Development And Evaluation Of Neem Oil Nanoemulsions: Enhanced Antifungal Activity Against Trichophyton Rubrum With Improved Stability

Maryam Iman[1]*, Shahin Mojra[2]

[1]Department of Pharmaceutics, Faculty of Pharmacy, Baqiyatallah University of Medical Sciences, Tehran, Iran
[2]Student Research Committee, Baqiyatallah University of Medical Sciences, Tehran, Iran.
*Correspondence

ABSTRACT

Background and Objectives: Neem oil, Azadirachta indica is an effective antifungal agent; however, its hydrophobic nature is one of the limiting factors for conventional formulation. Nanoemulsion systems offer a promising approach to enhance its solubility, stability, and antifungal activity. This study was carried out to formulate neem oil nanoemulsions with optimal droplet size, investigate their stability, and assess their antifungal efficacy against Trichophyton rubrum.

Methods: Nanoemulsions were prepared by high-energy emulsification at different oil-to-surfactant ratios (1:2, 1:3, 1:4) and two sonication times (10 and 15 minutes). The particle sizes were determined by DLS. Antifungal activity and minimum inhibitory concentration-MIC-by the broth microdilution method of the nanoemulsions were compared with neem oil macroemulsions and nystatin as a control.

Results: The minimum droplet size was 11.95 nm with an oil-to-surfactant ratio of 1:4, which was subjected to a sonication time of 15 minutes. Further, the optimized nanoemulsion showed higher antifungal efficiency (MIC: 1562.5 µg/mL) than the corresponding macroemulsion (MIC value 3125 µg/mL). The stability study exhibited only slight increase in droplet diameter within 60 days of evaluation, and thus it remained stable (<20 nm).

Conclusion: The neem oil nanoemulsion significantly enhanced antifungal efficacy and stability, which could be due to the enhancement in droplet's surface area and solubility. This study presents the potential of nanoemulsion technology in the development of efficient antifungal formulations and, therefore, is worthy of further clinical exploration.

Keywords: Neem Oil, Nanoemulsions, Antifungal Activity, Trichophyton rubrum.

INTRODUCTION

Neem (Azadirachta indica) is a plant of great importance in traditional medicine, exhibiting a wide spectrum of therapeutic properties, including strong antifungal, antibacterial, and anti-inflammatory activities (1-3). Among its bioactive constituents, azadirachtin, nimbin, and limonoids are the major players in inhibiting microbial growth and enhancing immune responses (4, 5). However, neem oil suffers from hydrophobicity, low bioavailability, and instability in conventional formulations, which limits its clinical application. Addressing these challenges requires innovative drug delivery systems that optimize the neem oil therapeutic potential (5-8).

Nanoemulsion technology represents a major breakthrough in drug delivery, especially for hydrophobic compounds such as neem oil. These systems, characterized by droplet sizes less than 100 nm, provide a greater surface area, better solubility, and enhanced bioavailability for the efficient delivery of drugs. Nanoemulsions also possess superior stability, less aggregation, and controlled release compared to macroemulsions (9-12). Despite growing interest, there is limited research on the formulation of neem oil nanoemulsions and their potential to enhance antifungal efficacy.

This study was thus designed to bridge this gap by formulating and characterizing neem oil nanoemulsions using high-energy emulsification techniques. The research optimizes parameters like oil-to-surfactant ratios and sonication times to develop a stable nanoemulsion with superior antifungal activity against Trichophyton rubrum, one of the most common fungal pathogens. The stability of the nanoemulsion upon storage and its minimum inhibitory concentration (MIC) against a macroemulsion

formulation and a standard antifungal agent were further explored in this study. The outcome of this study could lead to the furtherance of plant-based antifungal therapies by offering a more efficient and stable delivery system; it would address critical challenges in fungal infection treatment and contribute to the growing topic of nanotechnology-enabled pharmaceuticals.

Materials and Methods

Neem seeds were purchased from a local supplier in Iran, and neem oil was extracted using the cold-press method. The extracted oil served as the primary ingredient for nanoemulsion preparation. Tween 20 (Code: 822184) was used as the surfactant and sourced from Merck, Germany. Double-distilled water was prepared in the pharmaceutics laboratory of Baqiyatallah University, Iran. Nystatin (Sigma-Aldrich, USA) was used as the positive control for antifungal activity assays. The fungal strain Trichophyton rubrum (ATCC 28188) was procured from the Iran Biological Research Center (IBRC). The RPMI-1640 medium (code:R8758) was used as culture media and sourced from Sigma-Aldrich,USA.

