

# Eucalyptus camaldulensis, Citrus aurantium, and Citrus sinensis Essential Oils as Antifungal Activity against Aspergillus flavus, Aspergillus niger, Aspergillus terreus, and Fusarium culmorum

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Keywords: essential oils, Citrus sinensis, Citrus aurantium, Eucalyptus camaldulensis, antifungal activity

## Abstract:

Several molds are able to colonize wood and many building products or solid wood causing losses for their valuable uses. Essential oils (EOs) from aromatic plants can be used as an ecofriendly biofungicide against the growth of several molds. EOs from Eucalyptus camaldulensis, Citrus aurantium, and C. sinensis have a broad-spectrum antimicrobial activity. EOs from of E. camaldulensis air-dried aerial parts, C. aurantium leaf and C. sinensis peel, and their combinations (1:1 v/v) were evaluated for their antifungal activity against the growth of four common mold fungi (Aspergillus flavus, A. niger, A. terreus, and Fusarium culmorum). The chemical compositions of the EOs were analyzed with GC/MS. The main compounds in EO from E. camaldulensis were spathulenol (20.84%), eucalyptol (12.01%), and sabinene (9.73%); in C. aurantium were linalyl acetate (42.29%), and linalool (29.76%); and in C. sinensis were D-limonene (73.4%) and ?-terpinene (22.6%). At 50  $\mu$ L/mL, C. sinensis EO showed the highest fungal mycelial growth inhibition (FMGI) percentage (86.66%) against A. flavus. C. sinensis, E. camaldulensis, and E. camaldulensis/C. sinensis showed FMGI values of 96%, 91.66%, and 75.66% respectively, against A. niger. EOs from C. aurantium and C. sinensis showed potent activity against A. terreus (100% FMGI), while C. aurantium/E. camaldulensis and E. camaldulensis/C. sinensis showed FMGI values of 74.33% and 70.66%, respectively. Potent activity against F. culmorum with 100% was observed as the application of E. camaldulensis and C. sinensis EOs at 50  $\mu$ L/mL, while E. camaldulensis/C. sinensis (50  $\mu$ L/mL) showed FMGI value of 65.66%. The results suggest using the EOs and their combinations from E.camaldulensis, C. aurantium, and C. sinensis as a biofungicide against molds. The potent properties of EOs offer the possibility of using them as eco-friendly, safe, and cost-effective antimicrobials for molds that could cause discoloration of the wood packaging or food spoilage.

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Article

# *Eucalyptus camaldulensis*, *Citrus aurantium*, and *Citrus sinensis* Essential Oils as Antifungal Activity against *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus terreus*, and *Fusarium culmorum*

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**Abstract:** Several molds are able to colonize wood and many building products or solid wood causing losses for their valuable uses. Essential oils (EOs) from aromatic plants can be used as an ecofriendly biofungicide against the growth of several molds. EOs from *Eucalyptus camaldulensis*, *Citrus aurantium*, and *C. sinensis* have a broad-spectrum antimicrobial activity. EOs from of *E. camaldulensis* air-dried aerial parts, *C. aurantium* leaf and *C. sinensis* peel, and their combinations (1:1 v/v) were evaluated for their antifungal activity against the growth of four common mold fungi (*Aspergillus flavus*, *A. niger*, *A. terreus*, and *Fusarium culmorum*). The chemical compositions of the EOs were analyzed with GC/MS. The main compounds in EO from *E. camaldulensis* were spathulenol (20.84%), eucalyptol (12.01%), and sabinene (9.73%); in *C. aurantium* were linalyl acetate (42.29%), and linalool (29.76%); and in *C. sinensis* were *D*-limonene (73.4%) and  $\gamma$ -terpinene (22.6%). At 50  $\mu$ L/mL, *C. sinensis* EO showed the highest fungal mycelial growth inhibition (FMGI) percentage (86.66%) against *A. flavus*. *C. sinensis*, *E. camaldulensis*, and *E. camaldulensis*/*C. sinensis* showed FMGI values of 96%, 91.66%, and 75.66% respectively, against *A. niger*. EOs from *C. aurantium* and *C. sinensis* showed potent activity against *A. terreus* (100% FMGI), while *C. aurantium*/*E. camaldulensis* and *E. camaldulensis*/*C. sinensis* showed FMGI values of 74.33% and 70.66%, respectively. Potent activity against *F. culmorum* with 100% was observed as the application of *E. camaldulensis* and *C. sinensis* EOs at 50  $\mu$ L/mL, while *E. camaldulensis*/*C. sinensis* (50  $\mu$ L/mL) showed FMGI value of 65.66%. The results suggest using the EOs and their combinations from *E. camaldulensis*, *C. aurantium*, and *C. sinensis* as a biofungicide against molds. The potent properties of EOs offer the possibility of using them as eco-friendly, safe, and cost-effective antimicrobials for molds that could cause discoloration of the wood packaging or food spoilage.

**Keywords:** antifungal activity; *Eucalyptus camaldulensis*; *Citrus aurantium*; *Citrus sinensis*; essential oils

## 1. Introduction

Essential oils (EOs) extracts from different parts of medicinal and aromatic plants (leaves, bark, branches, flowers, roots, fruits, seeds, and peels) are complex mixtures with their compositions of terpenes, terpenoids, carotenoids, coumarins, ketonic bodies, curcumins, aldehydes, and phenols. They are classified as plant secondary metabolism and responsible for their characteristic aroma. Plant EOs have a several uses as antibacterial/antifungal activities [1–7]; insecticidal effects [8–14], antioxidant activity [15–18]; and in food preservatives, perfume, and cosmetic industries [19,20].

The EOs from Eucalyptus species have been studied in dozen research for their promising in industrial and pharmaceutical applications, which recommended as antimicrobial agents as well as potential insecticidal [21,22]. 1,8-Cineol is the main compound in the leaf EO of Eucalyptus, which can constitute 77% of the leaf EO, and some other constituents of cuminal, aromadendren, D-limonene, 4-terpineol, aromadendral, phellandrene, geraniol, cymene, and phellandral can be identified [1,11,16,22,23].  $\alpha$ -Pinene, *p*-cymene,  $\alpha$ -phellandrene, 1,8-cineole,  $\gamma$ -terpinene, and limonene were found in *E. camaldulensis* EO collected from eastern Taiwan [24], while *p*-cymene,  $\beta$ -phellandrene, 1,8-cineole, spathulenol, and cryptone were the main compounds from the aerial parts of *E. camaldulensis* grown in Italy [25]. In *E. camaldulensis* var. *obtusa*, its leaf EO contained *p*-cymene, spathulenol, cryptone, 1,8-cineole, 4-terpineol, and cuminal as the main chemotype compounds [22]. The main constituents in the seed EOs of *E. camaldulensis* var. *nancy* and *E. camaldulensis* var. *petford* were characterized with 1,8-cineole as main compound [26].

