

Article

Phytochemical Characterization and In Vitro Anti-Tyrosinase Activity of a Traditional *Hypericum perforatum* L. Olive Oil Macerate for Cosmetic Applications

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Abstract

Hypericum perforatum L. olive-oil macerate is a traditional topical remedy whose wound-healing efficacy is well documented, yet the phytochemical and mechanistic basis of its cosmetic-relevant activity remains poorly defined. This study provides an integrated experimental and in silico characterization of a traditionally prepared macerate of defined origin. The fatty acid composition, oxidative-stability indices, total phenolic and flavonoid contents, naphthodianthrone level, antioxidant capacity, and tyrosinase inhibition were determined, and the principal constituents were examined by molecular docking and in silico skin-permeation and toxicity profiling. The olive-oil matrix was oleic acid-dominated (73.9%) and oxidatively stable (iodine value 86.0; oxidizability index, COX, 1.84), while the macerate showed modest phenolic (36.84 µg pyrocatechol equivalents, PCE/mg) and naphthodianthrone (0.044 mg/mL hypericin equivalents) contents. It showed limited antioxidant activity but moderate tyrosinase inhibition (38.75% at 200 µg/mL). Docking revealed that, despite high affinity scores, the naphthodianthrone lay 7–9 Å from the catalytic copper centre (an artefact of the scoring function), whereas fatty acids accessed the active site more closely, implicating the matrix in the observed activity. In silico profiling indicated that the photoactive naphthodianthrone is largely retained at the skin surface. These findings link this centuries-old preparation to a mechanistically informed understanding of its cosmetic potential.

Keywords: *Hypericum perforatum*; olive oil macerate; tyrosinase inhibition; molecular docking; skin permeability; naphthodianthrone; phytochemical characterization; cosmetic application



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1. Introduction

Hypericum perforatum L. (St. John's Wort, Hypericaceae) is among the most extensively studied medicinal plants worldwide, best known for the antidepressant activity of its standardized hydroalcoholic extracts. Beyond this systemic use, however, the plant has a long and distinct dermatological tradition: an olive-oil macerate of its fresh flowering tops, known as *Oleum hyperici* or “red oil”, has been applied topically for centuries across the Mediterranean and Anatolia to treat wounds, burns, bruises, and inflammatory skin conditions [1,2]. Prepared by steeping the flowers in olive oil under sunlight until a deep red coloration develops, this preparation remains a widely used folk remedy in Türkiye and the broader region [3,4].

Unlike many traditional remedies, the topical efficacy of *H. perforatum* oil is supported by robust experimental and clinical evidence [5]. At the cellular level, hydroalcoholic extracts increase the proportion of polygonal fibroblasts and the number of collagen granules, indicating that the wound-healing effect involves stimulation of fibroblast migration and collagen synthesis [6]. In controlled animal models, the traditionally prepared Turkish olive-oil macerate significantly accelerates wound closure in both excision and incision models [3], and topical *H. perforatum* promotes re-epithelialization and preserves epidermal structures in experimental burns [4]. These preclinical findings are reinforced by clinical evidence: olive-oil macerates of the flowering tops reduced wound area in pressure ulcers [7], accelerated epithelial reconstruction of caesarean surgical wounds [8,9], and improved episiotomy wound healing [10]. The therapeutic value of the preparation is therefore well established; what remains comparatively underexplored is the detailed phytochemical and mechanistic basis underlying its bioactivity [5].

The chemistry of *H. perforatum* is dominated by several classes of bioactive constituents: naphthodianthrone (hypericin, pseudohypericin), acylphloroglucinols (hyperforin), flavonoids, and phenolic acids. Their transfer into an oil matrix is selective and strongly dependent on the lipophilicity of each compound and on the maceration conditions [11]. As a result, the composition of an oil macerate differs substantially from that of the alcoholic extracts on which most pharmacological data are based, and the relationship between this distinctive composition and the preparation's cosmetic-relevant activities is not well defined. This gap is particularly relevant given the growing interest in *Hypericum* extracts as functional ingredients in cosmetic and skincare formulations [12–15].

Among the activities relevant to cosmetic use, inhibition of tyrosinase (the rate-limiting, copper-dependent enzyme of melanin biosynthesis) is a key target for skin-brightening and anti-hyperpigmentation applications. Such inhibitory activity has previously been reported for *H. perforatum* extracts [16,17]. However, this activity has rarely been examined specifically for the traditional oil macerate, and the constituents likely responsible for it remain unidentified. Naphthodianthrone illustrate this complexity well: they are the visually defining, characteristically red pigments of the preparation, yet they are also potent photosensitizers [18], raising questions about their behaviour and safety upon topical application that cannot be addressed by activity measurements alone [19,20].

The present study therefore aimed to provide an integrated phytochemical and mechanistic characterization of a traditionally prepared *H. perforatum* olive-oil macerate of defined botanical and geographical origin, with a focus on properties relevant to cosmetic application. The fatty acid profile and derived oxidative-stability indices, total phenolic and flavonoid contents, naphthodianthrone level, antioxidant capacity, and tyrosinase inhibitory activity were determined experimentally. These data were then combined with molecular docking and in silico physicochemical, skin-permeation, and toxicity profiling to interpret the likely active constituents, their interaction with the tyrosinase active site, and their predicted cutaneous behaviour, following integrated experimental–computational

approaches recently applied to other *Hypericum* species [21]. Rather than seeking to demonstrate efficacy already established in vivo, this work sought to clarify what the preparation contains and how its principal constituents are likely to act, thereby linking a centuries-old traditional remedy to a contemporary, mechanistically informed understanding of its cosmetic potential.

2. Materials and Methods

2.1. Plant Material and Preparation of the Oil Macerate

Fresh flowering tops of *Hypericum perforatum* L. were collected from Yazır village (Korkuteli district, Antalya, Türkiye) in July 2023. A representative specimen was air-dried and taxonomically authenticated by Prof. Dr. Serpil Demirci Kayıran (Department of Pharmaceutical Botany, Çukurova University). A voucher specimen was deposited in the Herbarium of the Faculty of Pharmacy, Çukurova University (Voucher No: CUEF 1793).

The oil macerate was prepared according to traditional procedures [22]. A commercially available cold-pressed olive oil from a local producer in the Antalya region (Türkiye) was used as the maceration matrix. Fresh flowering tops (weighed) and olive oil (measured by volume using a graduated cylinder) were combined at a 1:4 ratio (*w/v*) in a transparent glass container. The mixture was kept under sunlight at ambient temperature for six weeks with periodic agitation until a characteristic red coloration developed. The macerate was then filtered through cotton gauze followed by paper filtration and stored in amber glass bottles at 4 °C until analysis. A single macerate batch, prepared as described above, was used for all subsequent analyses; the triplicate (*n* = 3) measurements reported throughout this study represent technical replicates from this single preparation rather than independently prepared biological batches.

2.2. Fatty Acid Composition by GC-FID/MS

The fatty acid composition of the plain olive oil, analyzed prior to maceration to chemically characterize the maceration matrix independently of the plant material, was determined by gas chromatography coupled with flame-ionization detection and mass spectrometry (GC-FID/MS), as fatty acid methyl esters, with sample derivatization carried out according to the Turkish Food Codex method for olive oil and olive-pomace oil analysis [23]. Analyses were performed on a gas chromatograph (Agilent 7890A) coupled to both a mass-selective detector (Agilent 5975C) and a flame-ionization detector (FID), using an HP-Innowax capillary column (60.0 m × 0.25 mm × 0.25 µm). The oven temperature was programmed from 150 °C to 230 °C at 2 °C/min, followed by a 10 min hold at 230 °C; this ramp-and-hold temperature program had a duration of 50 min. The complete chromatographic run additionally included a short initial isothermal hold prior to the temperature ramp, so that total retention times of up to approximately 55 min (Table 1) are consistent with the full run rather than with the temperature program alone. Individual fatty acids were identified by comparison of their mass spectra with the WILEY7N, NIST05, and OIL ADAMS library data, and their relative proportions (%) were calculated from the FID signal. The analytical procedure and instrumental configuration corresponded to those previously applied to regional olive oil by Gölükcü et al. [24].