Extraction of Neem Oil

Neem oil was extracted from the seed kernels of the Azadirachta indica tree. The outer shells of the seeds were removed, and the kernels were subjected to cold-press extraction to obtain oil. This method preserves the bioactive compounds of neem oil by minimizing thermal degradation.

Preparation of Neem Oil Nanoemulsion

Nanoemulsions were formulated using the high-energy emulsification technique. Three formulations were prepared with different oil-to-surfactant ratios: 1:2, 1:3, and 1:4. For each formulation:

- Neem oil and Tween 20 were mixed in the specified ratios.
- Double-distilled water was added dropwise while stirring to form a preliminary emulsion.
- The emulsion was vortexed for 5 minutes at room temperature to ensure homogenization.
- The emulsions were then subjected to probe sonication (UW 200, Bandelin, Germany) in a conical centrifuge tube. The depth of the sonicator probe was maintained at 1 cm from the bottom of the tube. Sonication was performed at two amplitudes (25% and 50%) and two durations:

5 minutes at 25% amplitude, followed by 5 minutes at 50% amplitude.
5 minutes at 25% amplitude, followed by 10 minutes at 50% amplitude.
To prevent overheating during sonication, the tube was placed in an ice bath.

Particle Size Analysis

The droplet size of the nanoemulsions was measured using dynamic light scattering (DLS) (SZ-100, Horiba, Japan). Each sample was diluted 1:100 with double-distilled water prior to analysis. Measurements were performed in triplicate for each formulation to ensure accuracy.

Stability Testing

The optimized nanoemulsion (1:4 oil-to-surfactant ratio, sonicated for 15 minutes) was stored at room temperature (25°C) for 60 days. Particle size was measured at 0, 30, and 60 days using DLS to assess physical stability.

Antifungal Activity and Minimum Inhibitory Concentration (MIC)

The antifungal activity of neem oil nanoemulsions was evaluated using the M38-A2 broth microdilution method, as outlined by the Clinical and Laboratory Standards Institute (CLSI, 2008).

Preparation of Test Solutions:

Eight two-fold serial dilutions (100–0.781 µL/mL) of the nanoemulsion and macroemulsion (neem oil with methanol:DMSO 1:1) were prepared in RPMI-1640 medium.

Nystatin (positive control) was prepared at concentrations of 1–5 µg/mL.

Negative controls included the solvent with inoculum and solvent alone.

Fungal Inoculum Preparation:

T. rubrum was cultured on potato dextrose agar at 25°C for 24 hours to form colonies.

A fungal suspension was prepared in RPMI-1640 medium and adjusted to 2.5×10^3 CFU/mL using a Neubauer chamber.

Microdilution Assay:

A sterile 96-well microplate was used for testing.

Each well was initially filled with 100 µL of RPMI-1640.

Test substances were added to the first well of each column and serially diluted across the plate.

Fungal inoculum (100 µL) was added to each well.

Plates were incubated at 25°C for 48 hours.

The MIC was determined as the lowest concentration of the test substance that visually inhibited fungal growth. Experiments were performed in triplicate for each test substance.

Statistical Analysis

All experiments were performed in triplicate, and results were expressed as mean ± standard deviation (SD).

Results

Particle Size Analysis

The particle size distribution of the prepared neem oil nanoemulsions was determined using dynamic light scattering (DLS). Three different oil-to-surfactant ratios (1:2, 1:3, and 1:4) and two sonication conditions (5 minutes at 25% amplitude + 5 minutes at 50% amplitude, and 5 minutes at 25% amplitude + 10 minutes at 50% amplitude) were evaluated (Fig 1 A). The particle size of Formulation A (1:2 oil-to-surfactant ratio) was measured as 153.22 nm (SD:13.052) for the 5-minute sonication at 25% + 5 minutes at 50% amplitude. With an increased sonication time to 15 minutes, the particle size increased to 177.92 nm (SD:4.5047). On the other hand, The initial particle size of Formulation B (1:3 oil-to-surfactant ratio) was 129.77 nm (SD:10.094) with the shorter sonication time, which increased to 172.24 nm (SD:7.5713) with the longer sonication time. Moreover, for the Formulation C (1:4 oil-to-surfactant ratio) the smallest initial particle size was observed at 16.64 nm (SD:5.4778) with 5 minutes at 25% + 5 minutes at 50% amplitude, and a significant reduction in size to 11.95 nm (SD:0.7228) was noted with the 15-minute sonication.

