Study of lavender essential oil encapsulated chitosan-derivative nanoparticles on shampoo applications

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Abstract. Since lavender essential oil (LEO) contains antibacterial, anti-inflammatory, and calming qualities, it is commonly utilized in hair care products. However, adding LEO conventionally results in quick evaporation and a shorter product shelf life due to its high volatility. In this study, chitosan-derivative nanoparticles (CSNPs) were initially prepared by degradation, modification, and purification. LEO was then encapsulated to create LEO-CSNPs, and FTIR, DLS, TEM, and ^{1}H-NMR were used to evaluate both CSNPs and LEO-CSNPs. The effectiveness of the shampoos was next assessed by clinical trials and in vitro tests, such as malassezia inhibition, sebocyte lipid secretion, DPPH radical scavenging as well as qPCR for inflammatory markers with varying concentrations of LEO-CSNPs. The findings confirmed successful encapsulation since LEO-CSNPs (352.27 nm) had a higher particle size than CSNPs (218 nm). With a 15.5 mm Malassezia inhibition zone, 87.71% sebocyte lipid inhibition, 79.03% DPPH scavenging rate, and a notable downregulation of IL-6 and IL-8 expression. Meanwhile, the 0.5% LEO-CSNPs group demonstrated concentration-dependent efficacy. In terms of clinical results, fragrance longevity was increased to 7.2 hours, scalp flaking was decreased by 77.73%, and itching was also decreased by 69.05%. In conclusion, encapsulating LEO in chitosan-derived nanoparticles resolved its volatility problem and improved the antifungal, sebum-regulating, antioxidant, and anti-inflammatory properties in shampoo. Finally, the ideal concentration of LEO-CSNPs is between 0.3% and 0.5%, laying the groundwork for multipurpose shampoo.

1 Introduction

The integration of nanotechnology in cosmetic formulations has revolutionized the personal care industry, particularly in the development of advanced shampoo systems. One of the most promising innovations involves the encapsulation of essential oils into biopolymeric nanoparticles to enhance their stability and efficacy[1]. Lavender essential oil (Lavandula angustifolia) has gained significant attention in hair care products due to its

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pleasant aroma and potential therapeutic properties, including antimicrobial, anti-inflammatory, and relaxation effects[2, 3]. However, the volatile nature of essential oils presents substantial challenges for formulators, as conventional incorporation methods often result in rapid evaporation and reduced shelf-life of the final product[4].

Recent advancements in encapsulation technologies have enabled the development of sophisticated delivery systems that not only preserve the integrity of essential oils but also enhance their performance through improved scalp penetration and targeted delivery. This comprehensive analysis examines the current research on lavender essential oil encapsulated in chitosan-derivative nanoparticles, focusing on preparation methods, characterization techniques, functional properties, and specific applications in shampoo products, along with discussing future perspectives and challenges in commercial implementation[5, 6].

Chitosan, a natural biopolymer derived from chitin, has emerged as an excellent encapsulation material due to its biodegradability, non-toxicity, and mucoadhesive properties[7, 8]. The molecular structure of chitosan, containing reactive amino and hydroxyl groups, allows for various chemical modifications to enhance its functionality for specific applications. When combined with lavender essential oil through nanoencapsulation techniques, chitosan-derivative nanoparticles can effectively protect the volatile compounds while providing controlled release mechanisms that extend the fragrance duration and therapeutic benefits in shampoo formulations[9, 10]. Due to the insoluble of natural chitosan in water, various dydrophilic groups such as Polyethylene Glycol(PEG), carbohydrate, trimethyl ammounium, phosphoryl groups and sulfuryl have been utilized for developing a amphiphilic derivative to form nanoparticles in aqueous solution. Chitosans can form nanopaticles because of interacting with polyanionic biomolecules such as proteins, nucleic acids and mucins. The amphiphilicity of chitosans prompted the micell formation and the cation-anion interations help anionic compounds wrapped in the cavity of chitosans. In our study, the cationic chitosan binds with a PEG group with one end of the hydroxyl group to increase its compability with lipids in skin and the other end of amino group was replaced by arginine to increase its positive charge content.

The polysaccharide network of chitosan can physically encase lavender essential oil. Their long, flexible chains form a tight coating in cross - linking, confining the oil molecules. Besides electrostatic interactions, chitosan and lavender essential oil may form hydrogen bonds to enhance encapsulation. Hydroxyls on chitosan and polar groups in the oil can form hydrogen bonds, improving microcapsule stability and oil encapsulation efficiency[11].

The integration of chitosan-coated lavender essential oil into shampoo formulations represents a significant advancement in cosmetic science and hair care technology[12]. This innovative approach combines the beneficial properties of lavender essential oil with the enhanced stability and controlled release provided by chitosan encapsulation. Lavender essential oil, derived primarily from Lavandula angustifolia, has been widely recognized for its therapeutic and cosmetic properties. Lavender oil is known for its anti-depressant, stress-relieving, and soothing properties, which help in calming the scalp and reducing irritation[13, 14]. It exhibits antibacterial and anti-inflammatory effects, making it effective in addressing scalp conditions such as dandruff, itching, and redness. Moreover, lavender oil has been traditionally used to promote hair growth, reduce hair loss, and improve overall scalp health[15].

In conclusion, the use of chitosan-coated lavender essential oil in shampoos represents an innovative and promising approach to combine the benefits of natural

ingredients with advanced encapsulation technology. This not only addresses the limitations of direct incorporation but also enhances the overall efficacy and consumer experience of hair care products.