Table 1. Fatty acid composition of the olive oil used for maceration, determined by GC-FID/MS.

Fatty Acid	Carbon Chain	Classification	RT (min)	Relative Composition (%)
Palmitic acid	C16:0	SFA	34.012	11.75
Palmitoleic acid	C16:1	MUFA	35.209	1.01
Myristic acid	C14:0	SFA	38.394	0.12
Stearic acid	C18:0	SFA	43.220	2.63
Oleic acid	C18:1	MUFA	44.741	73.92
Linoleic acid	C18:2	PUFA	46.927	9.18
Linolenic acid	C18:3	PUFA	50.584	0.72
Arachidic acid	C20:0	SFA	54.607	0.33
Unidentified	—	—	—	0.35
Total SFA				14.83
Total MUFA				74.93
Total PUFA				9.90

SFAs, saturated fatty acids; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; RT, retention time.

2.3. Calculation of Lipid Quality and Oxidative Stability Indices

Lipid quality and oxidative stability indices were calculated from the fatty acid composition obtained by GC-FID/MS, without additional experimental measurement. The total proportions of saturated (SFA), monounsaturated (MUFA), and polyunsaturated (PUFA) fatty acids were summed, and the ratio of unsaturated fatty acids (UFA, the sum of MUFA and PUFA) to SFA (UFA/SFA) was derived. The iodine value (IV), reflecting the overall degree of unsaturation, was calculated according to the standard relationship $IV = \sum[(\% \text{FA}) \times D \times 253.81 / M]$, where D is the number of double bonds and M is the molecular weight of each fatty acid [25]. The calculated oxidizability (COX) value was obtained from the proportions of unsaturated C18 fatty acids using the equation of Fatemi and Hammond [26]: $COX = (1 \times [C18:1] + 10.3 \times [C18:2] + 21.6 \times [C18:3]) / 100$.

2.4. Determination of Total Phenolic Content

Total phenolic content (TPC) was determined by the Folin–Ciocalteu method and expressed as μg pyrocatechol equivalent (PCE)/mg sample [27]. Briefly, the oil macerate was accurately weighed and dissolved in ethanol to prepare a stock solution at 1 mg/mL concentration; due to the low working concentrations used in the assays, the macerate could be dispersed homogeneously in ethanol, and the stock was vortex-mixed (2 min) and sonicated (15 min) at room temperature prior to dilution. For the TPC assay, 8 μL of this stock solution was mixed with 176 μL of distilled water and 4 μL of Folin–Ciocalteu reagent. After 3 min, 12 μL of 2% sodium carbonate solution was added, and the mixture was incubated at room temperature for 2 h. Absorbance was then recorded at 760 nm using a microplate reader. Calibration was performed using pyrocatechol at five concentration levels, and the calibration curve was defined by the equation $y = 0.0128x + 0.0324$ ($R^2 = 0.9984$), where y is the absorbance at 760 nm and x is the concentration of pyrocatechol ($\mu\text{g}/\text{mL}$). All measurements were carried out in triplicate, and the results were expressed as mean $\pm$ SD.

2.5. Determination of Total Flavonoid Content

Total flavonoid content (TFC) was determined by an aluminum nitrate colorimetric assay and expressed as μg quercetin equivalent (QE)/mg sample [28]. Briefly, 20 μL of the same ethanolic oil macerate stock solution (1 mg/mL; Section 2.4) was mixed with 4 μL of 10% aluminum nitrate, 4 μL of 1 M potassium acetate, and 172 μL of methanol. After incubation at room temperature for 40 min, the absorbance was measured at 415 nm using a microplate reader. Calibration was performed using quercetin at five concentration levels, and the calibration curve was defined by the equation $y = 0.1701x - 0.0778$ ($R^2 = 0.9939$),

where y is the absorbance at 415 nm and x is the concentration of quercetin ($\mu\text{g/mL}$). All measurements were carried out in triplicate, and the results were expressed as mean $\pm$ SD.

2.6. Quantification of Naphthodianthrone by UV Spectrophotometry

Naphthodianthrone content was determined by UV–Vis spectrophotometry at 590 nm and expressed as hypericin equivalents (mg/mL oil). The wavelength of 590 nm corresponds to the characteristic visible absorption maximum of hypericin and is in line with UV-based approaches reported for *Hypericum* preparations and oil macerates [11,29]. A calibration curve was constructed using an authentic hypericin standard (high-performance liquid chromatography, HPLC, grade) dissolved in methanol at four concentration levels prepared by two-fold serial dilution (0.0039–0.0313 mg/mL), with methanol as the corresponding blank. Hypericin concentration was calculated from absorbance using the linear regression equation $C = 0.0197 A - 0.0012$ ($r = 0.9939$), where C is the hypericin concentration (mg/mL) and A is the absorbance at 590 nm.

To account for the intrinsic absorbance of the lipid matrix, the macerate measurements were referenced against a blank prepared from the same olive oil used for maceration, and the matrix absorbance was subtracted from that of the macerate. For analysis, the oil macerate was diluted with blank olive oil (900 μL macerate + 1100 μL blank oil; dilution factor 2.22) to bring the absorbance within the calibrated range, and recorded at 590 nm using a 1 cm quartz cuvette. The naphthodianthrone content of the original oil was obtained by multiplying the measured concentration by the dilution factor. All measurements were carried out under light-protected conditions owing to the photosensitive nature of naphthodianthrone, performed in triplicate, and expressed as mean $\pm$ SD.

2.7. Antioxidant Activity Assays

Working solutions were freshly prepared from the ethanolic oil macerate stock solution (1 mg/mL; Section 2.4) at 10, 25, 50, and 100 $\mu\text{g/mL}$. The corresponding solvent (ethanol) was included as a blank in all assays. All assay solutions were prepared immediately before measurements and visually inspected to ensure the absence of phase separation during the assay period. All measurements were performed in triplicate.

2.7.1. DPPH Free Radical Scavenging Assay

2,2-Diphenyl-1-picrylhydrazyl (DPPH) solution (0.1 mM in methanol) was mixed with equal volumes of the ethanolic sample solutions (10, 25, 50, and 100 $\mu\text{g/mL}$). After 30 min incubation in the dark at room temperature, absorbance was measured at 517 nm. Radical scavenging activity was calculated as percentage inhibition relative to the blank. Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and α -tocopherol were used as positive controls [30]. The IC_{50} value (the concentration required to scavenge 50% of radicals) was calculated from the dose–response curve constructed using the four concentrations. Where 50% inhibition was not reached within the tested range, the value was reported as not detected (nd).

2.7.2. ABTS Radical Cation Decolorization Assay

2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation ($\text{ABTS}^{\bullet+}$) was generated by reacting 7 mM ABTS with 2.45 mM potassium persulfate. After overnight incubation in the dark at room temperature, the solution was diluted to an absorbance of 0.70 ± 0.02 at 734 nm. Equal volumes of the sample solutions (10, 25, 50, and 100 $\mu\text{g/mL}$) were added, and absorbance was measured after 6 min in the dark. Results were expressed as percentage inhibition relative to the blank. BHA, BHT, and α -tocopherol were used as positive controls [31]. The IC_{50} value was calculated from the dose–response curve

constructed using the four concentrations. Where 50% inhibition was not reached within the tested range, the value was reported as not detected (nd).