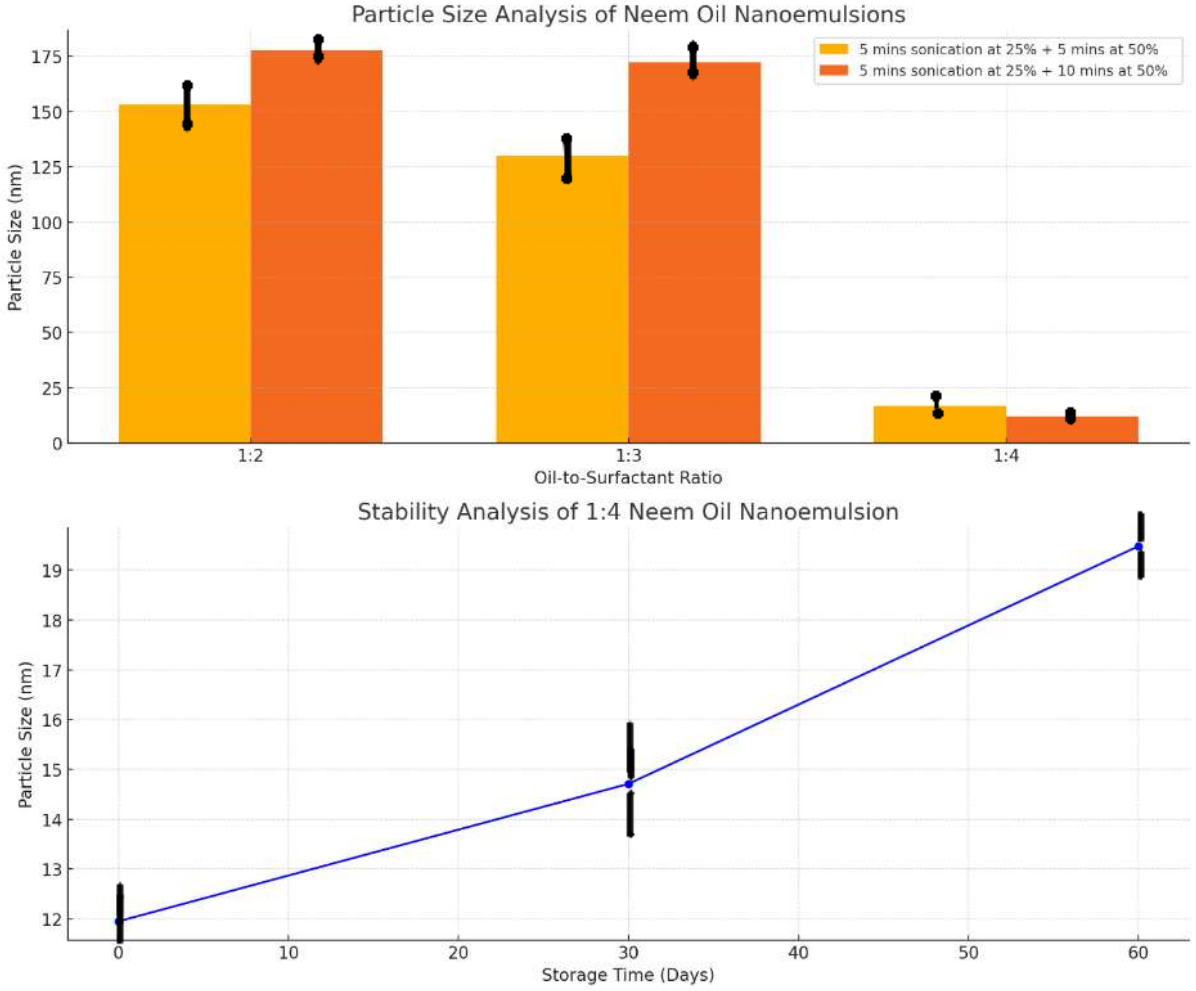

Figure 1: (A) Particle size distribution of neem oil nanoemulsions with different oil-to-surfactant ratios and sonication times; (B) Stability of neem oil nanoemulsion over 60 days (particle size over time).