2 Materials and Methods

2.1 Preparation and characterization of CSNPs and LEO-CSNPs

Chitosan (MW ~ 100 kDa, degree of deacetylation ≥ 90%, Macklin, China) 3 g, dissolved in 300 mL 10% acetic acid (≥ 98%, Sigma, USA), reacted with 20mM hydrogen peroxide (30%, Macklin, China) for degradation 6 h at 60-70°C by ultrasonic (KH-500D, Kunshan Hechuang Ultrasonic Instrument Co., Ltd., China). Then, the solution was adjusted to pH ~ 12 (NaOH, AR, Macklin, China) and the degraded chitosan was modified by using 20 mL ethlyene oxide (>99%, Macklin, China) for 8h at 60-70°C by stirring at 1300-1600 rpm (r/min, IKA RW 20, Germany). The formed Chitosan-derivative nanoparticles (CSNPs) were purified with PBS solution (Phosphate Buffered Saline, 1X, pH 7.4, Macklin, China) for 48 hours to eliminate the residual reactants.
In the process of LEO-CSNPs preparation, 10 g of lavender essential oil was added to an aqueous solution containing 1% of the surfactant Tween 85 to form an emulsion. Next, this emulsion was slowly added to a 10% CSNPs (Chitosan Nanoparticles) solution to create a water-in-oil emulsion. During emulsification, maintain a stirring speed of 5,000 revolutions per minute for 30 minutes. Finally, the moisture was removed within spray drying or other methods to obtain the microcapsule powder.
The structure of CSNPs was characterized by Nuclear Magnetic Resonance of Hydrogen atoms (NMR-H, Bruker, Germany) and Fourier Transform Infrared Spectroscopy (FITR, Cary630, Agilent, USA). The particle sizes for CSNPs and fibronectin-encapsulated CDN (LEO-CSNPs) were determined by Dynamic Light Scattering (DLS, Malvern Zetasizer Nano ZS90, England). Morphologies for both were visualized by Transmission Electron Microscopy (TEM, Tecnai F20, USA).

2.2 Shampoo preparation

The ingredients used included Sodium Laureth Sulfate (SLES, 12.0 wt%), Cocamide Diethanolamine (CDEA, 2.0 wt%), Cocamidopropyl Betaine (CAPB, 6.0 wt%), Deionized Water (q.s. to 100 wt%), Glyceryl Stearate (&) PEG-100 Stearate Pearlizing Agent (2.5 wt%), Glycerin (2.0 wt%), Polyquaternium-10 conditioning agent (0.5 wt%), Phenoxyethanol&Caprylyl Glycol preservative system (0.3 wt%), Sodium Chloride (2.0 wt%), and self-made Hydroxyethyl Chitosan-Encapsulated Lavender Essential Oil (LEO-CSNPs) at variable concentrations (0, 0.1, 0.2, 0.3, 0.5 wt%).

A base shampoo formulation was initially prepared without the active ingredient (LEO-CSNPs) and salt. The required quantity of deionized water was heated to 70 ± 5 °C and transferred to a suitable mixing vessel equipped with a mechanical stirrer (anchor or propeller impeller, 300-500 rpm).The pearlizing agent and glycerin were slowly dispersed into the warm water under constant stirring until a homogeneous mixture was obtained. The anionic surfactant (AES) was added slowly to avoid excessive foaming. Stirring was continued until the mixture was clear and uniform. The amphoteric surfactant (CAPB) and the conditioning agent (Polyquaternium-10) were then incorporated sequentially, ensuring complete dissolution and homogeneity after each addition. Finally, the foam

booster/viscosity modifier (CDEA) was added. The batch was allowed to cool to 40 ± 5 ° C with continuous gentle stirring. Upon reaching the target temperature, the preservative was added and mixed thoroughly. The homogeneous base formulation was divided into five equal parts. The LEO-CSNPs was incorporated into four of these batches at different concentration levels to create a gradient including 0%(F0), 0.1% (F1), 0.2% (F2), 0.3% (F3), and 0.5% (F4). The F0 batch remained without LEO-CSNPs to serve as the control. Each batch was stirred gently but thoroughly for 15 minutes to ensure uniform distribution of the encapsulated oil without breaking the microcapsules. Sodium Chloride was added as a 10% aqueous solution to each batch to adjust the final viscosity to the desired range (~5000-8000 cP at 25 ° C). Mixing was continued until the viscosity stabilized. Finally, the pH of each formulation was adjusted to 5.5 - 6.0 using citric acid solutions. The finished shampoos were allowed to stand for 24 hours at room temperature to allow viscosity stabilization and de-aeration before further evaluation.

2.3 Malassezia inhibitory assay

The inhibition zone test is a widely used method to evaluate the antimicrobial efficacy of chemical compounds or formulations[16]. The underlying mechanism involves the diffusion of the tested agent from a reservoir (e.g., paper disc or well) into the solid growth medium inoculated with the target microorganism. As the agent diffuses outward, it creates a concentration gradient[17]. Where the concentration exceeds the minimum inhibitory concentration (MIC) of the agent against the microorganism, microbial growth is suppressed, resulting in a clear, circular zone of inhibition surrounding the reservoir. The diameter of this zone is inversely proportional to the MIC and directly proportional to the diffusivity and potency of the antimicrobial agent[18]. Firstly, adjust the Malassezia suspension to a standardized density (e.g., 1–5 × 10^6 CFU/mL) using a spectrophotometer or hemocytometer. Secondly, evenly spread the fungal suspension onto the surface of the lipid-supplemented agar plates and allow the surface to dry. Thirdly, aseptically place sterile filter paper discs onto the inoculated agar surface. Apply a standardized volume (e.g., 20 μL) of the test formulations or blank control onto the respective discs. Next, allow the plates to stand at room temperature for 1–2 hours to facilitate pre-diffusion. Subsequently, incubate the plates at 32°C for 48–72 hours. Finally, after incubation, measure the diameter of the inhibition zones (including the disc diameter) in millimeters (mm) using a calibrated caliper or digital imaging software. In the result, the blank control (placebo formulation without active anti-Malassezia agents) produced an inhibition zone of 7.8 mm. This zone represents the basal level of inhibition attributable solely to the physical and chemical properties of the formulation vehicle (e.g., surfactants, solvents) and does not reflect specific antimicrobial activity. Additionally, any inhibition zone significantly larger than 7.8 mm observed for the active test formulations is indicative of true anti-Malassezia activity inherent to the incorporated actives (e.g., sophorolipids, hairy tea polyphenols).