2.7.3. Cupric Ion Reducing Antioxidant Capacity (CUPRAC) Assay

Cupric reducing antioxidant capacity was evaluated using CuCl_2 , ammonium acetate buffer (pH 7.0), and neocuproine solution. The sample solutions (10, 25, 50, and 100 $\mu\text{g/mL}$) were added to the reaction mixture. After 1 h incubation at room temperature, absorbance was recorded at 450 nm. The cupric reducing antioxidant capacity was expressed as the $A_{0.5}$ value, defined as the concentration of sample or standard producing an absorbance of 0.5. BHA, BHT, and α -tocopherol were used as reference standards [32].

2.8. Tyrosinase Inhibition Assay

Tyrosinase inhibitory activity was determined using mushroom tyrosinase with L-3,4-dihydroxyphenylalanine (L-DOPA) as the substrate. The enzyme was prepared in sodium phosphate buffer (50 mM, pH 6.8). Sample solutions were pre-incubated with the enzyme for 10 min at room temperature, and the reaction was initiated by adding L-DOPA (final concentration 0.5 mM). Dopachrome formation was monitored at 475 nm and 37 °C. Kojic acid was used as a positive control, and the corresponding solvent (ethanol) was included as a vehicle control. Inhibitory activity was evaluated at a single concentration of 200 $\mu\text{g/mL}$, expressed as percentage inhibition relative to the enzyme control (without inhibitor). All measurements were performed in triplicate [33]. A sample-only blank (macerate and substrate without enzyme) was not included, so a possible contribution of the macerate's own colour or turbidity to the absorbance reading at 475 nm cannot be ruled out.

2.9. Molecular Docking

Molecular docking was performed on the complete set of constituents characterized by the analytical methods above: the four naphthodianthrone generally reported as the principal pigments of *H. perforatum* (hypericin, pseudohypericin, protohypericin, and protopseudohypericin), whose combined content was quantified as total hypericin equivalents by UV spectrophotometry (Section 3.3), and all eight fatty acids identified by GC-FID/MS (Table 1), together with the reference tyrosinase inhibitors kojic acid and tropolone, against mushroom tyrosinase. We emphasize that the identity and relative proportions of the four individual naphthodianthrone in this specific macerate were not experimentally confirmed by a compound-specific chromatographic method (e.g., HPLC-DAD or LC-MS); their inclusion in the docking study rests on their well-established presence in *H. perforatum* in the literature rather than on direct identification in the present sample, and this should be considered when interpreting the docking results for these four compounds specifically. No additional compound pre-selection or filtering was applied beyond this correspondence with the analytically characterized constituents, consistent with the exploratory, mechanistic aim of relating the measured bioactivity to the identifiable matrix and plant-derived compounds rather than to a targeted subset. The crystal structure of *Agaricus bisporus* tyrosinase (Protein Data Bank, PDB, entry 2Y9X) was selected because it contains a well-defined dicopper catalytic center and has been widely used in docking studies of tyrosinase inhibitors [34]. Chain A was retained, and the receptor was prepared by removing the co-crystallized ligand, water molecules, and other non-essential heteroatoms while preserving the two catalytic copper ions, and was treated as a rigid receptor. Docking calculations were carried out with AutoDock Vina v1.2.7 [35,36] using a grid box of $22 \times 22 \times 22$ Å (grid spacing 0.375 Å) centered on the dicopper catalytic site (center: $x = -9.8$, $y = -24.5$, $z = -39.8$). The exhaustiveness was set to 16, generating up to 10 binding modes per ligand. Kojic acid and tropolone were included as reference

ligands to confirm the suitability of the selected docking region. The lowest-energy (highest-affinity) pose of each ligand was retained for analysis. Docking poses were visualized in ChimeraX [37], and post-docking interaction analysis (including predicted hydrogen-bond contacts, active-site residues within 4 Å of the ligand, and minimum distances between ligand heavy atoms and the catalytic copper ions, Cu400/Cu401) was performed using an in-house Python script based on Euclidean interatomic distances.

The docking protocol was validated by re-docking the co-crystallized inhibitor tropolone into the prepared receptor using identical grid parameters; the re-docked pose was compared with the crystallographic ligand position by calculating the centroid displacement and the heavy-atom root-mean-square deviation (RMSD).

2.10. In Silico Physicochemical, Skin-Permeation, and Toxicity Prediction

The physicochemical properties, skin-permeation potential, and predicted toxicity of the principal constituents of the macerate were evaluated in silico. The compound set comprised the four *Hypericum*-derived naphthodianthrones (hypericin, pseudohypericin, protohypericin, and protopseudohypericin), the representative olive-oil fatty acids identified by GC-FID/MS, and kojic acid as a reference tyrosinase inhibitor. To ensure consistency with the molecular docking study, the same canonical structures (retrieved from PubChem) were used for all predictions.

Physicochemical descriptors and skin permeability were calculated using the SwissADME web server [38]. The parameters considered included molecular weight, topological polar surface area (TPSA), gastrointestinal absorption, and the skin-permeation coefficient ($\log K_p$), the latter estimated from the Potts–Guy model. More negative $\log K_p$ values correspond to lower predicted skin permeability.

Predicted toxicity endpoints were obtained using the pkCSM web server [39]. The endpoints evaluated included AMES mutagenicity, hepatotoxicity, and skin sensitization. Because the pkCSM framework does not include a phototoxicity endpoint, the well-documented photosensitizing properties of the naphthodianthrones were considered separately on the basis of the published literature and their characteristic UV–visible absorption.

3. Results and Discussion

3.1. Fatty Acid Composition and Oxidative Stability of the Olive Oil Matrix

The fatty acid composition of the olive oil used as the maceration matrix was determined by GC-FID/MS following conversion to fatty acid methyl esters (Table 1). The major fatty acids were oleic acid (73.92%), palmitic acid (11.75%), and linoleic acid (9.18%), with minor contributions from stearic acid (2.63%), palmitoleic acid (1.01%), and linolenic acid (0.72%). This profile, dominated by monounsaturated oleic acid, is characteristic of olive oil and confirms the identity of the maceration matrix; the composition closely matched that reported for regional olive oil from the same production area [24]. The corresponding GC-FID chromatogram is shown in Figure 1.

To assess the oxidative behaviour of the matrix, lipid quality and oxidative stability indices were calculated from the fatty acid profile (Table 2). The oil showed a high unsaturated-to-saturated fatty acid ratio (UFA/SFA = 5.72), reflecting its predominantly monounsaturated character (MUFA 74.93%; PUFA 9.90%). The calculated iodine value (86.0 g I₂/100 g) fell within the range established for olive oil by the International Olive Council (75–94 g I₂/100 g) [40], confirming the identity of the matrix, while the low oxidizability value (COX = 1.84) matched values reported for extra-virgin olive oil [41]. These indices together indicate a relatively low intrinsic susceptibility to autoxidation, arising from the predominance of oleic acid over the more readily oxidized polyunsaturated fatty acids, particularly the low linolenic acid content (0.72%). This supports the suitability of

the olive-oil matrix as a comparatively stable carrier for a topical preparation. The same fatty acids were subsequently examined in the molecular docking and skin-permeation analyses (Sections 3.6 and 3.7), where they were found to interact with the target enzyme and to contribute to the predicted permeability profile of the macerate.

