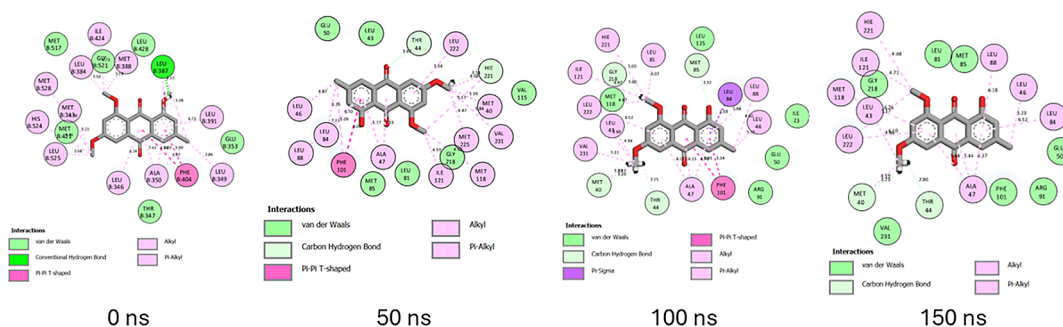


Figure 15. a: 2D interaction plot of ESR1 and afzelechin over 150 ns simulation. b: 2D interaction plot of ESR1 and cassipourol over 150 ns simulation. c: 2D interaction plot of ESR1 and decahydroretinol over 150 ns simulation. d: 2D interaction plot of ESR1 and emodin 6,8 dimethyl ether over 150 ns simulation. e: 2D interaction plot of ESR1 and lupeol over 150 ns simulation. f: 2D interaction plot of ESR1 and sitosterol glycoside over 150 ns simulation. g: 2D interaction plot of ESR1 and tricin over 150 ns simulation. h: 2D interaction plot of ESR1 and tranexamic acid over 150 ns simulation. i: 2D interaction plot of ESR1 and tamoxifen over 150 ns simulation.

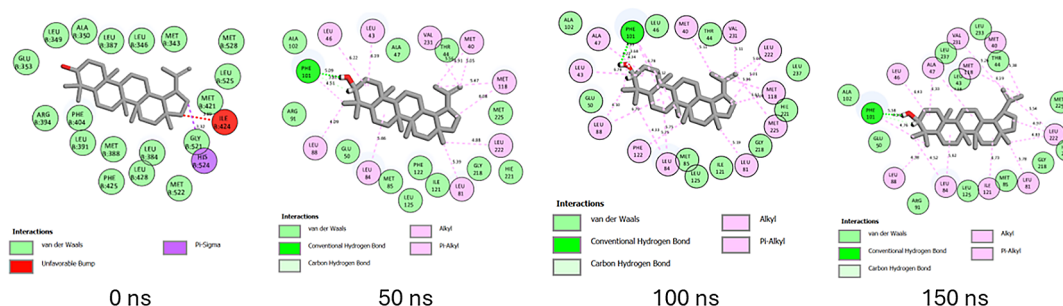
processes, such as cellular response to estrogen and estradiol stimulus, estrogen receptor signalling pathway, and regulation of inflammatory response, have also been implicated in skin pigmentation. However, steroid hormones, such as estrogen, estradiol, and progesterone, have been implicated in the control of skin pigmentation via certain receptors.⁵⁰ These hormones, as well as the overexpression of tyrosinase and melanocyte-stimulating hormone, are attributed to the excessive pigmentation observed during pregnancy.⁵¹ The interaction of these steroid hormones and signalling pathways is suggestive of the possible role of *Cassipourea* in preventing pregnancy-induced melasma. Interestingly, the signalling pathways and genes identified in this study are in line with the study by Yin et al.,²⁵ who studied the mechanism of action of *Croci stigma* on melasma.

The hyperstimulation of melanocytes by UV irradiation also induces high melanin secretion and ROS generation, resulting in cellular damage.⁴⁴ Vitamin D plays an important role in skin functions, including skin homeostasis and anti-inflammatory, immune, and cell proliferation responses that are essential for wound healing and restoration of skin