EO of peels from Citrus species has a complex combination of compounds with potential applications in food industries and pharmaceutical purposes as well as for their natural antioxidant and antimicrobial properties [27–30]. Orange fruits are usually refers to *Citrus × sinensis* (belongs to Rutaceae family) or Sweet Orange Group. The EO extracted from orange peels can be used as a green insecticide and have potential effects against microbes [31,32].

The peel EO was composed of 97% monoterpenes, while other compounds of aldehydes, alcohols, and esters appeared with the lowest percentage (1.8 to 2.2%) [33]. Limonene can be pointed out as distinct compound with percentages could be reached 99% in the EOs from orange peels [33,34]. Limonene with other bioactive monoterpenes such as  $\alpha$ -terpinene,  $\alpha$ -pinene, and sabinene were the major compounds in peels EO of different varieties of sweet oranges (*C. sinensis*) from Kenyan [35]. EO of peels from Citrus species contain ~92% D-limonene was reported to cause 68 and 96% death of termites (*Coptotermes formosanus* Shiraki and *Formosan subterranean*) at 5 ppm (*v/v*) concentration [36]. EO from Citrus species with their high concentration of limonene is reported for their potential control of some phyto-pathogenic fungi [37].

To control fungal growth, biosynthesis of mycotoxin, and food contamination, three main approaches (physical, biological, and chemical treatments) were recognized and used against mycotoxigenic fungi [7,23,38–45]. The crude EO was more effective than the major compounds, for example, the EO extracted from some Eucalyptus species such as *C. camaldulensis* was more active against *Pseudomonas aeruginosa* than the major constituents such as  $\alpha$ -pinene, 1,8-cineole, and *p*-cymene [46]. In addition, the EO reduced the growth and inhibited the production of spores and germination [47]. Additionally, under both in vitro and in vivo conditions, the EO from *C. camaldulensis* was more effective against *Penicillium digitatum*, the causative agent of fruit rot of mandarin cv. “Kinnow” [48]. Application of EO from *E. camaldulensis* leaves with its major compounds (eucalyptol (60.32%),  $\alpha$ -pinene (13.65%), and  $\gamma$ -terpinene (8.77%)) to some wood samples showed good antifungal activity against *Chaetomium globosum* and little inhibition against *F. subglutinans* [1].

Therefore, the aim of the present work was to evaluate the antifungal toxicity of the EOs from *E. camaldulensis* air-dried aerial parts, *C. aurantium* leaves and *C. × sinensis* peels singly or in combinations against the growth of common four fungi.

## 2. Materials and Methods

### 2.1. Hydrodistillation Method for Isolation of Essential Oils

*Eucalyptus camaldulensis* air-dried aerial parts, *Citrus aurantium* green old leaves, and *C. sinensis* fresh peels were collected during January 2019, from Alexandria, Egypt. The raw materials were transferred to small pieces then approximate 100 g from each of them were inserted in a flask (2 L capacity) that contained 1500 mL of distilled water (DW). The flask with its contents was heated under refluxing in terms to hydrodistillate the material and extract the essential oil (EO) using a Clevenger apparatus for 3 h [6]. The collected EOs were stored in brown glass bottles in a refrigerator at 4 °C. Oils were prepared in equal ratio (1:1 v/v) [49] as presented in Table 1.

**Table 1.** The essential oils and their mixtures used in this study.

Source or Composition
<i>Eucalyptus camaldulensis</i> oil
<i>Citrus aurantium</i> oil
<i>C. sinensis</i> peels oil
<i>E. camaldulensis</i> oil + <i>C. aurantium</i> oil (1:1 v/v)
<i>E. camaldulensis</i> oil + <i>C. sinensis</i> oil (1:1 v/v)
<i>C. aurantium</i> oil + <i>C. sinensis</i> oil (1:1 v/v)

### 2.2. GC-MS Analysis of Essential Oils and Their Combinations

The chemical constituents of the EOs from *E. camaldulensis* aerial parts, *C. aurantium* leaves, and *C. sinensis* peels were performed using GC-TSQ Quantum mass spectrometer (Thermo Scientific, Austin, TX, USA) with a direct capillary column TG-5MS (30 m × 0.25 mm × 0.25 µm film thickness). The conditions of the separation and identification of the EOs can be found in the previous works [12,50–53].

### 2.3. Antifungal Activity of Essential Oils and Their Combinations

The antifungal activity was evaluated against four fungi, *Aspergillus flavus* AFI375, *A. niger* FC24771, *A. terreus* Y.H. Yeh V0103, and *Fusarium culmorum* CBS128,537, isolated and identified with ITS gene [54], with their accession numbers of MH355,958, MH355,955, MH355,953, and MH355,954, respectively. The bioassay was evaluated using the radial growth technique method [5,14,55].

The EOs and their combinations were dissolved in a dimethyl sulfoxide (DMSO 10%), Tween 40, and DW mixture in the ration of 1:0.5:1. The dissolved EO and prepared at the concentration of 50, 25, and 12.5 µL/mL were added to warm PDA medium (40 to 45 °C), before immediately pouring into 9 cm Petri dishes. The standard antibiotic Sertaconazol (3 g/L) was used as a control, the dilution mixture were used as positive and negative controls, respectively. From a 7-day-old colony, the fungus with discs of 9 mm diameter was transferred to the center of the treated PDA plates and controls. All the plates were incubated at 26 ± 1 °C for 14 days. All the tested concentrations as well as positive and negative controls were measured in triplicate.

After the fungal growth reached the edges in the negative control plates, the percentage of fungal mycelial growth inhibition (FMGI) was calculated using the following equation [56]; FMGI (%) = [(DC – DT)/DC] × 100, where DC and DT represent the average diameters of the fungal colony of control and treatment, respectively. The minimum inhibitory concentrations (MICs) of the EOs were prepared at concentrations of 4–50 µL/mL and were assessed using the broth dilution method according to CLSI [55].

