OPEN ACCESS Plant Protection, 10 (05) 2026. 901-913 DOI:10.33804/pp.010.05.6260

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Available Online at EScience Press

Plant Protection

ISSN: 2617-1287 (Online), 2617-1279 (Print)

http://esciencepress.net/journals/PP

Research Article

Grinding-Induced Chemical Shifts in Clove (*Syzygium aromaticum*) Essential Oil Differentially Modulate Antifungal and Antibacterial Activity

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ARTICLE INFO

Article history
Received: 7th July, 2026
Revised: 30h September, 2026
Accepted: 2nd October, 2026

Keywords
Syzygium aromaticum
Clove essential oil
Grinding pretreatment
Antimicrobial activity
Postharvest disease management

ABSTRACT

Grinding is a common pretreatment before hydrodistillation of clove (*Syzygium aromaticum*) buds, but its effect on essential oil quality and bioactivity is not uniformly beneficial. This study compared ground essential oil (GEO) and unground essential oil (UEO) of clove bud for extraction yield, physicochemical properties, chemical composition (GC-MS), and antifungal and antibacterial activity. Grinding significantly increased extraction yield (1.19 ± 0.01% vs. 0.52 ± 0.02%, $p < 0.001$) and shifted the oil composition, raising the sesquiterpene fraction (6.96% vs. 3.39%) at the expense of phenylpropanoids (92.65% vs. 96.32%), while eugenol remained dominant in both oils (88.09% and 89.82%, respectively). This modest compositional shift produced a target-dependent, rather than uniform, effect on antifungal potency across three complementary assays. On disc diffusion, GEO gave larger inhibition zones against *Alternaria* sp. and *Penicillium* sp. II, whereas UEO was more active against *Botrytis* sp. and *Geotrichum* sp. UEO reached a low fungicidal concentration (≤ 25 µL/mL) against six of eight fungal species, versus three for GEO. In a volatile-phase (vapor) assay, however, GEO inhibited four species by vapor contact alone, while UEO was active only against *Geotrichum* sp. Antibacterial potency was unaffected by grinding for all five bacterial strains tested ($p > 0.45$), and both oils matched or exceeded tetracycline against *Bacillus* sp. and *Pseudomonas* sp. These results show that grinding does not uniformly enhance clove essential oil bioactivity: the appropriate processing choice depends on the target organism and the intended mode of application, whether by direct contact or fumigation.

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Introduction

Clove (*Syzygium aromaticum* (L.) Merr. & L.M. Perry, syn. *Eugenia caryophyllata*), a member of the Myrtaceae family, is among the most economically important spices traded worldwide. Its dried flower buds yield an essential oil dominated by eugenol, together with eugenyl acetate and β-caryophyllene as major secondary constituents. Clove essential oil is used across the food, pharmaceutical, and cosmetic sectors because of its antioxidant, antimicrobial, analgesic, and insecticidal properties (Haro-González et al., 2021).

Postharvest fungal decay is a major cause of quantitative and qualitative losses in fresh produce worldwide, with *Aspergillus*, *Penicillium*, *Botrytis*, *Fusarium*, and

Alternaria among the most destructive genera (Shahbazi et al., 2025). Synthetic fungicides raise growing concern over chemical residues, environmental persistence, and the selection of resistant strains, which has driven the search for plant-derived alternatives (Giménez-Santamarina et al., 2025). In parallel, the global rise of antibiotic-resistant foodborne and clinical bacteria has renewed interest in essential oils as antibacterial agents, either alone or as adjuncts to conventional antibiotics (Valarezo et al., 2025).

Clove essential oil has already demonstrated activity on both fronts: it inhibits several postharvest fungal pathogens (Giménez-Santamarina et al., 2025) and shows bactericidal effects against Gram-positive and Gram-negative bacteria, including *Staphylococcus aureus* and *Escherichia coli* (Valarezo et al., 2025). These two lines of evidence are rarely combined in a single, directly comparable dataset obtained from the same oil batch.

A technical detail that is often overlooked, yet strongly influences essential oil yield and composition, is the pre-treatment applied to the plant material before hydrodistillation. Ben Hassine et al. (2021) compared ground and unground clove buds and reported that grinding nearly doubled the extraction yield (from 7.1 ± 0.8% to 14.3 ± 0.6%), lowered the relative eugenol content (from 87.4% to 68.7%), and increased sesquiterpene compounds such as cyperene. The same study found that ground clove oil had a stronger antioxidant capacity and a larger inhibition zone against *E. coli* than the unground oil (Ben Hassine et al., 2021).

Ben Hassine et al. (2021), however, screened a single bacterial species by disc diffusion and a single antioxidant assay; they did not test any postharvest fungal pathogen, nor did they determine quantitative, target-specific endpoints such as the minimum fungicidal concentration or the minimum inhibitory and bactericidal concentrations. A compositional shift in eugenol and sesquiterpene content, such as the one grinding produces, does not necessarily translate into a proportional change in biological potency against a defined microbial target: this link has not been tested.

The present study therefore addresses a specific and, to our knowledge, still unresolved research gap: whether grinding clove buds before hydrodistillation changes not only the yield and chemical profile of the resulting essential oil, but also its quantitative antifungal and antibacterial efficacy. Ground essential oil (GEO) and unground essential oil (UEO) of clove were obtained from the same raw material and compared side by side against a panel of postharvest fungal pathogens and clinically relevant bacterial strains, using quantitative endpoints (minimum fungicidal concentration; minimum inhibitory and bactericidal concentrations) that were absent from earlier work on this topic.

Building on this gap, the present study aimed to: (i) compare the physicochemical properties and GC-MS chemical profile of ground (GEO) and unground (UEO) clove essential oil; (ii) evaluate and compare the antifungal potency of GEO and UEO against a panel of postharvest fungal pathogens, including determination of the minimum fungicidal concentration; and (iii) evaluate and compare the antibacterial potency of GEO and UEO against clinically relevant bacterial strains through the minimum inhibitory and bactericidal concentrations.

