KAVAKA 61(2): 78-84 (2025) DOI: 10.36460/Kavaka/61/2/2025/78-84

Nanoemulsion Formulation Enhancing the Antifungal Activity of *Eucalyptus globulus* essential oil against *Aspergillus tamarii In Vitro* Test

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(Submitted on December 10, 2024; Accepted on May 22, 2025)

ABSTRACT

Post-harvest losses in *Citrus sinensis* (L.) Osbeck, attributed to fungal pathogens like *Aspergillus spp*., poses significant economic and agricultural challenges. Nanoemulsions have emerged as promising carriers for essential oils, offering enhanced stability, solubility, and enhanced bioactivity. This research aims to fabricate and characterize a nanoemulsion formulation containing *Eucalyptus globulus* essential oil (EEOs) and evaluate its antifungal potential. The nanoemulsion was prepared using a low energy method and characterized for droplet size, polydispersity index (PI), zeta potential, FESEM and stability. The antifungal activity of the nanoemulsion was assessed against *Aspergillus tamarii.* MTCC 13583 through agar well diffusion assays and the microdilution method. The results demonstrated the successful fabrication of a stable nanoemulsion with an average droplet size of 169.5 nm and a zeta potential of -19.5 mv. FTIR stretching vibration signifies the presence of the O-H, N-H, C=C, C-H stretch. FESEM analysis indicates that the diameter of some droplets reaches up to 169.5 nm. The nanoemulsion exhibited potent antifungal activity, inhibiting the growth of *Aspergillus tamarii*. The nanoemulsion also showed a significant zone of inhibition and MIC of 10 µl/ml. These findings suggest that the nanoemulsion of *Eucalyptus globulus* essential oil holds great potential as an effective antifungal agent with possible applications in various therapeutic formulations.

Keywords: Essential oil, Fabrication, Post-harvest losses

INTRODUCTION

Citrus sinensis (L.) Osbeck, commonly known as sweet orange, holds global significance due to its nutritional richness, delightful taste, and versatile applications in both culinary and industrial domains (Kundu *et al*., 2020). However, the post-harvest phase presents significant challenges, with the fruit's susceptibility to various physiological issues and microbial threats, notably fungal infections induced by *Aspergillus* species (Strano *et al*., 2022). These challenges lead to substantial post-harvest losses during storage, transportation, and marketing. This impacts stakeholders globally and raises concerns about economic setbacks and food security (Wang *et al.,* 2022). A*spergillus* fungi, known for their ability to colonize various substrates, including citrus fruits, can cause mold formation, decomposition, and the production of harmful mycotoxins, rendering the fruits unfit for consumption and trade (Strano *et al*., 2022). Conventional control methods using synthetic fungicides, though effective, pose risks to the environment, food safety, and the emergence of fungicide-resistant strains. Consequently, there is a growing demand for eco-friendly alternatives to mitigate post-harvest losses in *Citrus sinensis* (Sharma and Bhandari, 2020).

Eucalyptus essential oil (EO), primarily derived from *Eucalyptus globulus*, is renowned for its potent antifungal properties, largely attributed to its key components such as cineole (eucalyptol), alpha-pinene, and beta-pinene (Ngo *et al*., 2020). Cineole, the major constituent, disrupts fungal cell membranes and inhibits spore germination, while alpha-pinene and beta-pinene contribute additional antimicrobial effects (Yu *et al.,* 2015). Its broad-spectrum antifungal activity, coupled with its natural origin, positions Eucalyptus EO as a valuable alternative to conventional chemical fungicides. However, incorporating essential oils into therapeutic formulations poses challenges due to their hydrophobic nature and poor stability in aqueous environments. Nanoemulsions have emerged as a promising solution to overcome these limitations. Nanoemulsions, a type of nanotechnology-based delivery system, disperse droplets of essential oil in water or other suitable solvents, with droplets typically in the nanometer range, offering enhanced stability due to reduced surface area and the use of surfactants (Barradas and Holanda, 2021).