Stability Analysis

The stability of the optimized neem oil nanoemulsion (Formulation C) was assessed over 60 days at room temperature (25°C). Particle size measurements were taken at 0, 30, and 60 days to evaluate changes in size over time (Fig 1 B). The particle size at the initial measurement (Day 0) was 11.95 nm (SD:0.7228).The average particle size at 30-day increased to 14.71 nm (SD:1.213), indicating slight aggregation. Whilte at 60-day the particle size further increased to 19.48 nm (SD:0.914), but remained below the critical threshold of 20 nm, suggesting that the nanoemulsion maintained physical stability over the storage period.

Antifungal Activity and Minimum Inhibitory Concentration (MIC)

The antifungal activity of the neem oil nanoemulsion was assessed using the M38-A2 broth

microdilution method, and the MIC was determined for both nanoemulsion and macroemulsion formulations against Trichophyton rubrum

The MIC for Formulation C was found to be 1562.5 µg/mL, demonstrating significant antifungal activity against Trichophyton rubrum. On the other hand, the macroemulsion showed a higher MIC of 3125 µg/mL, indicating lower efficacy compared to the nanoemulsion. Meanwile the MIC for nystatin was 2 µg/mL, confirming its potent antifungal properties.

Table 1: MIC values of neem oil nanoemulsion, macroemulsion, and nystatin.

Sample	MIC (µg/mL)
Neem oil nanoemulsion	1562.5
Neem oil macroemulsion	3125
Nystatin (positive control)	2

Discussion

The present study reports the successful formulation of neem oil nanoemulsion by high-energy emulsification techniques and its increased antifungal efficacy against Trichophyton rubrum. These results point out the potential of nanoemulsion technology to solve delivery challenges of hydrophobic plant-based bioactives. Moreover, optimization of oil-to-surfactant ratio and sonication time has shown that droplet size decreases with an increase in the concentration of surfactant and sonication time. The minimum droplet size of 11.95 nm was obtained by using a 1:4 oil-to-surfactant ratio with 15-minute sonication, which clearly indicates that effective emulsification is responsible for the nanoscale particle size. The main role of the surfactant Tween 20 in this regard was to decrease interfacial tension and prevent coalescence, maintaining droplet stability (13, 14). These results are in agreement with other studies related to nanoemulsion systems, where increased surfactant concentration and optimization of sonication led to smaller particle sizes and better homogeneity (15-17).

There was no considerable instability in the nanoemulsion after 60 days of storage at 25°C, with only a slight increase in droplet size from 11.95 nm to 19.48 nm. Such an increase probably indicates minor physical coalescence or minor chemical interactions. However, the droplet size was maintained below 20 nm, which indicated good physical and chemical stability. Further enhancement of the antioxidant content in the formulation or optimization of the storage conditions may improve stability. Similar trends of moderate size increases without significant losses of stability have been reported in other plant-based nanoemulsion studies (18-20).

The neem oil nanoemulsion presented a MIC value of 1562.5 µg/mL, which was better than that of the macroemulsion (MIC value of 3125 µg/mL). The enhancement might be attributed to improved solubility, increased surface area, and better bioavailability of nanoemulsion formulations. Smaller droplet size improves interaction with the fungal cell membrane, hence increasing permeability and further disturbing cellular functions (21-24). More importantly, the encapsulation of neem oil bioactives into nanoemulsion systems increases their transport across hydrophilic barriers and amplifies their antifungal action. Bioactive compounds in neem oil, such as azadirachtin and nimbin, are known to interfere with fungal cell wall synthesis and membrane integrity, inhibit spore germination, and induce oxidative stress in fungal cells (25, 26).