d) ESR1 + Emodin 6,8 dimethyl ether



e) ESR1 + Lupeol



f) ESR1 + Sitosterol-glycoside

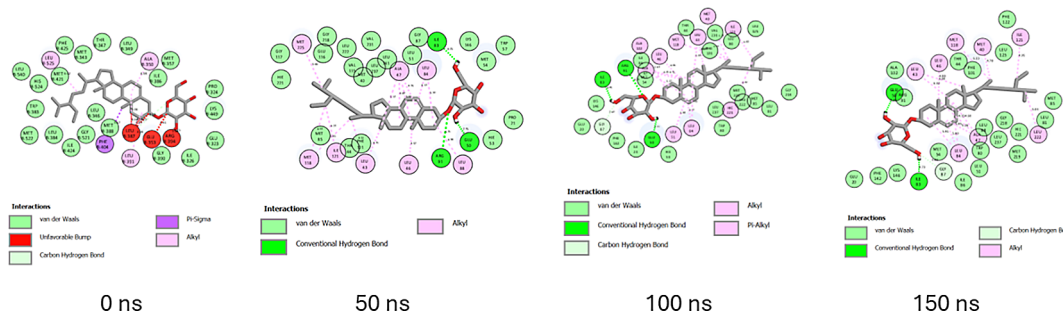


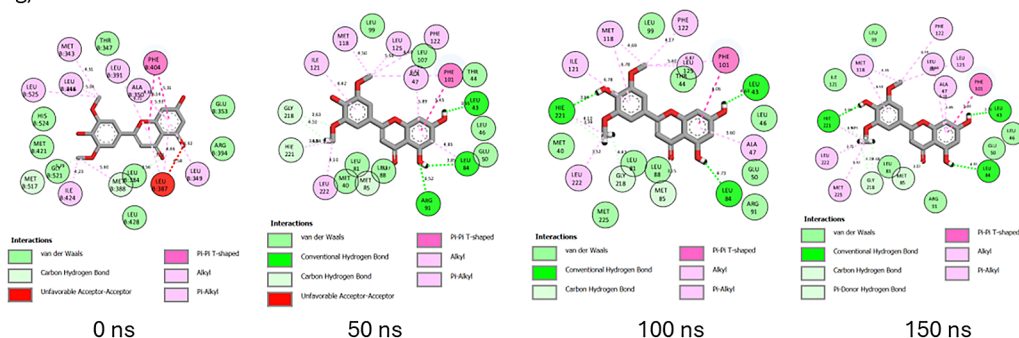
Figure 15. (continued)

pigmentation anomalies.⁵² The observation that response to vitamin D was one of the most enriched biological processes denotes the ability of *Cassipourea* metabolites to stimulate vitamin D production, which is necessary to prevent UV-induced melasma.

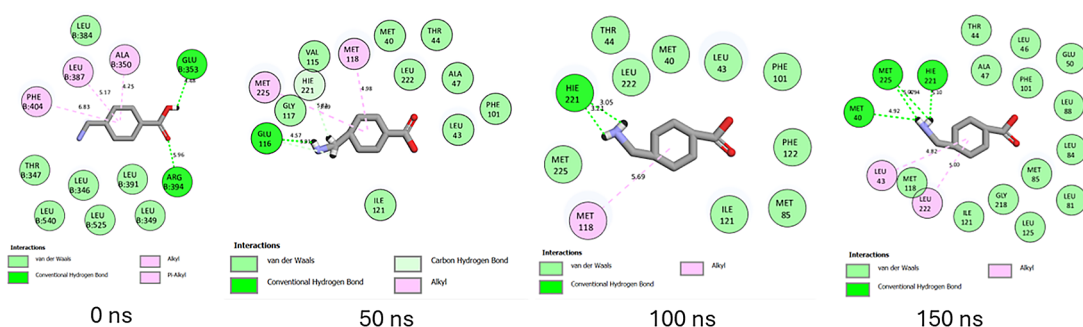
However, the macromolecular complex was the only enriched cellular component. This complex is vital to cellular processes. It is composed of assemblies of proteins and nucleic acids involved in transcriptional and translational processes that convert DNA to RNA and protein, respectively. In signaling pathways, these assemblies are transient,⁵³ which might imply that melasma temporarily affects signaling pathways and that skin anomalies could be restored to normalcy upon appropriate treatment.

