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Litsea cubeba Essential Oil Reduces Postharvest Blueberry Rot by Inducing Cell Membrane Damage and Reactive Oxygen Species Accumulation in *Alternaria alternata*

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ABSTRACT

Fruit rot caused by *Alternaria alternata* infection is one of the primary diseases responsible for postharvest losses in blueberries. *Litsea cubeba* essential oil (LCEO) exhibits potent control efficacy against *A. alternata*, but its antifungal mechanisms and effects on the postharvest quality of blueberries remain unclear. In vitro text showed that LCEO treatment induced severe developmental and hyphal morphology impairments of *A. alternata*. Treatment with 2×MFC LCEO notably reduced blueberry fruit rot incidence while decreasing fruit respiration rate and maintaining fruit quality such as higher fruit weight, firmness, total soluble solids, total acidity, total phenolic, and flavonoid. Mechanistic studies revealed that LCEO treatment severely impaired the cell membrane integrity of *A. alternata* mycelium, manifesting as markedly increased intracellular propidium iodide (PI) fluorescence intensity, pronounced intracellular solute leakage, and increased extracellular electrical conductivity. However, LCEO treatment did not alter cell wall calcofluor white (CFW) fluorescence intensity and alkaline phosphatase activity. Additionally, LCEO application triggered a sharp and sustained increase in ROS accumulation and consequent lipid peroxidation, while leading to a progressive decline in mitochondrial membrane potential. Therefore, LCEO exerts its antifungal activity by impairing the normal growth and development of *A. alternata* through multi-target damage, thereby reducing postharvest rot losses in blueberries.

1 | Introduction

Blueberries, a berry crop of significant economic value and nutritional function, are rich in various bioactive compounds, including anthocyanins, polyphenols, ascorbic acid, flavonoids, and tannins (D. Li et al. 2017; Ma et al. 2018). In recent years, both global blueberry production and consumption have surged dramatically, with China experiencing the fastest growth in blueberry cultivation area and yield (Y. Duan et al. 2022). However, blueberry fruits are highly susceptible to decay owing to their small size, soft skin, and vulnerability to both mechanical damage and fungal infection (Beg et al. 2025; Zhou et al. 2025). Reports indicate that blueberries are prone to infection by fungal pathogens, such as *Botrytis cinerea* (Saito et al. 2022), *Erysiphe vaccinii* (Bradshaw et al. 2025), and *Alternaria* spp. (Neugebauer et al. 2024). Among these, *Alternaria alternata* is a common, aggressive, necrotrophic fungus that widely colonizes the leaves and fruits of various plants, including tomatoes (Carmello et al. 2025), tobacco (Cai et al. 2023), and sweet cherries (Cao et al. 2024). In blueberries, the natural decay rate during storage ranges from 23% to 74%. Specifically, decay caused by *Alternaria* spp. (mainly *A. alternata* and *A. arborescens*) accounts for 11%–46% of the losses (Cong et al. 2024). Current chemical fungicides targeting *A. alternaria* include azoxystrobin, boscalid, and difenoconazole (Schmey et al. 2024). These fungicides are predominantly

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applied during preharvest cultivation and are unsuitable for post-harvest blueberry fruits. However, postharvest diseases frequently occur during storage, indicating that pre-harvest treatments have limited efficacy in controlling blueberry postharvest diseases. Therefore, developing new measures suitable for blueberry preservation is necessary.

Essential oils (EOs) are natural secondary metabolites produced by aromatic plants. Due to their antioxidant properties, broad-spectrum antibacterial activity, environmental friendliness, and low or no toxicity, they are widely used in fruit and vegetable storage preservation research (Gutiérrez-Pozo et al. 2023; Prakash et al. 2024). Furthermore, because EOs target a wide range of mechanisms, the risk of pathogens developing resistance to them is very low (Cardile et al. 2009). *Litsea cubeba* essential oil (LCEO) is a natural aromatic oil extracted from the *Litsea cubeba* plant and its fruit. It contains high levels of citral, imparting a strong lemon scent, along with components such as limonene, methyl heptenone, caryophyllene, geraniol, vanillin, and camphene, which confer broad-spectrum antimicrobial activity to LCEO (Y. Chen et al. 2013). In recent years, LCEO has demonstrated promising applications for controlling postharvest diseases in fruits and vegetables. For instance, studies have indicated that low-concentration LCEO treatments significantly inhibit the growth of *Penicillium digitatum* (Sun et al. 2024) and *B. cinerea* (Tancinova et al. 2022), thereby maintaining high fruit quality in postharvest citrus and strawberries. Similarly, previous research study demonstrated that LCEO treatment effectively inhibits four postharvest pathogens in blueberries (*Penicillium crustosum*, *Aspergillus tubingensis*, *A. alternata*, and *Fusarium verticillioides*), reducing the incidence of blueberry fruit rot; for *A. alternata*, the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of LCEO were 0.5 and 1.0 μL/mL (Wan et al. 2025). However, how LCEO effectively inhibits *A. alternata* growth remains to be elucidated. LCEO reportedly exerts antimicrobial effects by disrupting the integrity of the cell wall membrane of *Colletotrichum scovillei*, leading to cytoplasmic leakage, lipid peroxidation, and disruption of ergosterol biosynthesis (Huang et al. 2023). Similarly, LCEO exhibited antifungal activity by damaging the cell membrane of *Penicillium oxalicum* and enhancing intracellular ROS production (Yu et al. 2024). Furthermore, citral, the primary component of LCEO, damages the mitochondrial morphology and function of *P. digitatum*. It inhibits respiratory metabolism by reducing the activity of TCA-related enzymes and altering TCA metabolic capacity, thereby suppressing the growth of *P. digitatum* (Zheng et al. 2015). This implies that LCEO's mechanism of action varies with pathogen type; however, a systematic investigation of its effects within an individual fungal species is still lacking. Moreover, the preservation mechanisms of LCEO in a wider range of fruits and vegetables, including blueberries, still require further refinement.

Therefore, this study systematically investigated the antifungal mechanism of LCEO against *A. alternata* and its effects on blueberry quality, aiming to provide theoretical references for the application of LCEO in blueberry preservation.

2 | Materials and Methods

2.1 | Materials

The *A. alternata* strain was isolated from decayed blueberry fruit and preserved in the lab after identification through morphology and ITS-rDNA sequence analysis (Wan et al. 2025). The spores' concentration was adjusted to 1×10^5 spore/mL using a hemocytometer. Potato dextrose broth (PDB) was prepared by boiling 200 g of peeled sliced potatoes in 1 L distilled water until soft. The decoction was filtered through double-layered cheesecloth, supplemented with 20 g glucose, and adjusted to a final volume of 1 L with distilled water. To prepare potato dextrose agar (PDA), 1.5%–2% (w/v) agar powder was added to the PDB. Blueberry (Crisp Sweet Floral No. 25) fruit were harvested from Yuxi City, Yunnan Province, and transported through a cold chain to the laboratory immediately after picking. Fruits exhibiting uniform ripeness, size, color, and firmness, with no mechanical damage or pest/disease infestation were selected for the experiment. LCEO, with a purity of 99% (CAS: 68855-99-2), was obtained from Jiangxi Wanhua Flavors & Fragrances Co. Ltd., China. Our previous investigation, employing the agar dilution method, established the MIC and MFC of LCEO against *A. alternata* at 0.5 and 1.0 μL/mL, respectively (Wan et al. 2025). These predetermined values were adopted for the experimental treatments in the current study.

