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A supramolecular caffeic acid with enhanced water solubility, stability, and bioactivity

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1. Introduction

Caffeic acid (CA) is a phenolic acid compound widely found in various plants and is known for its diverse biological activities, including free radical scavenging, anti-inflammatory, antioxidant, antibacterial, antiviral, antitumor, UV absorption, and collagen regulation activities. [1] These properties make CA an attractive, active ingredient in cosmetics, potentially delaying skin aging, reducing pigmentation, and promoting skin repair. [2] The two phenolic hydroxyl groups in CA confer strong antioxidant capabilities, while the unsaturated carboxylic acid chain increases the conjugation effect of the molecule, further stabilizing free radicals and enhancing antioxidant efficacy. However, the molecular structure of CA also makes it susceptible to oxygen, light, and temperature, leading to reduced biological activity. Moreover, CA's inherent poor water solubility (<1 mg mL^{-1}) limits its application in cosmetic formulations.

In recent years, supramolecular technology has garnered significant attention in drug delivery due to its designability, green procedure, and minimal impact on precursor properties. [3, 4] Supramolecular crystal technology is an efficient drug modification strategy that can precisely control the structure and properties of drugs at the molecular level. [5] In transdermal delivery, supramolecular crystal technology can be used to balance the water solubility and lipophilicity of drugs to achieve higher bioavailability. [6-8] Moreover, non-covalent interactions in supramolecular crystals, especially hydrogen bonds, can modulate the antioxidant activity of phenolic compounds. [9] Matrine is a plant-derived alkaloid with relatively weak basicity and polarity. [10] In previous work, we prepared a supramolecular crystal of CA and matrine (SupCA, CCDC 2046794) and demonstrated its superior water solubility and anti-inflammatory, antioxidant, and antibacterial capabilities than CA in vitro experiments. [11] However, the lack of research on its stability, structure-activity relationship, and in vivo experiments has hindered its clinical application. Here, we systematically studied its structure-activity relationship through density functional theory (DFT), molecular docking, and molecular dynamics (MD) simulations and comprehensively investigated its skincare benefits through clinical trials, including

soothing, anti-wrinkle, acne-fighting, repairing, and whitening effects, to prove the mechanisms further. This study provides data and theoretical support for the commercial application of SupCA, which is supposed to be a natural, safe, efficient, and stable novel active ingredient for the cosmetic and pharmaceutical fields.

2. Materials and Methods

2.1. Synthesis and characterization of SupCA

First, three mM caffeic acid (98% Aladdin) was dissolved in 50 mL ethanol under continuous stirring at 300 rpm. Subsequently, three mM matrine (98% Aladdin) was slowly added to the caffeic acid solution in batches to ensure complete dissolution. After reacting at 60 ℃ under N_2 in darkness for 24 h, SupCA was collected by removing the solvent of the mixture solution via vacuum drying. Finally, we washed the product using cold ethanol to obtain SupCA of high purity. The stability of SupCA was tested with the assistance of a Shimadzu 1900i UV-vis spectrophotometer (Tokyo, Japan). The CA concentration was determined using its absorbance peak at around 290 nm, which corresponds to the phenolicπ-π transition.

2.2. Calculations

The molecular and electron structure of SupCA was studied using the ORCA 5.0.4 software based on the density functional theory (DFT). [12] The molecular structure and single-point energies were calculated at the B3LYP 6-31G(d,p) and M062X 6-311++G(d,p) levels, respectively, with the implicit solvation model (SMD) simulating aqueous solutions. [13] The obtained wave functions were analyzed and visualized using the Multiwfn 3.8 dev and VMD 1.9.3 programs. [14, 15]

The anti-inflammatory mechanism of SupCA was investigated through molecular docking and dynamics simulations using AutoDock and GROMACS tools based on the Molecular Mechanics-Poisson-Boltzmann Surface Area (MM-PBSA) method. [16] The docking studies were conducted with three target proteins: IκB kinase β (IKKβ, PDB ID: 3RZF), cyclooxygenase-2 (COX2, PDB ID: 5F1A), and extracellular signal-regulated kinase 1 (ERK1, PDB ID: 4QTB). The optimized docking structures were subjected to MD simulations for 100 ns for better accuracy and reliability. The interactions between supCA and the target proteins were analyzed based on the MD trajectories after equilibrium.