Materials and Methods

Plant material collection and preparation

Healthy and mature clove buds were purchased from a local market in Bordj Bou Arreridj (north of Algeria). To remove dust and surface impurities, the buds were thoroughly washed with tap water, air-dried, and dehydrated at 30°C in a ventilated oven until constant weight was reached. The dried material was stored in hermetic plastic bags at room temperature until further use. The dried clove buds were then divided into two experimental groups: (i) whole clove buds, kept in their original, intact form (unground, UEO); and (ii) ground clove buds, finely pulverized with an electric laboratory grinder (Moulinex AR 1105, France) immediately before extraction (ground, GEO).

Bacterial strains

Bacterial strains including *Staphylococcus aureus* (ATCC 6538), *Bacillus* sp., *Escherichia coli* O157:H7 (ATCC 10536), *Pseudomonas* sp., and *S. epidermidis* were sourced from the Setif University Research Laboratory collection. Cultures were grown in MRS broth at 37°C for 24 h and subsequently adjusted to 8 log CFU/mL.

Hydrodistillation and extraction yield

Ground and unground buds were subjected separately to hydrodistillation for 6 h using a Clevenger-type apparatus. The plant material was submerged directly in 500 mL distilled water in a 1 L round-bottom flask containing a few pumice stones to regulate boiling; extraction was carried out at atmospheric pressure. Because the density of clove bud essential oil (1.062 at

20°C) exceeds that of water (1.00), the organic phase settled below the aqueous phase, which complicated separation. The distillate was therefore placed in a decanter, salt-saturated water was added, and the mixture was shaken, degassed, and decanted to separate the essential oil from the aqueous phase. The recovered oil was dried over anhydrous Na_2SO_4, filtered, and stored in amber glass bottles at 4°C until analysis.

The extraction yield was calculated as:

$$\text{Oil yield (\%)} = \frac{w(EO)}{w(P)} \times 100$$

Where w(EO) is the weight of the essential oil and w(P) is the weight of the plant material (Kalagatur et al., 2018).

Gas chromatography–mass spectrometry (GC-MS) analysis

The chemical composition of each essential oil was determined using a Shimadzu GC-2010 gas chromatograph coupled to a GCMS-QP2020 mass spectrometer equipped with an AOC-20i+s autosampler. Separation was performed on a DB-5MS fused-silica capillary column (5% phenyl-methyl-polysiloxane, 30 m × 0.25 mm i.d., 0.25 µm film thickness). A 1 µL sample was injected in split mode (split ratio 50:1); the injector temperature was 250°C. Helium was used as carrier gas at a constant linear velocity of 35.9 cm/s (column flow 0.98 mL/min; purge flow 3.0 mL/min). The oven temperature was held at 50°C for 2 min, ramped to 310°C at 3°C/min, and held at 310°C for 2 min (total run time 90.67 min). The transfer line and ion source were set at 250°C and 200°C, respectively. Mass spectra were recorded in electron ionization (EI) scan mode (event time 0.30 s; scan speed 1666 u/s) over m/z 35–500, with a solvent delay of 3 min.

Compounds were identified using a dual-criteria approach: (i) linear retention indices, calculated relative to a homologous series of n-alkanes (C5–C24) injected under identical conditions and compared with literature or in-house reference standards; and (ii) mass spectral matching against the NIST 08 library and other published MS databases. The relative percentage of each constituent was determined by peak-area normalization, assuming equal mass response factors for all detected compounds.

Physicochemical properties of the essential oils

Refractive index, density, pH, and alcohol solubility of each essential oil were determined in triplicate following standard pharmacopoeial methods (Pharmacopoeia Commission, 2011).

Acid value

The acid value was calculated according to Paez et al. (2016):

$$\text{Acid value} = \frac{\text{N (0.1)} \times \text{mL of alkali}}{\text{Weight of sample (g)}}$$

Antifungal activity

Fungal isolates

Fungal isolates were recovered from naturally infected fruits and vegetables on potato dextrose agar (PDA) supplemented with chloramphenicol. Slow-growing species were incubated at 28°C. Colonies were identified from growth rate, texture, and colony color on the obverse and reverse sides. Microscopic identification used the Scotch-tape method and lactophenol cotton blue staining.

***In vitro* antifungal susceptibility test**

Antifungal susceptibility of GEO and UEO was screened by the Kirby-Bauer disc diffusion method against each fungal isolate. Sterile 6 mm paper discs were loaded with the pure essential oil (GEO or UEO); DMSO-soaked discs served as the negative control. Assays were performed on Sabouraud dextrose agar (SDA), in triplicate, and inhibition zone diameters (mm) were measured after incubation.

Volatile-phase (vapor) inhibition assay

The antifungal activity of GEO and UEO in the absence of direct oil–mycelium contact was evaluated using an inverted Petri dish vapor assay, according to standard protocols with minor modifications. Standard polystyrene Petri dishes (90 mm diameter × **15** mm height, with a free air headspace volume of approximately 60 mL) containing **15** mL of PDA were used. The center of each PDA plate was inoculated with a **5** mm diameter mycelial plug taken from the active margin of a 7-day-old fungal culture. A sterile filter paper disc (6 mm diameter, Whatman No. 1) was placed on the inner center of the upper lid of the Petri dish and impregnated with a fixed volume of 20 µL of pure essential oil (GEO or UEO). Discs treated with 20 µL of pure DMSO served as negative controls. Plates were immediately inverted to prevent direct contact between the oil droplets and the agar surface, hermetically sealed with Parafilm M® to prevent volatile leakage, and incubated at 28°C in the dark. Radial mycelial growth (mm) was measured perpendicularly using a digital caliper after 7 days of incubation (or when the growth in the negative control plate reached the edge of the dish). The percentage inhibition of mycelial growth relative to the negative control was calculated in independent

triplicates (n=3) using the following formula:

$$\text{Inhibition (\%)} = \left(\frac{d_c - d_t}{d_c - d_0}\right) \times 100$$

Where d_c is the mean colony diameter in the negative control plate, d_t is the mean colony diameter in the treatment plate (GEO or UEO), and d_0 is the initial diameter of the mycelial plug (5 mm).