Encapsulation within the nanoemulsion likely augmented these effects by ensuring efficient delivery and sustained release. Other recent studies also indicated increased antifungal effectiveness of nanoemulsions loaded with plant-based oils, such as clove and tea tree oil, which may be attributed to mechanisms involving improved membrane disruption and ergosterol inhibition (27, 28). While nystatin showed the lowest MIC value, as low as 2 µg/mL, the performance of the neem oil nanoemulsion was an indication that it could serve as an alternative agent. Considering its botanical origin and less possibility of resistance development, neem oil nanoemulsion might serve as a natural, renewable choice for the treatment of fungal infections, particularly in developing countries where synthetic drugs may be inaccessible. The results indicate that the neem oil nanoemulsions may be useful in fields other than antifungal applications, targeting bacterial pathogens and parasitic infections; they can also be an ingredient in agrochemical and cosmetic preparations.

Further research is recommended to be done on cytotoxicity studies of the formulations against mammalian cells to establish their toxicity profile and their efficacy in vivo.

Optimization of formulation variables such as the addition of co-surfactant or antioxidants may further improve stability and bioactivity. Moreover, the translation clinically and commercially will importantly need exploration of scalable production techniques.

Conclusion

This study demonstrates the effectiveness of nanoemulsion technology in enhancing the antifungal efficacy and stability of neem oil. The results underscore the potential of plant-based nanoformulations

as sustainable, effective therapeutic agents. By addressing challenges in the delivery of hydrophobic compounds, this research contributes to advancing nanotechnology applications in pharmaceutical and healthcare fields.

Ethics approval: The Code of proposal acceptance is 402000522

Consent for publication: Not applicable Availability of data and material: Financial support and sponsorship: Self sponsorship

Acknowledgements: We want to express our gratitude to all the individuals and institutions who helped us conduct this study. We appreciate the management and the staff of pharmacy department where this study has been conducted.we would like to thank the guidance and support from research and technology comprehensive laboratory(RTCL) of baqiyatallah university of medical sciences.

Conflict of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results

References

1. Alzohairy MA. Therapeutics Role of Azadirachta indica (Neem) and Their Active Constituents in Diseases Prevention and Treatment. Evidence-based complementary and alternative medicine : eCAM. 2016;2016:7382506.
2. Wylie MR, Merrell DS. The Antimicrobial Potential of the Neem Tree Azadirachta indica. Frontiers in pharmacology. 2022;13:891535.
3. Reddy IVS, Palagani N. Neem (Azadirachta indica): A Review on Medicinal Kalpavriksha. International Journal of Economic Plants. 2022;9.
4. Sarkar S, Singh RP, Bhattacharya G. Exploring the role of Azadirachta indica (neem) and its active compounds in the regulation of biological pathways: an update on molecular approach. 3 Biotech. 2021;11(4):178.
5. Islas JF, Acosta E, G-Buentello Z, Delgado-Gallegos JL, Moreno-Treviño MG, Escalante B, et al. An overview of Neem (Azadirachta indica) and its potential impact on health. Journal of Functional Foods. 2020;74:104171.
6. Ashfaq R, Rasul A, Asghar S, Kovács A, Berkó S, Budai-Szűcs M. Lipid Nanoparticles: An Effective Tool to Improve the Bioavailability of Nutraceuticals. International journal of molecular sciences. 2023;24(21).
7. Bonaccorso A, Cimino C, Manno DE, Tomasello B, Serra A, Musumeci T, et al. Essential Oil-Loaded NLC for Potential Intranasal Administration. Pharmaceutics. 2021;13(8).
8. Nemati S, Mohammad Rahimi H, Hesari Z, Sharifdini M, Jalilzadeh Aghdam N, Mirjalali H, et al. Formulation of Neem oil-loaded solid lipid nanoparticles and evaluation of its anti-Toxoplasma activity. BMC complementary medicine and therapies. 2022;22(1):122.
9. Preeti, Sambhakar S, Malik R, Bhatia S, Al Harrasi A, Rani C, et al. Nanoemulsion: An Emerging Novel Technology for Improving the Bioavailability of Drugs. Scientifica. 2023;2023:6640103.
10. Bamisaye A, Adegoke KA, Alli YA, Bamidele MO, Idowu MA, Ogunjinmi OE. Recent advances in nanoemulsion for sustainable development of farm-to-fork systems. Journal of Cleaner Production. 2023;429:139226.
11. Kumar A, Kanwar R, Mehta SK. Nanoemulsion as an effective delivery vehicle for essential oils: Properties, formulation methods, destabilizing mechanisms and applications in agri-food sector. Next Nanotechnology. 2025;7:100096.
12. Azmi NA, Elgharbawy AAM, Motlagh SR, Samsudin N, Salleh HM. Nanoemulsions: Factory for Food, Pharmaceutical and Cosmetics. Processes [Internet]. 2019; 7(9).
13. Samanta S, Ghosh P. Coalescence of bubbles and stability of foams in aqueous solutions of Tween surfactants. Chemical Engineering Research & Design - CHEM ENG RES DES. 2011;89:2344-55.
14. Shen W, Koirala N, Mukherjee D, Lee K, Zhao M, Li J. Tween 20 Stabilized Conventional Heavy Crude Oil-In-Water Emulsions Formed by Mechanical Homogenization. Frontiers in Environmental Science. 2022;10:873730.
15. Modarres-Gheisari SMM, Gavagsaz-Ghoachani R, Malaki M, Safarpour P, Zandi M. Ultrasonic nano-emulsification – A review. Ultrasonics Sonochemistry. 2019;52:88-105.
16. Tahir I, Millevania J, Wijaya K, Mudasir, Wahab RA, Kurniawati W. Optimization of thiamine chitosan nanoemulsion production using sonication treatment. Results in Engineering. 2023;17:100919.
17. Ali E, Shakil N, Rana V, Sarkar DJ, Majumder S, Kaushik P, et al. Antifungal activity of nano emulsions of neem and citronella oils against phytopathogenic fungi, Rhizoctonia solani and Sclerotium