2.3. Clinical trials

The clinical trials (Project No. ZY-CE-242041 and ZY-CE-242042, by the Medical Ethics Committee of Shanghai Zhengyan Technology Service Co., Ltd) were performed on no less than 30 subjects according to relevant Chinese standards: "Safety and technical standards for cosmetics (2015 Edition)", "TB/ZGKSL 001-2022 Evaluation criteria for human skin aging", "T/CAFFCI 67-2023 Test methods for anti-acne efficacy of cosmetic products", and "T/CAB 0152-2022 Seven efficacy test methods for anti-wrinkle, firming, moisturizing, oil control, repairing, nourishing and soothing of cosmetics."

We evaluated the clinical efficacy of SupCA from multiple dimensions and compared it to an equivalent mixture of matrine and CA, with a molar ratio of 1:1. To simulate the practical application condition, we added 2 wt% of SupCA to a basic emulsion formulation. The control sample was the basic emulsion containing 1.09 wt% matrine and 0.79 wt% CA. In the efficacy tests, the subjects' right and left faces were randomly divided into control and test areas, which were treated with the mixture and SupCA samples, respectively, twice a day, once in the morning and once in the evening. The skin parameters of subjects' right and left faces

were recorded before the application of samples, 14 days after treatment, and 28 days after treatment.

2.4. Statistical analysis

With the assistance of the GraphPad Prism 9.5 software, the clinical data were expressed as the mean ± (standard deviation) SD and analyzed using the paired t-test. $p < 0.05$ was defined as a significant difference.

3. Results and discussion

3.1. Structure of SupCA

The independent gradient model based on the Hirshfeld partition (IGMH) map of CA single crystals (CCDC 780650) reveals abundant hydrogen bonding between CA molecules (**Figure 1a**). [17] The atoms-in-molecules (AIM) analysis reveals that two relatively strong hydrogen bonds exist between adjacent carboxyl groups, unfavorable for CA's water solubility. Meanwhile, the phenolic hydroxyl groups are only involved in weak hydrogen bonds, making them potential active sites and unstable. There are also extensive hydrogen bonds in SupCA single crystals (Figure 1b). The introduction of matrine prevents hydrogen bonding between CA carboxyl groups and enhances the strength of hydrogen bonds involving CA's phenolic hydroxyls, probably leading to enhanced solubility and stability. Compared to CA, SupCA's meta-phenolic hydroxyl group (m-OH) features similar charge distribution but a shorter bond length, while the para-phenolic hydroxyl group (p-OH) exhibits reduced charge difference and bond length, which is probably the reason for higher stability and antioxidant activity (Figures 1c and 1d). As shown in the electrostatic potential (ESP) isosurface (Figures 1e and 1f), the supramolecular interactions from matrine increase the negative charge distribution of CA's p-OH, reducing its charge difference. Moreover, the polarity of SupCA is significantly higher than that of CA, which is likely the reason for its increased water solubility.