2.2 | Optical Microscope

A. alternata was grown on PDA medium at 28°C for 5 days. A 6 mm disc of *A. alternata* was placed on PDA medium supplemented with LCEO at 0.25 μL/mL (1/2 MIC) and 0.5% (v/v) Tween 80, and PDA medium without LCEO served as the control. After incubation at 25°C for 5 days, a small amount of mycelium was harvested for methylene blue staining. Subsequently, the microscopic morphology of the mycelium and spores was observed under an NE620 optical microscope (Yongxin, China) and documented photographically.

2.3 | Scanning Electron Microscope (SEM) Observation

An appropriate amount of spore suspension was added to PDB to achieve a final concentration of 1×10^5 spore/mL. The mixture was incubated at 25°C on a shaking incubator (160 rpm) for 48 h to obtain mycelium. 0.5 g mycelium was harvested, washed with sterile water, and then incubated in PDB containing MIC LCEO for 120 min. After washing with phosphate-buffered saline (PBS), the hyphae were fixed in glutaraldehyde solution (4°C) for 24 h, rinsed three times with distilled water, dehydrated using a gradient of 30%, 50%, 70%, and 95% (v/v) ethanol, and treated with absolute ethanol for 45 min (Tan et al. 2022). The samples were freeze-dried and gold-sputtered, followed by JSM-6610LV scanning electron microscopy (JEOL, Japan) to observe the hyphal ultrastructure.

2.4 | Blueberry Fruit Infection Experiment

Blueberries were randomly divided into control, 2×MFC, and 8×MFC LCEO treatment groups (all solutions contained 0.5% [v/v] Tween 80 to improve the solubility of LCEO), each containing 60 fruit and three replicates. A 2 mm × 2 mm wound was created in the equatorial region of the fruit and inoculated with 5 μL of *A. alternata* spore suspension (1×10^5 spore/mL). After air-drying at room temperature, the treated berries were immersed in the corresponding LCEO concentrations for 3 min, while the controls were immersed in sterile water. Disease incidence was monitored continuously for 5 days at 25°C and 95% relative humidity.

2.5 | Blueberry Quality Assessment

Blueberry fruit from the control and LCEO-treated groups described in Section 2.4 were selected for daily quality evaluation over a 5 days period. The following parameters were measured:

2.5.1 | Relative Weight Change

Daily determination of the relative weight changes of blueberry fruits was performed following the protocol of Tan et al. (2021), with slight adjustments, with the calculation formula shown in Equation (1).

$$\text{Relative weight change} = \frac{W_x - W_0}{W_0} \times 100\%, \quad (1)$$

where W_0 represents the initial mass of the blueberries; W_x denotes the mass of the blueberries after storage.

2.5.2 | Color Difference

Measurements were performed according to the method described by Chu et al. (2018), using a CR-400 colorimeter (Konica Minolta, Japan) on the sound (unblemished) areas of the fruit.

2.5.3 | Firmness

According to the method described by X. F. Duan et al. (2016), firmness was determined by vertically inserting a GY-2 probe (Minghan, China) approximately 2 mm into a healthy area of the fruit at a constant speed and recording the dial indicator value.

2.5.4 | Total Soluble Solids (TSS)

According to the method described by Fan et al. (2014), with slight adjustments, fruits were ground into a slurry, filtered through gauze, and the filtrate was analyzed using a PAL-1 digital handheld refractometer (ATAGO, Japan).

2.5.5 | Titratable Acidity (TA)

According to the method described by Q. Ouyang et al. (2025), with slight adjustments, a 10 g blueberry sample was homogenized in 10 mL ultrapure water. Subsequently, 10 mL of the homogenate was transferred to a 50 mL volumetric flask, diluted to volume with ultrapure water, and mixed thoroughly. The mixture was titrated with 0.1 M NaOH using phenolphthalein as the indicator.

2.5.6 | Respiration Rate

Following the method of Wang et al. (2022), the respiratory rate of blueberry fruits was determined using a fruit and vegetable respirometer (3051H, Zhejiang Top Yunnon Technology Co. Ltd.). Each treatment group consisted of five blueberry fruits, and the experiment was performed with three replicates per treatment. The CO_2 emission rate was recorded over a 30-min period, with the calculation formula shown in Equation (2):

$$\text{Respiration rate} = \frac{(a - a_0)\frac{44}{22.4} \times \frac{273}{273+T}}{m \times h}, \quad (2)$$

where a = final CO_2 emission rate; a_0 = initial CO_2 emission rate; T = measurement temperature; m = weight of blueberry fruit; h = duration of measurement.

2.5.7 | Anthocyanin, Total Phenolics, and Flavonoids

According to the method described by Lian et al. (2022). A 2 g sample of blueberry pulp was homogenized in 1% HCl-methanol solution (1:25, m/v) and extracted in the dark at 4°C for 20 min with occasional shaking. After filtration, 0.1 mL of the filtrate was mixed with 9.9 mL of buffer solutions at pH 1.0 (HCl/KCl) and pH 4.5 (acetate/sodium acetate). The mixture was scanned over the full wavelength range using a UV-2802S spectrophotometer (Unico, China) to identify the maximum absorption wavelength of the anthocyanins. After equilibration, the absorbance was measured at this wavelength to quantify the anthocyanin content. According to the method described by B. Duan et al. (2023), absorbance was also measured at 280 and 325 nm to assess the total phenolic and flavonoid contents, respectively.

2.6 | Calcofluor White (CFW) and Alkaline Phosphatase (AKP) Assay

CFW staining experiment was measured according to Q. Ouyang et al. (2025) with minor alterations. The collected *A. alternata* mycelium was treated with 1/2 MIC and MIC of LCEO for 30, 60, and 120 min. After rinsing with PBS, a small amount of mycelium was spotted onto slides and stained with 10 μL CFW (Catalog No. G3235, Beijing Solarbio Science & Technology Co. Ltd, China) for 10 s. Subsequently, 10 μL of 10% KOH solution was added for decolorization of the samples. Changes in cell wall fluorescence intensity were observed under a fluorescence microscope (Nikon, Japan) and quantified using the ImageJ software. The AKP activity assay procedure follows the

established protocol (Xiao et al. 2023). The filtrate was mixed with 0.1 g/dL p-nitrophenyl phosphate disodium salt in a 2:1 (v/v) ratio to obtain a total volume of 1.5 mL. The samples were then incubated in the dark at room temperature for 30 min. Then, 0.2 mL of 1 mol/L NaOH was added to terminate the reaction. The AKP activity was ultimately reflected by measuring the absorbance of the assay solution at a wavelength of 405 nm.