Nanoemulsions stabilize essential oils, preventing their separation or degradation over time, which is crucial for maintaining the effectiveness of antimicrobial compounds. The small droplet size increases the surface area of the essential oil, promoting better absorption and bioavailability in therapeutic formulations. Nanoemulsions improve the penetration of essential oils through biological barriers, potentially enhancing their antimicrobial effects (Saberi *et al*., 2014). They can be designed for controlled release of essential oil compounds, prolonging their activity and reducing the application frequency.

MATERIALS AND METHODS

Materials

Eucalyptus globulus leaves were collected from G.G.V. Campus, Bilaspur, (C.G, India), infected fruits were collected from the fruit market Bilaspur, (C.G, India), Potato dextrose agar (PDA), potato dextrose broth (PDB), Tween 80 was purchased from HI-Media, Bilaspur (C.G.), India, and distilled water was used.

Collection and isolation of fungal pathogen from *Citrus sinensis* (L.) Osbeck

Fungus infected *Citrus sinensis* were collected from the fruit market of Bilaspur (Chhattisgarh, India) in December 2022 and the fungal pathogen was subsequently cultured on Potato Dextrose Agar (PDA) medium. Pure cultures were obtained and designated as LGFO-1.

Identification of isolated fungi based on ITS sequencing

Genomic DNA was extracted from the isolated fungal strain (LGFO-1), and a segment of approximately 700 base pairs corresponding to the ITS-rDNA region was amplified using high-fidelity PCR polymerase. For amplification, 177 nanograms of the extracted DNA were utilized along with 10 picomoles of each primer. The ITS primers, namely ITS-Forward (5'- TCCGTAGGTGAACCTGCGG - 3') and ITS-Reverse (5'- TCCTCCGCTTATTGATATGC -3'), were employed to target the fungal 5.8S rDNA and its flanking ITS regions. The PCR amplification protocol followed the methodology outlined by Kumaresan *et al*. (2021). Subsequently, the PCR products were purified and subjected to sequencing using an ABI 3500 DNA Analyzer. The resulting sequence data were aligned and analyzed to identify the fungal species and its closest genetic relatives.

Phylogenetic analysis and deposition of culture

The sequence data underwent alignment and analysis to determine the identity of the fungal species and its closest genetic matches. Sequences showing significant similarities were retrieved using NCBI Blast and subsequently aligned using Clustal W (Hung and Weng, 2016). A phylogenetic tree was constructed using sequences aligned with the System Software aligner. The distance matrix was generated using the Jukes-Cantor corrected distance model, considering only alignment model positions and ignoring alignment inserts. The minimum comparable position for generating the distance matrix was set at 200. The phylogenetic tree was built using Weighbor with an alphabet size of 4 and a length size of 1000 (Blanke, 2023). After identification, the fungus sample was deposited in MTCC (Assession no. MTCC 13583) and in the NCBI database (GenBank: PP506143).

Collection of *Eucalyptus* leaves and Extraction of essential oil

Eucalyptus leaves were collected from the G.G.V. campus, Bilaspur (Chhattisgarh, India). The extraction procedure involved the hydro-distillation method using Clevenger's apparatus, as outlined by Elyemni *et al*. (2019). The separated essential oil was collected in a clean glass container. To ensure purity and longevity, dehydration was carried out using anhydrous sodium sulfate to remove any residual water content. The extracted essential oil was stored in the refrigerator at 4°C to preserve its stability and freshness for further investigation.

Preparation of *Eucalyptus* essential oil Nanoemulsion

Nanoemulsions were produced using the low energy method following a two-step process (Shokri *et al*., 2020) with some modifications. Initially, the aqueous phase was created by combining non-ionic surfactant Tween 80 (v/v) with double-distilled water (v/v) and the organic phase by Essential oil (v/v). All these preparations were carried out at room temperature, specifically within the range of 25 to 30 degrees Celsius. Controlling the temperature during the preparation process is critical to achieving stable nanoemulsions with desired properties.