## 2.4. Statistical Analysis

FMGI (%) values of the fungi diameter growth were statistically analyzed based on two factors (EO type or EO mixture and EO concentration) using analysis of variance in SAS system [57]. The differences between the mean of each treatment were recorded using  $LSD_{0.05}$  and compared with positive control (Sertaconazol 3 g/L) and negative control (DMSO 10%).

## 3. Results

### 3.1. Chemical Constituents of the Essential Oils

Tables 2–4 present the chemical composition of the EOs from *Eucalyptus camaldulensis*, *Citrus aurantium*, and *C. sinensis*, respectively, analyzed by GC/MS apparatus (Figure 1). The major compounds in *E. camaldulensis* EO, spathulenol (20.84%), eucalyptol (12.01%), sabinene (9.73%),  $\alpha$ -phellandrene (8.18%), crypton (7.69%), terpinen-4-ol (3.69%), phellandral (3.54%) and *D*-limonene (2.28%) (Table 2). Linalyl acetate (42.29%), linalool (29.76%),  $\alpha$ -terpineol (7.38%), geranyl acetate (5.23%), neryl acetate (3.27%), and caryophyllene (2.35%), can be pointed out as distinct compounds in *C. aurantium* leaf EO (Table 3). *D*-limonene (73.4%) and  $\gamma$ -terpinene (22.6%) were the abundant compounds in the EO from *C. sinensis* peels (Table 4).

**Table 2.** Phytochemical composition of *E. camaldulensis* essential oil by GC/MS.

RT (min.)	Compound	Percentage in the Oil (%)	RSI-SI *
6.68	2-Thujene	1.12	(944–905)
6.98	$\alpha$ -Pinene	1.14	(938–919)
8.63	$\beta$ -pinene	0.77	(931–897)
9.44	$\alpha$ -Phellandrene	8.18	(939–938)
9.70	4-Terpinenyl acetate	0.38	(913–882)
10.10	<i>D</i> -Limonene	2.28	(924–916)
10.39	Sabinene	9.73	(940–936)
10.53	<i>p</i> -Cymene	15.16	(923–906)
10.68	Eucalyptol	12.01	(921–902)
11.16	$\gamma$ -Terpinene	1.09	(919–879)
12.06	( <i>E</i> )- $\alpha$ -Ocimene	0.7	(871–860)
14.11	<i>cis</i> - $\beta$ -Terpineol	0.64	(918–885)
14.87	<i>cis</i> -para-2-menthen-1-ol	0.38	(913–863)
16.11	Terpinen-4-ol	3.69	(928–923)
16.83	$\alpha$ -Terpineol	0.42	(869–853)
17.85	Crypton	7.69	(952–932)
19.36	Cuminaldehyde	1.81	(949–855)
20.19	Phellandral	3.54	(952–884)
20.94	2-ethylidene-6-methyl-3,5-Heptadienal	1.54	(823–807)
23.61	Aromadendrene	1.71	(932–847)
24.85	Nerolidyl acetate	0.41	(797–787)
27.59	Spathulenol	20.84	(947–922)
28.26	2-Methylene-5 $\alpha$ -cholestan-3 $\beta$ -ol	0.41	(843–781)
28.54	Linoleic acid ethyl ester	1.65	(743–735)
28.82	Oleic acid	0.27	(808–792)
29.06	$\alpha$ -Vetivol	1.12	(772–759)
29.23	$\alpha$ -Sinensal	0.21	(793–759)
29.30	( <i>E,E,E</i> )-9-Octadecenoic acid, 1,2,3-propanetriyl ester	0.23	(845–773)
29.54	( <i>Z,Z</i> )-1,3-Dioctadecenoyl glycerol	0.17	(836–816)
29.77	(11 <i>Z</i> )-12-(2-Oxiranyl)-11-dodecenyl acetate	0.18	(805–766)

RT: Retention time (min.); \* (RSI: Reverse Standard index; SI: Standard Index).

**Table 3.** Phytochemical composition of *C. aurantium* essential oil by GC/MS.

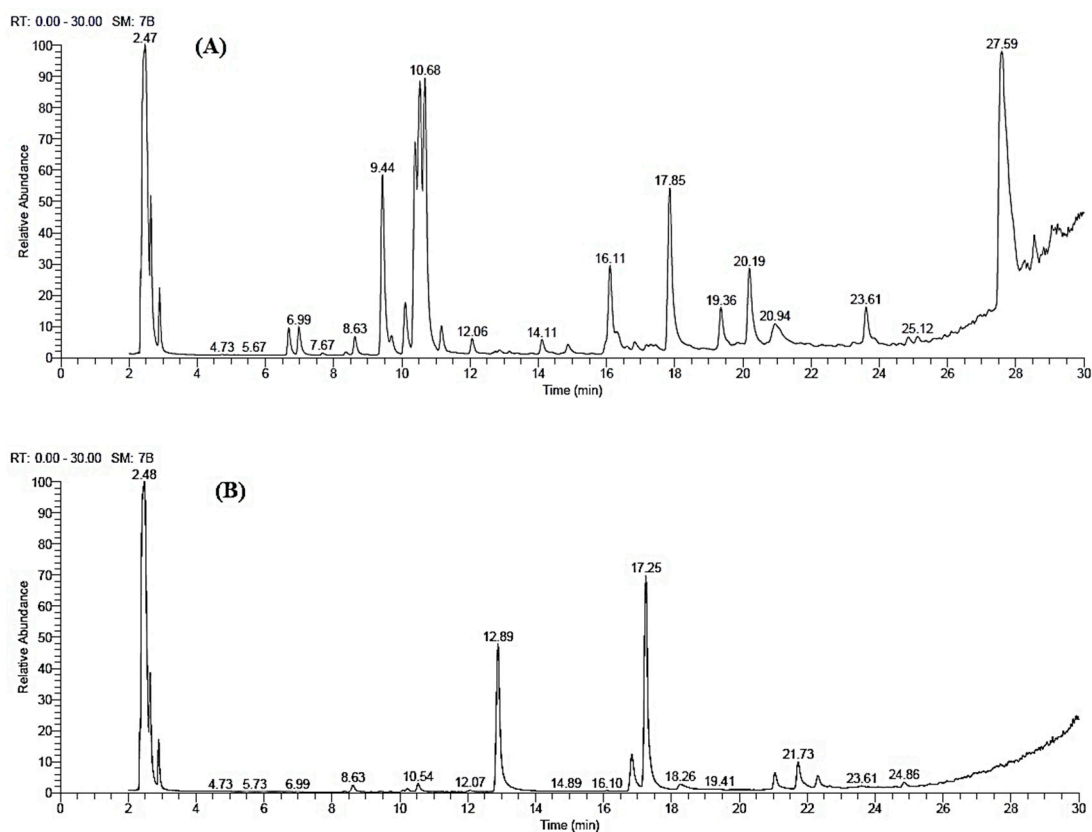
RT (min.)	Compound	Percentage in the Oil (%)	RSI-SI
8.63	$\beta$ -pinene	1.27	(931–889) *
10.54	( <i>E</i> )- $\alpha$ -Ocimene	1.4	(919–884)
12.89	Linalool	29.76	(968–960)
16.83	$\alpha$ -Terpineol	7.38	(934–930)
17.25	Linalyl acetate	42.29	(959–957)
18.25	nerol	1.18	(928–871)
21.05	Neryl acetate	3.27	(939–894)
21.73	Geranyl acetate	5.23	(933–903)
22.31	Caryophyllene	2.35	(895–861)
24.86	Nerolidyl acetate	1.08	(797–772)
27.69	Oleic acid	0.69	(835–817)
29.91	Z-(13,14-Epoxy)tetradec-11-e n-1-ol acetate	0.72	(812–767)