2.7 | Propidium Iodide (PI) Staining

Experiment was measured according to Zhang et al. (2023) with minor alterations. *A. alternata* mycelium (0.05 g wet weight) was collected after treatment with 1/2 MIC or MIC LCEO for 30, 60, or 120 min. The harvested mycelium was then resuspended in 1 mL of PBS (pH 7.4) and then mixed with 10 μg/mL PI (Catalog No. P8080, Beijing Solarbio Science & Technology Co. Ltd., China) at a 100:1 (v/v) ratio and incubated at 37°C for 10 min. The residual dye was removed by washing with PBS, and the samples were observed under a fluorescence microscope. Fluorescence intensity was analyzed using the ImageJ software.

2.8 | Cell Membrane Permeability

The experiment was performed to measure intracellular leakage according to Huang et al. (2023), with minor alterations. An appropriate amount of *A. alternata* mycelia was resuspended in PDB medium containing 0 (control), 1/2 MIC, or MIC of LCEO and incubated at 25°C with shaking at 160 rpm. Culture filtrates were collected after 0, 30, 60, and 120 min of treatment and centrifuged at 10,000 r/min for 5 min. The absorbance of the supernatant was measured at 260 and 280 nm using a UV-2802S spectrophotometer (Unico, China) to assess the leakage of intracellular nucleic acids and proteins, respectively. 0.05 g of *A. alternata* mycelia were incubated in 10 mL PBS (pH 7.0) with different concentrations of LCEO (0, 1/2 MIC, and MIC) at 25°C with shaking at 160 rpm. Samples were collected at 0, 30, 60, and 120 min, centrifuged at 10,000 r/min for 5 min, and the supernatant was equilibrated to approximately 25°C. The electrical conductivity of the supernatant was measured directly using a DDSJ-308A conductivity meter (Leici, China), with each sample tested in triplicate.

2.9 | Total Lipid Content Determination

An appropriate quantity of *A. alternata* mycelium was collected and subjected to freeze-drying. Subsequently, 0.01 g of the dried mycelium powder was weighed and homogenized in 4 mL of distilled water under low-temperature conditions to form a uniform slurry. Then, 2 mL of the slurry was combined with 4.2 mL of a pre-chilled extraction solvent (methanol: chloroform: water = 1:1:1, v/v/v) and vigorously vortexed for 10 min. The mixture was centrifuged at 8000 r/min for 10 min. Following centrifugation, 0.5 mL of the lower organic phase was collected, mixed with 200 μL of concentrated sulfuric acid, and heated in a water bath for 10 min. After cooling to room temperature, 3 mL of phosphorescent vanillin reagent (1.2 g/L) was added and mixed thoroughly. The absorbance was measured at 520 nm. All experiments were performed in triplicate, and the average value was reported (Tao, Jia, and Zhou 2014; Tao, OuYang, and Jia 2014).

2.10 | Reactive Oxygen Species (ROS) and Mitochondrial Membrane Potential (MMP) Determination

ROS and MMP in *A. alternata* mycelium treated with LCEO at different time points were measured according to the protocols of the DCFH-DA Kit (Catalog No. ID31309, Beijing Solarbio Science & Technology Co. Ltd, China) and JC-10 Kit (Catalog No. CA1310, Beijing Solarbio Science & Technology Co. Ltd, China). Fluorescence intensity was analyzed using the ImageJ software.

2.11 | Malondialdehyde (MDA) Content Determination

MDA was measured according to the procedure described by Tan et al. (2020), with some modifications. Five grams of *A. alternata* mycelium were homogenized with 5.0 mL of 10% trichloroacetic acid solution using an ice bath and grinding at 8000 rpm. The homogenate was centrifuged at 4°C for 10 min. Then, 2 mL of the supernatant was collected and mixed with an equal volume of 10% trichloroacetic acid containing 0.67% barbituric acid. The mixture was boiled in a water bath for 15 min, cooled, and centrifuged. The absorbance of the resulting supernatant was measured at 450, 532, and 600 nm. In the blank control group, the sample was replaced with distilled water. Each experimental group was repeated thrice.

2.12 | Statistical Analysis

All experiments were performed in triplicate. Data were processed and analyzed using SPSS 26. Statistical significance between groups was evaluated using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test, with significance defined at $p < 0.05$ or $p < 0.01$. Data are presented as mean ± standard error (SE). All graphs were prepared with Origin 2024 and Ps 2023.

3 | Result

3.1 | LCEO Treatment Induced Developmental Abnormalities and Altered Hyphal Morphology in *A. alternata*

After 5 days of cultivation on PDA medium, the control group exhibited normal colony morphology (Figure 1A), with an intact mycelium structure and relatively plump smooth mycelium surface morphology under both light microscopy and SEM (Figure 1C,E). In contrast, colonies treated with 0.5 μL/mL LCEO showed slowed growth rate and lighter pigmentation

(Figure 1B). Under optical microscopy, hyphae exhibited wrinkled and twisted morphology with irregular branch diameters, and unstainable regions within the hyphal structure (Figure 1D). SEM analysis revealed surface collapse, shrinkage, and deformation of hyphae (Figure 1F). These findings indicate that LCEO treatment induces developmental abnormalities and morphological alterations in *A. alternata* hyphae.

3.2 | Effects of LCEO Treatment on the Occurrence of Postharvest Rot in Blueberry Fruit

At 2 days of storage after inoculation with *A. alternata*, fruits in the control group exhibited water-soaked lesions at the wound sites, with some fruits showing cracking, resulting in a disease incidence of 41.66%. As storage continued, both cracking and water-soaked lesions expanded further, culminating in a disease incidence of 100% at 5 days of storage.

In contrast, LCEO treatment reduced the occurrence of blueberry fruit rot. At 2 days of storage, water-soaked lesions were observed in the 2×MFC and 8×MFC LCEO-treated groups, with disease incidence rates of 16.66% and 18.02%, respectively, both lower than that of the control group at the same time point. As the infection progressed, the 2×MFC LCEO-treated group showed enlarged water-soaked lesions but no wound cracking. The disease incidence in this group was significantly lower than that in the control group on 3–4 days. At 5 days of storage, the disease incidence in the 2×MFC LCEO-treated group was 78.33%, significantly lower than that in the control group (100%). In comparison, fruits treated with 8×MFC LCEO exhibited darkening in color and expansion of water-soaked lesions. Although disease incidence in this group was significantly lower than the control group within 2–3 days, after 5 days of storage, the disease incidence in the 8×MFC group reached 100%, matching that of the control group (Figure 2). The results indicate that LCEO can reduce the incidence of postharvest blueberry fruit rot within a certain concentration range.