Prolactin, a hormone secreted during lactation and reproduction, is involved in metabolism, immunoregulation, protection, growth, and development. The binding of prolactin to the prolactin receptor initiates the prolactin signaling pathway.⁵⁴ The observation that the prolactin signalling pathway was the most enriched in the KEGG analysis signifies that the functions of prolactin, its predominance in females who are largely affected by melasma, and the activation of its signalling pathway during the reproductive process, the regulation of this pathway could also serve as a target for the action of *Cassipourea* metabolites. Furthermore, the most significant genes (ESR2 and ESR1) in this pathway were analyzed for their binding affinity, stability, and interaction with top-ranked metabolites.

g) ESR1 + Tricin



h) ESR1 + Tranexamic acid



i) ESR1 + Tamoxifen

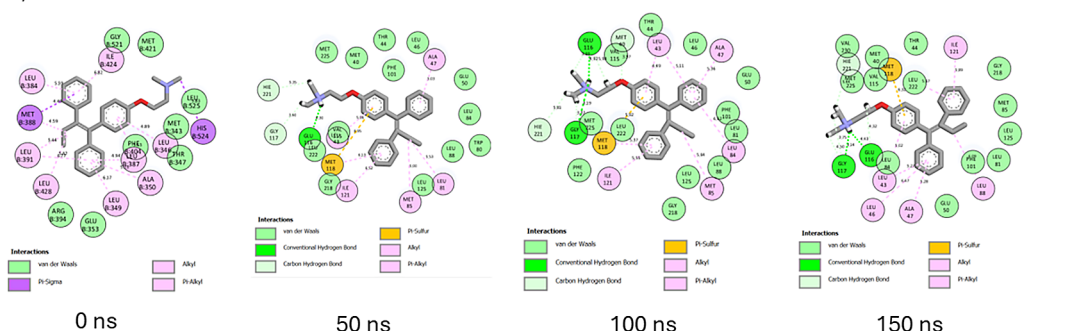


Figure 15. (continued)

Molecular docking identifies the top therapeutic metabolites by assessing their binding affinity for the active site of selected receptors to further augment the understanding of their bioactive mechanisms of action.⁵⁵ Higher negative docking scores are associated with better docking poses and fitness.⁵⁶ Except for ESR2-bound hexose, lupeol, sitosterol-glycoside, and ESR1-bound decahydroretinol, the higher negative docking scores of receptor-bound *Cassipourea* metabolites, relative to tamoxifen and tranexamic acid, are suggestive of their better biological activity.⁵⁷ Afzelechin acts as a dual modulator of both ESR2 and ESR1, with the highest negative docking scores within each receptor-bound system, further highlighting its potential therapeutic application. However, molecular docking merely assesses the crystallographic binding orientation of ligands, while molecular dynamic (MD) simulations help to elucidate binding stability.⁵⁸

To better understand the biomolecular affinity and efficacy of *Cassipourea* metabolites as therapeutic candidates, their thermodynamic binding free energies were analyzed over 150 ns,⁵⁹ with higher negative values indicative of greater binding affinity. The higher negative binding free energy of tamoxifen than that of ESR2-bound metabolites suggests that tamoxifen offers marginal superiority as an ESR2 modulator. The lower negative binding free energy of tranexamic acid relative to that of ESR2-bound metabolites indicates that *Cassipourea* metabolites are more thermodynamically stable. Comparatively, the higher negative binding free energies of ESR1 bound cassipourol, decahydroretinol, lupeol, triclin,

and sitosterol-glycoside complexes with tamoxifen and tranexamic acid were presumed to be stable and had better modulatory effects on ESR1. Moreover, MD simulations identified sitosterol-glycoside as a dual modulator of ESR2 and ESR1 because of its high negative binding free energy and superior thermodynamic stability among all other *Cassipourea* metabolites, further augmenting its biological activity in melasma-based therapeutics.

Thereafter, the post-dynamic parameters RMSD, RMSF, RoG, and SASA of the ESR2 and ESR1 bound systems were assessed at 150 ns for better insight into their thermodynamic stability.⁶⁰ RMSD measures the stability of protein-ligand systems by quantifying the change between their initial and final structural conformation, with lower deviations (values) denoting greater stability.⁶¹ According to Umar et al.,⁶² an RMSD value of ≤ 3 Å is an acceptable measurement of protein-ligand stability, validating the stability of ESR2 and ESR1 bound and unbound systems. The lower RMSD of the ESR2-bound tranexamic acid and ESR1-bound tamoxifen complexes is presumed to be due to their better stability as modulators of their respective genes. Conversely, the higher RMSD of ESR2-bound tamoxifen and ESR1-bound tranexamic acid systems compared to *Cassipourea* metabolites suggests their potential stability with the ESR2 and ESR1 genes. Comparatively, the lower RMSD of ESR2-bound emodin ethyl ether and sitosterol-glycoside and ESR1-bound afzelechin, cassipourol, decahydroretinol, emodin 6,8 dimethyl ether, and lupeol against their apo-genes indicated that these metabolites formed stable complexes with their relative genes.