Minimum fungicidal concentration (CMF)

The minimum fungicidal concentration (MFC) was determined using the agar dilution method. Essential oils (UEO and GEO) dissolved in DMSO (<1% v/v final concentration) were incorporated into molten PDA to yield final concentration series of 100 (c_1), 50 (c_2), 25 (c_3), 12.5 (c_4), and 6.25 μL/mL (c_5). Plates were inoculated with 10 µL of standardized fungal spore suspension (10^5 spores/mL) and incubated at 28°C for 72 h. Control plates included PDA without essential oil (growth control) and PDA supplemented with 1% DMSO (solvent control). The MFC was recorded as the lowest concentration that completely inhibited visible fungal colony formation on the agar surface. All tests were executed in independent triplicates ($n = 3$).

Antibacterial activity

Disc diffusion assay

Bacterial inocula were prepared from 18–24 h log-phase cultures and standardized to a 0.5 McFarland turbidity standard ($A_{625} = 0.08$--0.10, corresponding to approximately 1.5×10^8 CFU/mL for bacteria) using a visible spectrophotometer. A sterile cotton swab was dipped into the standardized suspension and evenly spread onto Mueller–Hinton Agar (MHA) plates for bacteria to form a uniform lawn. Sterile Whatman No. 1 filter paper discs (6 mm diameter) were impregnated with 10 µL of each essential oil samples (UEO and GEO) and allowed to dry briefly under sterile conditions before placement onto the inoculated plates. Pure DMSO-impregnated discs served as negative controls, while standard antibiotic discs (tetracycline 30 µg, amoxicillin 25 µg, penicillin 10 UI) served as positive controls. Plates were incubated at 37°C for 24 h. Inhibition zone diameters (IZD, mm) including the disc diameter were measured in triplicates (n = 3).

Minimum inhibitory and bactericidal concentrations (MIC/MBC)

The minimum inhibitory concentration (MIC) was determined using the broth tube macrodilution method following Doughari et al. (2007) with minor modifications. Essential oil samples (UEO and GEO) were dissolved in DMSO and diluted in Nutrient Broth to obtain five test concentrations: $c_1 = 100$, $c_2 = 50$, $c_3 = 25$, $c_4 = 12.5$, and $c_5 = 6.25$ µL/mL. The final concentration of DMSO in each tube was maintained below 1% (v/v) to prevent solvent toxicity. Each tube containing 1 mL of broth-essential oil mixture was inoculated with 100 µL of standardized microbial suspension (0.5 McFarland standard, corresponding to $\sim 1.5 \times 10^8$ CFU/mL). The control tubes included:

- Growth control: broth + inoculum (without essential oil or DMSO);
- Solvent control: broth + DMSO (1% v/v) + inoculum;
- Sterility control: broth + essential oil (without inoculum).

Tubes were incubated at 37°C for 24 h for bacteria and visually inspected for turbidity. The MIC was defined as the lowest concentration exhibiting no macroscopic visible growth.

To determine the minimum bactericidal concentration (MBC), a 10 µL aliquot from each clear (non-turbid) MIC tube was subcultured onto Mueller-Hinton Agar plates divided into sectors. Plates were incubated under the conditions described above. The MBC was recorded as the lowest concentration showing no visible colony growth on subculture (≥ 99.9% killing efficiency). All experiments were performed in independent triplicates ($n = 3$).

Statistical analysis

All experiments were performed in triplicate (n = 3 independent replicates per condition), and results are expressed as mean ± standard deviation (SD). Statistical analyses were performed in Python (version 3.11) using SciPy (Virtanen et al., 2020), statsmodels (Seabold and Perktold, 2010), and scikit-posthocs (Terpilowski, 2019). The normality of data distribution was assessed with the Shapiro-Wilk test and the homogeneity of variances with Levene's test. Physicochemical parameters (extraction yield, refractive index, density, pH, acid value) were compared between ground (GEO) and unground (UEO) essential oil using an independent-samples t-test, or the Mann-Whitney U test when the normality or homogeneity-of-variance assumptions were not satisfied. For each fungal or bacterial species/strain, inhibition zone diameters (disc-diffusion assays) or percentage mycelial growth inhibition (volatile-phase assay) across treatments (GEO, UEO,

positive control, and negative control for the antifungal disc-diffusion assay; GEO, UEO, and negative control for the volatile-phase assay; GEO, UEO, reference antibiotics, and negative control for the antibacterial assay) were compared by one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) post hoc test, cross-checked by the Kruskal-Wallis test followed by Dunn's post hoc test with Bonferroni correction. Minimum fungicidal, inhibitory, and bactericidal concentrations (CMF, MIC, and MBC) were determined from serial two-fold dilution series as the lowest concentration producing no visible growth in all replicates, cross-checked programmatically against the raw growth/turbidity readings. Differences were considered statistically significant at $p < 0.05$.

Results

Extraction yield and physicochemical properties

Extraction yield and physicochemical properties of ground (GEO) and unground (UEO) clove essential oil have been provided in Table 1. The extraction yield was significantly higher for GEO (1.19 ± 0.01%) than for UEO (0.52 ± 0.02%; $t(4) = 46.47$, $p < 0.001$), consistent with the direction reported by Ben Hassine et al. (2021), where grinding increased essential oil yield. Density was significantly higher for UEO (1.047 ± 0.006 g/mL) than for GEO (1.020 ± 0.010 g/mL; $t(4) = -4.00$, $p = 0.016$); both values fall within the range reported for clove bud essential oil (1.03-1.06 g/mL) and are consistent with the value cited in section "Bacterial strains" (1.062 at 20°C). Refractive index (GEO 1.514 ± 0.005 vs. UEO 1.537 ± 0.006; $p = 0.006$) and acid value (GEO 0.205 ± 0.001 vs. UEO 0.135 ± 0.001 mg KOH/g; $p < 0.001$) also differed significantly between the two oils. pH did not differ between GEO and UEO (8.07 ± 0.21 in both groups; $p = 1.000$); this parameter was recorded as numerically identical across all three replicate pairs, which, while not physically implausible for two neat oils of the same botanical origin, is worth a bench-notebook cross-check before submission, since it is a stronger coincidence than most replicate measurements of a continuous variable would be expected to produce.

Table 1. Extraction yield and physicochemical properties of ground (GEO) and unground (UEO) clove essential oil (mean ± SD, n = 3; independent-samples t-test).

