Lemongrass (*Cymbopogon flexuosus*) Essential Oil Nano-Biofilm for Postharvest Management of Orange (*Citrus sinensis*): Ultrasonic Extraction Optimization, Colloidal Characterization, and Antifungal Efficacy against *Alternaria alternata*

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Abstract. Postharvest fungal spoilage, principally caused by *Alternaria alternata*, results in significant losses of citrus fruits during storage and transport. This study developed and evaluated a biodegradable nano-biofilm based on lemongrass essential oil (LEO) from *Cymbopogon flexuosus*, rich in citral, as a sustainable alternative to synthetic fungicides for the postharvest preservation of oranges (Citrus sinensis). Preliminary screening compared the Soxhlet-derived hexane extract and the hydrodistilled essential oil; based on extraction yield and antifungal efficacy, hydrodistillation was selected for further optimization via ultrasonic pretreatment (UPHD), yielding 1.25% under optimized conditions (30 °C, 25 min), compared with 0.91% for the untreated control. The nano-biofilm was formulated with sodium alginate and Tween 80; dynamic light scattering (DLS) characterization revealed a Z-average droplet diameter of 178.6 nm (PDI = 0.269), confirming a stable, narrow-distribution nano-emulsion. In vitro antifungal assessment using the poisoned food technique demonstrated complete inhibition of *A. alternata* at 1000 ppm, whereas the control and biopolymer treatments showed unrestricted fungal growth. In vivo application of 5% and 2.5% LEO coatings on inoculated oranges prevented visible fungal infection and maintained fruit quality; 1.25% LEO provided partial protection with minor shrinkage. Shelf life was extended by up to 10 days under ambient storage. These findings establish LEO-based nano-biofilm as a physicochemically stable, biologically active, and ecologically sound postharvest coating system.

1 Introduction

The significant scale of postharvest losses of fresh fruit and vegetables leads to poor global food security and affects 30–40% of total fruit and vegetable production in developing countries [1]. Oranges (*Citrus sinensis* L. Osbeck) are one of the most popular and widely traded fruit crops. They also suffer from postharvest losses due to senescence and damage from transport and handling, which can worsen with fungal infections [2]. Among the fungal pathogens, *Alternaria alternata* (Fr.) Keissl. has been known to cause black rot and stem-end decay of citrus and can also cause infections by entering through wound openings and even by ambient storage [3].

Thiabendazole and imazalil are synthetic fungicides used to control postharvest losses, but their continued use has been very challenging due to the fungicides leaving significant chemical residues on fresh produce, the emergence of resistant fungi, and increasingly strict regulations in major export markets. This has created a very urgent need to search for alternative, bio-based and biodegradable solutions [4]. Essential Oils (EOs) have been studied for their antifungal properties because they are GRAS, biodegradable, and have a broad-spectrum antifungal activity [5].

Lemongrass EO, obtained from *Cymbopogon flexuosus* (Nees ex Steud.) Watson (family Poaceae) is composed mostly of citral (geranial + neral, 70-85%) which gives it a prolonged potential for antifungal applications [6]. Citral is also unique because it disrupts cells and membranes of fungi. It can integrate dissipation of the electrochemical gradient of membranes and synthesis of ergosterol, which minimizes cellular leakage. This justifies the lower potential for citral-based fungicides than other single-target synthetic fungicides [6, 7]. Due to citral's chemical instability and volatility, citral cannot be applied directly to post-harvest treatments. It needs to be encapsulated and/or emulsified to be stabilized and to allow for controlled release at the surface of the fruit [8].

Biopolymer in nano-emulsion bio-films can effectively be used for controlled release of citral and other essential oils. Sodium alginate, a biopolymer derived from brown algae, is an ideal candidate for the biopolymer framework of nano-emulsions. Sodium alginate, in addition to forming a strong structure that holds essential oils, forms a film on the surface of the fruit that allows gas exchange and is non-toxic and fully biodegradable [9]. Formulations in the nano-scale range (< 200 nm) confer superior surface adhesion, increased uniformity o f coating coverage, and enhanced

antimicrobial activity per unit mass relative to conventional emulsions [8, 10].