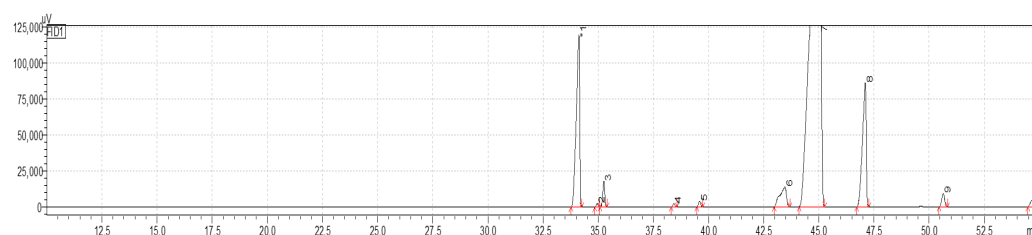


Figure 1. GC-FID chromatogram of the olive oil used as the maceration matrix, showing the fatty acid methyl ester peaks quantified in Table 1 (peak assignments, in order of retention time: 1, palmitic acid; 2/3, palmitoleic acid; 4, myristic acid; 5, unidentified minor peak; 6, stearic acid; 7, oleic acid; 8, linoleic acid; 9, linolenic acid; 10, arachidic acid).

Table 2. Lipid quality and oxidative stability indices of the olive oil used for maceration, calculated from the fatty acid composition.

Index	Value
Saturated fatty acids, SFAs (%)	14.83
Monounsaturated fatty acids, MUFAs (%)	74.93
Polyunsaturated fatty acids, PUFAs (%)	9.90
UFA/SFA ratio	5.72
MUFA/PUFA ratio	7.57
Iodine value, IV (g I ₂ /100 g)	86.0
Calculated oxidizability, COX	1.84

SFAs, MUFAs, PUFAs, and UFAs (unsaturated fatty acids) were obtained by summing the corresponding fatty acids in Table 1. The iodine value was calculated as $IV = \sum[(\% FA) \times D \times 253.81 / M]$, where D is the number of double bonds and M the molecular weight. The COX value was calculated as $(1 \times [C18:1] + 10.3 \times [C18:2] + 21.6 \times [C18:3]) / 100$ [26].

Beyond confirming the identity of the matrix, this fatty acid profile has direct relevance to the preparation's topical use. Oleic acid, the dominant component, is a well-recognized skin-penetration enhancer that fluidizes the stratum corneum lipid bilayers and can facilitate the permeation of co-administered compounds, while the high monounsaturated content contributes to the emollient and occlusive properties valued in cosmetic carriers [42,43]. The low oxidizability of the matrix (COX = 1.84) is equally relevant from a formulation standpoint, as it implies a comparatively low tendency toward rancidity and a longer functional shelf life, an important consideration for a lipid-based cosmetic preparation. The close agreement between the present profile and that reported by Gölükcü et al. [24] for olive oil from the same region (oleic 73.9%, linoleic 10.1%, palmitic 11.7%) further supports the consistency of the regional matrix. Although the fatty acid composition was determined on the olive oil prior to maceration, the maceration process is not expected to substantially alter the triglyceride profile, since the transfer of plant-derived constituents into the oil involves predominantly minor lipophilic and pigment compounds rather than the bulk fatty acids [11].

3.2. Total Phenolic and Flavonoid Content

The total phenolic and flavonoid contents of the oil macerate were 36.84 ± 2.33 μg PCE/mg and 35.49 ± 0.55 μg QE/mg, respectively (Table 3). These relatively modest values reflect the lipophilic nature of the preparation, in which only a limited fraction of the polar phenolic constituents of the plant material partitions into the olive-oil matrix during maceration. This selective transfer is a recognized characteristic of oil-based *Hypericum* preparations, which retain substantially lower phenolic levels than the corresponding hydroalcoholic or water-miscible cosmetic extracts of the aerial parts [11,12]. Direct numerical comparison with previous reports is limited not only by differences in extraction matrix, solvent, and the units of expression employed, but also by differences in plant geographical origin, chemotype, and harvest season, which are known to substantially affect the phytochemical starting material in *Hypericum* species; nevertheless, the present results fall within the qualitative expectation for a traditionally prepared lipophilic macerate.

From a cosmetic standpoint, the modest phenolic and flavonoid contents indicate that the macerate's functional potential is unlikely to derive primarily from a high concentration of polar antioxidants. Instead, its activity is more plausibly attributable to the lipophilic constituents transferred during maceration (including naphthodianthrone and less polar flavonoid aglycones), together with the intrinsic properties of the olive-oil matrix itself. This interpretation agrees with the comparatively low antioxidant capacity observed for the preparation (Section 3.4) and points to the olive-oil matrix, rather than a high polar-antioxidant load, as a key determinant of the preparation's behaviour.

Table 3. Phenolic content, antioxidant activity, and tyrosinase inhibitory activity of the *H. perforatum* oil macerate and reference compounds (mean $\pm$ SD, $n = 3$).

Sample	TPC (μg PCE/mg)	TFC (μg QE/mg)	DPPH IC ₅₀ ($\mu\text{g/mL}$)	ABTS IC ₅₀ ($\mu\text{g/mL}$)	CUPRAC A _{0.5} ($\mu\text{g/mL}$)	Tyrosinase Inhib. (%) ^a
Oil macerate	36.84 ± 2.33	35.49 ± 0.55	nd ^b	nd ^b	163.48 ± 3.44	38.75 ± 0.17
BHA	—	—	3.22 ± 0.08	2.74 ± 0.03	4.14 ± 0.17	—
α -Tocopherol	—	—	1.41 ± 0.00	8.48 ± 0.43	13.64 ± 0.32	—
BHT	—	—	16.71 ± 0.80	4.44 ± 0.30	3.93 ± 0.24	—
Kojic acid	—	—	—	—	—	95.05 ± 0.37

TPC, total phenolic content; PCEs, pyrocatechol equivalents; TFC, total flavonoid content; QEs, quercetin equivalents. A_{0.5}, concentration giving an absorbance of 0.5 in the CUPRAC assay. ^a Measured at a single concentration of 200 $\mu\text{g/mL}$. ^b nd, not detected (50% inhibition not reached within the tested concentration range).

3.3. Naphthodianthrone Content

The naphthodianthrone content of the oil macerate, determined by UV spectrophotometry at 590 nm and expressed as hypericin equivalents, was 0.044 ± 0.001 mg/mL (relative standard deviation, RSD, =2.9%). Because absorbance at 590 nm captures the combined contribution of hypericin, pseudohypericin, and related naphthodianthrone, this value represents a semi-quantitative estimate of total naphthodianthrone rather than the concentration of hypericin alone. UV-spectrophotometric quantification at this wavelength is an established approach for naphthodianthrone in *Hypericum* oil macerates [44].

This relatively low naphthodianthrone level reflects the known instability of these compounds during oil maceration. Naphthodianthrone is sensitive to light and prolonged processing, and their concentration in oil macerates has been reported to decline after extended maceration periods [24,44]. Indeed, Gölükcü et al. [24] observed that naphthodianthrone levels in regional olive-oil macerates peaked at around 30 days of maceration and decreased markedly thereafter, attributing this decline to the limited stability of these compounds. Given that the present macerate was prepared by traditional sunlight maceration over approximately six weeks, some degradation of the initially extracted naphthodi-

anthrones is therefore expected, which likely accounts for the modest content observed. This behaviour also reflects the well-documented photosensitivity of naphthodianthrone: the same chromophore responsible for the characteristic absorption at 590 nm renders these molecules photoactive, a property examined further in relation to their predicted skin behaviour and safety in Section 3.7.

More broadly, because this preparation follows a traditional protocol governed by natural, batch-dependent variables (plant material composition across harvests, ambient sunlight intensity and duration, and maceration temperature), batch-to-batch variability in these parameters is expected to affect not only naphthodianthrone content, as illustrated above, but also total phenolic and flavonoid levels, and consequently the consistency of the biological effects reported here (tyrosinase inhibition, antioxidant activity). This is an inherent limitation of traditionally prepared macerates relative to standardized industrial extracts, and underscores the need for defined preparation protocols and quality-control markers before consistent cosmetic performance can be assured across batches.