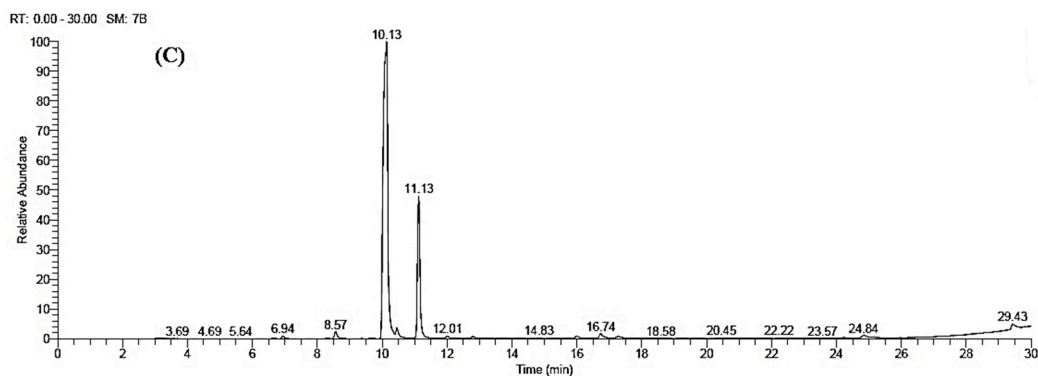
RT: Retention time (min.); \* (RSI: Reverse Standard index; SI: Standard Index).

**Table 4.** Phytochemical composition of *C. sinensis* essential oil by GC/MS.

RT (min.)	Compound	Percentage in the oil (%)	RSI-SI *
8.57	Myrcene	1.13	(952–944)
10.13	<i>D</i> -Limonene	73.4	(945–944)
10.46	<i>p</i> -Cymene	1.02	(923–840)
11.13	$\gamma$ -Terpinene	22.6	(950–949)
16.74	$\alpha$ -Terpineol	0.81	(931–923)
29.42	Ylangenal	1.04	(803–783)

RT: Retention time (min.); \* (RSI: Reverse Standard index; SI: Standard Index).

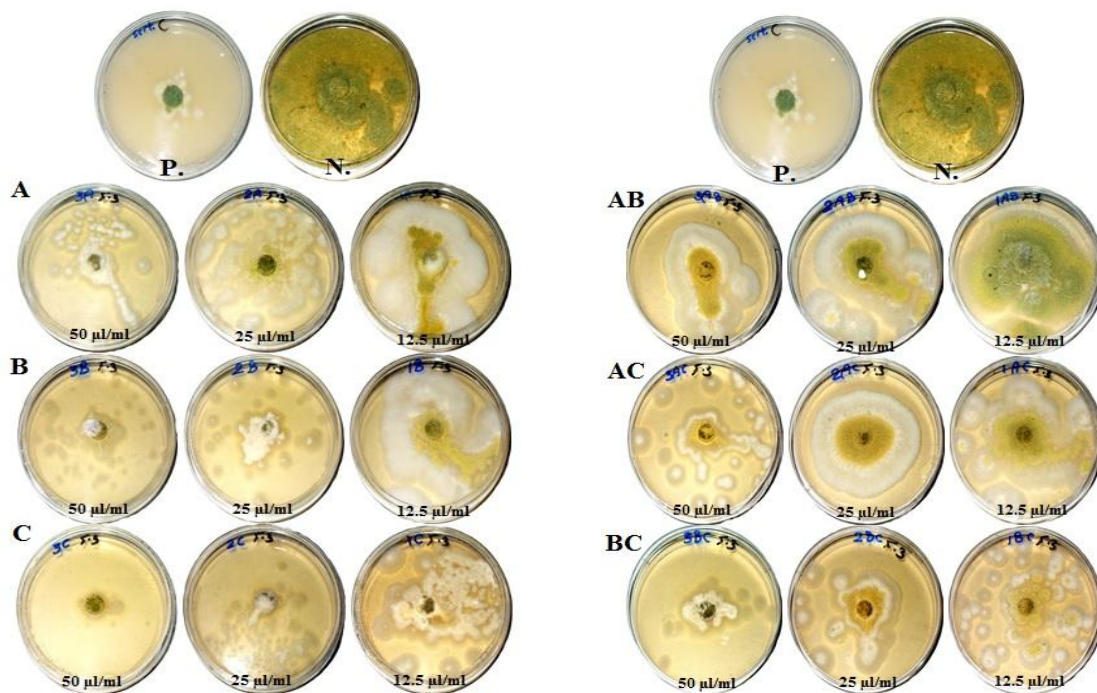
**Figure 1.** Cont.