Parameter	Unit	GEO	UEO	t-test p	Sig
Extraction yield	%	1.185 ± 0.013	0.523 ± 0.021	<0.001	***
Refractive index	-	1.514 ± 0.005	1.537 ± 0.006	0.006	**
Density	g/mL	1.020 ± 0.010	1.047 ± 0.006	0.016	*
pH	-	8.067 ± 0.208	8.067 ± 0.208	1.000	ns
Acid value	mg KOH/g oil	0.205 ± 0.001	0.135 ± 0.001	<0.001	***

GC-MS chemical composition

Nineteen compounds were identified in both oils by GC-MS (Table 2), together accounting for 99.99% (GEO) and 100.00% (UEO) of the total peak area. Eugenol was the major constituent in both oils (88.09% in GEO vs. 89.82% in UEO), followed by eugenol acetate (4.04% vs. 5.57%) and E-β-caryophyllene (5.68% vs. 2.69%). Grouped by chemical class (Figure 1b), phenylpropanoids accounted for 92.65% of GEO and 96.32% of UEO, while sesquiterpene hydrocarbons and oxides made up 6.96% of GEO but only 3.39% of UEO, grinding was associated with a relatively higher sesquiterpene fraction and a slightly lower phenylpropanoid fraction, consistent with the direction (though not the magnitude) reported by Ben Hassine et al. (2021). trans-Cinnamaldehyde (0.37%) was detected only in UEO.

Antifungal activity

Disc-diffusion assay

One-way ANOVA showed a significant effect of treatments for seven of the eight fungal species tested ($p < 0.001$ in all cases; Table 3); no treatment produced any inhibition of *Fusarium* sp., including the positive control, indicating this isolate was not susceptible to any agent tested under the conditions used. Tukey's HSD post hoc comparison of GEO against UEO specifically showed a significantly larger inhibition zone for GEO against *Penicillium* sp. II (45.3 ± 5.0 vs. 37.3 ± 2.5 mm; $p = 0.040$) and *Alternaria* sp. (53.3 ± 2.9 vs. 25.0 ± 5.0 mm; $p < 0.001$), and a significantly larger zone for UEO against *Botrytis* sp. (49.7 ± 2.9 vs. 33.3 ± 7.6 mm; $p = 0.005$) and *Geotrichum* sp. (38.7 ± 5.1 vs. 22.3 ± 2.5 mm; $p = 0.0005$). GEO and UEO did not differ significantly against

Aspergillus sp. (p = 0.069), *Penicillium* sp. I (p = 0.811), or *Paecilomyces* sp. (p = 0.717). No single oil was uniformly more potent: which oil produced the larger inhibition zone depended on the fungal species.

Table 2. GC-MS chemical composition of ground (GEO) and unground (UEO) clove essential oil.

Compound	RT (min)	RI	Chemical class	% GEO	% UEO
Eugenol	27.8	1407	Phenylpropanoid (phenol)-major marker	88.09	89.82
Caryophyllene (E-beta-caryophyllene)	30.0	1460	Sesquiterpene hydrocarbon	5.68	2.69
Eugenol acetate	34.4	1571	Phenylpropanoid ester	4.04	5.57
alpha-Humulene	31.4	1492	Sesquiterpene hydrocarbon	0.63	0.36
Chavicol (4-allylphenol)	22.7	1288	Phenylpropanoid (phenol)	0.26	0.34
trans-Anethole (reassigned, see note)	24.1	1320	Phenylpropanoid (ether)	0.26	0.22
Caryophyllene oxide	36.5	1626	Sesquiterpene oxide	0.20	0.21
alpha-Copaene	28.2	1415	Sesquiterpene hydrocarbon	0.15	0.05
Methyl salicylate	19.9	1226	Phenolic ester	0.15	0.13
delta-Cadinene	34.2	1565	Sesquiterpene hydrocarbon	0.14	0.08
Undecan-2-one (Nonyl methyl ketone)	24.5	1329	Aliphatic ketone	0.10	0.06
alpha-Farnesene	33.6	1549	Sesquiterpene hydrocarbon	0.06	0.00
alpha-Cubebene	26.9	1386	Sesquiterpene hydrocarbon	0.06	0.00
Sesquiterpene hydrocarbon (tentative: Cubenene / Cadina-1,4-diene isomer)	34.5	1574	Sesquiterpene hydrocarbon (unresolved isomer)	0.04	0.00
2-Nonanol, acetate	13.0	1072	Aliphatic ester	0.03	0.05
Benzyl acetate	18.6	1196	Aromatic ester	0.03	0.00
2-Nonanone	15.3	1123	Aliphatic ketone	0.03	0.03
2-Heptanone	7.0	920	Aliphatic ketone	0.00	0.02
trans-Cinnamaldehyde (tentative, see note)	23.5	1306	Phenylpropanoid (aldehyde)	0.00	0.37