3.4. Antioxidant Activity

The antioxidant activity of the oil macerate was evaluated by the DPPH, ABTS, and CUPRAC assays, with BHA, BHT, and α -tocopherol as reference antioxidants (Table 3). In the DPPH and ABTS assays, the macerate did not reach 50% inhibition within the tested concentration range, whereas the reference antioxidants were active at low concentrations ($IC_{50} = 1.41$ – $16.71 \mu\text{g/mL}$). In the CUPRAC assay, the macerate showed measurable but comparatively low reducing capacity ($A_{0.5} = 163.48 \pm 3.44 \mu\text{g/mL}$), more than an order of magnitude higher than the reference compounds ($A_{0.5} = 3.93$ – $13.64 \mu\text{g/mL}$).

This limited radical-scavenging and reducing activity is in line with the low total phenolic content of the macerate (Section 3.2), since phenolic compounds are the principal contributors to the antioxidant capacity of *Hypericum* preparations. The result is expected for a traditionally prepared lipophilic macerate, in which only a small fraction of polar antioxidant constituents is transferred into the oil matrix, rather than a phenolic-enriched extract. Accordingly, the antioxidant contribution of the macerate should be regarded as modest, and its traditional topical use is more plausibly related to other constituents and to the properties of the olive-oil matrix itself than to a strong direct radical-scavenging effect.

A further consideration in interpreting these results is the contribution of the olive-oil matrix itself. Olive oil contains its own phenolic constituents (principally hydroxytyrosol, tyrosol, and their secoiridoid derivatives such as oleuropein), which are recognized antioxidants and are detectable in common radical-scavenging and reducing assays [45]. Consequently, the total phenolic content and antioxidant signal measured here reflect the combined contribution of *H. perforatum*-derived constituents and the intrinsic phenolics of the olive oil, rather than the plant material alone. This overlap, together with the modest overall activity observed, underscores that a plain olive-oil matrix control would be required to unambiguously attribute the measured antioxidant capacity to the macerated plant constituents: an important consideration for the design of future studies. Such matrix-controlled comparisons remain uncommon, although some earlier clinical evaluations of *Hypericum* oil did include plain olive oil as a control [7].

It should also be emphasized that DPPH, ABTS, and CUPRAC are chemical, cell-free assays that quantify electron- or hydrogen-atom-transfer capacity toward synthetic radicals or metal ions under non-physiological conditions, and do not by themselves establish antioxidant activity in living skin [46,47]. Under physiological conditions, cutaneous oxidative stress is buffered by an integrated system of enzymatic (superoxide dismutase, catalase, glutathione peroxidase) and non-enzymatic defenses, and many phytochemicals act indirectly, for instance by inducing endogenous antioxidant enzymes via the Nrf2/ARE

pathway, rather than through direct radical scavenging alone [48]. Bioavailability and penetration through the stratum corneum, which depend on the lipophilicity and molecular size of the active constituents, further determine whether a compound reaches cellular targets in sufficient concentration to exert a biological effect [42,43]. The modest chemical antioxidant activity reported here should therefore be interpreted as an indication of limited direct radical-scavenging capacity in vitro, rather than as a definitive statement about the preparation's antioxidant behaviour in skin; cell-based or ex vivo skin models would be needed to establish this translation.

3.5. Tyrosinase Inhibitory Activity

Tyrosinase is the rate-limiting enzyme in melanin biosynthesis, and its inhibition is a common target in the development of skin-whitening and anti-hyperpigmentation cosmetic agents. The oil macerate produced $38.75 \pm 0.17\%$ inhibition of mushroom tyrosinase at $200 \mu\text{g/mL}$, compared with $95.05 \pm 0.37\%$ for the reference inhibitor kojic acid at the same concentration (Table 3). This represents a measurable but moderate anti-tyrosinase effect, corresponding to roughly 40% of the inhibition produced by kojic acid.

As the activity was assessed at a single concentration, the present data should be regarded as a preliminary screening rather than a full potency determination; a complete dose–response characterization with IC_{50} determination remains to be carried out. Nevertheless, the observed inhibition is consistent with previous reports of tyrosinase inhibitory activity in *H. perforatum* preparations [17], and indicates that the macerate retains a modest depigmenting potential of possible relevance to cosmetic applications. Notably, this value is somewhat lower than the 49.75% inhibition reported at the same concentration ($200 \mu\text{g/mL}$) for a methanolic extract of *H. perforatum* [16], a difference in line with the lower transfer of polar active constituents into the lipophilic oil matrix compared with a polar solvent extract. To explore the molecular basis of this activity, the principal constituents of the macerate were subsequently examined by molecular docking against the tyrosinase active site (Section 3.6).

As for the antioxidant assays (Section 3.4), this measurement was performed without a plain olive-oil matrix control, so the extent to which the observed inhibition is attributable to *Hypericum*-derived constituents versus the olive-oil matrix itself cannot be determined from the present data. This is a non-trivial consideration here specifically, rather than a generic caveat: the docking results below indicate that several olive-oil fatty acids access the tyrosinase active site considerably more closely than the naphthodianthrone (Section 3.6), raising the concrete possibility that part of the 38.75% inhibition measured for the whole macerate originates from the olive-oil matrix itself rather than exclusively from *Hypericum*-derived compounds. Resolving this would require testing a plain olive-oil control prepared under identical conditions (sunlight exposure, storage, agitation, and filtration) but without plant material.

3.6. Molecular Docking

To explore the molecular basis of the observed tyrosinase inhibition and to assess the potential contribution of individual macerate constituents, the principal naphthodianthrone (hypericin, pseudohypericin, protohypericin, protopseudohypericin) and the major fatty acids were docked into the active site of mushroom tyrosinase (PDB: 2Y9X), alongside the reference inhibitors kojic acid and tropolone. Binding affinities and minimum distances to the catalytic copper ions are summarized in Table 4.

Table 4. Binding affinities, minimum distances to the catalytic copper ions, and number of catalytic histidine residues contacted (≤ 4 Å) for the docked compounds. Despite their higher affinity scores, the naphthodianthrone are positioned substantially farther from the dicopper centre and engage only a single catalytic histidine, in contrast to the reference inhibitors.

Compound	Affinity (kcal/mol)	Min. Cu400 (Å)	Min. Cu401 (Å)	Catalytic His (<i>n</i>)	Class
Hypericin	−7.76	7.05	7.71	1	Naphthodianthrone
Pseudohypericin	−7.73	8.67	8.36	1	Naphthodianthrone
Protopseudohypericin	−7.42	6.80	9.95	1	Naphthodianthrone
Protohypericin	−7.37	6.96	7.82	1	Naphthodianthrone
Linolenic acid	−6.23	4.10	4.03	2	Fatty acid
Tropolone	−6.00	4.11	3.87	4	Reference inhibitor
Kojic acid	−5.74	3.02	2.23	5	Reference inhibitor
Arachidic acid	−5.63	4.85	3.83	3	Fatty acid
Linoleic acid	−5.36	2.61	2.23	5	Fatty acid
Palmitic acid	−5.31	4.35	4.13	2	Fatty acid
Stearic acid	−5.28	4.64	4.08	3	Fatty acid
Palmitoleic acid	−5.27	4.60	3.57	3	Fatty acid
Oleic acid	−5.20	3.90	3.94	4	Fatty acid
Myristic acid	−4.43	3.10	2.57	4	Fatty acid

Catalytic His (*n*) denotes the number of the six active-site copper-coordinating histidine residues (His61, His85, His94, His259, His263, His296) within 4 Å of the ligand. Compounds are sorted by binding affinity. Full contact details, including the specific catalytic histidine residues engaged by each ligand, are provided in Table S1.