RMSF analysis of ESR2 and ESR1 bound and unbound systems measures the flexibility of protein-ligand interactions. Higher RMSF values and fluctuations denote greater flexibility but less stability, whereas lower RMSF values and fluctuations indicate greater stability.⁶³ Generally, an RMSF value <2 Å is an optimal measure of ligand flexibility.⁶⁴ Thus, all metabolites bound to ESR2 and ESR1 exhibited suitable flexibility, with ESR2-bound emodin 6,8 dimethyl ether and ESR1-bound afzelechin, decahydroretinol, and lupeol exerting stabilizing effects on their relative apo-genes. However, among *Cassipourea* metabolites, tranexamic acid may potentiate greater stability as an ESR2 modulator, and tamoxifen may exhibit comparable flexibility as an alternate ESR1 modulator.

The RoG measures the time-dependent compactness of protein-ligand systems, with lower values indicating greater overall compactness and stability.⁶⁵ The similar RoG values of the ESR2 and ESR1 bound systems relative to their apo-genes are suggestive of the marginal impact exerted by *Cassipourea* metabolites on ESR2 and ESR1 protein folding. The lower RoG values of ESR2-bound decahydroretinol, emodin 6,8 dimethyl ether, and hexose complexes and ESR1-bound afzelechin, cassipourol, decahydroretinol, sitosterol-glycoside, lupeol, and tricin complexes relative to tranexamic acid, tamoxifen, and their apo-genes are indicative of their greater compactness, stability, and suitability as ESR2 and ESR1 modulators.

The SASA plot was used to measure the rate of interaction between complexes and their surrounding hydrophobic environment, with higher values denoting greater environmental exposure and lower values depicting more stable systems.⁶⁶ The lower SASA values of ESR2-bound metabolites (excluding lupeol) and ESR1-bound metabolites (excluding emodin 6,8 dimethyl ether, cassipourol, and lupeol) relative to their apo-genes suggest *Cassipourea* metabolites induce protein-ligand stability and compactness through protein folding. The minor fluctuations experienced by ESR2-bound lupeol at 125 ns and ESR1-bound tricin and cassipourol at 85 ns and 105 ns, respectively, suggest less interaction during that period. The lower SASA values of ESR2 bound afzelechin, hexose, emodin 6,8 dimethyl ether, decahydroretinol, and tricin complexes and ESR1 bound afzelechin, decahydroretinol, sitosterol-glycoside, and tricin complexes relative to tamoxifen and tranexamic acid are presumptive of their weaker interactions with the surrounding hydrophobic environment, resulting in better stability and modulation of ESR2 and ESR1.

The presence of hydrogen bonds significantly induces protein-ligand stability, binding specificity, and several pharmacokinetic properties such as metabolism and adsorption.⁶⁷ The 2D interaction plots of ESR2 and ESR1 bound systems also formed van der Waals and hydrophobic interactions, both of which augment their stability and affinity. In both ESR2 and ESR1 systems, sitosterol-glycoside could retain and steadily increase its hydrogen bond formation, corroborating its highest negative binding free energy among *Cassipourea* metabolites in both systems. The higher negative binding free energy of ESR2-bound tamoxifen can be attributed to the formation of multiple hydrophobic interactions throughout the 150 ns simulation.

5. Conclusions

The results of this study provide pharmacological support for the development of an alternative treatment for the management of hypermelanosis disorders such as melasma. The constituents of *Cassipourea* have potential commercial value. The finding that several sex hormones implicated in melasma, transcription processes, and vitamin D are highly involved in this study is a strong indication of the multi-target pathways by which *Cassipourea* metabolites potentially exert effects on melasma. The potential regulation of estrogen synthesis by these metabolites, depicted by their binding

interactions, stability, and compactness, is suggestive of melanogenesis control for normal skin pigmentation and the possible restoration of melasma skin. Further studies are recommended to validate and assess the safety of these metabolites.

Ethical statement

The study was conducted following the approval of the University of KwaZulu-Natal Biomedical Research Ethics Committee (UKZN BREC) (protocol reference number: BREC/00002721/2021).

Statement of contribution

Conceptualization: NM; methodology: NM; CP and SS; software: SS.; formal analysis: SS, CP, and HYL; investigation: NM, CP, and SS.; resources: SS; writing: original draft preparation: NM, CP, and HYL; writing: review and editing, NM, CP, HYL, SS, and MUM.; visualization, SS; supervision: NCD, NG, and AH; funding acquisition: NM and NG. All authors have read, agreed to, and approved the final version of the manuscript.