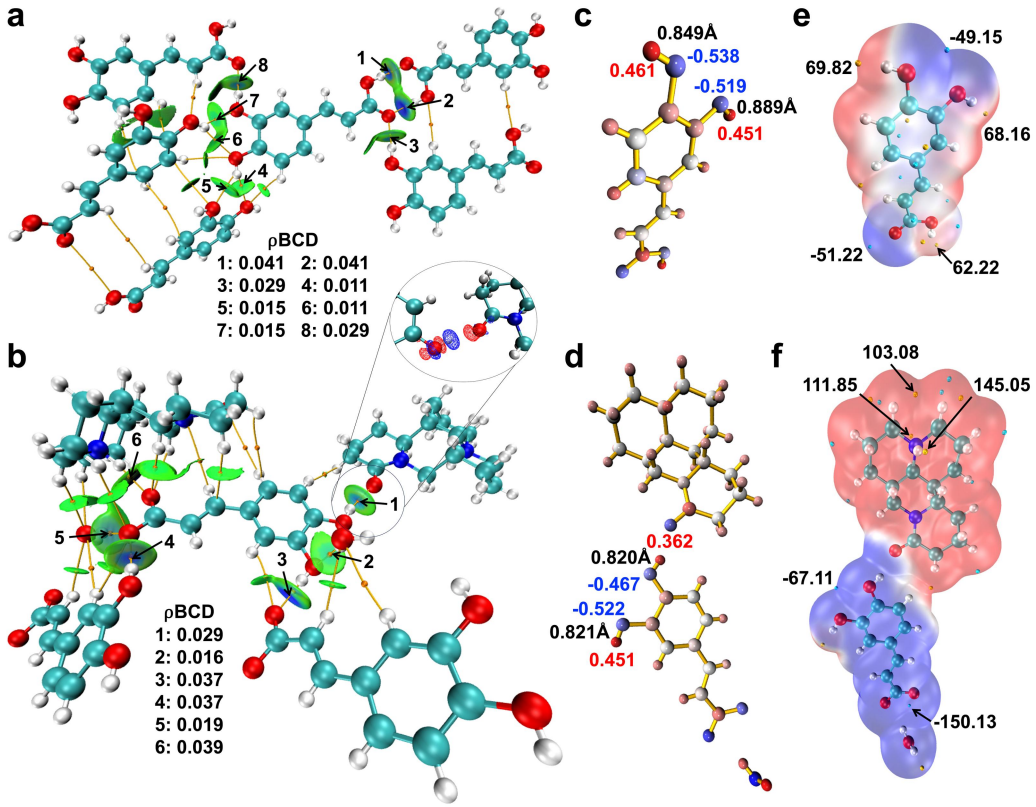

Figure 1. Structure of SupCA and CA crystals. (a-b) IGMH and AIM analysis of SupCA and CA crystals. (c-d) Charge distribution and bond length of the phenolic hydroxyl groups in SupCA and CA. (e-f) ESP analysis of SupCA and CA molecules.

3.2 Structure-activity relationship of SupCA

Compared to CA, matrine exhibits a significantly larger energy gap (Δ*E*g), indicating lower reactivity (**Figure 2a**). In SupCA, both the the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO) reveal that CA is the active site in SupCA. Compared to CA, SupCA has higher HOMO and LUMO energies, indicating easier to lose electrons and harder to gain electrons, consistent with lower Vertical Ionization Potential (VIP) and Vertical Electron Affinity (VEA) (Figure 2b). The lighter color of SupCA than CA implies higher stability (Figure 2c). NaCA's absorbance spectrum is significantly different from those of CA and SupCA, indicating a huge electronic distribution change of its CA moiety (Figure 2d). The lower loss of SupCA after UV and heating treatments than CA and NaCA highlights its superior stability (Figure 2e).