2.4 Inhibition of sebum secretion in sebaceous gland cells

Materials include SZ95 cells (from Germany), Adhesive Slides (Paraffin Sections, Servicebio, G6012-1), 6-Well Plate Servicebio (CCP-6H), Upright Optical Microscope Olympus (Japan OLYMPUS CK31), Imaging System (Mshot TVO.63XC-MO), Digital Whole Slide Scanner (3DHISTECH), Pannoramic (SCAN). The SZ95 sebum secretion assay is a specialized experimental method used to study sebum production in sebaceous

glands, particularly using the SZ95 sebocyte cell line[19, 20]. SZ95 cells are an immortalized human sebaceous gland cell line that closely similar the behavior of primary sebocytes, which is a valuable tool for researching sebum production, lipid metabolism, and the effects of various compounds on sebaceous gland activity[21]. The assay is widely used in dermatological and cosmetic research to evaluate the efficacy of anti-acne treatments, moisturizers, or other skincare products that target sebum regulation. Melt and culture SZ95 sebocytes in an appropriate medium (DMEM/F12 supplemented with 10% FBS and growth factors) until they reach 70–80% confluence. Transfer cells into multi-well plates at a density of 1.2×10^4 cells per well and allow them to adhere overnight. Prepare dilutions of the test compounds in the culture medium. Replace the medium in the wells with the treatment medium containing the test compounds. Include control wells (untreated or vehicle-treated cells). Next,incubate the cells for 48 hours. After treatment, wash the cells with PBS to remove any residual medium. Fix the cells with 4% paraformaldehyde for 15 minutes at room temperature. Stain the cells with a lipid-specific dye called Oil Red O[22]. Wash the cells again with PBS to remove excess dye. For quantitative analysis, extract the stained lipids using an appropriate solvent (isopropanol for Oil Red O) and measure the fluorescence intensity using a microplate reader. In the process of data analysis, compare the lipid content of treated cells to control cells to determine the effect of the test compounds on sebum production.

2.5 DPPH radical scavenging

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay is a widely used method to evaluate the antioxidant activity of compounds and formulations. The mechanism involves the donation of a hydrogen atom or an electron by an antioxidant substance to the stable, purple-colored DPPH• radical, reducing it to a yellow-colored 1,1-diphenyl-2-picrylhydrazine compound. The degree of discoloration, measured spectrophotometrically at 517 nm, is proportional to the radical scavenging activity of the test sample[23, 24].

To avoid interference from shampoo surfactants and excipients, firstly, dilute 1 g of shampoo sample in 10 mL of methanol, vortex vigorously for 2 minutes, and then centrifuge at 10,000 × g for 10 minutes. Secondly, collect the supernatant containing the extracted antioxidants, prepare a 0.1 mM DPPH solution in methanol which need be protected from light and use within 2 hours. Thirdly, add each sample into a 96-well plate, including 50 μL of each shampoo+150 μL DPPH solution as sample group, 50 μL shampoo+ 150 μL methanol (to correct for sample color) as sample blank, 50 μL methanol + 150 μL DPPH solution as negative control and 50 μL standard antioxidant (e.g., ascorbic acid) + 150 μL DPPH solution for positive control group. Next, mix thoroughly and incubate the plate in darkness at room temperature for 30 minutes. Subsequently, measure the absorbance at 517 nm using a microplate reader. Finally, calculate the DPPH radical scavenging activity (%) using the formula,

$$\text{Scavenging Activity } (\%) = [1 - A\text{negative control}(A\text{sample} - A\text{sample blank})] \times 100$$

Where, Asample=Absorbance of test sample with DPPH, Asample blank=Absorbance of sample without DPPH and Anegative control=Absorbance of DPPH solution without sample.

2.6 qPCR assay for inflammatory factors inhibition

To evaluate the anti-inflammatory effects of each shampoo, the mRNA expression levels of key pro-inflammatory cytokines (IL-6 and IL-8) were quantified using quantitative real-time polymerase chain reaction (qPCR).

Human immortalized keratinocyte cells (Hacat) were cultured in standard conditions. Upon reaching 80-90% confluence, cells were pre-treated with the test formulations: 1) vehicle control (0.1% DMSO in culture medium), and 2) each shampoo sample at a non-cytotoxic, clinically relevant dilution (1:500 in culture medium). After a pre-treatment period within 1 hour, inflammation was induced by stimulating the cells with Lipopolysaccharide (LPS, 100 μg/mL) for a predetermined period (6-8 hours)[25].

Total RNA was isolated from the treated cells using a commercial kit (RNeasy Mini Kit, Qiagen) according to the manufacturer's instructions. Genomic DNA was eliminated by on-column DNase I digestion. RNA concentration and purity were assessed spectrophotometrically (NanoDrop) by ensuring A260/A280 and A260/A230 ratios were >1.8. Equal amounts of RNA (500 ng - 1 μg) from each sample were reverse-transcribed into complementary DNA (cDNA) using a high-capacity cDNA reverse transcription kit (from Applied Biosystems) with random hexamer primers[26].

qPCR reactions were performed in triplicate using a SYBR Green or TaqMan-based master mix on a real-time PCR detection system (Applied Biosystems StepOnePlus™). Each reaction contained cDNA template, gene-specific forward and reverse primers, and the master mix. The primer sequences for human genes were as follows[27]:

IL-6:
Forward: 5'-ACTCACCTCTTCAGAACGAATTG-3'
Reverse: 5'-CCATCTTTGGAAGGTTCAGGTTG-3'

IL-8:
Forward: 5'-ACTGAGAGTGATTGAGAGTGGAC-3'
Reverse: 5'-AACCCTCTGCACCCAGTTTTC-3'

Reference Gene (GAPDH):
Forward: 5'-GGAGCGAGATCCCTCCAAAAT-3'
Reverse: 5'-GGCTGTTGTCATACTTCTCATGG-3'

The thermal cycling conditions were: initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. A melt curve analysis was performed at the end of each run to confirm the specificity of the amplification and the absence of primer-dimers.