Various extraction approaches have been reported for the recovery of bioactive constituents from lemongrass, including solvent extraction and hydrodistillation. Solvent extraction generally yields a broader spectrum of lipophilic compounds, whereas hydrodistillation yields a volatile essential oil fraction suitable for food and postharvest applications. Therefore, a preliminary screening was conducted to identify the most suitable extraction method before optimization and nano-biofilm development.

Despite several reports on EO-based edible coatings for citrus fruits, a comprehensive study that integrates (i) systematic optimization of LEO extraction yield via ultrasonic pretreatment, (ii) colloidal characterization of the resulting nano-biofilm, and (iii) quantitative antifungal evaluation in both in vitro and in vivo settings remains limited. The present study addresses this gap by developing an ultrasonic-assisted extraction protocol to maximize LEO recovery from *C. flexuosus*; formulating and characterizing a sodium alginate-based LEO nano-biofilm; and evaluating its antifungal efficacy against *A. alternata* in vitro and on inoculated oranges.

2 Materials and Methods

2.1 Plant material and fungal culture

Fresh, mature oranges (*Citrus sinensis*) of uniform size were procured from a local market, Noida, Uttar Pradesh, India. High-grade dried lemongrass (*Cymbopogon flexuosus*) was obtained from an online supplier. All experiments were conducted in the Ripening and Processing Laboratory, Amity University, Noida.

The fungal pathogen *A. alternata* (Fr.) Keissl., a causative agent of postharvest black rot, was maintained on Potato Dextrose Agar (PDA; HiMedia, India) and incubated at 28 ± 1 °C for 5–7 days prior to each assay. PDA was prepared at 39 g L^{-1} in distilled water and sterilized at 121 °C for 15 min.

2.2 Extraction of lemongrass essential oil

2.2.1 Ultrasonic-assisted Soxhlet extraction

Dried lemongrass leaves (20 g) were ground and mixed with hexane (100 mL; CDH, India) at a 1:5 (w/v) ratio. As a pretreatment step, the contents were sonicated in an ultrasonic water bath at 40 °C for 30 min (53 kHz). After sonication, the mixture was filtered, and the residue was dried at 40 °C for 30 min in a hot air oven. The dried residue was packed into extraction thimbles and subjected to Soxhlet extraction with n-hexane [4]. The resulting hexane extract was concentrated using a rotary evaporator. The untreated control was subjected to direct Soxhlet extraction without ultrasonic pretreatment. The hexane extract obtained was used only for preliminary bioactivity screening.

2.2.2 Ultrasonic-assisted hydro-distillation (UPHD)

Dried lemongrass leaves (20 g) were ground and hydrodistilled in distilled water (1:5, w/v). For the UPHD treatment, the contents were sonicated at 30 °C for 25 min in an ultrasonic bath. The ultrasonicated sample was transferred to the Clevenger apparatus for hydrodistillation. Essential oil was extracted at 80–90 °C for approximately 2 h. In the control, only hydrodistillation was performed without sonication pretreatment. Two replicates were maintained for the experiment. Based on preliminary screening results, the hydrodistillation-derived essential oil was selected for further optimization and formulation studies.

2.3 Preliminary antifungal screening of extracts

The hexane extract (Soxhlet) and hydrodistilled essential oil were each tested at 1000 ppm against *A. alternata* using the poisoned food technique. Potato dextrose agar was prepared at 40 g L−1, and 500 µL of each extract was incorporated into 25 mL agar to achieve the target concentration. Plates were inoculated with a spore suspension and incubated at 28 ± 1 °C for 7 days. Percentage inhibition of radial fungal growth relative to an untreated PDA control was calculated to determine the superior extract for further study.

2.4 Development of LEO-based nano-biofilm

2.4.1 Biofilm emulsion preparation

To prepare the nano-emulsion, 9.5 mL distilled water and 10 drops of Tween 80 were mixed, and the mixture was magnetically stirred for emulsion preparation at 100-200 rpm. After the stirring, 0.5 mL of LEO was added, and the mixture was sonicated for 5 minutes for initial dispersion. Then, 6 drops of Tween 20 were added, and the mixture was sonicated for 5 minutes for preparation of the nano-emulsion.