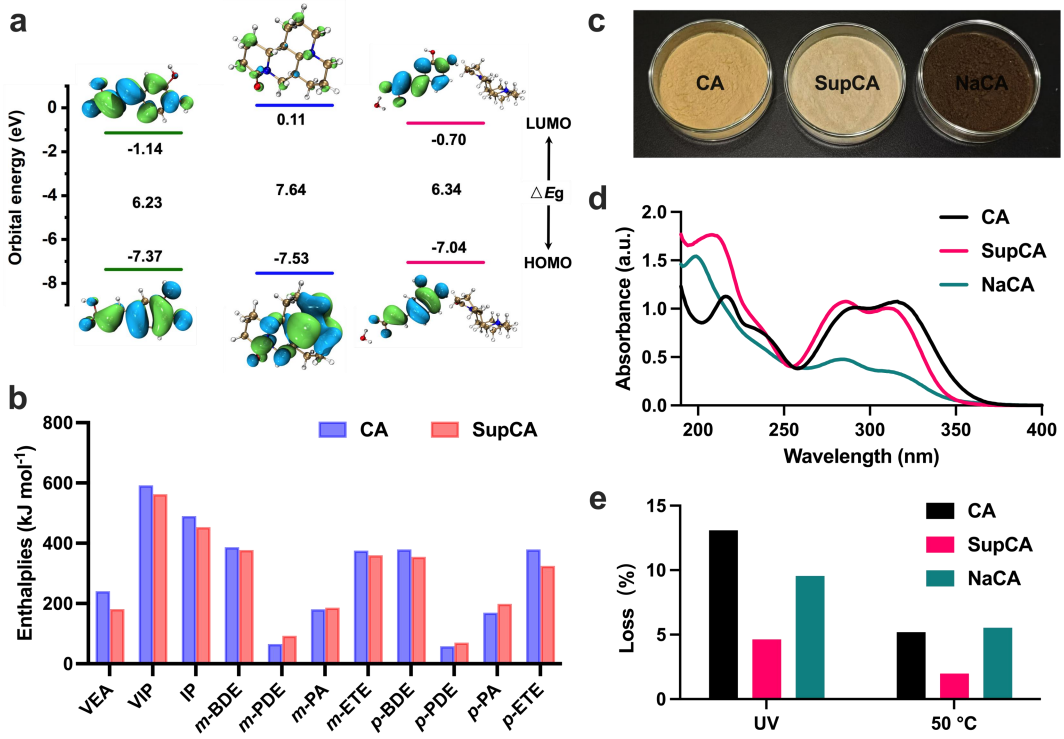

Figure 2. Antioxidant mechanism and stability of SupCA. (a) HOMO and LUMO analysis of CA, matrine, and SupCA. (b) Relative enthalpy energies of CA and SupCA in different antioxidant procedures. (c) Photographs of CA, SupCA, and NaCA. (d) Absorbance spectra of 0.08 mM CA, SupCA, and NaCA in aqueous solutions. (e) CA loss of 0.08 mM CA, SupCA, and NaCA in aqueous solutions after 3 d UV irradiation (254 nm) or 50 ℃ treatment.

Hydrogen atom transfer (HAT), single electron transfer-proton transfer (SET-PT), and sequential proton loss-electron transfer (SPLET) are the three primary mechanisms by which phenolic acids scavenge free radicals. [18] To elucidate the reasons for the superior antioxidant activity of SupCA compared to CA, we calculated the bond dissociation energies (BDEs), ionization potentials (IPs), protonation energies (PDEs), proton affinities (PAs), and electron transfer energies (ETEs) of m-OH and p-OH (Figure 2b). Notably, the PAs of CA and SupCA are significantly lower than their IPs and BDEs, suggesting that they primarily exert

antioxidant effects through the SPLET procedure. [19-21] In SupCA, the PAs of the p- and m-OH groups are 199.33 and 186.55 kJ mol^{-1}, higher than those of CA, especially for the p-OH, indicating enhanced stability. However, the total SPLET energies, BDEs, and IP of SupCA are lower than those of CA, indicating enhanced antioxidant activity. In summary, by forming hydrogen bonds with CA, SupCA inhibits the proton transfer of the most reactive p-OH group of CA, thereby enhancing its stability. Simultaneously, by altering the electron distribution of CA, SupCA enhances its electron-donating and O-H bond dissociation ability, thereby improving antioxidant activity.

CA exerts anti-inflammatory effects by modulating multiple cellular signaling pathways. The total binding free energy (ΔG_t) between SupCA and IKKβ was more negative than between CA and IKKβ, indicating stronger interactions (**Figure 3a**). Compared to CA, the modified van der Waals free energy (ΔG_{vdw}), electrostatic free energy (ΔG_{ele}), polar solvation free energy (ΔG_{pb}), and nonpolar solvation free energy (ΔG_{np}) of SupCA with IKKβ resulted in weaker polar interactions ($\Delta G_p = \Delta G_{ele} + \Delta G_{pb}$) and enhanced nonpolar interactions ($\Delta G_n = \Delta G_{vdw} + \Delta G_{np}$). The fewer hydrogen bonds (H-bonds) (Figure 3b) and more negative ΔG_{pb} for SupCA suggest that coulombic interactions between SupCA and IKKβ are stronger than between CA and IKKβ, which helps to counteract solvation effects. The interactions between CA and IKKβ are complex, involving polar, nonpolar, and charged residues (Figure 3c). These residues exhibit higher binding affinities for SupCA than for CA (Figure 3d), indicating that the supramolecular interactions of SupCA enhanced the binding strength between CA and IKKβ without altering their coordination mode.