The comparative threshold cycle ($2^{(-\Delta\Delta Cq)}$) method was used to calculate the relative fold changes in gene expression. The Cq values of the target genes were normalized to the Cq value of the housekeeping gene GAPDH (ΔCq). The $\Delta\Delta Cq$ was then calculated by comparing the normalized expression of the treated groups to the vehicle control (unstimulated) group. Data are presented as the mean fold change $\pm$ standard deviation (SD) from at least three independent experiments. Statistical significance was determined using one-way ANOVA followed by a suitable post-hoc test (Tukey's test), with a p-value of < 0.05 considered significant.

2.7 Clinical assay

Clinical testing is essential to substantiate the efficacy, safety, and consumer acceptability of a new shampoo formulation[28]. For a shampoo containing LEO-CSNPs, the clinical study should be designed to evaluate its primary claimed benefits, which likely include scalp soothing, anti-dandruff efficacy, hair conditioning, and aromatherapeutic effects. Randomized, double-blind, parallel-group study (comparing the test shampoo against a placebo control shampoo identical in base formulation but without LEO-CSNPs). For the selected volunteers, a total of 15 subjects who met the inclusion criteria were enrolled in the study, including 2 males and 13 females, with an age range of 22 to 42 years and a mean age of 29.87 $\pm$ 5.78 years. Duration is typically 8 weeks of product use[24]. Wash Frequency is set as 3 times per week. Participants are selected based on specific inclusion criteria and exclusion criteria as follows in Table 1.

Table 1. Inclusion and exclusion criteria for volunteers in the clinical assay

Inclusion criteria	Exclusion criteria
Be in good general health with no significant systemic diseases. Present with a defined dandruff condition, with a severity rating (e.g., Adherent Scalp Flaking Score [ASFS]) meeting the pre-defined criteria for mild-to-moderate or higher. Exhibit mild scalp erythema, pruritus, or inflammation, with a severity rating meeting the pre-defined standard. Agree to adhere to a uniform hair-washing frequency and procedure for the duration of the study (washing hair every 2 days with the provided shampoo). Be able to understand and willing to comply with all requirements of the study protocol and attend all scheduled follow-up visits.	Presence of other scalp conditions that could interfere with the assessment, such as psoriasis, seborrheic dermatitis, tinea capitis, scalp eczema, folliculitis, etc. (Unless the target population for this study is specifically defined as subjects with seborrheic dermatitis). Have active scalp lesions, ulcerations, infections, or wounds requiring medical treatment. Use of topical medications for treating dandruff or scalp inflammation within 1 month prior to screening. Suffer from severe, uncontrolled systemic diseases. Be taking medications that may significantly affect scalp condition or the immune system.

Adherent scalp flaking score:
Method: The scalp is divided into sections (e.g., frontal, parietal, occipital). A trained dermatologist or evaluator visually assesses the degree of scaling in each area using a standardized scale (e.g., 0-10, where 0=no flakes and 10=severe scaling). This is performed at baseline (D0), and after 2, 4, and 8 weeks of use. High-resolution digital imaging under controlled lighting conditions can be used for documentation or computerized image analysis[29].

Itchiness and scalp sensation:
Method: Subjective assessment by participants using a self-assessment questionnaire (e.g., Visual Analog Scale - VAS or 5-point Likert scale) to rate the intensity of scalp itching, burning, or tightness before and during the study period[30].

Erythema (Redness) assessment:
Method: Clinical evaluation of scalp redness by a dermatologist using a standardized scale. Can be supplemented with instrumental measurements like colorimetry (using a Chroma Meter®) to objectively quantify changes in redness (a* value in the L*a*b* color space)[31].

Fragrance perception and duration:

Method: Participants rate the intensity, pleasantness, and longevity of the lavender fragrance during and after washing using questionnaires[32].

Subjective relaxation and sensory experience:
Method: Assessment of the product's perceived relaxing effect using validated psychological scales (e.g., Profile of Mood States - POMS, or a simple VAS for relaxation/stress) administered pre- and post-wash[33].

2.8 Statistical analysis

Statistical analysis was performed using Graphpad Prism 12.0 software. Data are presented as Mean±SD. For normally distributed data, paired samples t - test was used, while for non - normally distributed data, Wilcoxon signed - rank test for two related samples was applied. The significance level was set at $\alpha = 0.05$. In the figure legend, $P \geqslant 0.05$, $P < 0.05$, $P < 0.01$ and $P < 0.001$ are represented by “n.s.”, “*”, “**” and “***” respectively.

3 Results

3.1 Characterization of CSNPs andLEO-CSNPs

The IR spectra showed that the peaks of the stretching vibration of O-H and N-H of CSNPs occurred nearby 3513cm^{-1}, the peak of the stretching vibration of saturated carbon (including formyl group) C-H occurred at 2873cm^{-1}, and the peak of 1868cm^{-1} represented the stretching vibration of C=O on the amido bond. The peak nearby 1647cm^{-1} indicated C=N stretching vibration. C-H bending vibration occurred at 1395cm^{-1}. C-H in-plane bending vibration and C-N stretching vibration appeared around 1021cm^{-1}. Multi-peaks nearby 613cm^{-1} declared C-H out-of-plane bending vibration (Figure 1a). Figure 1b represented the 400MHz 1H NMR spectrum of CSNPs with solvent DMSO. 1H NMR (400 MHz, DMSO-d6) spectrum peaks included δ 8.19 (s, 1H), 6.14-6.19 (m, 1H), 5.09 (s, 1H), 4.81 (s, 3H), 4.58 (s, 5H), 4.34 (s, 3H), 3.61-3.77 (m, 7H), 3.54 – 3.51 (m, 4H), 3.48 (s, 7H), 3.42 (d, J = 5.8 Hz, 7H), 3.39 (s, 15H), 3.13-3.22 (m, 2H), 2.81 (s, 5H), 2.39 – 2.35 (m, 2H), 2.24-2.30 (t, 1H). Particularly, the single peak at $\delta = 8.19$ represented the hydrogen atom in amino bond. $\delta = 6.14 \sim 6.19$ represented the H atom in =NH. $\delta = 4.81 \sim 5.09$ indicated H atoms in -NH2. It seems like H atoms in the arginine structure. $\delta = 3.61\text{-}3.77$ represented H atoms in the hex-atomic rings, indicating structural features of chitosan.