Characterization of *Eucalyptus* essential oil Nanoemulsion

Dynamic light scattering

The size and distribution of emulsion droplets were assessed using a particle size analyzer (Malvern-UK, 4700 model) utilizing the dynamic light scattering (DLS) technique. Before conducting the experiments, the nanoemulsion formulations were diluted with water to eliminate multiple scattering effects. The measurements included determining the droplet size and the polydispersity index (PDI) of the formulated nanoemulsion. The hydrodynamic diameter (dz) represents the average particle size, while the polydispersity index (PI) indicates the distribution width of the particles, reflecting their homogeneity or heterogeneity. All measurements were done in triplicates for accuracy, and the reported values are the mean of three measurements.

Zeta potential measurements

Zeta potential measurements were conducted at 25°C using laser doppler anemometry with a zeta sizer nano instrument Prior to measurement, the samples were diluted with a sample buffer that had been filtered through filters. The zeta potential was determined through three measurements, each comprising ten subruns, and the reported data represents the mean value obtained.

Fourier-transform infrared spectroscopy (FTIR) analysis

Fourier-transform Infrared spectroscopy serves as a robust technique for discerning the structure of both inorganic and organic substances. It operates on the principle that the interaction between infrared radiation and a sample alters the vibrational energy of molecular bonds, offering valuable insights into the functional groups and overall molecular arrangement.

Field emission scanning electron microscopy (FESEM) analysis

Field emission scanning electron microscopy analysis or FESEM analysis, is one of the most widely used methods in material sciences to take images from the surface of the nanomaterials with high resolution and magnification.

Turbidity and % transmittance

Turbidity and % transmittance of the nano-formulations was assessed using a UV-VIS spectrophotometer (Systronics double beam spectrophotometer 2203). Each sample was measured three times, and the average values were reported. For analysis, one milliliter of the nano-formulations was diluted 100 times with double distilled water and analyzed at a wavelength of 100 to 700 nm, using water as the blank reference**.**

Antifungal Activity of *Eucalyptus* essential oil Nanoemulsion (EGE NEs)

The *in vitro* antifungal activity of *Eucalyptus* essential oil and nanoemulsions was evaluated using the agar well diffusion method against *Aspergillus tamarii*. MTCC 13583. *The* procedure involved spreading a swab of fungal suspension on petri plates containing potato dextrose agar (PDA) medium. Forming a well with the help of corn borer and putting bioactive nanoemulsion, essential oil, and fluconazole within the well (Magaldi *et al*., 2004). The plates were incubated at 37°C for 24 hours. During incubation, the bioactive compound diffused into the PDA plates, leading to the inhibition of fungal growth. All the experiment were done in triplicate. The antifungal activity was observed as a clear zone of inhibition surrounding the well.

Determination of MIC through microdilution method using 96-well microtiter and resazurin dye.

The microdilution method was employed using a 96-well microtiter plate along with resazurin dye to determine the MIC of the nanoemulsion was performed according to the CLSI guidelines (CLSI M602020) (Teh *et al*., 2017). Each well contains 100 µl of liquid media potato dextrose broth, and 50 µl of fungal inoculum. Column 12 served as a control to confirm the absence of contamination during plate preparation. In Column 11, a negative control without nanoemulsion. Serial dilutions of the nanoemulsion were prepared, ranging from the highest concentration of 10 µl/ml to the lowest concentration of 0.01 µl/ml achieved through double serial dilution. Prepared microtiter plate placed in an incubator set at 25 °C for 48 hr for Fungi. Following the incubation period, 10 µl of resazurin indicator solution was added to each well, and further incubation took place for 2–4 hours to observe colour changes. Any colour changes from purple to pink or colourless indicate the growth of fungus.