2.4.2 Sodium Alginate Matrix Preparation

A 2% (w/v) sodium alginate stock solution was prepared by dissolving 0.6 g of sodium alginate in 30 mL of distilled water and was stirred magnetically until dissolved completely. For the preparation of antifungal formulation, 2 mL of LEO, 2 mL of Tween 80, and 1 mL of Tween 20 were added to 17 mL of the stock sodium alginate solution, thoroughly stirred, and sonicated for 5 minutes to prepare the working solution of 5% LEO biofilm.

2.4.3 Dilution Series

To prepare the 2.5% and 1.25% (v/v) LEO biofilm formulations, the 5% (v/v) LEO biofilm formulation was diluted with distilled water. Each of the formulations was stirred vigorously prior to use to achieve homogenous dispersion of LEO.

2.5 Physicochemical characterization

Droplet size (Z-average diameter), polydispersity index (PDI), and the correlation function intercept were

determined by Dynamic Light Scattering (DLS) using a Malvern Zetasizer Nano S90 (Malvern Instruments, UK), with a 90° detector angle and a maximum sample volume of 30 mL. Results were taken as the mean of three consecutive measurements at 25 °C (Table 5).

2.6 Stability assessment

Phase stability was assessed by vigorously shaking the biofilm formulation in a graduated cylinder for 15 s and observing for phase separation over 30 min. Miscibility was evaluated by mixing 1 mL of the formulation with 10 mL of distilled water. Thermal stability was assessed by storing samples at ambient temperature and at 40 °C for 15 days and monitoring for creaming, phase inversion or visible precipitation.

2.7 In vitro antifungal efficacy — poisoned food technique

Four treatments were evaluated on PDA plates (Table 1). PDA was prepared at 40 g L^{-1}, distributed into sterile beakers in 25 mL aliquots, and autoclaved at 121 °C for 15 min. After cooling to ~50 °C, 500 µL of the respective formulation or control was aseptically added to achieve a final concentration of 1000 ppm (poisoned food technique). The poisoned medium was poured into sterile Petri dishes under laminar airflow and allowed to solidify.

Table 1. Treatment descriptions for in vitro antifungal assay.

No.	Group	Treatment	Inoculation
T1	LEO 5%	Biofilm 5% LEO	*A. alternata spores*
T2	LEO 2.5%	Biofilm 2.5% LEO	*A. alternata spores*
T3	LEO 1.25%	Biofilm 1.25% LEO	*A. alternata spores*
C1	Blank control	Sodium alginate (no LEO)	*A. alternata spores*
C2	Pathogen control	No coating	*A. alternata spores*
C3	Negative control	No coating	None

A spore suspension of *A. alternata* was prepared by transferring a morphologically intact colony fragment into sterile distilled water containing 0.05% Tween 80 and mixing gently until uniform suspension was achieved. After solidification of the poisoned media, 5 µL of the spore suspension was inoculated at five equidistant points on each plate. Plates were sealed with parafilm and incubated at 28 ± 1 °C for 7 days. Fungal colony growth was assessed visually at 3, 5, and 7 days post-inoculation.

2.8 In vivo application and postharvest assessment

A 20 µL suspension of *A. alternata* spores was inoculated into uniformly created wounds on the orange surface to simulate postharvest wound infection [12]. After 2 h of incubation at room temperature to allow spore adhesion, oranges were dipped in the respective coating solutions (Table 2) for 60 s, allowed to drain, and air-dried. Coated fruits were stored at ambient temperature (25 ± 2 °C) and evaluated at 0, 3, 5, 7, and 10 days for fungal growth, surface appearance, shrinkage, and overall quality.

Table 2. Treatment groups and controls used in the in vivo postharvest study.

No.	Treatment	Composition
C1	PDA blank	PDA medium only; no additives (negative control)
C2	Biopolymer control	PDA + sodium alginate solution; no LEO
T1	LEO emulsion	LEO + deionised water + Tween 80 @ 1000 ppm
T2	LEO-based biofilm	LEO + Tween 80 + sodium alginate (5%) @ 1000 ppm

3 Results and Discussion

3.1 Preliminary screening of extraction methods

Soxhlet extraction and hydrodistillation were initially evaluated as extraction approaches for lemongrass biomass (Table 3). While both methods recovered biologically active constituents, hydrodistillation produced a solvent-free essential oil and exhibited superior antifungal activity (97.2%) against *A. alternata* at 1000 ppm. The hexane extract obtained by Soxhlet extraction is a lipophilic solvent fraction containing waxes and non-volatile lipids, in addition to bioactive compounds, and is not equivalent to an essential oil. Therefore, hydrodistillation was selected for further optimization and subsequent development of a nano-biofilm.