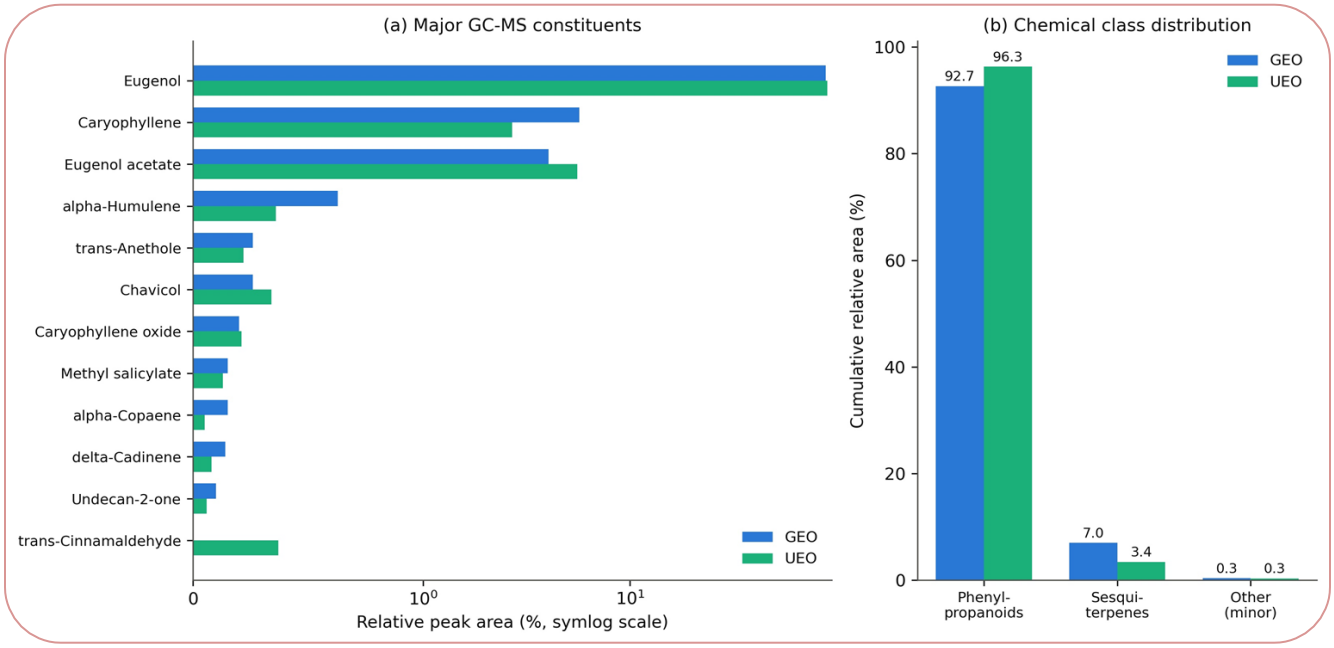


Figure 1. GC-MS chemical composition of ground (GEO) and unground (UEO) clove essential oil. (a) Major GC-MS constituents; (b) Chemical class distribution.

Table 3. Inhibition zone diameters (mm, mean ± SD, n = 3) of GEO and UEO against postharvest fungal pathogens, disc-diffusion assay (one-way ANOVA per species).

Fungal species	GEO	UEO	Positive control	Negative control	ANOVA p
Aspergillus sp.	48.3 ± 3.8	43.3 ± 1.5	32.7 ± 0.6	0.0 ± 0.0	<0.001
Penicillium sp. I	48.0 ± 1.7	50.0 ± 5.0	37.7 ± 1.5	0.0 ± 0.0	<0.001
Penicillium sp. II	45.3 ± 5.0	37.3 ± 2.5	40.3 ± 1.5	0.0 ± 0.0	<0.001
Alternaria sp.	53.3 ± 2.9	25.0 ± 5.0	31.0 ± 1.0	0.0 ± 0.0	<0.001
Botrytis sp.	33.3 ± 7.6	49.7 ± 2.9	8.7 ± 0.6	0.0 ± 0.0	<0.001
Fusarium sp.	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	n/a
Geotrichum sp.	22.3 ± 2.5	38.7 ± 5.1	10.0 ± 0.0	0.0 ± 0.0	<0.001
Paecilomyces sp.	50.0 ± 0.0	53.3 ± 7.6	43.0 ± 0.0	0.0 ± 0.0	<0.001

Minimum fungicidal concentration (CMF)

In contrast to the disc-diffusion results, UEO achieved a measurable fungicidal endpoint (CMF ≤ 25 µL/mL) against six of the eight species, whereas GEO did not reach complete fungicidal inhibition within the tested concentration range (CMF > 100 µL/mL) for five of the eight species, including *Aspergillus* sp., *Penicillium* sp. I, *Alternaria* sp., *Botrytis* sp., and *Fusarium* sp (Table 4a). The two endpoints therefore diverge for several species: GEO produced comparatively large inhibition zones on disc diffusion (a fungistatic effect at the disc's local, largely undiluted concentration) but did not fully suppress growth at any diluted concentration tested, while UEO was fungicidal at low concentrations for most species. *Penicillium* sp. II, *Geotrichum* sp., and *Paecilomyces* sp. were the only three species where both oils reached a fungicidal endpoint within the tested range (CMF 6.25-25 µL/mL for both), with *Paecilomyces* sp. the only species where GEO and UEO reached an identical CMF (25 µL/mL).

Volatile-phase (vapor) inhibition assay

In the volatile-phase assay, where the oil was not in direct contact with the fungal culture, GEO produced significant mycelial growth inhibition against four of the eight species, *Aspergillus* sp. (40.3 ± 20.7%; p = 0.027), *Penicillium* sp. II (34.4 ± 21.5%; p = 0.022), *Alternaria* sp. (54.1 ± 18.5%; p = 0.001), and *Paecilomyces* sp. (37.8 ± 11.1%; p < 0.001), while UEO produced no measurable volatile-phase inhibition against any of these four species (Table 4b). UEO, conversely, was the only oil active against *Geotrichum* sp. (38.9 ± 12.7%; p < 0.001), the reverse of the pattern for the other four active species. Neither of the oils produced any volatile-phase inhibition of *Penicillium* sp. I, *Botrytis* sp., or *Fusarium* sp. Overall, GEO was active by the volatile route against a broader range of species than UEO. This is a distinct pattern from the direct-contact disc-diffusion and CMF assays, where UEO was often the more consistently effective oil; the volatile-phase result cannot be trivially explained by the sesquiterpene/phenylpropanoid balance, since GEO is the more sesquiterpene-enriched (and less eugenol-enriched) of the two oils and sesquiterpene hydrocarbons are generally less volatile than eugenol. Which specific constituents drive vapor-phase activity in this system was not addressed by the present design and would require headspace-GC-MS or single-compound vapor assays to resolve.

Table 4a. Minimum fungicidal concentration (CMF, µL/mL) of GEO and UEO against postharvest fungal pathogens.

Fungal species	Oil type	CMF (µL/mL)
Aspergillus sp.	GEO	> 100
Aspergillus sp.	UEO	12.5
Penicillium sp. I	GEO	>100
Penicillium sp. I	UEO	6.25
Penicillium sp. II	GEO	25
Penicillium sp. II	UEO	6.25
Alternaria sp.	GEO	> 100
Alternaria sp.	UEO	> 100
Botrytis sp.	GEO	>100
Botrytis sp.	UEO	12.5
Fusarium sp.	GEO	> 100
Fusarium sp.	UEO	> 100
Geotrichum sp.	GEO	12.5
Geotrichum sp.	UEO	6.25
Paecilomyces sp.	GEO	25
Paecilomyces sp.	UEO	25

Table 4b. Mycelial growth inhibition (%, mean ± SD, n = 3) of GEO and UEO in the volatile-phase (vapor) assay against postharvest fungal pathogens (one-way ANOVA per species).