The docking protocol was first validated by re-docking the co-crystallized inhibitor tropolone into the prepared active site. The re-docked pose reproduced the binding location of the crystallographic ligand, with a centroid displacement of 0.91 Å from the original position, confirming correct placement within the catalytic pocket. The heavy-atom RMSD was 2.9 Å; this relatively high atom-level value, despite the close centroid agreement, reflects the small, near-symmetric structure of tropolone, for which minor in-plane reorientations produce disproportionately large RMSD values. Overall, the re-docking confirmed that the grid configuration correctly targeted the dicopper catalytic site.

The four naphthodianthrone returned the most favourable affinity scores (−7.37 to −7.76 kcal/mol), exceeding those of the reference inhibitors kojic acid (−5.74 kcal/mol) and tropolone (−6.00 kcal/mol). On the basis of affinity alone, this might suggest strong inhibitory binding. However, examination of the binding geometry reveals that this interpretation would be misleading. The minimum distances from the naphthodianthrone to the catalytic copper ions ranged from 6.80 to 9.95 Å, placing them well outside the dicopper coordination sphere, and each contacted only a single catalytic histidine (His85). In contrast, kojic acid and tropolone coordinated directly to the copper centre (2.23 and 3.87 Å, respectively) and each engaged four to five catalytic histidine residues (among His61, His85, His259, His263, and His296), consistent with genuine active-site binding.

Because tyrosinase inhibition is mediated through interaction with the dicopper active site, the spatial positioning of the naphthodianthrone is incompatible with direct catalytic inhibition: a molecule located 7–9 Å from the copper centre cannot coordinate the metal ions required for catalysis. Their high affinity scores are therefore most plausibly an artefact of the size- and contact-dependent bias of the AutoDock Vina scoring function, which tends to assign more favourable scores to large, polycyclic molecules with extensive van der Waals surface area, irrespective of their proximity to the catalytic site [35,36]. Such scoring bias is a recognized limitation of empirical docking functions and has been shown to produce misleading affinity rankings when not accompanied by inspection of the binding geometry [49,50]. The naphthodianthrone instead occupied a peripheral region of the active-site cleft, stabilized by contacts with second-shell residues such as His244 and Val283 (Figure 2A,B), reflecting surface-level accommodation rather than catalytic engagement.

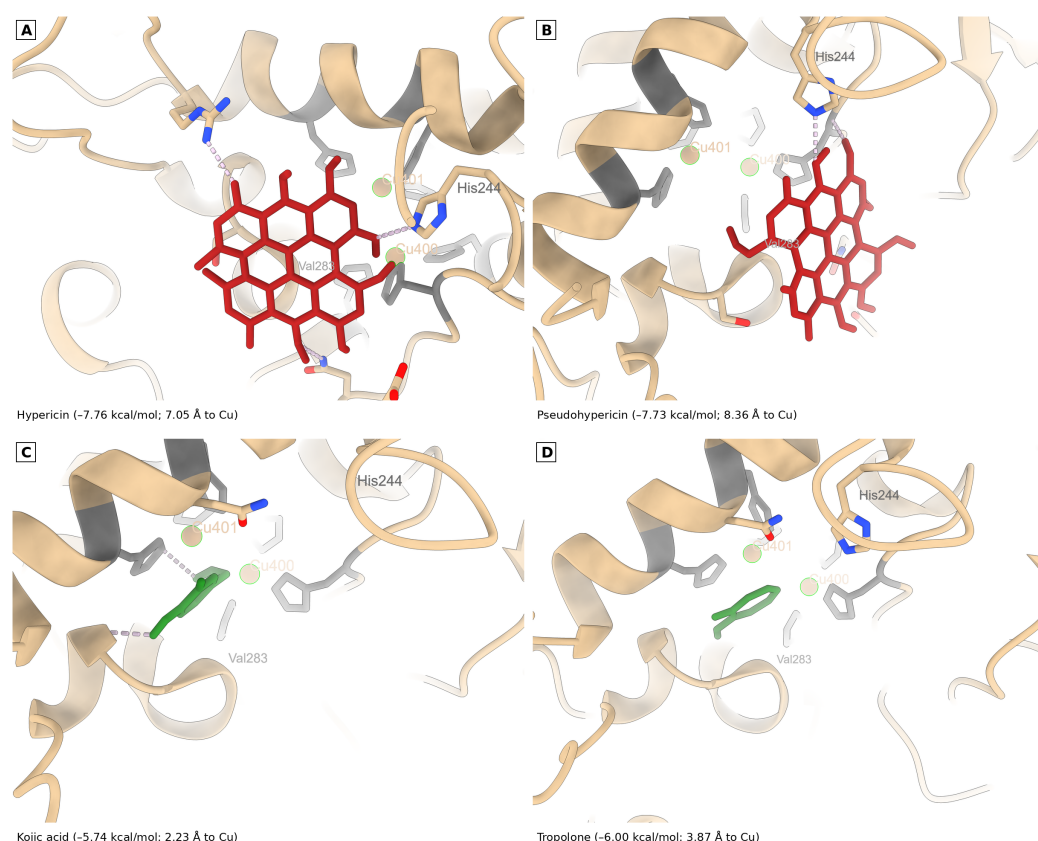


Figure 2. Representative docking poses in the active site of mushroom tyrosinase (PDB: 2Y9X). (A) Hypericin (−7.76 kcal/mol; 7.05 Å to Cu) and (B) pseudohypericin (−7.73 kcal/mol; 8.36 Å to Cu) (red) occupy a peripheral region of the active-site cleft, positioned 7–9 Å from the catalytic copper ions (Cu400/Cu401), despite their high affinity scores. (C) Kojic acid (−5.74 kcal/mol; 2.23 Å to Cu) and (D) tropolone (−6.00 kcal/mol; 3.87 Å to Cu) (green), the reference inhibitors, coordinate directly to the dicopper centre. Copper ions are shown as spheres; the second-shell residues His244 and Val283 are labelled, and hydrogen bonds are indicated by dashed lines.

Several olive-oil fatty acids approached the copper centre far more closely than the naphthodianthrones (minimum Cu distances of 2.2–4.6 Å) and engaged multiple catalytic histidine residues, comparable to the reference inhibitors (Table 4). Although their absolute affinity scores were modest (−4.43 to −6.23 kcal/mol), their proximity to the catalytic dicopper site indicates that matrix fatty acids are geometrically capable of accessing the active site. We note, however, that our GC-FID/MS analysis (Table 1) quantified total fatty acid content following derivatization of the lipid extract, reflecting fatty acids that are predominantly esterified as triglycerides in olive oil; it does not establish the free (non-esterified) fatty acid fraction available to interact directly with the enzyme, which was not separately determined (e.g., by free acidity/acid value titration) in this study. The docking results for these fatty acids should therefore be interpreted as an exploratory, geometry-based hypothesis regarding their potential to access the active site if present in free form, rather than as confirmation that free fatty acids at biologically relevant concentrations directly inhibit tyrosinase in this preparation. This is of particular interest given that unsaturated fatty acids, including oleic, linoleic, and α -linolenic acids, are well-documented modulators of melanogenesis and have been reported to suppress melanin synthesis and tyrosinase activity, with linoleic acid even applied topically against hyperpigmentary disorders [51–53]. In cellular systems this effect has been attributed largely to accelerated proteasomal degradation of the tyrosinase protein rather than to direct active-site inhibition; the present docking results suggest that a complementary mechanism, namely direct interaction with the catalytic

dicopper site, may also be geometrically feasible for these fatty acids. Computational approaches of this kind have previously been applied to other *Hypericum* species, including docking analyses that identified chlorogenic acid as a favourable tyrosinase binder [21]; the present work extends this strategy to the naphthodianthrone and matrix fatty acids of a traditional oil macerate. These observations support the view that the olive-oil matrix is not merely an inert carrier but an active participant in the preparation's measured bioactivity. It should be noted that our docking and in vitro assays each examined either individual compounds (docking) or the bulk macerate (bioactivity assays), rather than testing pairwise or multi-component combinations; potential synergistic or antagonistic interactions among the major constituents, such as competitive occupancy of the tyrosinase active site between fatty acids and naphthodianthrone, therefore cannot be resolved by the present data. The measured tyrosinase inhibition of the whole macerate (38.75%) reflects the net outcome of all such interactions rather than the simple additive contribution of its individual constituents; dissecting this further would require fractionation studies or combination assays testing defined mixtures of the identified constituents.