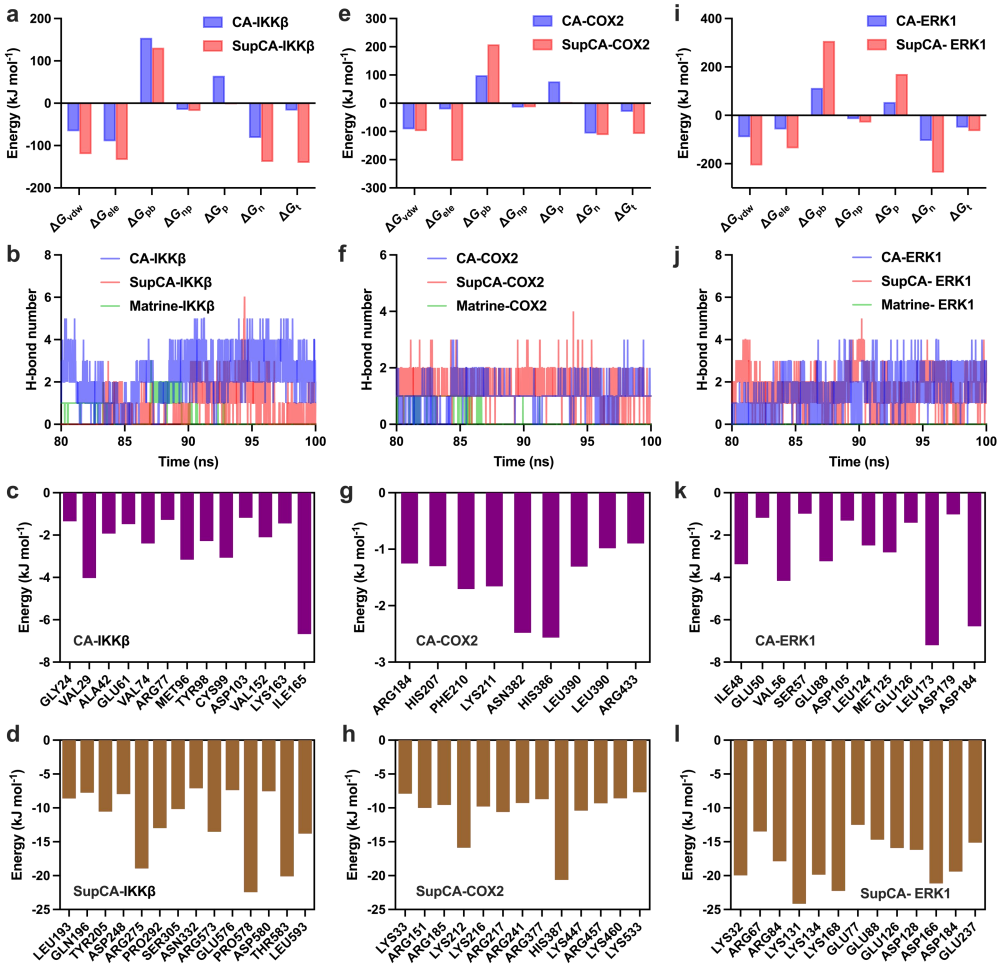

Figure 3. Anti-inflammatory mechanism of SupCA. (a-d) Binding energies, H-bond numbers, and residue contributions between ligands (CA or SupCA) and IKKβ. (e-h) Binding energies,

H-bond numbers, and residue contributions between ligands (CA or SupCA) and COX-2. (i-l) Binding energies, H-bond numbers, and residue contributions between ligands (CA or SupCA) and ERK1.

The more negative ΔG_t of SupCA-COX2 reveals stronger binding interactions than CA-COX2 (Figure 3e). The mean H-bond number between matrine and COX2 was 0.63, with increased H-bond number between CA and COX2, indicating stronger hydrogen binding between SupCA and COX2 than between CA and COX2 (Figure 3f). In SupCA-COX2, the increased contribution of positively charged residues indicates enhanced ionic interactions between CA segment and COX-2 (Figures 3g and 3h). The coordination of SupCA-ERK1 exhibited a more negative ΔG_t than CA-ERK1 due to modified ΔG_{vdw}, ΔG_{ele}, and ΔG_{np} (Figure 3i). The more negative ΔG_{ele} and similar H-bond number of SupCA-ERK1 compared to CA-ERK1 suggest enhanced ionic interactions (Figure 3j). The interactions between CA and ERK1 mainly involved hydrophobic and negatively charged residues (Figure 3k), probably due to the hydrophobicity of CA and its hydrogen binding with ERK1. For SupCA-ERK1, the positively charged residues contributed more than the negatively charged and hydrophobic residues (Figure 3l), probably due to the increased negative charge distribution of CA in SupCA.
In summary, the stronger anti-inflammatory capacity of SupCA compared to CA is attributed to its enhanced interaction with key proteins in multiple inflammatory signaling pathways, particularly the NF-κB pathway. The small H-bond numbers between matrine and proteins and the minor contribution of negatively charged residues demonstrate that the superior anti-inflammatory ability of SupCA over CA stems primarily from the modulation of the electronic structure of CA by supramolecular interactions between matrine and CA, rather than from the additive effects of the two.