Average diameters of self-assembled CSNPs and LEO-CSNPss were 218 nm and 352.27 nm respectively (Figure 1c). The particle size increased probably caused by encapsulating FN into the cavity of CSNPs. It is the same result of TEM showing that the diameter was higher after encapsulation (Figure 1d,1e).

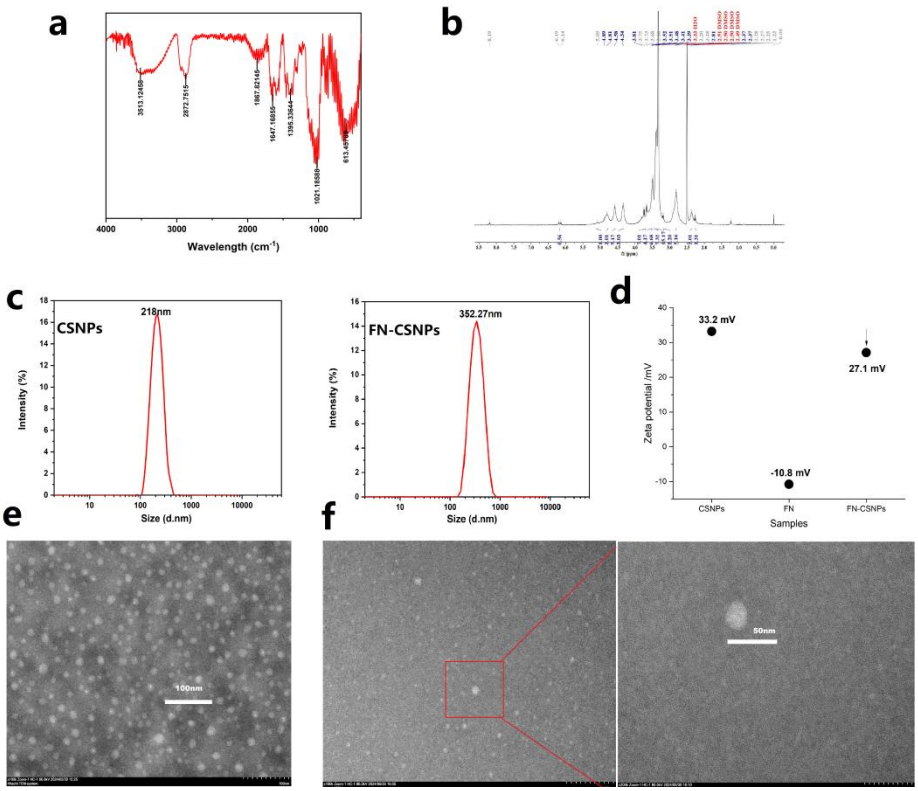


Fig. 1. Characterizations of FN, CSNPs andLEO-CSNPss. (a) FITR spectrum of CSNPs; (b) 1H-NMR spectrum of CSNPs; (c) Particle sizes of CSNPs andLEO-CSNPss with DLS; (d) TEM image of CSNPs; (e) TEM images ofLEO-CSNPss. Scale bar = 50nm, 100nm.

3.2 Malassezia inhibitory efficiency

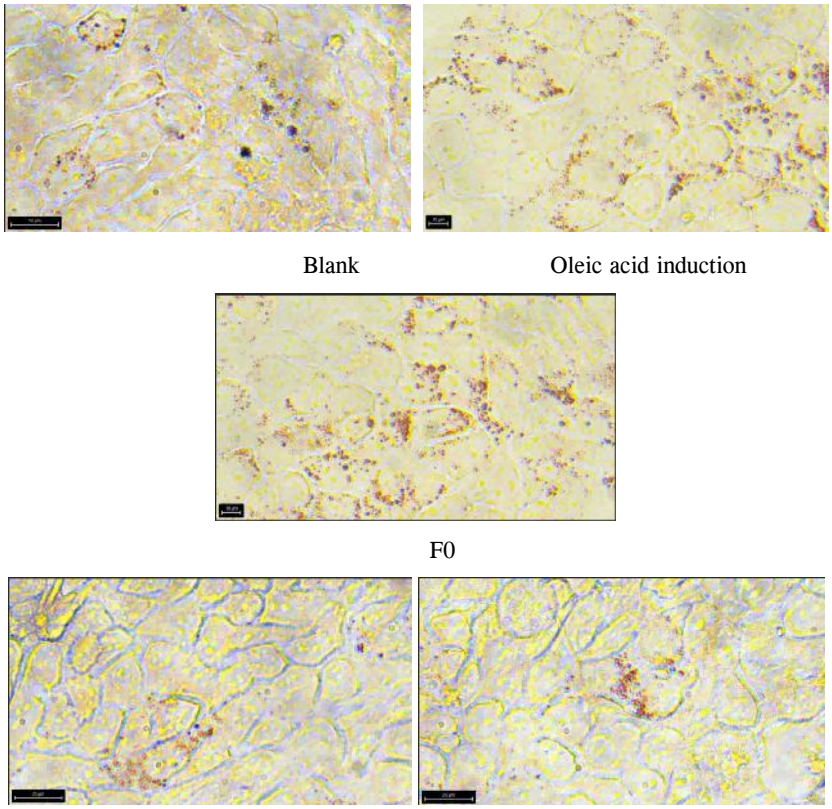
Based on the provided data, the zones of inhibition against Malassezia spp. demonstrate a clear and concentration-dependent antimicrobial effect for the LEO-CSNPs in the shampoo formulation. The results in Table 2 show a direct positive correlation between the concentration of the LEO-CSNPs and the size of the inhibition zone. Blank control (0% LEO-CSNPs): the 7.8 mm zone is attributable to the baseline activity of the shampoo base formula. Certain surfactants (e.g., AES) and cationic polymers (e.g., M550) can exhibit mild intrinsic antimicrobial properties. 0.1% LEO-CSNPs: the increase to 9.0 mm indicates a slight improvement over the baseline. This suggests a minimal effective concentration where the active compounds from the oil begin to contribute notably to the antimicrobial effect. 0.2% LEO-CSNPs: the zone expands to 11.0 mm, representing a moderate enhancement. This signifies a more pronounced effect as the concentration of bio-active compounds reaches a more valid threshold. 0.3% LEO-CSNPs: a zone of 12.5 mm reflects significant activity. The shampoo formulation at this concentration releases a sufficient quantity of antimicrobial agents to effectively suppress fungal growth. 0.5% LEO-CSNPs: the largest zone of 15.5 mm confirms strong inhibition. This result demonstrates that the highest tested concentration provides a potent antifungal effect, making it highly effective against Malassezia.