3.3 | Effects of LCEO Treatment on the Quality of Blueberries Inoculated With *A. alternata*

During storage, the control group exhibited a gradual increase in its weight. The 2×MFC LCEO treatment group showed a transient weight reduction at 1 day, followed by gradual recovery and stabilization, although the value at 5 days remained lower than that of the control group (−0.35 ± 1.79b). In

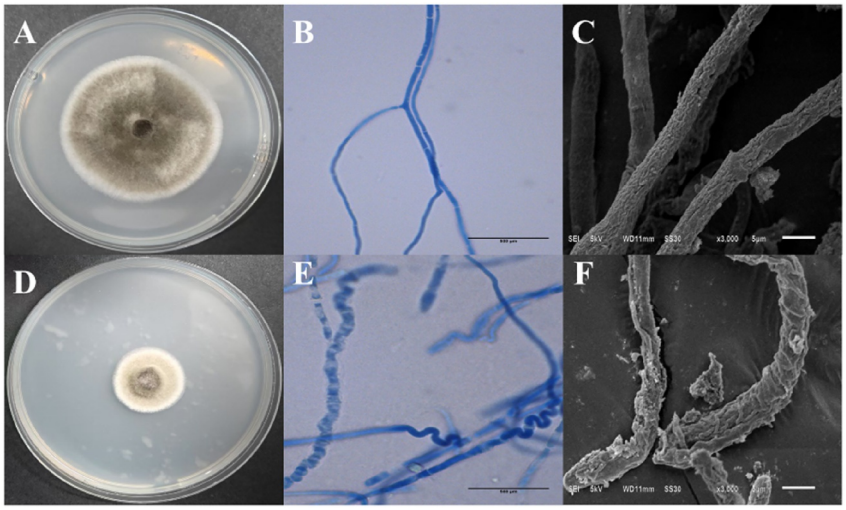


FIGURE 1 | Effects of LCEO treatment on *Alternaria alternata*. Colony morphology of control (A) and treated (B) groups after 5 days culture on PDA; hyphal morphology of control (C) and treated (D) samples after 5 days culture on PDA; hyphal morphology of control (E) and treated (F) groups after 120 min of exposure in PDB. Scale bars in panels (C) and (D) represent 500 and 2 μm for panels (E) and (F), respectively.

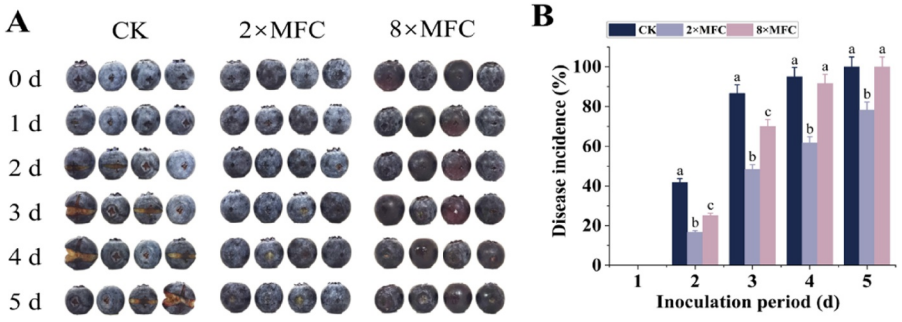


FIGURE 2 | Effect of LCEO treatment on disease incidence in blueberry fruit inoculated with *Alternaria alternata*. (A) Appearance; (B) disease incidence. Different lowercase letters at each time point denote significant differences, as determined by Duncan's test ($p < 0.05$).

contrast, the 8×MFC group displayed significant weight loss early in storage, which continued throughout the period, reaching −1.18% (−1.18 ± 0.39b) at 5 days.

Low-concentration LCEO maintained fruit firmness. The firmness of the control fruit decreased gradually during storage, whereas that of the 2×MFC group remained relatively stable and was significantly higher than that of the control at 5 days. However, the 8×MFC group showed a sharp decline in firmness from the beginning, which continued until 5 days, reaching only 1.58 ± 0.73b—significantly lower than both the control (1.98 ± 0.41b) and the 2×MFC group (2.82 ± 0.52a).

The lightness (L^* value) of the control group remained stable throughout the storage period. In comparison, LCEO-treated fruit showed a significant drop in the L^* value early on; despite some fluctuation, it remained significantly lower than that of the control by 5 days. The green–red chromaticity (a^* value) of the control changed only slightly during storage. The 2×MFC group showed no significant difference in a^* value compared to the control, whereas the 8×MFC group exhibited a marked increase from the start, continuing to rise until reaching 4.34 ± 0.86a at 5 days, which was significantly higher than that of the control (2.96 ± 0.14b) and the 2×MFC group (2.80 ± 0.10b). The blue–yellow chromaticity ($-b^*$ value) of the control declined gradually from 5.64 ± 0.58a to 4.74 ± 0.60a. The LCEO-treated groups showed an abrupt decrease in $-b^*$ value early in storage and maintained lower levels than the control throughout the storage period.

LCEO helped maintain TSS content, whereas a high concentration accelerated the metabolic conversion of organic acids. No significant differences were observed in the TSS among the groups during storage. Similarly, TA did not differ significantly between the control and 2×MFC group, whereas the 8×MFC group showed significantly higher TA content (1.00 ± 0.04a) than the control (0.53 ± 0.20b) on 5 days.

LCEO effectively suppressed fruit respiration. The respiration rate of the control remained stable, whereas both the 2×MFC and 8×MFC groups showed a significant reduction early in storage, remaining lower than that of the control thereafter. High-concentration LCEO accelerated the decline in the total phenolic content. No significant difference was observed between the control and 2×MFC group, but the 8×MFC group showed a pronounced decrease from the beginning, falling to 8.50 ± 0.25b by 5 days, which was significantly lower than both the control (9.75 ± 0.25a) and the 2×MFC group (9.50 ± 0.25a).

Different LCEO concentrations maintained flavonoid content at different times. The control group exhibited fluctuating flavonoid levels during storage. The 2×MFC group had a significantly higher flavonoid content than the control on 1, 3 and 4 days. The 8×MFC group showed a significant increase early in storage, with values higher than those of the control on 1 and 4 days.

Only the 2×MFC group exhibited a higher anthocyanin content than that of the control group on 4 days. The 8×MFC group exhibited a markedly reduced anthocyanin content from the first day onward, with a consistent decline observed throughout the storage period (Figure 3). The results above indicate that treatment with 2×MFC LCEO not only significantly reduced the decay rate and respiration rate of blueberry fruits but also maintained excellent postharvest commodity quality. In contrast, although the 8×MFC treatment increased the total acidity and maintained soluble solids content in blueberries, it promoted the decline of other quality indicators.

3.4 | LCEO Treatment did Not Affect the Integrity of the Cell Wall in *A. alternata*

The fluorescence intensity of CFW in the mycelia remained strong and stable in both the control and LCEO-treated groups over the 120 min period, with no significant difference observed between them (Figure 4A,B). Extracellular AKP activity showed an increasing trend over time in both groups, but no statistically significant differences were detected (Figure 4C). These results indicate that LCEO had no significant effect on the cell wall integrity of *A. alternata*.

3.5 | LCEO Treatment Compromised the Cell Membrane Integrity of *A. alternata*

The fluorescence intensity of PI in the mycelia increased in a concentration- and time-dependent manner in the LCEO-treated groups and was significantly higher than that in the control group. At 30 min, faint red fluorescence was observed in the control mycelia, whereas bright red fluorescence was detected in both the 1/2 MIC and MIC LCEO-treated groups, with fluorescence intensities 1.1 and 2.6 times higher than that of the control, respectively. With prolonged treatment and higher concentrations, red fluorescence in the LCEO-treated groups increased markedly, accompanied by a continuous increase in fluorescence intensity (Figure 5A,B).