Statistical analysis

The experiments were performed in triplicate, and data are expressed as the mean ± standard deviation ($n = 9$). Statistical analysis was carried out using MS Excel, with statistical significance determined at $p \leq 0.05$. Antifungal agents were evaluated by measuring their zones of inhibition relative to the control.

RESULTS & DISCUSSION

The isolated fungus was successfully cultured (**Figure 1A**) and identified based on Internal Transcribed Spacer rRNA gene sequencing. The constructed phylogenetic tree confirms that the isolated fungus was *Aspergillus tamarii* (**Figure 1B**). The essential oil was extracted from *Eucalyptus* leaves with a yield of 2-3% (**Figure 1C**). The nanoemulsion of *Eucalyptus* essential oil was successfully prepared (**Figure 1D**) with an average droplet size of 169.5 nm (**Figure 1E**), indicating its potential for improved solubility and bioactivity. The low polydispersity index indicated uniform droplet size distribution, contributing to the stability of the nanoemulsion. The zeta potential of (-19.5 mv) confirmed the electrostatic stability of the nanoemulsion (**Figure 1F**). **Figure 1H** shows that the vibrations and stretches observed in the FTIR analysis correspond to specific molecular bonds and functional groups within the nanoemulsion. At 3338.66 cm^{-1}, 1634.12 cm^{-1}, 603.62 cm^-1 the stretching vibration signifies the presence of the O-H bond typically found in the Hydroxyl group, C=C double bond is the characteristic of alkenes or aromatic compounds, and Metal Oxides (M-O) in Inorganic compound respectively (Nandiyanto *et al*., 2019). The utilization of a UV-VIS spectrophotometer enabled the observation of formulated nanoemulsions at a wavelength of 100 to 700 nm. This analytical technique allowed for both qualitative and quantitative assessments of the samples and maximum absorbance was shown in 220 to 240 nm range. According to the absorbance (Abs600) data, the % Transmittance of TAEOs (bioactive) nanoemulsion was found to be 95%, mentioned that the optical properties of nanoemulsions are highly sensitive to variations in size, shape, and concentration, highlighting the importance of such considerations in their characterization (Tomaszewsk *et al*., 2013). The formulation also exhibited excellent stability over 90 days without any visible phase separation or significant changes in droplet size (**Figure 1G**). The FESEM images of the nanoemulsion revealed the presence of spherical and semi-spherical particles, exhibiting a notable degree of aggregation. Within these aggregates, the diameter of some particles reached up to 169.5 nm (**Figure 2i**). **Table 1** shows the antifungal activity of nanoemulsions and antifungal agent (Fluconazole) at different concentration, 1% EO NEs showing a zone of inhibition of about 19.2 ± 0.20 mm the maximum inhibition zone. 1% essential oil showed an inhibition zone of 16.2 ± 0.10 mm and 0.5% showed 9.6 ± 0.10 mm. Remarkably, even the well-known antifungal drug 1% fluconazole demonstrated a smaller inhibition zone of 5.7 ± 0.10 mm compared to the 1% nanoemulsion.