Table 2. Inhibition zone for each shampoo

LEO-CSNPs Concentration (%)	Zone of Inhibition (mm)	Antimicrobial Activity
0 (Blank Control)	7.8	Baseline (no active essential oil)
0.1	9.0	Slight improvement
0.2	11.0	Moderate enhancement
0.3	12.5	Significant activity
0.5	15.5	Strong inhibition

3.3 Inhibition of sebum secretion in sebaceous gland cells

As shown in Figure 2, in the Oil Red O staining assay using SZ95 sebaceous gland cells, oleic acid induction significantly increased lipid droplet accumulation in the blank control group. Treatment with test samples (shampoo F0-F4) resulted in a dose-dependent inhibition of sebum secretion. The inhibition rates were calculated for each sample F0 (withou LEO-CSNPs)=3.25%, F1=65.67%, F2=71.03%, F3=83.33% and F4=87.71%. These results demonstrate that formulations containing LEO-CSNPs (F1-F4) markedly suppress lipid production compared to the non-active base formula (F0).



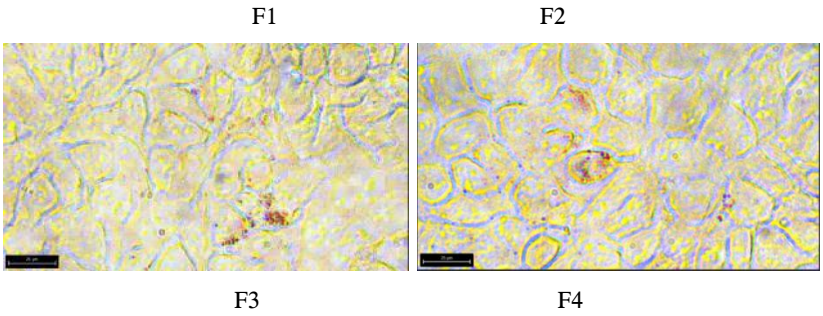


Fig. 2. Oleic acid O staining images

3.4 DPPH radical scavenging rate

The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay was employed to evaluate the antioxidant potential of shampoo formulations containing varying concentrations of LEO-CSNPs. The results demonstrate a clear concentration-dependent increase in antioxidant activity (Table 3). F0 (0% LEO-CSNPs) exhibited a baseline inhibition rate of 12.31%, attributable to minor inherent antioxidant properties of the shampoo base ingredients. F1 (0.1% LEO-CSNPs) showed 30.13% inhibition, indicating a noticeable antioxidant effect. F2 (0.2% LEO-CSNPs) achieved 52.34% inhibition, representing moderate antioxidant activity. F3 (0.3% LEO-CSNPs) displayed 65.17% inhibition, indicating high antioxidant capacity. F4 (0.5% LEO-CSNPs) demonstrated the highest inhibition at 79.03%, confirming very strong antioxidant activity.These results establish a direct positive correlation ($R^2 > 0.95$) between LEO-CSNPs concentration and free radical scavenging capacity.

Table 3. DPPH inhibition rate for each shampoo

Formulation	**LEO-CSNPs Concentration (%)**	**DPPH Inhibition Rate (%)**	**Antioxidant Activity**
F0	0.0	12.31	Very Low / Baseline
F1	0.1	30.13	Low
F2	0.2	52.34	Moderate
F3	0.3	65.17	High
F4	0.5	79.03	Very High

3.5 qPCR results for inflammatory factors inhibition

Quantitative PCR (qPCR) analysis was performed to evaluate the effect of formulations F0-F4 on the mRNA expression of pro-inflammatory cytokines IL-6 and IL-8 in an LPS-stimulated cellular model. The results in Figure 3 demonstrated a clear concentration-dependent inhibitory effect. For IL-6 expression, LPS Control group was significantly induced IL-6 expression (Avg: 2.12 ± 0.01). F0 (without LEO-CSNPs) showed minimal inhibition (Avg: 2.02 ± 0.02, 4.7% inhibition vs LPS), F1 (0.1% LEO-CSNPs) moderate inhibition (Avg: 1.84 ± 0.03, 13.2% inhibition), F2 (0.2% LEO-CSNPs) significant inhibition (Avg: 1.63 ± 0.01, 23.1% inhibition), F3 (0.3% LEO-CSNPs) strong inhibition (Avg: 1.41 ± 0.01, 33.5% inhibition) and F4 (0.5% LEO-CSNPs) potent inhibition (Avg: 1.22 ± 0.02, 42.5% inhibition). For IL-8 expression, LPS Control strongly induced IL-8 expression (Avg: 3.53 ± 0.06). F0 negligible effect (Avg: 3.51 ± 0.01, 0.6% inhibition), F1 (0.1% LEO-CSNPs) mild inhibition (Avg: 3.22 ± 0.01, 8.8% inhibition), F2 (0.2% LEO-CSNPs) noticeable inhibition (Avg: 2.92 ± 0.07, 17.3% inhibition), F3 (0.3% LEO-CSNPs) substantial inhibition (Avg: 2.75 ± 0.02, 22.1% inhibition) and F4 (0.5% LEO-CSNPs) marked inhibition (Avg: 2.32 ± 0.03, 34.3% inhibition).