Table 3. Preliminary screening of extraction methods for recovery of bioactive constituents from lemongrass.

Extraction method	Product obtained	Yield (%)	Antifungal inhibition (%) at 1000 ppm
Soxhlet extraction	Hexane extract	0.93	86.8 ± 1.2
Hydrodistillation	Essential oil	0.91	97.2 ± 1.8

3.2 Optimization of hydrodistillation

Following the selection of hydrodistillation as the preferred extraction method, ultrasonic pretreatment was evaluated to improve oil recovery (Table 4). UPHD (30 °C, 25 min sonication) increased essential oil yield from 0.91% to 1.25%, representing a 37.4% enhancement, while simultaneously reducing extraction time from 2.2 h to 1.8 h.

Table 4. Optimization of hydrodistillation by ultrasonic pretreatment

Treatment	Sonication condition	Yield (%)	Extraction time (h)
Hydrodistillation (Control)	None	0.91	2.2
UPHD	30 °C, 25 min	1.25	1.8

The yield improvement is mechanistically consistent with ultrasonic cavitation-mediated disruption of lemongrass leaf cell walls and secretory glandular trichomes, thereby reducing the diffusional barrier for intracellular volatile compounds and accelerating their transfer into the extraction medium [11]. The low pretreatment temperature (30 °C) further preserves the thermolabile citral fraction. Silva and Bárbara [11] similarly observed that ultrasound pretreatment substantially reduced extraction time while maintaining or improving the recovery of volatile compounds in hydrodistillation systems.

3.3 Colloidal characterization of the nano-biofilm

The formulated LEO-based nano-biofilm had a Z-average droplet diameter of 178.6 nm, PDI of 0.269 and a correlation function intercept of 0.788 (Table 5). The droplet size of less than 200 nm is a characteristic of nano-emulsions, which are known to have higher kinetic stability, lower creaming rates and better uniformity of surface coverage than conventional macro-emulsions [8, 10]. It is again seen that there are no aggregation or bimodal droplet size distributions, as there is a single peak in the size-distribution spectrum, and the PDI is low (< 0.3). The value of the intercept (0.788) is greater than the recommended 0.6 for high quality DLS measurements which indicates that the signal-to-noise ratio was sufficient and that the size value obtained is reliable. It is expected that by encapsulating LEO at the nano scale within the alginate matrix, surface adhesion, citral release kinetics, and maximizing the surface area of bioactive contact with the fungal cells will be enhanced on the fruit surface [8].

Table 5. The nano-biofilm in the LEO is characterized by DLS. Z-average corrected (typographic error) in preliminary data. .

Parameter	Value	Interpretation
Z-Average diameter (d, nm)	178.6	Nano-scale; optimal for coating
Polydispersity Index (PDI)	0.269	Narrow, uniform distribution
Intercept (quality factor)	0.788	Good signal quality (>0.6)

3.4 Appearance and stability of the biofilm

The nano-biofilm made using LEO showed a thin, flexible film which was slightly translucent and had a slight milky appearance caused by the light scattering from the dispersed LEO nano-droplets. The film was smooth in surface, uniform in thickness and had a characteristic citrus-herbaceous aroma characteristic of successful incorporation of citral. Phase stability testing showed no separation after standing for 30 min and the formulation was totally miscible with water. No phase inversion, creaming, or precipitation was observed after 15 days of thermal storage at both ambient and 40 °C, suggesting the thermal stability for ambient post-harvest application conditions.

3.5 Antifungal efficacy

3.5.1 In vitro

The treatments with LEO (emulsion + sodium alginate biofilm – T1 and T2) completely inhibited *A. alternata* colony formation at all five inoculation points, indicating effective antifungal activity consistent with the properties of citral in disrupting the fungal membrane, as reported previously [6, 7]. The PDA-only blank (C1) and the sodium alginate control (C2) exhibited no antimicrobial activity in the growth media and the bioactivity observed is only due to the LEO component.