Data availability statement

Underlying data

FIGSHARE: Compounds elucidated from the three selected *Cassipourea* species as well as phytochemical comparison, <https://doi.org/10.6084/m9.figshare.26418361.v1>.⁶⁸

The project contains the following underlying data:

- Compounds elucidated from the three selected *Cassipourea* species
- Phytochemical comparison of the compounds

Data are available under the terms of the [Creative Commons Attribution 4.0 International license](#) (CC-BY 4.0).

Reporting guidelines

All the methods used in the study were adequately detailed in the “Methodology Section” of the manuscript and the followed the standard guidelines on reporting computational biology studies.

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Abstract: The background, methods, results, and conclusion are briefly described. The authors clearly state the study objectives and a concise rationale for the study. The experimental results and conclusions are well represented, giving readers a good sense of the research and the key takeaways. In the methods section of the abstract, the information provided is general and does not provide sufficient detail to readers. Additional information regarding the three specific metabolites, how the molecular docking and simulation were performed, what softwares were utilized and structures used, etc. would help provide substance and clarity.

Introduction: The authors present a thorough background on the known pathogenesis of melasma, the role of melanin, and existing treatment options. An epidemiological background is also provided but could benefit from including the disproportionate impact and disease burden on populations with skin of color, as well as their more limited treatment options. Additionally, since melasma is known as the 'mask of pregnancy,' the association with pregnancy should be included. This would offer a more robust introduction and more effectively communicate the impact of this research. The sentences addressing the value and history of secondary metabolites could be strengthened by providing concrete example(s) of previously discovered secondary metabolites that have played 'protection and treatment roles against various diseases.' We also recommend the authors provide more comprehensive details on the current evidence for the three *Cassipourea* species. This would help strengthen the justification for their analyses. Lastly, the authors should grammatically revise the first sentence of the sixth paragraph: 'Studies have explored natural products that inhibit UV-induced ROS, suppress enzymes, and reduce melanin formation as potential alternatives to current treatments have been conducted.'

Methods: The methods effectively support the goals of the experiment. The organization and division into subsections make the protocol easy to follow. The purpose of each analysis is clear and well justified. However, additional context would strengthen the robustness of the methodology. Specifically, in section 2.1, we recommend that the authors list the three *Cassipourea* species. In section 2.2, we suggest briefly describing Lipinski's Rule of Five (as

referenced later in Section 3.2) and its purpose within the context of this study.

Results: The results are presented clearly with supporting tables and figures. The figures are well displayed and effectively convey the data in a concise and logical manner. The information supports the goals of the experiment and the conclusions drawn. However, in all figure legends, the authors should not only describe the methods used but also briefly summarize the main findings from the figures and their corresponding experiments. Additionally, the authors should redefine all abbreviations used within the figures for clarity. Lastly, in Figure 2, the GeneCards/OMIM Venn diagram requires clarification and correction, as the number of genes identified as common to both GeneCards and OMIM exceeds the number of metabolite genes identified by GeneCards alone.

Discussion: The discussion highlights the novelty and value of this research, efficiently yet comprehensively discussing each of the study's results. The authors effectively convey how each analysis and result builds upon the previous one, providing sufficient contextual information for readers to fully appreciate the implications of the findings. While Figures 14 and 15 are helpful visual representations, we recommend adjusting the sizing and formatting of the keys for better readability. Additionally, we suggest examining any limitations of the study, including those related to the technologies the authors relied on to perform the analyses.

Conclusion: The conclusion briefly and effectively summarizes the study's purpose and the key takeaways from the discussion. We recommend expanding upon potential future directions, including future research and clinical applications.

General comments: The paper is well-written overall. The information is presented clearly and completely. The writing style is scientific and professional. The goals of the study, the methods, and results/conclusions are clear, justified, and strong. Our specific comments are as stated above.

Decision: Minor revisions

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Not applicable

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: melasma, phototherapy, keloids, basic science research, AN, cosmetics, skin of color

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 19 Sep 2024

Nomakhosi Mpofana

- **Reviewer comment: Are sufficient details of methods and analysis provided to allow replication by others?:** Partly
- **Response:** Thank you for your feedback. To address your concern about the sufficiency of details provided for replication, we would like to ask for your help in identifying specific methods or aspects of our analysis that you found unclear, in order for us to amend the sections as required.
- **Are all the source data underlying the results available to ensure full reproducibility?:** Partly
- **Response:** Thank you for your comment. To better address this comment, please advise which specific aspects of the results you found unclear or inadequately supported by the source data. Identifying these areas will allow us to provide the necessary information and increase the transparency of our results.

Competing Interests: Authors declare no competing interests .

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