The total lipid content in the control group showed minimal overall change. After 60 min of treatment, the 1/2 MIC LCEO group showed a significant increase in mycelial total lipids (177.90 ± 3.50 mg/g DW), whereas the MIC LCEO group showed a marked decrease (145.09 ± 3.95 mg/g DW). By 120 min, both treatment groups displayed opposite directional changes compared to their earlier responses, although neither differed significantly from the control (Figure 5C). These results suggest that LCEO may exert its effects on the cell membrane integrity of *A. alternata* by inducing dynamic fluctuations in the mycelial composition.

3.6 | LCEO Treatment Induced Intracellular ROS Accumulation in *A. alternata*

The control group exhibited minimal fluorescence throughout the treatment period, with only minor fluctuations in the fluorescence intensity. In contrast, after 30 min of treatment, visible fluorescence was observed in both the 1/2 MIC and MIC LCEO-treated groups (Figure 6A), with fluorescence intensities 1.1 and 2.9 times higher than that of the control, respectively. As the treatment duration and concentration increased, the

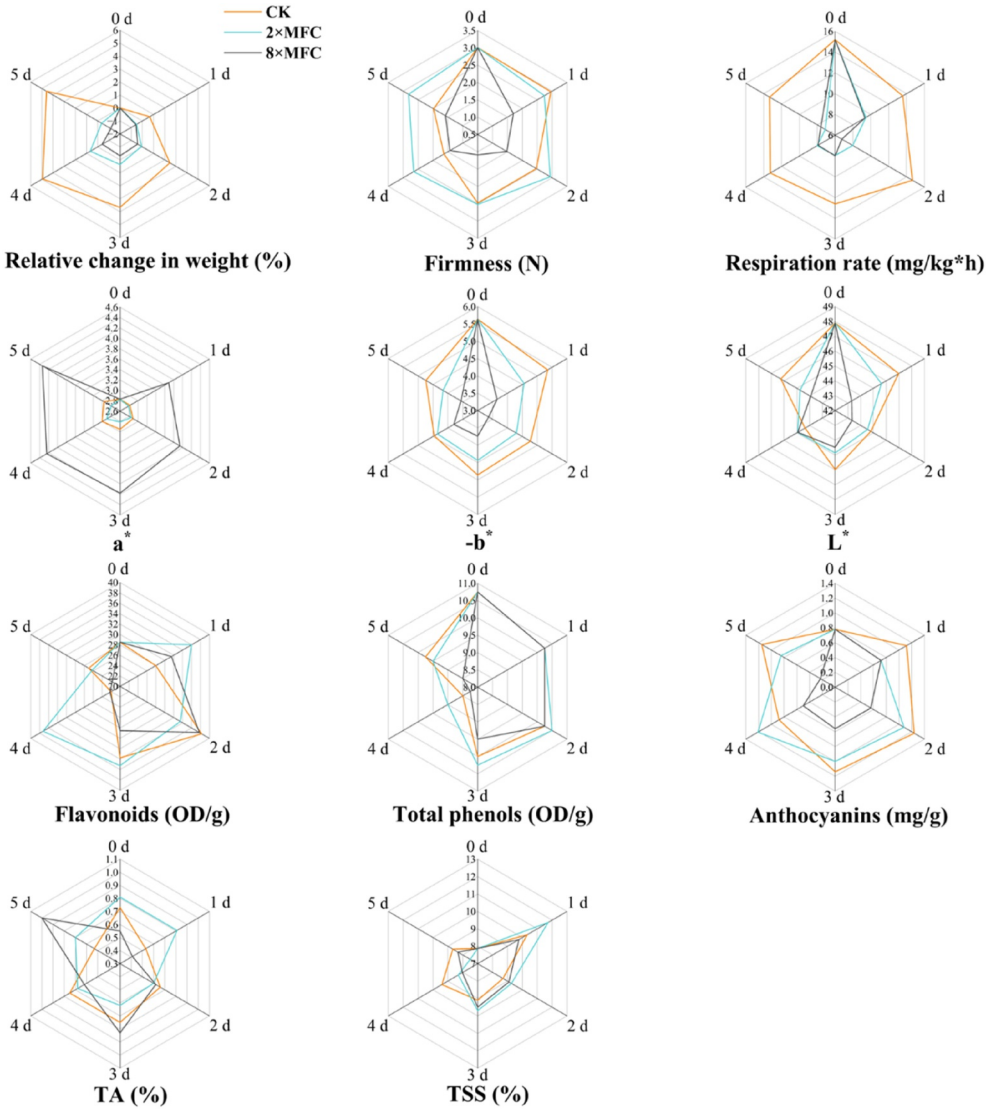


FIGURE 3 | Impact of LCEO treatment on the postharvest quality maintenance of blueberry fruit inoculated with *Alternaria alternata*.

fluorescence intensity increased rapidly. After 120 min, the fluorescence intensity in the LCEO-treated groups was 3.1 and 5.8 times that of the control group (Figure 6B). These results demonstrate that LCEO treatment may mediate its antifungal effect through inducing intracellular ROS accumulation.

3.7 | LCEO Stimulated MDA Accumulation and Induced Intracellular Leakage of *A. alternata* Mycelium

The MDA content in both the control and LCEO-treated groups initially increased, followed by a decrease. Throughout the treatment period, the MDA levels in the LCEO-treated groups were significantly higher than those in the control group. At 60 min, the MDA content peaked in the control, 1/2 MIC, and MIC LCEO-treated groups, with values of 0.19 ± 0.0071, 0.21 ± 0.00, and 0.25 ± 0.0062 μmol/L, respectively, LCEO-treated groups were 11% and 32% higher than that in the control group (Figure 7A). These results indicate that LCEO treatment induced membrane lipid peroxidation in *A. alternata*.

The nucleic acid leakage, protein leakage, and extracellular conductivity in the LCEO-treated groups were significantly higher than those in the control group (Figure 7B,D). Furthermore, both protein leakage and extracellular conductivity increased with rising LCEO concentrations. These results indicate that LCEO treatment compromises the cell membrane permeability of *A. alternata* hyphae.