Fungal species	GEO	UEO	Negative control	ANOVA p
Aspergillus sp.	40.3 ± 20.7	11.9 ± 10.9	0.0 ± 0.0	0.027
Penicillium sp. I	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	n/a
Penicillium sp. II	34.4 ± 21.5	0.0 ± 0.0	0.0 ± 0.0	0.022
Alternaria sp.	54.1 ± 18.5	0.0 ± 0.0	0.0 ± 0.0	0.001
Botrytis sp.	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	n/a
Fusarium sp.	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	n/a
Geotrichum sp.	0.0 ± 0.0	38.9 ± 12.7	0.0 ± 0.0	<0.001
Paecilomyces sp.	37.8 ± 11.1	0.0 ± 0.0	0.0 ± 0.0	<0.001

Antibacterial activity

Disc-diffusion assay

One-way ANOVA showed a significant effect of treatment for all five bacterial strains ($p < 0.01$ in all cases; Table 5). Tukey's HSD comparison of GEO against UEO specifically showed no significant difference for any strain (*Bacillus* sp., $p = 0.472$; *Pseudomonas* sp., $p = 0.452$; *E.* coli, $p = 1.000$; *S. epidermidis*, $p = 0.466$; *S. aureus*, $p > 0.999$): grinding did not significantly change the antibacterial potency of the oil, in contrast to the species-dependent pattern observed for the antifungal disc-diffusion assay. Both oils produced inhibition zones against *Bacillus* sp. and *Pseudomonas* sp. that were as large as, or larger than, the zones produced by tetracycline, and considerably larger than those produced by amoxicillin and penicillin, to which both strains were fully resistant (0 mm).

Minimum inhibitory and bactericidal concentrations (MIC/MBC)

MIC was identical (50 µL/mL, Table 6) for GEO and UEO against four of the five strains, and higher for UEO than GEO against *Pseudomonas* sp. (100 vs. 50 µL/mL). The MBC/MIC ratio classified the effect as bactericidal (ratio ≤ 4) for both oils against *Bacillus* sp., *E. coli*, and *S. aureus*, and for GEO against *S. epidermidis*; the ratio could not be determined for *Pseudomonas* sp. (MBC > 100 µL/mL for both oils) or for UEO against *S. epidermidis* (MBC > 100 µL/mL), i.e., outside the tested concentration range.

Table 5. Inhibition zone diameters (mm, mean ± SD, n = 3) of GEO and UEO against bacterial strains, compared with reference antibiotics (one-way ANOVA per strain).

Strain	GEO	UEO	Tetracyclin	Amoxicilli	Penicillin	Negative	ANOVA p
Bacillus sp.	24.0 ± 9.5	17.7 ± 3.8	18.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	<0.001
Pseudomonas	19.0 ± 3.6	15.0 ± 4.4	11.7 ± 2.9	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	<0.001
E. coli	22.7 ± 0.6	22.3 ± 2.5	21.3 ± 1.2	21.0 ± 1.7	18.3 ± 7.6	0.0 ± 0.0	<0.001
S. epidermidis	20.7 ± 3.8	14.7 ± 2.3	28.7 ± 5.8	7.3 ± 6.4	0.0 ± 0.0	0.0 ± 0.0	<0.001
S. aureus	17.7 ± 2.5	19.0 ± 2.6	12.3 ± 2.3	26.3 ± 1.2	15.0 ± 13.2	0.0 ± 0.0	0.003

Table 6. Minimum inhibitory (MIC) and bactericidal (MBC) concentrations (µL/mL) of GEO and UEO against bacterial strains.

Strain	Oil type	MIC (µL/mL)	MBC (µL/mL)	Interpretation
Bacillus sp.	GEO	50	50	bactericidal
Bacillus sp.	UEO	50	50	bactericidal
Pseudomonas sp.	GEO	50	>100	not determinable (out of tested range)
Pseudomonas sp.	UEO	100	>100	not determinable (out of tested range)
E. coli	GEO	50	100	bactericidal
E. coli	UEO	50	100	bactericidal
S. epidermidis	GEO	50	50	bactericidal
S. epidermidis	UEO	50	>100	not determinable (out of tested range)
S. aureus	GEO	50	100	bactericidal
S. aureus	UEO	50	100	bactericidal

Discussion

The central question raised in the "Introduction" was whether grinding clove buds before hydrodistillation changes not only the yield and chemical profile of the resulting essential oil, but also its quantitative antifungal and antibacterial efficacy. The results provide a nuanced answer: grinding shifted the chemical balance of the oil only modestly, yet this modest shift was accompanied by pronounced, target-dependent differences in antifungal potency, while antibacterial potency was essentially unaffected. Composition and bioactivity therefore did not change in a single, uniform direction, a finding that qualifies, rather than confirms, the assumption that grinding uniformly enhances the biological value of clove essential oil.

Extraction yield and physicochemical properties

Ben Hassine et al. (2021) reported that grinding nearly doubled clove bud essential oil yield. The present dataset reproduces the same direction. GEO gave a significantly higher extraction yield than UEO (1.19 ± 0.01% vs. 0.52 ± 0.02%; $p < 0.001$), even though the magnitude of the increase was smaller than the near-doubling reported previously. Density differed significantly between the two oils (UEO 1.047 ± 0.006 g/mL vs. GEO 1.020 ± 0.010 g/mL; $p = 0.016$), and both values fall within the range reported for clove bud essential oil (1.03-1.06 g/mL). A plausible explanation for the yield increase is that grinding increases the surface area of plant material exposed to steam during hydrodistillation, improving mass transfer of the essential oil out of the secretory cavities of the bud tissue and shortening the diffusion path for volatile compounds, an effect that has been proposed previously for ground versus whole spice material (Ben Hassine et al., 2021).

Refractive index and acid value both differed significantly between the two oils and remained within physically plausible ranges for a phenylpropanoid-rich essential oil, supporting the overall analytical quality of the GC-MS and titration measurements. The absence of a pH difference (8.07 in both groups) indicates that grinding, despite its clear effect on yield and density, did not alter the oil's acid-base behavior. The numerically identical triplicate pH readings for GEO and UEO are nonetheless worth a bench-notebook cross-check before submission, since this level of agreement is a stronger coincidence than most replicate measurements of a continuous variable would be expected to produce.