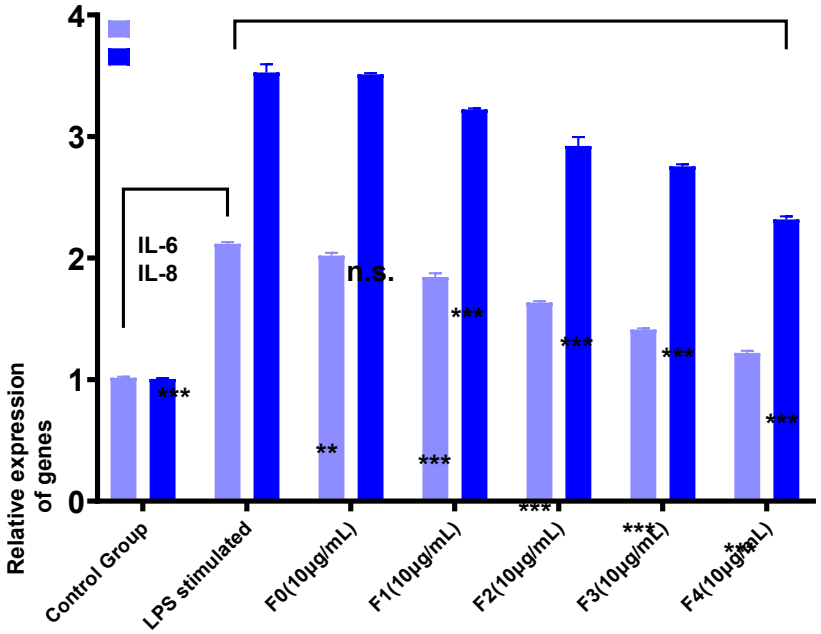


Fig. 3. Relative expression of IL-6 and IL-8 genes (In the graph, P≥0.05, P<0.05, P<0.01 and P<0.001 are represented by "n.s.", "*", "**" and "***" respectively.)

3.6 Clinical trial results

As shown in Table 4, the clinical evaluation of shampoo formulations F0-F4 demonstrates a clear and compelling concentration-dependent efficacy across all measured parameters. The results strongly support the therapeutic potential of LEO-CSNPs for improving scalp health. The clinical outcomes across all evaluation parameters show a

consistent ranking of efficacy: F4 (0.5% LEO-CSNPs) > F3 (0.3%) > F2 (0.2%) > F1 (0.1%) > F0 (0%). This demonstrates a direct positive correlation between the concentration of active ingredient and clinical improvement. F4 achieved the most significant reduction (77.73%), followed by F3 (68.03%), representing marked improvements over the base formula F0 (17.35%). A strong dose-response relationship was observed, with F4 showing 69.05% reduction in VAS score compared to 11% for F0. The 49.83% reduction in redness with F4 demonstrates potent anti-inflammatory effects. Both fragrance longevity and relaxation scores show substantial enhancement with increasing LEO-CSNPs concentration.

Table 4. Subjective evaluation summary (Group average: Mean±SD)

Formulation	Adherent Scalp Flaking Score Reduction (Baseline to Week 8)	Reduction in Itchiness (VAS Score)	Reduction in Erythema	Pleasantness longevity (Hours post-wash)	Relaxation Score Increase
F0	17.35%±1.24%	11%±1.03%	3.45%±0.58%	<1	Neutral
F1	35.13%±2.15%	25.30%±2.22%	12.35%±1.33%	1.30±0.12%	Mild
F2	55.23%±3.31%	44.25%±3.21%	22.35%±2.01%	2.40±0.22%	Noticeable
F3	68.03%±4.05%	53.81%±3.56%	35.64%±3.35%	5.50±0.35%	Significant
F4	77.73%±4.53%	69.05%±4.04%	49.83%±3.70%	7.20±0.65%	Pronounced

4 Discussion and conclusion

The LEO is encapsulated within the hydrophobic core cavity of the self-assembled CSNPs. The integration of LEO molecules into this core domain necessitates a structural expansion of the nanoparticle to accommodate the payload, leading to an increased core volume and, consequently, a larger overall hydrodynamic diameter. The incorporation of LEO molecules can cause the chitosan-based polymeric matrix to swell. This swelling occurs as the polymer chains adjust to encapsulate and stabilize the oil droplets, further contributing to the overall increase in nanoparticle size. The TEM results corroborate the DLS data, confirming that the process results in a well-defined nanostructure with a core (containing the LEO) and a shell (composed of hydroxyethyl chitosan). The consistency between the DLS and TEM data underscores the uniformity of the encapsulation process and the homogeneity of the resulting LEO-CSNPs. In conclusion, the physicochemical data conclusively demonstrate the successful loading of LEO into CSNPs. The mechanism is driven by the hydrophobic interaction-driven incorporation of the oil into the nanoparticle's core, resulting in a predictable and measurable expansion of the particle dimensions, as uniformly confirmed by both DLS and TEM techniques.

The observed dose-dependent antimicrobial activity can be attributed to several key mechanisms. The primary mechanism of LEO involves the penetration and disruption of the lipid bilayer of the fungal cell membrane. Key hydrophobic components like linalool and linalyl acetate accumulate in the membrane, increasing its permeability. This leads to the leakage of vital cellular contents (ions, ATP) and ultimately, cell lysis and death. The CSNPs carrier is not inert; it plays a crucial synergistic role. The nano-encapsulation system improves the stability and water dispersity of the hydrophobic oil. The positively charged chitosan shell can also electrostatically adhere to the negatively charged surfaces of fungal cells, ensuring targeted delivery and a higher local concentration of the active oil at the site of action. Chitosan itself possesses well-documented antifungal properties. It can penetrate cell walls, bind to DNA, and inhibit the synthesis of mRNA and essential proteins. The combination of chitosan's action with the membrane-disrupting effects of LEO creates a powerful multi-mechanistic attack on the fungus. In conclusion, the results conclusively demonstrate that the incorporation of LEO-CSNPs significantly enhances the antifungal efficacy of the shampoo formulation against malassezia in a concentration-dependent manner. The key significance of these findings lies in the successful development of an anti-dandruff shampoo prototype. The encapsulation technology effectively delivers the active ingredients, and concentrations of 0.3% to 0.5% show particularly promising results for strong inhibition of the fungus responsible for dandruff and seborrheic dermatitis. This provides a solid scientific rationale for selecting an optimal concentration (e.g., 0.3% or 0.5%) for further product development and in vivo efficacy testing. The study underscores the potential of combining natural antimicrobials with advanced delivery systems like CSNPs for effective cosmetic and personal care products.