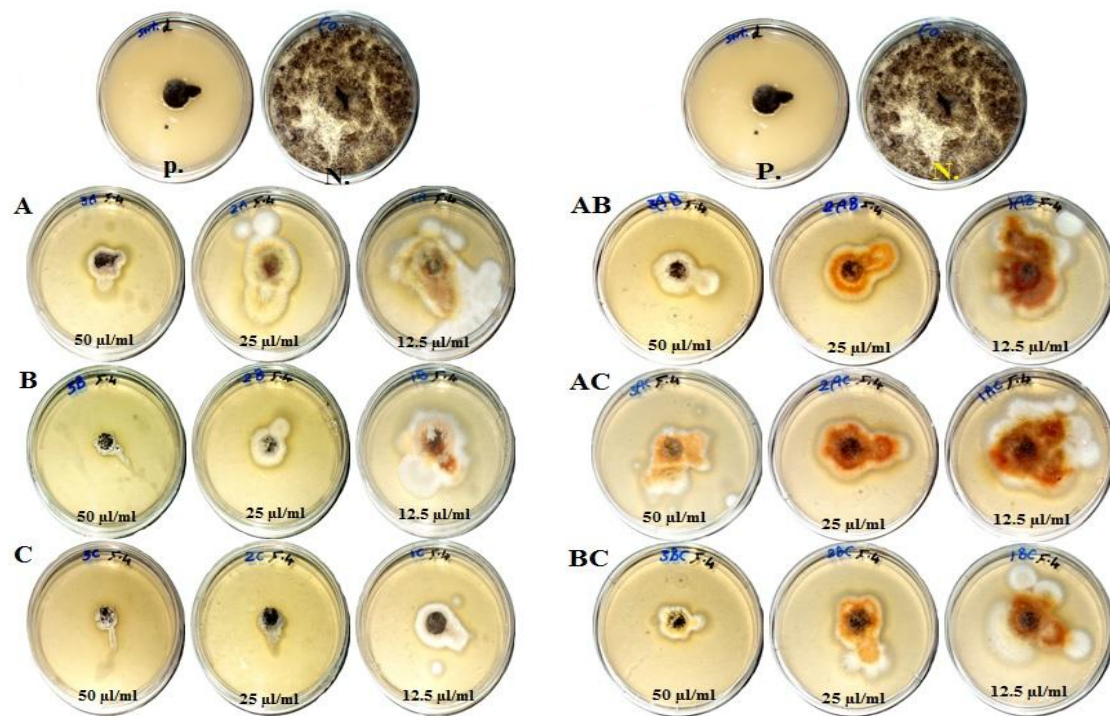
**Figure 1.** Chromatograms of GC/MS of the essential oils from (A) *E. camaldulensis* oil, (B) *C. aurantium* oil, and (C) *C. sinensis*.

### 3.2. Fungal Inhibition by Visual Observation

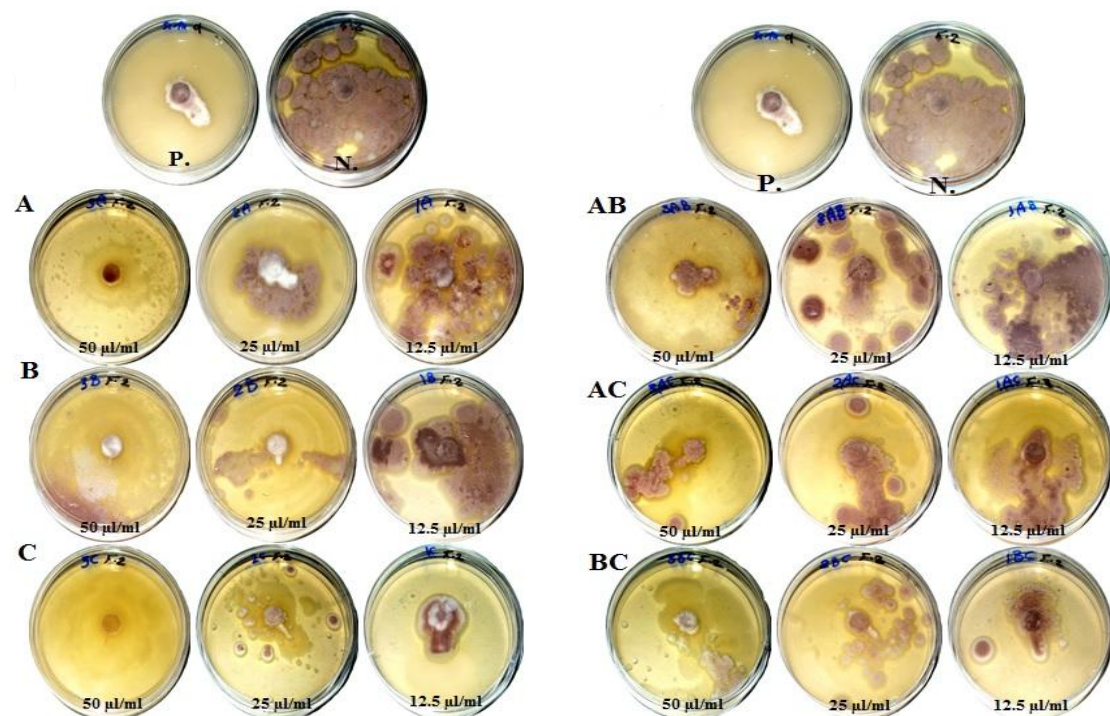
The visual observations of the fungal inhibition growth are shown in Figures 2–5 for *Aspergillus flavus*, *A. niger*, *A. terreus*, and *Fusarium culmorum*, respectively, as affected by the tested essential oils (EOs) from *C. aurantium* leaves, *E. camaldulensis* aerial parts, and *C. sinensis* peels, as well as their equal combinations. With increasing the concentration of EOs or their combinations, the fungal mycelial growth inhibition (FMGI) increased. No FMGI was shown in negative control (DMSO 10%) plates, while the positive control (Sertaconazol 3 g/L) of all the studied fungi showed good FMGI.



**Figure 2.** Visual observation of *A. flavus* growth inhibition as affected by the essential oils from (A) *C. aurantium* oil, (B) *E. camaldulensis* oil, and (C) *C. sinensis* oil and their combinations. (P) Positive control (Sertaconazol 3 g/L). (N) Negative control (DMSO 10%). (A,B) *C. aurantium* oil + *E. camaldulensis* oil. (A,C) *C. aurantium* oil + *C. sinensis* oil. (B,C) *E. camaldulensis* oil + *C. sinensis* oil.