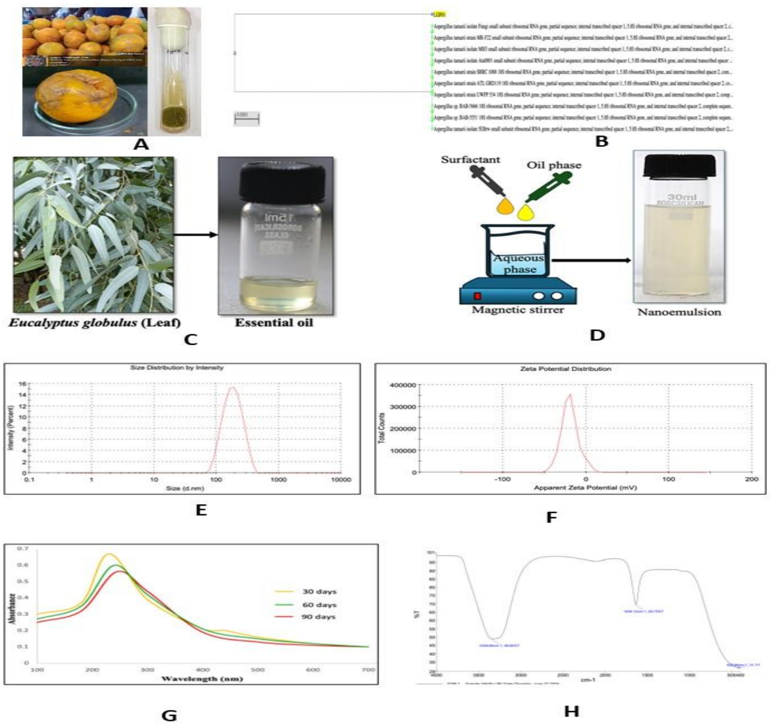

Figure 1: A, Fungi isolated from *Citrus sinensis*; B, Phylogenetic relationship of *Aspergillus tamarii* inferred from ITS sequences analysis by maximum likelihood method; C, Extracted essential oil from *Eucalyptus* leaves; D, Formulation of Essential oil; E, Particle size distribution of essential oil nanoemulsion; F, Zeta potential of essential oil nanoemulsion; G, UV-visible spectrum of essential oil nanoemulsion with time interval of 30,60, and 90 days; H, FTIR analysis of essential oil nanoemulsion.

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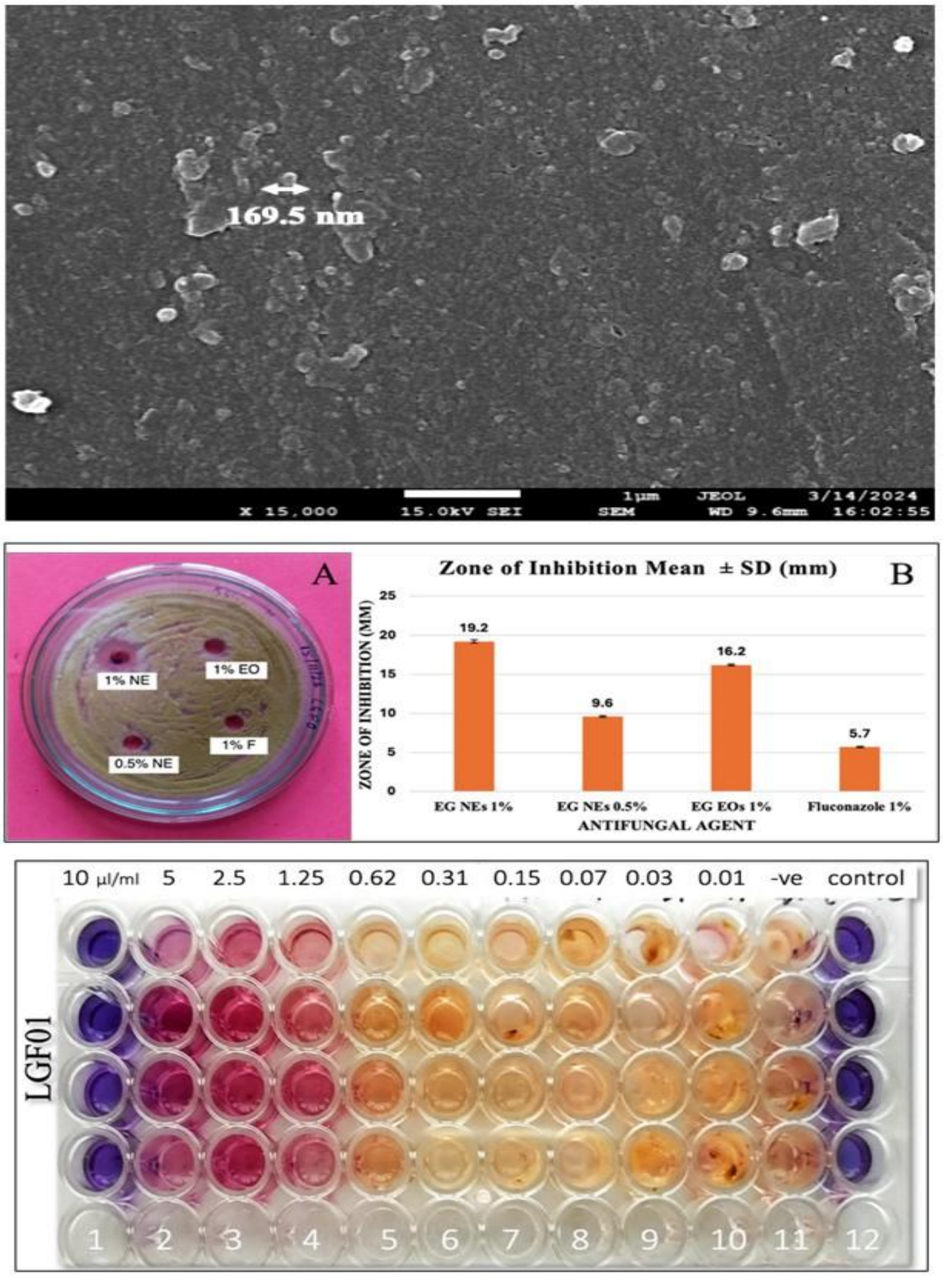