Chemical composition

Both oils were dominated by eugenol (88.09% in GEO, 89.82% in UEO), consistent with the chemotype typically reported for *S. aromaticum* bud oil (Haro-González et al., 2021). Grinding was associated with a relatively higher sesquiterpene fraction (6.96% vs. 3.39%, driven mainly by E-β-caryophyllene) and a correspondingly lower phenylpropanoid fraction (92.65% vs. 96.32%). This direction mirrors the shift reported by Ben Hassine et al. (2021), grinding lowering relative eugenol content in favor of sesquiterpene compounds, although the magnitude observed here was considerably smaller than in that study (which reported a shift from 87.4% to 68.7% eugenol). A plausible explanation is that grinding accelerates the loss of the most volatile, low-molecular-weight monoterpene-type fraction during hydrodistillation and the preceding handling of the powdered material, while the heavier sesquiterpene hydrocarbons are retained. The magnitude of this effect would then depend on particle size achieved, grinding duration, and the interval between grinding and distillation, none of which were standardized between the present study and Ben Hassine et al. (2021).

Antifungal activity: a target-dependent, not a uniform, effect of grinding

The disc-diffusion assay showed that neither of the oils was uniformly more potent: GEO produced significantly larger inhibition zones against *Alternaria* sp. and *Penicillium* sp. II, UEO produced significantly larger zones against *Botrytis* sp. and *Geotrichum* sp., and the two oils were equivalent against the remaining species. Because GEO was consistently the more sesquiterpene-enriched oil and UEO the more phenylpropanoid (eugenol)-enriched oil, this pattern suggests that different fungal genera respond preferentially to different fractions of the oil rather than to its eugenol content alone. Eugenol is known to act on fungal plasma membranes and, in *Penicillium* species, on chitin synthase activity (a dual membrane/cell-wall mechanism). Sesquiterpene hydrocarbons such as caryophyllene act through comparatively less-characterized, more lipophilic membrane-partitioning mechanisms. A shift of only a few percentage points between these two fractions may therefore be sufficient to change the outcome for a fungal species whose membrane or cell-wall composition makes it differentially sensitive to one fraction over the other, without requiring any change in overall extraction yield.

The lack of any inhibition of *Fusarium* sp. by either oil, or by the positive control, indicates that this isolate was broadly unresponsive under the conditions tested. This could reflect innate resistance of the specific isolate used, an inoculum size effect, or a technical failure specific to that assay run (e.g., disc saturation or diffusion medium); it cannot be resolved from the data alone and should be reported as an isolate-specific finding rather than generalized to the genus.

The comparison between the disc-diffusion assay and the minimum fungicidal concentration determination revealed a second, arguably more informative, pattern: GEO produced large inhibition zones for most species yet failed to reach a fungicidal endpoint within the tested concentration range (CMF > 100 µL/mL) for five of the eight species, while UEO reached a fungicidal endpoint at low concentrations (CMF ≤ 25 µL/mL) for six of the eight species. Disc diffusion and broth/agar dilution series do not measure the same property of an essential oil: the disc is loaded with a small volume of concentrated (undiluted or minimally diluted) oil, so the assay partly captures diffusion and vapor-phase contact of the most volatile constituents at a high local concentration near the disc, whereas the dilution series measures whether a defined, uniformly distributed concentration is sufficient to kill rather than merely suppress growth (Hulánková, 2024). A large disc-diffusion zone is therefore a fungistatic, concentration-gradient-dependent readout, and does not guarantee a low fungicidal concentration under uniform exposure. For an oil intended for postharvest use, where the practical question is what concentration must be applied to fully arrest decay, not what zone it produces on an agar plate, the CMF result is the more directly translatable endpoint, and on this criterion UEO, not GEO, was the more broadly effective oil against the fungal panel tested.

The dedicated volatile-phase (vapor) assay adds a third, partly independent, layer to this picture. Here, GEO, not UEO, was the more broadly active oil, producing significant mycelial growth inhibition against four species (*Aspergillus* sp., *Penicillium* sp. II, *Alternaria* sp., *Paecilomyces* sp.) with no direct contact between the oil and the fungal culture, whereas UEO was volatile-phase active only against *Geotrichum* sp. This pattern does not track either the disc-diffusion result (where GEO and UEO were each superior for two species) or the CMF result (where UEO was more broadly fungicidal on direct contact). Because volatile-phase activity depends on which constituents partition into the headspace above the growth medium rather than on the oil's bulk composition, and GEO is, if anything, relatively enriched in the less-volatile sesquiterpene fraction rather than depleted in it, the compositional basis of this third pattern cannot be inferred from the present bulk GC-MS data alone. Taken together, the three antifungal endpoints indicate that GEO and UEO are not simply "stronger" or "weaker" versions of one another: each is comparatively more effective under a specific mode of fungal exposure (contact-fungistatic, contact-fungicidal, or vapor-phase), a distinction with direct practical relevance for postharvest applications, where an oil may be applied by direct coating, dilution, or fumigation.

Antibacterial activity

Unlike the antifungal disc-diffusion results, grinding produced no significant change in antibacterial potency for any of the five strains tested (all GEO vs. UEO comparisons, $p > 0.45$). This asymmetry, a measurable, species-dependent effect on antifungal potency but no detectable effect on antibacterial potency, is consistent with the modest size of the compositional shift: a few percentage points of redistribution between eugenol and sesquiterpene fractions may be enough to tip the balance for some fungal targets, particularly given the wide spread of fungal responses observed, but was evidently insufficient to produce a statistically detectable difference against the bacterial strains under the conditions used here, where both oils already achieved near-maximal, saturating inhibition.