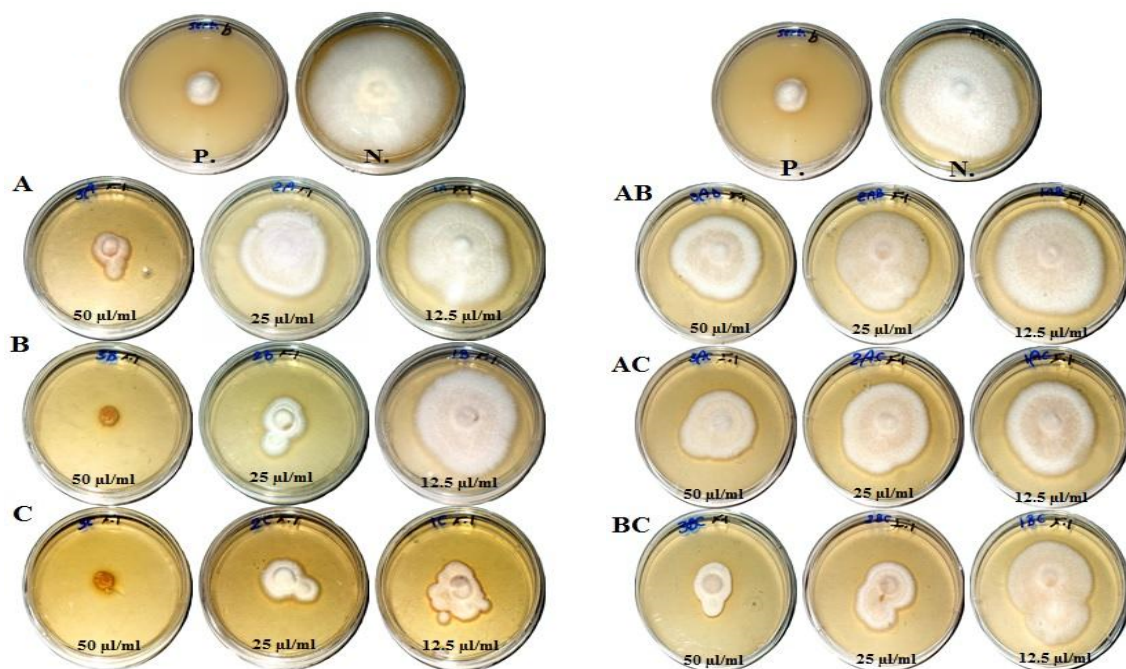


**Figure 3.** Visual observation of *A. niger* growth inhibition as affected by the EOs from (A) *C. aurantium* oil, (B) *E. camaldulensis* oil, and (C) *C. sinensis* oil and their combinations. (P) Positive control (Sertaconazol 3 g/L). (N) Negative control (DMSO 10%). (A,B) *C. aurantium* oil + *E. camaldulensis* oil. (A,C) *C. aurantium* oil + *C. sinensis* oil. (B,C) *E. camaldulensis* oil + *C. sinensis* oil.



**Figure 4.** Visual observation *A. terreus* growth inhibition as affected by the EOs from (A) *C. aurantium* oil, (B) *E. camaldulensis* oil, and (C) *C. sinensis* oil and their combinations. (P) Positive control (Sertaconazol 3 g/L). (N) Negative control (DMSO 10%). (A,B) *C. aurantium* oil + *E. camaldulensis* oil. (A,C) *C. aurantium* oil + *C. sinensis* oil. (B,C) *E. camaldulensis* oil + *C. sinensis* oil.





**Figure 5.** Visual observation of *F. culmorum* growth inhibition as affected by the EOs from (A) *C. aurantium* oil, (B) *E. camaldulensis* oil, and (C) *C. sinensis* oil and their combinations. (P) Positive control (Sertaconazol 3 g/L). (N) Negative control (DMSO 10%). (A,B) *C. aurantium* oil + *E. camaldulensis* oil. (A,C) *C. aurantium* oil + *C. sinensis* oil. (B,C) *E. camaldulensis* oil + *C. sinensis* oil.

### 3.3. Antifungal Activity of Essential Oils and Their Combinations In Vitro

The antifungal activity of the EOs and their combinations are presented in Table 5. *C. sinensis* peel EO showed the highest FMGI percentage of 86.66% against the growth of *A. flavus*, followed by *E. camaldulensis* EO (74.33%) at the concentration of 50 µL/mL. The bioactivity of EOs was decreased for all the combination treatments, but it was reached 64.66% as the EO combination of *E. camaldulensis*/*C. sinensis*. However, these values are lower than of FMGI value from the positive treatment (88.66%). All the EO combinations showed limit impact on the growth of *A. flavus*.

At the EO concentration of 50 µL/mL, the highest FMGI percentages of 96, 91.66, 75.66, 71.33, and 70.66%, against the growth of *A. niger* were observed with the application of *C. sinensis*, *E. camaldulensis*, *C. aurantium*, *E. camaldulensis*/*C. sinensis*, and *C. aurantium*/*E. camaldulensis* EOs compared to positive control treatment (87%). Additionally, *C. sinensis* peel EO at 25 µL/mL observed a FMGI value of 77.66%.

It is worth noting that the potent toxicity was observed against the growth of *A. terreus* with 100% FMGI percentage with the application of EOs from *C. aurantium* and *C. sinensis*. These values were higher from those obtained from the positive control (91%), while the EOs from *E. camaldulensis*, *C. aurantium*/*E. camaldulensis*, and *E. camaldulensis*/*C. sinensis* observed good FMGI values of 79, 74.33, and 70.66%, respectively, against the growth of *A. terreus*. *E. camaldulensis* and *C. sinensis* EOs showed potent activity with 100% FMGI against the growth of *F. culmorum*. EOs from *C. aurantium* (50 µL/mL), *C. sinensis* (25 µL/mL), and *E. camaldulensis*/*C. sinensis* (50 µL/mL) were shown FMGI values of 65.66, 66.33, and 65.66%, respectively, which lower than the value from positive control (89.66%). Table 6 presents the minimum inhibitory concentrations for the EOs ranged between 8 and 40 µL/mL, 6 and 8 µL/mL, 6 and 12 µL/mL, and 6 and 40 µL/mL against the growth of *Aspergillus flavus*, *A. niger*, *A. terreus*, and *Fusarium culmorum*, respectively, while it was 8 µL/mL, 6 µL/mL, 8 µL/mL, and 6 µL/mL as measured for Sertaconazol for the same order of fungi.