Figure 2: i, FESEM analysis of essential oil nanoemulsion; ii, A-Determining the Antifungal Activity of EO NEs, EOs, Fluconazol against *Aspergillus tamarii* Using well diffusion Method, B- Comparative zone of inhibition; iii) Determination of MIC through microdilution method.

Table 1: Antifungal activity of nanoemulsions and antifungal agent (Fluconazole) at different concentration

Antifungal agent concentration	ZOI Mean ± SD
EG NEs 1%	19.2 ± 0.20
EG NEs 0.5%	9.6 ± 0.10
EG EOs 1%	16.2 ± 0.10
Fluconazole 1%	5.7 ± 0.10

Figure 2ii demonstrates the Graphical representation of the comparative zone of inhibition. In microdilution method upon incubation, wells in Column 1 exhibited no change in color, indicating complete inhibition of microbial growth at that concentration. Thus, the concentration of the NEs in Column 1 (10 µl/ml) was determined as the MIC value, representing the lowest concentration at which no visible microbial growth occurred (**Figure 2iii**).

CONCLUSION

This research successfully fabricated and characterized a stable nanoemulsion of *Eucalyptus globulus* essential oil. The nanoemulsion demonstrated potent antifungal activity against *Aspergillus tamarii,* indicating its potential application in antimicrobial formulations. The findings highlight the significance of nanoemulsions as effective carriers for essential oils and emphasize the therapeutic potential of *Eucalyptus globulus* essential oil in combating fungal infections. The nanoemulsion formulated with Eucalyptus essential oil shows promise as a cost-effective and versatile solution. It holds significant potential for various applications across industries such as food, cosmetics, and pharmaceuticals. Its successful development suggests that it could become a valuable and economically feasible method for addressing a wide range of needs in the future. Further investigations are warranted to explore its application in various therapeutic products and evaluate its efficacy *in vivo*.

ACKNOWLEDGEMENT

We extend our sincere gratitude to the VRET Fellowship for their generous financial support, which greatly facilitated this research. We also express our appreciation to Prof. Sushil Kumar Shahi, Supervisor at the Bioresource Product Research Laboratory, Department of Botany, Guru Ghasidas Vishwavidyalaya, Bilaspur, C.G., India, for his invaluable guidance and mentorship throughout the course of this study.

CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

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