3.3 Skincare benefits of SupCA

In clinical trials, both the mixture and SupCA emulsions exhibited significant soothing ability after 14 d and 28 d applications (**Figure 4**). The red area portion of subjects' faces reduced by 28.38% and 10.26% after 14 d treatment with the SupCA and mixture emulsions, respectively, and by 65.62% and 31.17% at 28 d (Figure 4a). The a* value of subjects' faces reduced by 6.40% and 5.66% after 14 d treatment with the SupCA and mixture emulsions, respectively, and by 13.27% and 9.90% at 28 d (Figure 4c). After treatment with the mixture and SupCA emulsion, the red area portion and a* value decreased with increasing time, indicating a time-dependent soothing ability of SupCA and the mixture. The changes of the red area portion at 14 d and 28 d on the SupCA side, as well as the change of a* value at 28 d, were significantly higher than those on the mixture side (Figures 4b and 4d), demonstrating the superior anti-inflammatory and antioxidant ability of SupCA compared to mixture.

In clinical trials, both the mixture and SupCA emulsions exhibited significant anti-wrinkle ability after 14 d and 28 d applications (**Figure 5**). The area portion of fine lines under eyes on subjects' faces reduced by 23.74% and 20.71% after 14 d treatment with the SupCA and mixture emulsions, respectively, and by 45.07% and 32.80% at 28 d (Figure 5a). The area portion of crow's feet wrinkles on subjects' faces reduced by 24.17% and 18.04% after 14 d treatment with the SupCA and mixture emulsions, respectively, and by 38.09% and 30.98% at 28 d (Figure 5c). After treatment with the mixture and SupCA emulsion, the fine lines under eyes and crow's feet wrinkles decreased with increasing time, indicating a time-dependent anti-wrinkle ability of SupCA and the mixture. The area portion changes of fine lines under eyes and crow's feet wrinkles at 28 d on the SupCA side were significantly higher than those

on the mixture side (Figures 5b and 5d), demonstrating the superior anti-wrinkle ability of SupCA compared to the mixture.

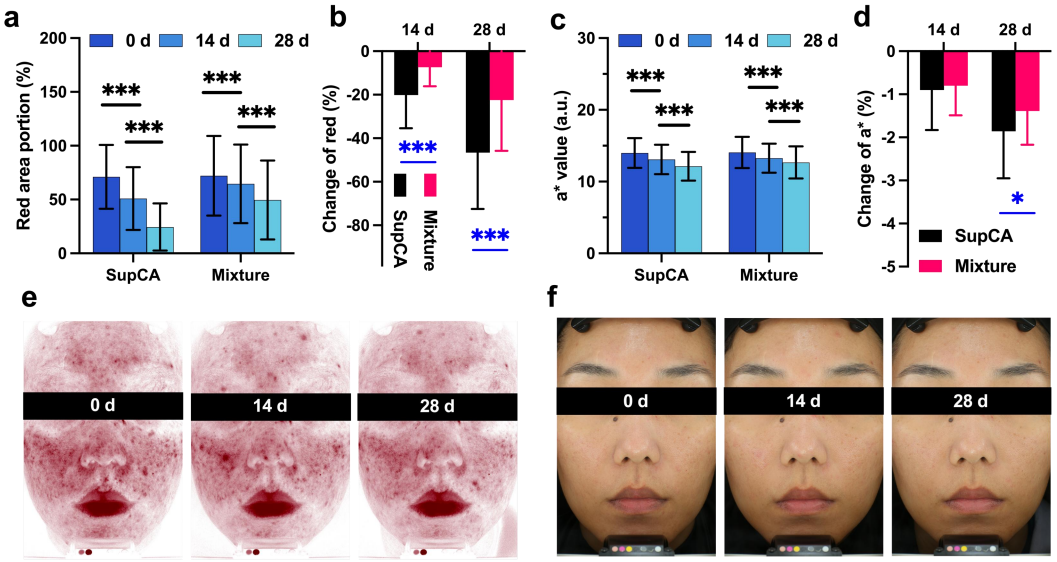

Figure 4. Soothing ability of SupCA. (a-b) Red area portion and its change of the subjects' faces treated with the mixture emulsion on one side and the SupCA emulsion on the other side. (c-d) a* value and its change of the subjects' faces treated with the mixture emulsion on one side and the SupCA emulsion on the other side. Results are shown as means ± SDs, n = 30, *p < 0.05, **p < 0.01, ***p < 0.001. (e-f) Red lesion and standard photographs of a representative subject's face treated with the mixture and SupCA emulsions on the left and right sides, respectively.