**Table 5.** Inhibition percentage of the diameter growth of *A. flavus*, *A. niger*, *A. terreus*, and *F. culmorum* as affected by essential oils from *C. aurantium*, *E. camaldulensis*, and *C. sinensis* and their combinations.

Oil Source.	Concentration (µL/mL)	Inhibition Percentage of Diameter Growth (%)			
		<i>Aspergillus flavus</i>	<i>Aspergillus niger</i>	<i>Aspergillus terreus</i>	<i>Fusarium culmorum</i>
<i>C. aurantium</i>	12.5	0.33 ± 0.33	22.33 ± 2.72	3.66 ± 0.88	5 ± 0.57
	25	2 ± 0.57	48.33 ± 0.88	48 ± 2	45 ± 0.57
	50	62.66 ± 1.21	75.66 ± 0.66	100	65.66 ± 0.33
<i>E. camaldulensis</i>	12.5	2.33 ± 0.33	48.66 ± 0.66	13 ± 1.15	21.33 ± 0.66
	25	63 ± 1.15	65.66 ± 0.33	57 ± 1.52	58 ± 1.52
	50	74.33 ± 0.88	91.66 ± 4.17	79 ± 2	100
<i>C. sinensis</i>	12.5	5 ± 0.57	65 ± 0.57	60.33 ± 0.33	60 ± 1.73
	25	61.33 ± 0.33	77.66 ± 0.33	70.33 ± 1.66	66.33 ± 0.88
	50	86.66 ± 0.33	96 ± 4	100	100
<i>C. aurantium</i> + <i>E. camaldulensis</i>	12.5	0.66 ± 0.33	51 ± 0.57	24 ± 1.15	14.33 ± 0.88
	25	9.33 ± 1.33	57 ± 1.52	40 ± 2	30.66 ± 1.45
	50	24.33 ± 1.21	70.66 ± 0.66	74.33 ± 1.45	41.66 ± 1.21
<i>C. aurantium</i> + <i>C. sinensis</i>	12.5	1.33 ± 0.33	32.66 ± 0.33	45.66 ± 1.20	27.33 ± 0.33
	25	22.66 ± 0.33	46.66 ± 0.33	53.66 ± 0.88	34 ± 0.57
	50	46.33 ± 1.85	53.33 ± 0.66	63.33 ± 1.45	47.66 ± 0.33
<i>E. camaldulensis</i> + <i>C. sinensis</i>	12.5	23.66 ± 1.21	43.33 ± 0.66	62.66 ± 1.66	34 ± 0.57
	25	35.33 ± 1.21	50 ± 1.15	67 ± 0.57	50.66 ± 0.66
	50	64.66 ± 0.66	71.33 ± 0.66	70.66 ± 0.66	65.66 ± 0.33
Negative control (DMSO)	10%	0.00	0.00	0.00	0.00
Sertaconazol	3 g/L	88.66 ± 0.66	87 ± 0.57	91 ± 0.57	89.66 ± 0.88
P value		<0.0001	<0.0001	<0.0001	<0.0001

Values are means ± SE.

**Table 6.** Minimum inhibitory concentrations (MICs) of the essential oil treatments.

Essential Oil	MIC (µL/mL)			
	<i>Aspergillus flavus</i>	<i>Aspergillus niger</i>	<i>Aspergillus terreus</i>	<i>Fusarium culmorum</i>
<i>C. aurantium</i>	25	8	12	12
<i>E. camaldulensis</i>	12	7	10	8
<i>C. sinensis</i>	12	6	6	6
<i>C. aurantium</i> + <i>E. camaldulensis</i>	40	6	12	40
<i>C. aurantium</i> + <i>C. sinensis</i>	30	8	10	40
<i>E. camaldulensis</i> + <i>C. sinensis</i>	8	6	8	20
Sertaconazol (reference fungicide)	8	6	8	6

#### 4. Discussion

Results of the study show the considerable values of antifungal activity of essential oils (EOs) of *Eucalyptus camaldulensis* air-dried aerial parts, *Citrus aurantium* leaves, and *C. sinensis* peels against *Aspergillus flavus*, *A. niger*, *A. terreus*, and *Fusarium culmorum* singly and in combination together with equal volume. The occurred effects are due to presence of components such as spathulenol, *p*-cymene, eucalyptol, linalyl acetate, linalool, sabinene,  $\alpha$ -phellandrene, crypton, terpinen-4-ol, *D*-limonene,  $\gamma$ -terpinene,  $\alpha$ -terpineol, longifolene, neryl acetate, *p*-cymene, phellandral, cuminaldehyde, and alloaromadendrene in the EOs [22,58,59].

The EO from aerial parts of *E. camaldulensis* showed the presence of *p*-cymene (27.8–42.7%), 1,8-cineole (4.1–39.5%), spathulenol (2.1–15.5%), and cryptone (3.2–10.2%) as main compounds [25].

The EOs with their chemical compositions of 1,8-cineole ( $\geq 60\%$ ), aromadendrene ( $\geq 5\%$ ), and limonene ( $\geq 4\%$ ) or *p*-cymene (10%),  $\beta$ -pinene (8%) and spathulenol (10%), were characterized some *E. camaldulensis* clones grown in Australia [60]. *p*-Cymene, cyptone, and spathulenol with 22.9%, 14.1%, and 16.5%, respectively, were found to be the abundant compounds of *E. camaldulensis* EO from Australia [61]. 1,8-Cineole (34.7%),  $\beta$ -pinene (7.7%), *p*-cymene (9.3%), and spathulenol (9.5%) were reported as main compounds in EO of *E. camaldulensis* from Greece [62]. *E. camaldulensis* leaf EO with its main compounds eucalyptol,  $\alpha$ -pinene, and  $\gamma$ -terpinene applied to wood showed good inhibition against *Chaetomium globosum*, moderate activity against *F. subglutinans*, and weak activity against *A. niger* and *T. viride* [1]. The EO produced complete in all the test pathogens at a minimum inhibitory concentration in the range of 7 to 8  $\mu\text{L/mL}$  and after five days of incubation, the mycelial growth inhibition was completely produced against *Fusarium solani*, *F. oxysporum*, *F. verticillioides*, *F. proliferatum*, and *F. subglutinans* commonly associated with maize [63].