rolfsii. Industrial Crops and Products. 2017;108:379-87.
18. Campolo O, Giunti G, Laigle M, Michel T, Palmeri V. Essential oil-based nano-emulsions: Effect of different surfactants, sonication and plant species on physicochemical characteristics. Industrial Crops and Products. 2020;157.
19. Jamir Y, Bhushan M, Sanjukta R, Robindro Singh L. Plant-based essential oil encapsulated in nanoemulsions and their enhanced therapeutic applications: An overview. Biotechnology and Bioengineering. 2024;121(2):415-33.
20. Ali MS, Alam MS, Alam N, Siddiqui MR. Preparation, Characterization and Stability Study of Dutasteride Loaded Nanoemulsion for Treatment of Benign Prostatic Hypertrophy. 2014;13(4):e125572.
21. Giro dos Santos A, Trigo Marquês J, Carreira A, Castro I, Viana A, Mingeot-Leclercq M-P, et al. The molecular mechanism of Nystatin action is dependent on membrane biophysical properties and lipid composition. Phys Chem Chem Phys. 2017;19.
22. Jacob S, Kather FS, Boddu SHS, Shah J, Nair AB. Innovations in Nanoemulsion Technology: Enhancing Drug Delivery for Oral, Parenteral, and Ophthalmic Applications. Pharmaceutics. 2024;16(10).
23. Nikolaev B, Yakovleva L, Fedorov V, Li H, Gao H, Shevtsov M. Nano- and Microemulsions in Biomedicine: From Theory to Practice. Pharmaceutics. 2023;15:1989.
24. Shams N, Sahari MA. Nanoemulsions: Preparation, structure, functional properties and their antimicrobial effects. Applied Food Biotechnology. 2016;3:138-49.
25. Harwansh R, Deshmukh R, Rahman M. Nanoemulsion: Promising nanocarrier system for delivery of herbal bioactives. Journal of Drug Delivery Science and Technology. 2019;51.
26. Lohith Kumar DH, Sarkar PJECL. Encapsulation of bioactive compounds using nanoemulsions. 2018;16:59-70.
27. Alghaith A, Alshehri S, Alhakamy N, Hosny K. Development, optimization and characterization of nanoemulsion loaded with clove oil-naftifine antifungal for the management of tinea. Drug Delivery. 2021;28.
28. Flores FC, Ribeiro RF, da Silva CdB. Antifungal activity of nanocarriers containing tea tree oil on the growth of C. albicans: an inhibition profile study. Ciência e Natura. 2015;37(5):1-6.