More generally, AutoDock Vina, like most standard empirical docking scoring functions, does not include an explicit potential for metal coordination chemistry (e.g., dative bonding, charge transfer, or the specific geometric constraints of copper coordination); it treats the copper ions as part of the rigid receptor and scores ligand poses using generic van der Waals, electrostatic, and hydrogen-bonding terms. Our use of minimum ligand-copper distance and catalytic-histidine contact count as criteria for active-site engagement is therefore a geometric proxy, qualitatively supported by re-docking of the co-crystallized ligand tropolone (Section 3.6), rather than a direct, physically validated measure of coordinate bond formation. Close proximity to the copper centre and contact with catalytic histidines are consistent with, but do not by themselves prove, genuine metal coordination and catalytic inhibition. Accordingly, the docking-based mechanistic interpretations in this study, for both the naphthodianthrone and the fatty acids, should be regarded as exploratory, hypothesis-generating findings rather than confirmed mechanistic evidence; direct confirmation would require methods better suited to metalloenzyme active sites, such as covalent/coordination-specific docking protocols, quantum mechanics/molecular mechanics (QM/MM) calculations, or site-directed mutagenesis and enzyme kinetics studies.

3.7. In Silico Physicochemical, Skin-Permeation, and Toxicity Profiling

To complement the in vitro findings and to interpret the likely cutaneous behaviour of the macerate constituents, the principal naphthodianthrone and major fatty acids were profiled in silico using SwissADME and pkCSM, with kojic acid included as a reference (Table 5). Skin permeability ($\log K_p$) was taken from SwissADME, as the pkCSM skin-permeability output was essentially invariant across all compounds (≈ -2.7) and therefore non-discriminating; SwissADME was thus used as the primary source for permeability and pkCSM for the toxicity panel.

A clear divergence in predicted skin permeability emerged between the two compound classes. The naphthodianthrone exhibited low predicted permeability ($\log K_p$ from -5.21 to -6.31 cm/s), in line with their high molecular weight (504–522 g/mol) and large polar surface area (TPSA 156–176 Å²), and all were predicted to have low gastrointestinal absorption. In contrast, the olive-oil fatty acids showed substantially higher predicted permeability ($\log K_p$ from -1.61 to -3.41 cm/s), in line with their lower molecular weight and minimal polar surface area (TPSA 37.3 Å²). The reference inhibitor kojic acid, a small hydrophilic molecule, showed the lowest predicted permeability ($\log K_p = -9.55$ cm/s). These predictions indicate that, upon topical application, the naphthodianthrone are

likely to remain at or near the skin surface, whereas the fatty acid matrix components are predicted to penetrate the stratum corneum more readily.

Table 5. Predicted physicochemical properties and skin permeability of the principal macerate constituents and the reference inhibitor kojic acid (SwissADME).

Compound	MW (g/mol)	TPSA (Å ²)	logK _p (cm/s)	GI abs.	Class
Hypericin	504.4	155.5	−5.32	Low	Naphthodianthrone
Protohypericin	506.5	155.5	−5.21	Low	Naphthodianthrone
Pseudohypericin	520.4	175.8	−6.31	Low	Naphthodianthrone
Protopseudohypericin	522.5	175.8	−6.19	Low	Naphthodianthrone
Myristic acid	228.4	37.3	−3.35	High	Fatty acid
Palmitic acid	256.4	37.3	−2.77	High	Fatty acid
Palmitoleic acid	254.4	37.3	−3.18	High	Fatty acid
Stearic acid	284.5	37.3	−2.19	High	Fatty acid
Oleic acid	282.5	37.3	−2.60	High	Fatty acid
Linoleic acid	280.5	37.3	−3.05	High	Fatty acid
Linolenic acid	278.4	37.3	−3.41	High	Fatty acid
Arachidic acid	312.5	37.3	−1.61	Low	Fatty acid
Kojic acid	142.1	70.7	−9.55	High	Reference inhibitor

MW, molecular weight; TPSA, topological polar surface area; logK_p, predicted skin permeability coefficient (more negative = lower permeability); GI abs., predicted gastrointestinal absorption.

The predicted toxicity profiles (Table 6) showed that the naphthodianthrone were negative for mutagenicity (AMES), hepatotoxicity, and skin sensitization. The fatty acids were likewise AMES-negative but were flagged as potential skin sensitizers and, in some cases, hepatotoxic; the skin-sensitization alerts for long-chain carboxylic acids are most plausibly false positives, as these are common dietary and endogenous compounds without established sensitizing activity, and should be interpreted with caution.

Importantly, the pkCSM toxicity panel does not include a phototoxicity endpoint, and the resulting “clean” profile of the naphthodianthrone must therefore not be interpreted as indicating photochemical safety. Hypericin and its congeners are well-established photosensitizers: upon absorption of light (including in the visible region responsible for the characteristic 590 nm absorption used for their quantification, Section 3.3), they generate reactive oxygen species, and excessive systemic exposure is known to cause the photosensitization syndrome termed hypericism [18]. This photochemical reactivity is an intrinsic property of the naphthodianthrone chromophore and is not captured by the in silico toxicity model.

Taken together, the in silico predictions and the established photochemistry converge on a coherent picture of the topical behaviour of the macerate. The low predicted skin permeability of the naphthodianthrone agrees with experimental evidence that hypericin penetrates poorly into the epidermis and produces only limited phototoxicity after topical application, whereas enhanced penetration markedly increases the phototoxic response [19]. Consistently, clinical evaluation of topical *Hypericum* oil has shown no severe phototoxic potential under standard conditions, with only a minor increase in photosensitivity detectable by sensitive instrumental measurement [20]. The macerate therefore emerges as a preparation whose principal photoactive constituents are largely retained at the skin surface: this limits systemic photosensitization under normal topical use, but, given the intrinsic photoreactivity of the naphthodianthrone, still warrants protection from light during storage and a recommendation to avoid intense sun exposure after application. We emphasize, however, that SwissADME and pkCSM model individual, isolated molecules and do not capture the behaviour of the actual oil formulation as a whole, including its true application concentration, the effect of occlusion (which can substantially increase percutaneous absorption relative to open, unoccluded conditions), or potential mixture

effects among co-formulated constituents. The cited clinical and experimental phototoxicity data [19,20] concern hypericin and *Hypericum* oil specifically, which supports but does not replace direct testing of the present preparation. Accordingly, the topical retention and safety picture presented here should be regarded as a plausible, literature- and prediction-supported hypothesis, and experimental skin-permeation (e.g., Franz diffusion cell) and phototoxicity studies on the actual macerate, under both occluded and non-occluded conditions, are required before firm conclusions can be drawn.

Table 6. Predicted toxicity profile of the principal macerate constituents and the reference inhibitor kojic acid (pkCSM).

Compound	AMES Toxicity	Hepato-Toxicity	Skin Sensitization ^a	Class
Hypericin	No	No	No	Naphthodianthrone
Protohypericin	No	No	No	Naphthodianthrone
Pseudohypericin	No	No	No	Naphthodianthrone
Protopseudohypericin	No	No	No	Naphthodianthrone
Myristic acid	No	No	Yes	Fatty acid
Palmitic acid	No	Yes	Yes	Fatty acid
Palmitoleic acid	No	Yes	Yes	Fatty acid
Stearic acid	No	No	Yes	Fatty acid
Oleic acid	No	No	Yes	Fatty acid
Linoleic acid	No	Yes	Yes	Fatty acid
Linolenic acid	No	No	Yes	Fatty acid
Kojic acid	No	No	No	Reference inhibitor

^a The skin-sensitization alerts for the long-chain fatty acids are most plausibly false positives, as these are common dietary and endogenous compounds without established sensitizing activity. Note that the pkCSM panel does not include a phototoxicity endpoint; the naphthodianthrone are established photosensitizers despite their otherwise clean predicted profile (see text).