3.8 | LCEO Treatment Impaired the MMP of *A. alternata*

The fluorescence intensity of the control group remained stable over the 120 min, whereas that of the LCEO-treated groups exhibited a continuous decline. The difference between the control and treatment groups became more pronounced over time. At

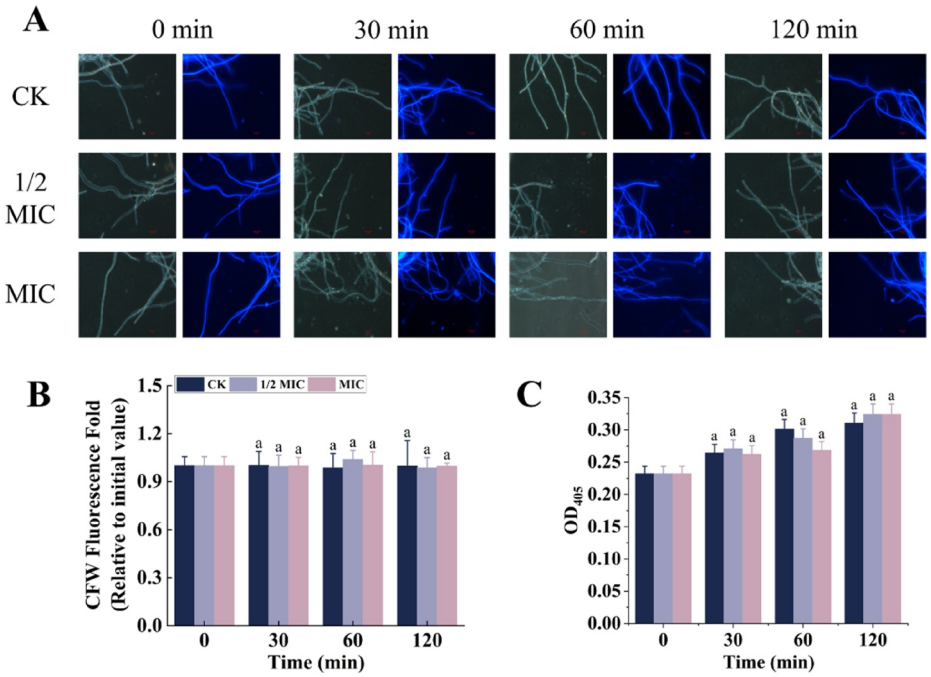


FIGURE 4 | Effects of LCEO on the cell wall integrity of *Alternaria alternata*. (A) Microscopic images of CFW-stained hyphae; (B) quantitative analysis of fluorescence fold changes; (C) the extracellular AKP activity of *A. alternata*. Scale bar in 4(A) was 10 μm. Different lowercase letters at each time point denote significant differences, as determined by Duncan's test ($p < 0.05$).

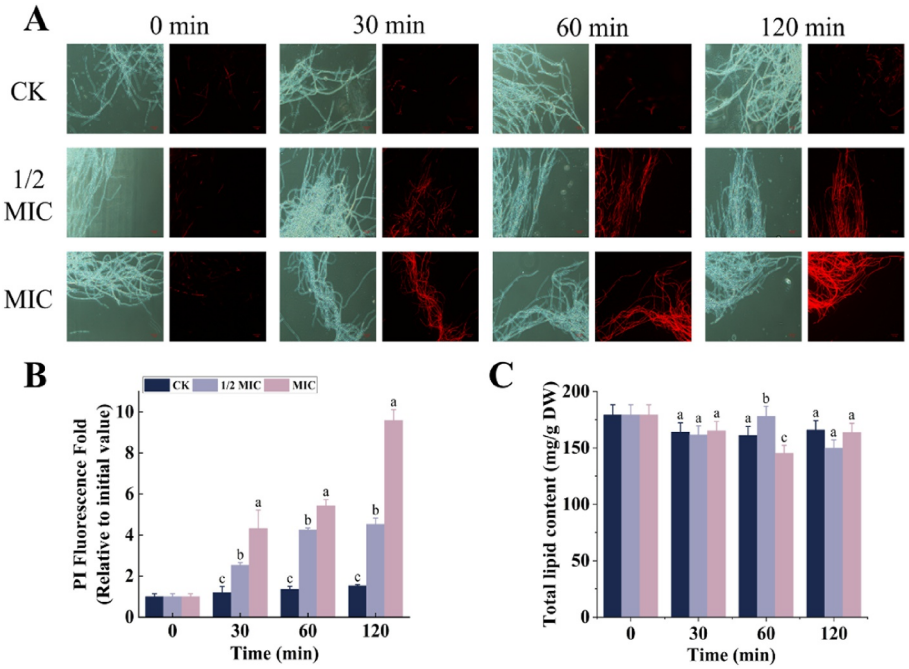


FIGURE 5 | Effects of LCEO on the cell membrane integrity of *Alternaria alternata*. (A) Microscopic images of PI-stained hyphae; (B) quantitative analysis of fluorescence fold changes; (C) total lipid content. Scale bar in 5(A) was 10 μm. Different lowercase letters at each time point denote significant differences, as determined by Duncan's test ($p < 0.05$).

30 min, LCEO treatment resulted in a reduction in MMP-related fluorescence; however, the difference between the 1/2 MIC group and the control was not statistically significant, whereas the MIC treatment caused a significant decrease in fluorescence. With prolonged exposure, both fluorescence intensity and relative fluorescence values in the treated groups continued to decline. After 120 min of treatment, the fluorescence intensities of the 1/2 MIC and MIC groups were (0.78 ± 0.028) and (0.57 ± 0.017), respectively, which were significantly lower than that of the control group (1.1 ± 0.089; Figure 8). These results indicate that LCEO treatment leads to damage to the mitochondrial membrane potential in *A. alternata* mycelia.

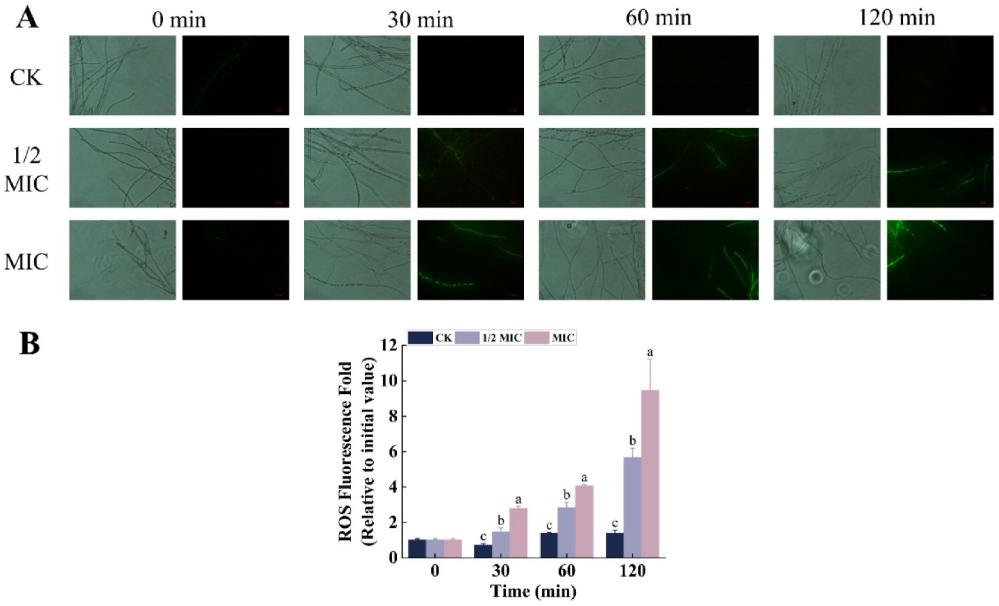


FIGURE 6 | Effects of LCEO on the ROS content of *Alternaria alternata*. (A) Microscopic images of DCFH-DA-stained hyphae; (B) quantitative analysis of fluorescence fold changes. Scale bar in 6(A) was 10 μm. Different lowercase letters at each time point denote significant differences, as determined by Duncan's test ($p < 0.05$).