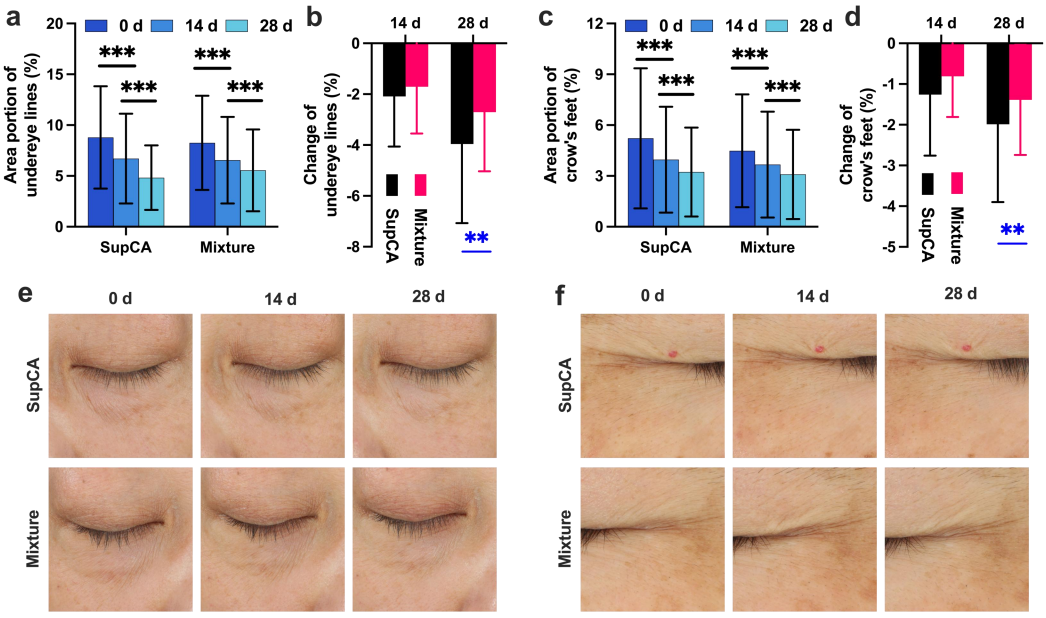

Figure 5. Anti-wrinkle ability of SupCA. (a-b) Area portion of fine lines under eyes and its change of subjects after treatment with the mixture and SupCA emulsions on each side of their faces. (c-d) Area portion of crow's feet wrinkles and its change of subjects after treatment with the mixture and SupCA emulsions on each side of their faces. Results are shown as means ± SDs, n = 32, *p < 0.05, **p < 0.01, ***p < 0.001. (e-f) Representative photographs of fine lines under eyes and crow's feet wrinkles of the subjects after treatment with the mixture and SupCA emulsions.

The sebum level on subjects' faces reduced by 29.22% and 43.47% after 14 d and 28 d treatment with the SupCA emulsion, respectively (**Figure 6a**). Since acne formation is closely associated with inflammatory responses and excessive sebum production, the SupCA emulsion effectively inhibited various acne lesions, resulting in a 57.00% reduction in the total acne number at 28 d (Figures 6f and 6g). The significant increase in skin hydration and decrease in TEWL demonstrate the SupCA emulsion's efficacy in repairing the skin barrier (Figures 6b and 6c). The significant increase in ITA° and decrease in MI values demonstrate the brightening effects of the SupCA emulsion (Figures 6d and 6e). Subjects' self-assessment of skin condition agreed with the above objective instrumental data (Figure 6h). Additionally, the SupCA emulsion achieved 100% satisfaction among subjects' sensory evaluations (Figure 6i), with no adverse reactions, making SupCA a safe, versatile, and efficient ingredient for cosmetics.