Both oils produced inhibition zones against *Bacillus* sp. and *Pseudomonas* sp. that matched or exceeded tetracycline, and were markedly larger than those of amoxicillin and penicillin, to which both strains were fully resistant in this assay. This observation is consistent with the growing literature positioning clove essential oil as a candidate adjunct or alternative to conventional antibiotics against strains with acquired resistance to beta-lactams (Valarezo et al., 2025). The relatively strong activity against *E. coli* and *Pseudomonas* sp., two Gram-negative organisms whose outer membrane is often considered a barrier to hydrophobic plant-derived antimicrobials, is nonetheless in line with reports that eugenol-rich oils can still cross or destabilize this membrane sufficiently to inhibit growth, even where full bactericidal clearance requires a higher concentration than for Gram-positive targets (Lopez-

Romero et al., 2015), a distinction that matches the present MIC/MBC data, where *Pseudomonas* sp. was the only strain for which no bactericidal endpoint could be established within the tested range for either oil, and where *E. coli* and *S. aureus* required a higher concentration to kill (MBC 100 µL/mL) than to inhibit (MIC 50 µL/mL) for both oils, giving an MBC/MIC ratio of 2, bactericidal by the conventional ≤ 4 threshold, but closer to the bacteriostatic boundary than *Bacillus* sp. or *S. epidermidis*/GEO, for which MBC equaled MIC.

Practical implications for postharvest and food-preservation applications

From an applied standpoint, these results argue against treating grinding as a generic potency-enhancing pretreatment for clove essential oil. Rather, the appropriate processing choice depends on the target organism: UEO would be the preferable source oil where a low fungicidal concentration against *Aspergillus* sp., *Penicillium* sp. I, or *Botrytis* sp. is the priority, species for which GEO failed to reach a fungicidal endpoint within the tested range (CMF > 100 µL/mL) while UEO did (CMF ≤ 12.5 µL/mL), whereas for *Alternaria* sp. and *Fusarium* sp. neither oil reached a fungicidal endpoint at all within the concentrations tested, so grinding status is irrelevant to that specific application choice. GEO offers no consistent advantage on the CMF criterion despite its often larger inhibition zones, and neither of the oils offers an advantage over the other for the antibacterial applications tested. This distinction matters for postharvest applications, where the active concentration that can be economically and organoleptically incorporated into a coating, fumigant, or packaging system is limited, and where a fungistatic zone on an agar plate is a poor predictor of performance at those working concentrations.

Methodological limitations

Several limitations qualify these conclusions. First, all assays used n = 3 replicates; while sufficient to power the one-way ANOVA comparisons reported here, this sample size makes the Shapiro-Wilk normality test largely uninformative and caps the resolution of the Mann-Whitney U test at a minimum two-tailed p-value of 0.100 for the physicochemical comparisons, so a non-significant Mann-Whitney result should not be over-interpreted where the parametric test disagrees. Second, both oils were obtained from a single batch of clove buds from a single geographic origin and a single extraction run per condition; batch-to-batch and seasonal variation in clove bud composition is well documented and was not captured here. Third, fungal isolates were characterized at the genus level only; genus-level identification is sufficient to demonstrate the pattern reported but limits direct comparison with studies using identified reference strains or species-confirmed field isolates. Fourth, two GC-MS peak assignments (trans-anethole and trans-cinnamaldehyde) remain tentative pending confirmation against authentic reference standards. Fifth, the volatile-phase assay protocol (headspace/inverted-plate exposure geometry, distance between oil and inoculum, exposure duration, incubation temperature) is described only in general terms pending confirmation of the exact parameters used, which should be completed before submission to allow independent replication. Finally, two data-consistency points identified during statistical analysis should be re-checked against the original laboratory records before submission: the essential-oil-implausible replicate-for-replicate identity of the pH readings between GEO and UEO, and the minimum bactericidal concentration of *E. coli*/GEO, where the raw subculture growth series supported a higher value (100 µL/mL) than the originally transcribed summary figure (50 µL/mL) and the raw-data-derived value was used throughout this article.

Conclusion

Grinding clove buds before hydrodistillation increased extraction yield and produced a modest redistribution between the phenylpropanoid and sesquiterpene fractions of the resulting essential oil, consistent with the direction previously reported by Ben Hassine et al. (2021). This modest compositional shift was accompanied by a clearly target-dependent, rather than uniform, effect on antifungal potency across three complementary assays: on direct-contact disc diffusion, ground oil (GEO) was more effective against *Alternaria* sp. and *Penicillium* sp. II while unground oil (UEO) was more effective against *Botrytis* sp. and *Geotrichum* sp.; on the fungicidal (CMF) endpoint, UEO reached a low fungicidal concentration more consistently across the panel; and in the volatile-phase assay, GEO was active by vapor contact against four species where UEO showed no such activity. Antibacterial potency, in contrast, was not significantly affected by grinding for any of the five

bacterial strains tested, and both oils performed comparably to, or better than, tetracycline, amoxicillin, and penicillin against strains resistant to the latter two antibiotics. Together, these findings indicate that the value of grinding as a pre-extraction treatment for clove essential oil cannot be generalized into a single "better" or "worse" oil: GEO and UEO differ consistently in yield and in which mode of antifungal exposure (direct-contact fungistatic, direct-contact fungicidal, or vapor-phase) each is more effective under, and the appropriate processing choice should be matched to the specific target organism and intended mode of application.

Acknowledgement

We thank two anonymous reviewers for their helpful comments on earlier drafts of this manuscript. We thank the Algerian Government for the provision of resources and Algerian Ministry of Higher Education and Scientific Research.

Authors' Contributions

AH, OS, MA, and BS conceptualized and designed the study. AH, OS, MA, and SA conducted the field investigations and experiments. AH provided overall supervision and ensured methodological and analytical consistency. AH, OS, MA, and SA contributed to experimental guidance, data interpretation, and research refinement. AH, OS, MA, BS, SA, HS, and KIE contributed to manuscript preparation. All authors critically reviewed and revised the manuscript, verified the data and analyses, and approved the final version for submission.

Research Funding

This research did not receive any grant from funding agencies.

Conflict of Interest

The authors declare no conflict of interest.

Sustainable Development Goals Targeted

SDG 2: Zero Hunger
SDG 3: Good Health and Well-Being
SDG 12: Responsible Consumption and Production

Policy Addressed

The findings support standardized processing and target-specific evaluation of clove essential oil for sustainable postharvest disease management. Regulatory frameworks should consider processing effects on oil composition and require pathogen-specific efficacy, chemical profiling, and food-safety validation before commercial use. Essential oils should be selected according to the target pathogen and intended delivery mode rather than assumed to have uniform antimicrobial activity.

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