The comparable efficacy between the free LEO emulsion (T1) and the alginate-encapsulated biofilm (T2) at the 1000 ppm test concentration suggests that nano-encapsulation did not attenuate the acute antifungal potency of citral under the in vitro test conditions, while its expected benefit—sustained release and improved stability over extended storage—would be more apparent in long-duration in vivo trials [5, 14].

3.5.2 In vivo — postharvest application

In vivo results demonstrated that LEO-based coatings applied at 5% and 2.5% concentrations effectively prevented visible fungal growth at the inoculation wound sites throughout the 10-day storage period, while also maintaining fruit appearance, firmness, and surface integrity without perceptible shrinkage (Figure 1).

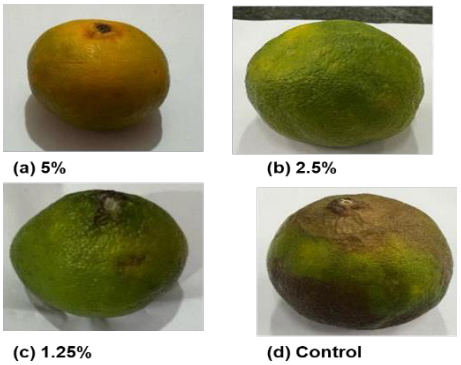

Figure 1. Oranges coated with 5 (a) 2.5 (b), and 1.25 (c) percentage biodegradable biofilm made from lemongrass essential oil, showing great reduction in fungal decay compared with (d) uncoated control.

By day 7, the colony formation of fungi and extent of shrinkage of the surface for the 1.25% LEO treatment were minor (fungistatic effect). The colonies of *A. alternata* were observed on the untreated (control) oranges and the degradation increased at a faster rate with the loss of LEO, which indicated that it played an important role as the active antifungal agent.

The results support the previous reports of accessorial mechanisms of citral rich EO coatings such as disruption of fungal membrane integrity leading to inhibit of spore germination and the physical oxygen barrier effect of alginate film on the fruit surface that led to reduce of the aerobic respiration rate and thus extending the shelf-life [5, 9]. The concentration-dependent efficacy gradient (5% > 2.5% > 1.25%) agrees with the dose dependent membrane disruption action of citral, reported by Gao et al. [6] in the case of the dual-species biofilms of LEO and Sahal et al. [13] with respect to easy disruption of the membrane by *C. citratus* EO in *Candida* biofilms.

4 Conclusion

The results of the preliminary screening indicated the best method used for release of the antifungal essential oil from lemongrass was through the process of hydrodistillation compared to hexane Soxhlet extraction. It is found from this study that the extraction ability of LEO by using UPHD method (30 °C, 25 min) of *C. flexuosus* was better than conventional method of hydrodistillation by 37.4 %. UPHD provides the greatest absolute yields of 1.25%, thus facilitating the subsequent biofilm formation. The droplet diameter of the nano-biofilm formulated with sodium alginate-Tween 80 was 178.6 nm (PDI = 0.269); suggesting that droplets were in the state of stable nano-scale encapsulation that provided the physicochemical basis to achieve effective controlled release of citral at the fruit surface. The antifungal evaluation had proved it to be useful in relation to the fungus *A. alternata*. Both LEO emulsion (free and biofilm) in vitro results complete inhibition of fungal infection in test tubes, but in vivo result showed that LEO formulation (5% and 2.5%) that was applied on oranges provided 10 days of good fruit quality under ambient storage conditions, while there were no fungal infection in test tubes under in vitro conditions. Partial protection was seen with a 1.25% concentration and 2.5% was deemed to be a practical minimum for post harvest materials applications. The shelf life was extended up to 10 days compared to uncoated ones. The simplicity and scalability of the ultrasonic extraction protocol along with an edible/replicable biopolymer matrix and intrinsic antifungal properties of LEO make it an exciting and promising alternative to current post harvest synthetic fungicides in the market. Future research work should concentrate on calculating the release kinetics of citral in simulated cold-chain and ambient storage environment under the nano-biofilm and sensory and regulatory test should be carried out for commercialization and the assessment of the coating for bigger citrus fruit/pathogens as well. The combination with other GRAS antimicrobials (e.g. carvacrol or thymol) can also extend the range of fungal inhibition without compromising the biodegradability benefits of using GRAS products in sustainable post-harvest management.

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