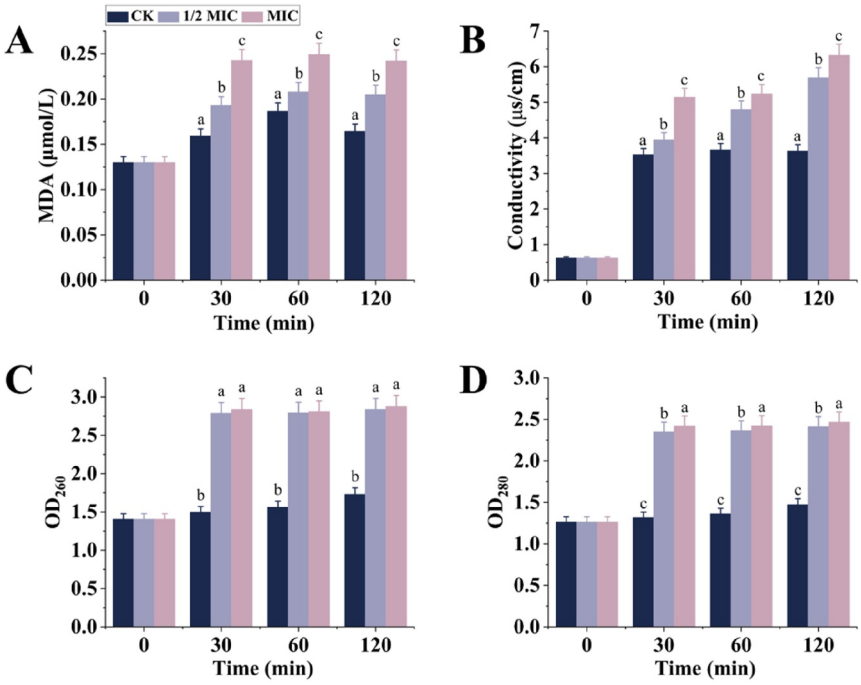


FIGURE 7 | Effects of LCEO treatment on intracellular MDA (A), extracellular conductivity (B), nucleic acid leakage (C), and soluble protein release (D) in *Alternaria alternata*. Different lowercase letters at each time point denote significant differences, as determined by Duncan's test ($p < 0.05$).

4 | Discussion

Essential oils (EOs) have been reported to enhance the defense responses of fruit and vegetable tissues against pathogens and to reduce physiological deterioration (El Khetabi et al. 2022). For instance, thyme essential oil significantly reduced the incidence of brown rot in peaches (Cindi et al. 2016), and clove oil at concentrations ranging from 0.05% to 0.8% (v/v) effectively suppressed *Penicillium italicum* in citrus fruits (C. Chen et al. 2019). In this study, 2×MFC LCEO markedly reduced the decay rate of blueberry fruits inoculated with *A. alternata*, demonstrating its efficacy in suppressing postharvest fruit rot. Furthermore, treatment with 2×MFC LCEO enhanced the firmness of infected blueberries and helped maintain levels of TSS, TA, total phenolics, and flavonoids, indicating that LCEO contributes to the preservation of fruit quality and extension of storage life. Similarly, a konjac glucomannan coating incorporated with 5.0 g of LCEO nanoemulsion completely inhibited

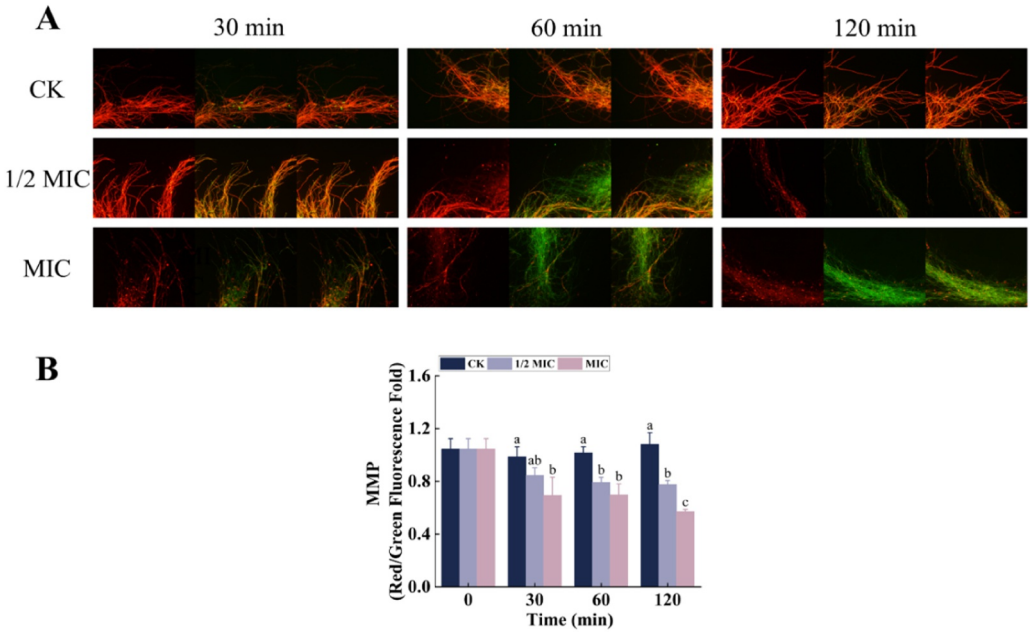


FIGURE 8 | Effects of LCEO on the MMP of *Alternaria alternata*. (A) Microscopic images of JC-10-stained hyphae; (B) the mycelia red/green fluorescence ratio. Scale bar in 8(A) was 10 μm. Different lowercase letters at each time point denote significant differences, as determined by Duncan's test ($p < 0.05$).

the growth of *P. italicum* without inducing phytotoxicity in citrus fruits, while also maintaining quality parameters such as firmness, TSS, and TA (Lou et al. 2024). In contrast, although the 8×MFC LCEO treatment increased TA and maintained TSS levels, it accelerated the deterioration of other quality attributes of blueberry fruits. This is consistent with previous findings that treatment with 500 μL sage essential oil reduced tomato firmness, increased respiration and ethylene production rates, and compromised antioxidant capacity (Chrysargyris et al. 2021). In this study, 8×MFC demonstrated inferior efficacy to 2×MFC in both delaying disease onset and maintaining fruit quality, likely due to phytotoxic symptoms induced by high LCEO concentrations. The antifungal activity of LCEO's primary component, citral, typically manifests through damage to fungal cell membranes. This mechanism may also cause disruption to peel tissue, thereby reducing fruit antimicrobial capacity and quality attributes (Rahman et al. 2023). Reports indicate that no phytotoxicity was observed in untreated citrus fruits or those exposed to 20 mL/L citral. However, peel damage symptoms were observed in fruits exposed to 60 mL/L citral and 150 mL/L citral (Wuryatmo et al. 2014). Together, these findings highlight the considerable potential of LCEO in reducing disease incidence and maintaining fruit quality, although the applied concentration must balance antimicrobial efficacy with tissue protection.