The study clearly indicates LEO, especially when delivered via CSNPs, significantly inhibits sebum production in SZ95 sebocytes in a concentration-dependent manner. The base formulation (F0) showed negligible activity, confirming that the anti-lipogenic effects are specifically attributable to the encapsulated lavender oil. The inhibition rates exceeding 65% for F1–F4 highlight the potential of these formulations as effective natural alternatives for managing seborrhea-related conditions, such as acne and oily skin. The mechanisms likely involve the modulation of lipogenic transcription factors, anti-inflammatory actions, and improved cellular delivery of active compounds. Further studies involving gene expression analysis (e.g., PPAR-γ, SREBP-1 FAS) are recommended to elucidate the precise molecular pathways involved. These findings support the development of LEO-CSNPs as a promising ingredient in dermatological and cosmetic products aimed at controlling sebum secretion.

The primary antioxidant compounds in LEO (linalool, linalyl acetate, camphor, and terpinen-4-ol) possess labile hydrogen atoms that can be donated to stabilize the DPPH radical (DPPH• → DPPH-H). This hydrogen atom transfer mechanism directly neutralizes free radicals, with efficiency increasing with bioactive compound concentration. The CSNPs system significantly contributes to the antioxidant efficacy through improved solubility of hydrophobic lavender oil compounds in aqueous assay conditions, stabilization of reactive components against degradation and evaporation controlled release kinetics that ensure sustained availability of active compounds. Synergistic effect between the chitosan matrix (with mild intrinsic antioxidant properties) and the encapsulated bioactive compounds. Moreover, chitosan derivatives that may chelate pro-oxidant metals and provide additional radical scavenging. The study conclusively demonstrates that the incorporation of LEO-CSNPs significantly enhances the antioxidant capacity of shampoo formulations in a concentration-dependent manner.

The nearly linear response (12.31% to 79.03% inhibition) across the concentration gradient indicates excellent dose-responsive behavior.

The dose-dependent suppression of IL-6 and IL-8 gene expression can be attributed to several key mechanisms. The primary mechanism involves suppression of the Nuclear Factor-kappa B (NF-κB) signaling pathway. Bio-active components in LEO (linalool, linalyl acetate) inhibit IκB kinase (IKK) activity, preventing IκB degradation and subsequent NF-κB nuclear translocation. This directly down-regulates transcription of IL-6 and IL-8 genes, which contain NF-κB binding sites in their promoters. Concurrent inhibition of MAPK pathways (particularly p38 and JNK) contributes to reduced cytokine production. Both LEO components and CSNPs interfere with phosphorylation events necessary for MAPK activation, thereby attenuating downstream inflammatory gene expression. The hydroxyethyl chitosan encapsulation enhances cellular uptake of hydrophobic active compounds and provides sustained release kinetics. The chitosan carrier itself exhibits intrinsic anti-inflammatory properties through electrostatic interactions with cell membranes, modulation of Toll-like receptor signaling and scavenging of reactive oxygen species that activate inflammatory pathways. The graded response across formulations (F1-F4) directly correlates with increased concentration of bioactive compounds, demonstrating classical receptor-mediated or enzyme-inhibition kinetics where higher concentrations achieve greater pathway saturation and inhibitory effects. The qPCR results provide compelling molecular evidence that hydroxyethyl chitosan-encapsulated lavender essential oil (LEO-CSNPs) effectively suppresses expression of key pro-inflammatory cytokines IL-6 and IL-8 in a concentration-dependent manner.

The exceptional performance in flake reduction (F4: 77.73%) can be attributed to the dual mechanism of action. Previously demonstrated anti-malassezia efficacy directly reduces the fungal trigger for dandruff formation. Suppression of IL-6 and IL-8 production prevents the inflammatory response that exacerbates scaling and flaking. The previously observed anti-sebum effects help create an unfavorable environment for malassezia proliferation. The significant reduction in erythema (49.83% with F4) and itchiness (69.05% with F4) results from NF-κB Pathway Inhibition: Bioactive components in lavender oil suppress the master regulator of inflammatory cytokine production, Neuropeptide Modulation: Linalool and linalyl acetate may directly affect sensory nerve endings, reducing itch perception and barrier function improvement: reduced inflammation and microbial load allow for restoration of normal scalp barrier function. The concentration-dependent improvement in sensory parameters demonstrates the chitosan encapsulation provides prolonged fragrance release, accounting for the 7.2-hour longevity with F4. The pronounced relaxation score with F4 confirms the psychophysiological effects of LEO through olfactory pathways. The strong correlation between active concentration and pleasantness scores suggests high consumer preference for the encapsulated formulation. The clinical results validate the hypothesis that LEO-CSNPs provides comprehensive scalp health benefits through multiple complementary mechanisms of action. Especially, all clinical parameters showed clear dose-response relationships, confirming that 0.3-0.5% LEO-CSNPs represent the optimal concentration range for efficacy. The formulations address dandruff through antifungal, anti-inflammatory, anti-pruritic, and sebum-regulating mechanisms simultaneously. F3 and F4 demonstrated clinically relevant improvements across all measured parameters, significantly outperforming the base formula. F3 (0.3% LEO-CSNPs) represents an excellent balance between efficacy and cost-effectiveness for general consumer use. F4 (0.5% LEO-CSNPs) is recommended for premium therapeutic applications targeting

more severe scalp conditions. The encapsulation technology should be maintained as it ensures both stability and sustained release of active components. These results support the use of LEO-CSNPs as a novel active ingredient system for the development of effective, multi-functional anti-dandruff and scalp-soothing shampoo products. Further studies should investigate long-term effects and consumer satisfaction in real-world usage conditions.

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