Linalool, the purified compound from the EO of *Ocimum basilicum*, observed a potent antimicrobial activity [64]. 1,8-Cineole, the most significant compounds in Eucalyptus EOs had strong antimicrobial activity against plant pathogens [65,66].  $\alpha$ -Pinene was reported to inhibit the growth of some fungi including *Alternaria* sp., *A. nidulans*, and *A. niger* [67]. Antifungal activities found with *Eucalyptus* EOs and their combinations with *Citrus* species might be attributed to spathulenol [68,69]. Leaves EO from *E. camaldulensis* var. *obtusata* showed the presence of *p*-cymene, spathulenol, crypton, 1,8-cineole, 4-terpineol, cuminal, phellandral, and aromadendrene, as main compounds with percentages of 19.38%, 18.37%, 16.91%, 9.27%, 6.26%, 5.56%, 1.96%, and 2.29%, respectively, with good antibacterial activity against *Escherichia coli* and *Agrobacterium tumefaciens* [5].

Limonene, the main compound in peels of *Citrus* species, with percentage of 96.62% and other compounds of  $\beta$ -pinene,  $\beta$ -myrcene,  $\alpha$ -pinene, and citral (*Z* and *E*) were identified in *Citrus sinensis* var. Valencia peel EO with good antifungal activity against of *A. flavus* [59]. The chemical composition peel EOs of *C. sinensis* from Uganda and Rwanda had limonene ranged from 87.9 to 92.5%, with small amounts of myrcene,  $\alpha$ -pinene, and linalool [58]. Other study showed that the *C. sinensis* peel EO had limonene,  $\beta$ -myrcene, decanal,  $\beta$ -pinene, and linalool, as major compounds with good antioxidant activity [70]. Limonene in the present study reached 73.4% in *C. sinensis*, while in previous investigation it was 77.49% the peel oil of sweet orange followed by myrcene 6.27% [71,72]. Limonene (80.9%) and  $\beta$ -myrcene (4.19%) were the main constituents in fresh peel EO of *C. sinensis* [73]. Limonene found in percentage of 87.9% and 92.5% from *C. sinensis* peels of Uganda and Rwanda, respectively [58]. *C. sinensis* peel EO with its main compound of limonene (98.54%) was observed potential of inhibition of mycelial growth (63.46%) of *Sclerotinia sclerotiorum* at the oil dose 300  $\mu\text{L}$  [74]. *D*-limonene is highly useful in agriculture as antibacterial agent against economic phyto-pathogenic bacteria *Ralstonia solanacearum* isolated from potato as well as for insect repellent [12,75,76]. Moreover, in the pure form, monoterpene was reported to exhibit promising antifungal activity against *Aspergillus niger*, *Fusarium oxysporum*, *Phytophthora digitatum*, *F. verticillioides*, *R. solani*, and *S. sclerotiorum* [77,78].

In this work, the main compounds, linalyl acetate,  $\alpha$ -terpineol, linalool, neryl acetate, geranyl acetate, and caryophyllene, were found in *C. aurantium* leaf EO. A typical composition of *C. aurantium* leaf essential oil would be linalyl acetate, geranyl acetate, and neryl acetate with 45%, 3%, and 0.5%, respectively, also present alcohols and terpenes of linalool, geraniol,  $\alpha$ -terpineol, nerol, myrcene, and *trans*-ocimene with values of 28%, 2.5%, 7.5%, 1%, 5%, and 3.5%, respectively [79]. *C. aurantium* var. *amara* from Tunisia (Nabeul) showed the presence of linalool, linalyl acetate, and  $\alpha$ -terpineol in percentages of 36.8%, 22.1%, and 11.7%, respectively, in petitgrain (leaves) EO [80]. *C. aurantium* flower and leaf EOs from south Croatia were qualitatively similar containing linalyl acetate, linalool, (*E,E*)-farnesol, and (*E*)-nerolidol up to 19.3%, 17.3%, 13.0%, and 12.4%, respectively. Moreover, it was reported that *C. aurantium* leaf EO belonged to linalool/linalyl acetate chemotype contained (*E,E*)-farnesol (13%) and (*E*)-nerolidol (12.4%) [81]. Major compounds found in leaf EOs of seven accessions of *C. aurantium* were linalool, linalyl acetate, and  $\alpha$ -terpineol in the range of 6.6 to 48.9%, 0.4 to 33.8%, and 0.3 to 10.8%, respectively, for most of the samples [82].

It was reported that the combination of EOs could be probably resulted in a more effective [83–85]. *E. globulus* and *Zingiber officinalis* EOs in combination showed considerable activity against *Giardia lamblia* cysts [85]. *C. maxima* and *C. sinensis* EOs, with their main compounds dl-limonene, alone or in combination (1:1), showed potential fungitoxic spectrum against food-contaminating molds including *A. flavus* and completely inhibited aflatoxin B<sub>1</sub> [49]. In addition, EOs mixture of thyme and oregano exhibited potent antifungal activity [86]. The EO mixture of *O. vulgare*/*Rosmarinus officinalis* observed synergism effects against some microbes [87]. Checkerboard EOs of *Lippia multiflora*/*Mentha piperita* showed broad-spectrum synergism antibacterial activity [88]. Mixture EOs of *S. aromaticum*/*R. officinalis* showed synergism effects against *Staphylococcus epidermidis*, *S. aureus*, *B. subtilis*, *E. coli*, *Proteus vulgaris*, *P. aeruginosa*, and *Candida albicans* and antagonism effects against *A. niger* [89].

For the generally accepted mechanisms of antimicrobial interaction that produce synergism, it was found that the combinations of EOs led to inhibition of the common biochemical pathway with inhibition of the protective enzymes, with subsequent use of cell wall-active agents to enhance the uptake of other antimicrobials [90–95].

## 5. Conclusions

This study revealed that the essential oils from *Eucalyptus camaldulensis* aerial parts, *Citrus aurantium* leaves, and *C. sinensis* peels singly and/or in combination showed qualitative differences in their chemical compositions. The results demonstrate that the essential oils possessed promising antifungal activity against *Aspergillus flavus*, *A. niger*, *A. terreus*, and *Fusarium culmorum*. Therefore, these essential oils could be considered for use as ecofriendly biofungicides to deter the growth of molds in food packaging or wood containers; however, for food preservatives, the toxicity test should be run before use is approved.

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