Given that LCEO treatment significantly reduced the incidence of postharvest blueberry fruit rot while maintaining fruit quality, we further investigated its inhibitory mechanism against *A. alternata* at the tissue and cellular levels. Morphological alterations in fungal hyphae following EOs treatment are often attributable to increased cell wall and membrane permeability, resulting in ion and metabolite leakage, membrane lesions, and disrupted cellular metabolism (Tao, Jia, and Zhuo 2014; Tao, OuYang, and Jia 2014). In this study, LCEO effectively inhibited the growth of *A. alternata*, and optical microscope and SEM revealed significant morphological abnormalities in LCEO-treated *A. alternata* hyphae, including surface wrinkling and structural distortion. This finding is consistent with previous reports that citral, the main component of LCEO, induced folding and rupture in the hyphae of *Magnaporthe grisea*, as observed under SEM (R.-Y. Li et al. 2014). The results confirm that LCEO exerts a strong inhibitory effect on *A. alternata* growth and damages the hyphal surface morphology. The fungal cell wall, a key component in maintaining cellular structural integrity, is primarily composed of polysaccharides such as chitin and glucan and is often considered a target of antifungal EOs (Dou et al. 2018; Nazzaro et al. 2017). CFW, a fluorescent dye that specifically binds to chitin and cellulose in the cell wall, is commonly used to assess cell wall integrity (Florczuk et al. 2023). AKP is a key enzyme involved in the hydrolysis of phosphate monoesters and plays important roles in fungal growth, metabolism, cell wall biosynthesis, biofilm formation, and signal transduction (Goulet and Saville 2017; Rocha and Wilson 2019). However, both CFW fluorescence and AKP activity assays showed no significant differences between the LCEO-treated and control groups within 120 min of treatment. This result aligns with the findings of Leite et al., who reported that citral did not target the cell wall in *Candida albicans* (Leite et al. 2014), suggesting that the antifungal mechanism of LCEO against *A. alternata* does not involve direct disruption of the cell wall or related enzymatic activity. The fungal cell membrane plays a crucial role in maintaining homeostasis, material exchange, and energy and information transduction, which are essential for cell viability (Mani-López et al. 2021). PI staining was used to detect membrane integrity, as PI penetrates only cells with severe membrane damage, resulting in increased red fluorescence (Khan et al. 2011; Lee et al. 2020). Lipids are critical structural components of the cell membrane, and measuring membrane integrity and lipid content can indicate

the impact of antifungal agents on fungal membranes (Q. Ouyang et al. 2025). PI staining analysis revealed that red fluorescence intensity in LCEO-treated hyphae increased in a concentration- and time-dependent manner and was consistently higher than that in the control; similarly, *Rhizopus stolonifera* mycelia treated with perillaldehyde and salicylaldehyde at a concentration of 10 mL/L exhibited enhanced red fluorescence after PI staining (L. L. Li et al. 2024). Additionally, the total lipid content of *A. alternata* hyphae displayed dynamic fluctuations following exposure to LCEO. This result is consistent with that of Q. L. OuYang et al. (2019), who reported that treatment with cinnamaldehyde induced an initial increase, followed by a decrease in the total lipid content of *Geotrichum citri-aurantii*. Reports also suggest that citral, the major active component of LCEO, appears to primarily target the fungal cell membrane, affecting its structure and disrupting its synthesis, ultimately leading to cell death (Leite et al. 2014). These findings indicate that LCEO treatment impairs the membrane integrity of *A. alternata* and may exert antifungal effects by inducing fluctuations in the lipid components of hyphae.

Oxidative stress, characterized by elevated levels of ROS, is a major cause of lipid peroxidation. Lipid peroxidation, compromises membrane integrity and function, yielding MDA, a recognized biomarker of lipid oxidation (Khan et al. 2011; Mani-López et al. 2021). Further, membrane lipid peroxidation can significantly increase cell membrane fluidity and permeability, thereby inducing the leakage of intracellular ions and other biological macromolecular contents. The results of this study demonstrated that after 30 min of LCEO treatment, the ROS levels and MDA contents in *Alternaria alternata* hyphae increased significantly, accompanied by rapid leakage of nucleic acids and proteins as well as a sharp rise in extracellular conductivity. This observation is consistent with previously reported findings that citral can induce ROS burst and MDA accumulation in *Aspergillus niger*, leading to intracellular nucleic acid leakage and elevated conductivity (Ju et al. 2020a; Ju et al. 2020b). These findings further support the conclusion that LCEO initially triggers an increase in ROS and MDA levels in *A. alternata* mycelia, impairs membrane permeability, and consequently causes the leakage of intracellular contents and enhanced electrolyte release. Under stable conditions, MMP remains relatively constant. MMP disruption is an early indicator of apoptosis (Zorova et al. 2018). A decline in MMP depletes intracellular ATP reserves, subsequently attenuating energy-dependent metabolic processes; this cascade of events is commonly associated with fungal cell death and sustained depletion or hyperpolarization can lead to cell death (Reymick et al. 2024). In this study, LCEO treatment resulted in a decrease in MMP, with higher concentrations and longer exposure times leading to a greater loss of MMP. These results indicate that LCEO inhibits the mitochondrial function of *A. alternata*. Similarly, citral has been shown to cause mitochondrial dysfunction in *Penicillium roqueforti* by altering its mitochondrial morphology and membrane potential (Ju et al. 2020a; Ju et al. 2020b). Collectively, this study demonstrates that LCEO exhibits effective control against *A. alternata* both in vitro and in vivo on blueberries. Treatment with an appropriate concentration of LCEO effectively reduced postharvest decay and maintained the commercial quality of postharvest blueberry. The antifungal mechanism results indicate that LCEO inhibits the growth of *A. alternata* through multi-target damage, including inducing intracellular ROS accumulation, disrupting the structure and function of the fungal cell membrane, and impairing mitochondrial membrane potential, demonstrating its potential as a green control measure for postharvest storage diseases. However, practical application of LCEO and other plant essential oils remains constrained by inherent challenges, such as their high volatility and short duration of activity. Future research should focus on optimizing application strategies to enhance the stability and sustained efficacy of LCEO in postharvest disease control.

5 | Conclusion

This study investigated the in vitro and in vivo efficacy of LCEO against *A. alternata* in blueberries and explored its underlying antifungal mechanism. The results demonstrated that LCEO treatment reduced the incidence of blueberry fruit rot and helped maintain a better postharvest quality during storage. The antifungal mechanism results indicate that LCEO inhibits the growth of *A. alternata* by causing hyphal damage through a multi-target mechanism involving the cell membrane, ROS accumulation, and mitochondria, thereby exerting a preservative effect on blueberry fruit. This indicates that LCEO holds potential as a green preservation measure for postharvest storage diseases in blueberries.

Author Contributions

Ruogu Cheng: methodology, software, data curation, formal analysis, visualization, validation, investigation, conceptualization. **Chunxiu Wan:** conceptualization, methodology, investigation, validation, formal analysis, data curation, visualization. **Ziyu Wang:** investigation, software, visualization. **Xiumei Chen:** project administration, supervision, writing – review and editing. **Qiuli Ouyang:** supervision, project administration, writing – review and editing. **Nengguo Tao:** writing – review and editing, project administration. **Xiaoli Tan:** funding acquisition, writing – review and editing, project administration, resources, supervision.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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