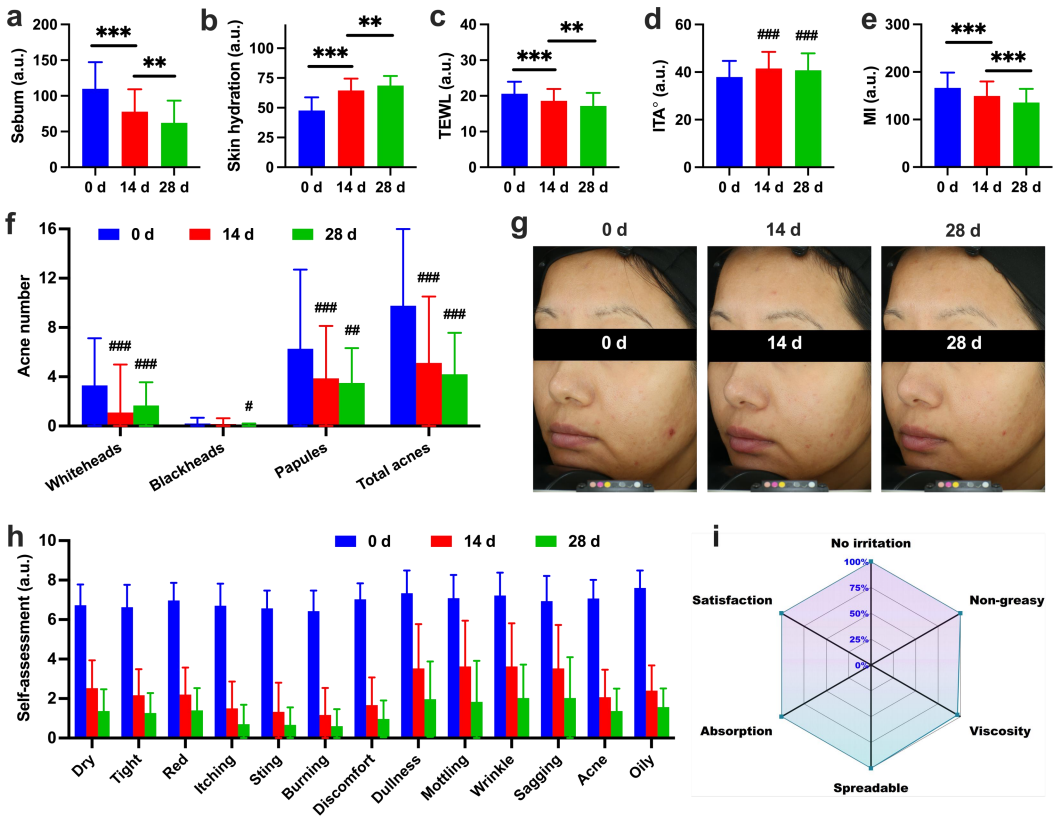

Figure 6. Anti-acne, repair, and whitening ability of SupCA. (a) Sebum level, (b) skin hydration, (c) TEWL, (d) ITA°, (e) MI, (f) acne number, (g) representative photographs, and (h) self-assessment of subjects after treatment with the SupCA emulsion on their faces. The self-assessment of skin condition is categorized into a 10-point scale (0-9 points): 0 indicates no such concern, and 9 represents a very severe issue; a higher score means a more severe condition. Results are shown as means ± SDs, n = 30, *p < 0.05, **p < 0.01, ***p < 0.001; #p < 0.05, ##p < 0.01 versus 0 d. (i) Sensory evaluation of the SupCA emulsion from subjects (scored using 1 to 5 points, indicating "strongly disagree," "somewhat disagree," "neutral" (neither agree nor disagree), "somewhat agree," and "strongly agree," respectively). The agreement rate among subjects is defined as the percentage of subjects who selected "strongly agree" and "somewhat agree" out of the total number of subjects.

5. Conclusion

This work provided a simple supramolecular strategy to enhance the stability and bioactivity of CA. The structure of SupCA was studied based on single crystal analysis, and its structure-activity relationship was investigated through DFT, molecular docking, and MD simulations. The skincare benefits of SupCA were determined in clinical trials and compared with the mixture of CA and matrine to prove the vital role of its supramolecular interactions on its bioactivities. Results show that SupCA exhibited significantly superior soothing and anti-wrinkle effects than the mixture, consistent with the calculation results. Meanwhile, SupCA has excellent anti-acne, skin repair, and whitening performance and can sustain skincare benefits. These findings provide a scientific basis for the broad application of SupCA in cosmetics, with new insights and approaches for CA's modification. Future research will further explore the potential applications of SupCA in addressing other skin concerns and optimize its formulations to enhance market competitiveness.

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