4. Conclusions

This study provided a detailed phytochemical and in silico-supported characterization of a traditionally prepared *Hypericum perforatum* olive oil macerate intended for cosmetic application. The olive oil used as the maceration matrix was an oleic acid-dominated, oxidatively stable lipid (iodine value 86.0 g I₂/100 g; COX 1.84), and the resulting macerate contained modest levels of phenolics, flavonoids, and naphthodianthrone, the last quantified semi-quantitatively as total hypericin equivalents (0.044 mg/mL). In vitro, the preparation showed limited antioxidant activity but a measurable, moderate tyrosinase inhibition (38.75% at 200 µg/mL), supporting a modest depigmenting potential relevant to cosmetic use.

Integrating the experimental data with molecular docking and in silico profiling yielded the principal mechanistic insight of this work. Although the naphthodianthrone returned the most favourable docking scores, distance- and contact-based analysis demonstrated that they are positioned 7–9 Å from the catalytic dicopper centre and engage only a single catalytic histidine, indicating that their high scores are an artefact of the scoring function rather than evidence of genuine inhibition. The olive-oil fatty acids, by contrast, accessed the catalytic site more closely, suggesting that the matrix is an active contributor to the measured activity rather than an inert carrier. In parallel, in silico skin-permeability predictions, the established photochemistry of the naphthodianthrone, and their characteristic 590 nm absorption converged on a coherent picture: the principal photoactive constituents are largely retained at the skin surface, which limits systemic photosensitization under normal topical use while still warranting protection from light during storage and avoidance of intense sun exposure after application.

Several limitations should be acknowledged. All experimental measurements were performed on a single macerate batch, with the reported triplicates representing technical replicates rather than independently prepared biological batches; consequently, the reported means and standard deviations reflect measurement precision on this one preparation and do not capture batch-to-batch biological variability in plant material or the maceration process, and should not be generalized as representative of the preparation's biological variability without independent replication. The antioxidant and tyrosinase assays were performed without a plain olive-oil matrix control, so the measured activities cannot be unambiguously attributed to the macerated plant constituents alone; the tyrosinase activity was assessed at a single concentration, without a sample-only blank to rule out a contribution of the macerate's own colour or turbidity to the 475 nm absorbance reading; and the naphthodianthrone content was determined semi-quantitatively by UV spectrophotometry rather than by compound-specific LC-MS. The identity and relative proportions of the four individual naphthodianthrone selected for molecular docking were likewise not experimentally confirmed in this macerate by a compound-specific chromatographic method (e.g., HPLC-DAD or LC-MS), but were instead assumed on the basis of their well-established presence in *H. perforatum* in the literature; and the docking scoring function used does not explicitly model metal coordination chemistry, so proximity-based evidence of active-site engagement with the catalytic copper centre should be regarded as an exploratory, hypothesis-generating finding rather than confirmed mechanistic evidence. In addition, phytochemical stability during the six-week maceration period was not monitored experimentally in this study: the present measurements represent a single end-point analysis of the finished preparation, and the extent of naphthodianthrone degradation or transformation during maceration was inferred from prior literature reports [24] rather than determined directly by serial sampling.

Accordingly, future work should replicate the preparation across independently prepared biological batches; incorporate a plain olive-oil control; establish full dose-response relationships with IC₅₀ determination for tyrosinase inhibition using appropriate sample blanks and colour correction to account for the matrix's own absorbance; monitor phytochemical stability through time-course sampling during maceration; quantify the free (non-esterified) fatty acid fraction of the matrix (e.g., by free acidity/acid value titration) to establish what proportion, if any, is available to interact directly with enzyme targets; perform molecular dynamics simulations of the best-scoring docked complexes to assess the dynamic stability of the predicted binding poses over time, complementing the static, geometry-based analysis presented here; and extend the chemical characterization through quantitative LC-MS.

The present work was deliberately scoped around tyrosinase inhibition, given the traditional use of this preparation for skin-brightening and anti-hyperpigmentation purposes; broader cosmeceutical assays, such as collagenase, elastase, and hyaluronidase inhibition, would help characterize its relevance to skin ageing and wound-healing applications and are recommended for future studies. Ultimately, these directions would support the development and stability assessment of standardized cosmetic formulations based on this traditional preparation, including formulation-compatibility and physicochemical stability testing once the macerate is incorporated into a topical dosage form, which has not been undertaken in the present phytochemical characterization study.

More broadly, the phytochemical and mechanistic data generated in this study are derived from chemical, cell-free, and in silico methods; they characterize the constituents and their plausible mode of action but do not, by themselves, establish clinical or consumer-relevant efficacy or safety. This is distinct from the pre-existing clinical evidence for the traditional preparation's wound-healing use cited in the Introduction, which pertains to

the whole product rather than to the specific mechanistic findings reported here. Direct extrapolation of the present in vitro/in silico results to clinical or consumer claims should therefore be avoided until supported by cell-based, ex vivo, or clinical validation.

Taken together, the sources of variability identified above point to concrete quality-control strategies for a standardized botanical cosmetic based on this traditional preparation. Botanical sourcing should be controlled through defined geographical origin, chemotype, and harvest timing, supported by voucher-specimen authentication, since these factors substantially affect the plant-derived phytochemical starting material. The olive-oil matrix itself should be characterized and certified batch to batch (fatty acid profile, iodine value) given its own contribution to the measured bioactivity. Maceration parameters, including light exposure, temperature, and duration, should be fixed and validated against a time-course stability profile, so that batches are consistently harvested at or near the naphthodianthrone content optimum rather than after post-peak degradation. Analytically, semi-quantitative UV spectrophotometry should be complemented, for release testing, by compound-specific quantification (e.g., LC–MS or HPLC–DAD) of the naphthodianthrone and principal phenolic constituents. Finally, a functional, bioactivity-based release specification, such as a full dose–response tyrosinase inhibition assay with defined IC₅₀ acceptance criteria, would provide a more direct quality marker than chemical composition alone, since it integrates the net effect of the matrix and plant-derived constituents together. Adopting such combined botanical, analytical, and functional controls would be a necessary step toward translating this traditional remedy into a reproducible, standardized cosmetic ingredient.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/cosmetics13040194/s1>, Table S1: Detailed molecular docking contact analysis for all docked compounds. File S1: Batch molecular docking script (run_bulk_docking.py). File S2: Post-docking contact analysis script (analyze_contacts.py). File S3: AutoDock Vina configuration file (config.txt).

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Abbreviations

The following abbreviations are used in this manuscript:

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
AMES	Ames mutagenicity test
COX	Calculated oxidizability
CUPRAC	Cupric ion reducing antioxidant capacity
DPPH	2,2-diphenyl-1-picrylhydrazyl
FID	Flame-ionization detector
GC	Gas chromatography
GI	Gastrointestinal
IV	Iodine value
LC–MS	Liquid chromatography–mass spectrometry
MS	Mass spectrometry
MUFA	Monounsaturated fatty acid
PCE	Pyrocatechol equivalent
PUFA	Polyunsaturated fatty acid
QE	Quercetin equivalent
RSD	Relative standard deviation
SFA	Saturated fatty acid
TFC	Total flavonoid content
TPC	Total phenolic content
TPSA	Topological polar surface area
UFA	Unsaturated fatty acid

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