Antifungal activity and mechanism of the essential oils from Litsea (*Litsea cubeba*), Melissa (*Melissa officinalis*), Palmarosa (*Cymbopogon martini*) and Verbena (*Verbena officinalis*) and their major active constituents against *Trametes hirsuta* and *Laetiporus sulphureus*

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Abstract

Antifungal activities of 37 essential oils (EOs) against two wood-decaying fungi, *Trametes hirsuta* and *Laetiporus sulphureus* were screened in vitro, and investigated the underlying mechanism. Of the 37 EOs, litsea (*Litsea cubeba*), melissa (*Melissa officinalis*), palmarosa (*Cymbopogon martini*), and verbena (*Verbena officinalis*) demonstrated strong antifungal activity, in which litsea oil exhibited the strongest antifungal property against *T. hirsuta* and *L. sulphureus*, with IC_{50} values of 72.3 and 40.2 μg/ml, respectively. The compositions of litsea, melissa, palmarosa, and verbena EOs were analyzed using a gas chromatography-mass spectrometry method and demonstrated geranial, geraniol, neral, and citral as their major active constituents. Among of them geranial exhibited the strongest antifungal activity against *T. hirsuta* and *L. sulphureus*, with IC_{50} values of 56.6 and 33.3 μg/ml, respectively. These EOs and their major active constituents increased the plasma membrane permeability of *T. hirsuta* and *L. sulphureus*, resulting in the leakage of nucleic acid, protein, and soluble sugar. Results indicate that the EOs of litsea, melissa, palmarosa, and verbena and its major constituents inhibited *T. hirsuta* and *L. sulphureus* growth by targeting its plasma membrane.

1. Introduction

The biodegradation of lignocellulosic materials occurs by beetles, fungi, marine borers, and termites; among these, fungi are recognized to cause the greatest financial loss of wooden products (Wu et al. 2012). Decay fungi, molds, and stain fungi are universally recognized as major wood-degradation fungi (Bakar et al. 2013). In general, traditional wood preservatives are ammoniacal copper quaternary (ACQ), chromated copper arsenates (CCAs), copper azole (CA), and copper (II) dimethyldithiocarbamate (CDDC), which significantly affect human health and the environment (Chen et al. 2014). Therefore, there is an urgent need to research and explore more eco-friendly, convenient, and highly effective benign wood preservatives for lignocellulosic materials.

In recent years, many natural plant products that are non-residual, biodegradable, and environmentally friendly have been shown to be excellent potential alternatives for preserving the wood industry (Xie et al. 2017a). Studies have shown that plant essential oils (EOs) from *Calocedrus fromosana*, *Cryptomeria japonica*, *Cinnamomum osmophloem*, *Ci. zeylanicum*, *Cymbopogon citratus*, *Cunninghamia konishii*, *Eucalyptus camaldulensis*, *Eugenia caryophyllata*, *Machilus philippinensis*, *Origanum vulgare*, *Pelargonium graveolens*, and *Thymus vulgaris* have antifungal properties (Cheng et al. 2005, 2006, 2011; Ho et al. 2010; Xie et al. 2015, 2017a; Salem et al. 2016). As is well known, the strong antifungal activity of EOs against wood decaying fungi were contributed to a rich of monoterpenes, sesquiterpenes and phenylpropanoids (Cheng et al. 2012; Zhang et al. 2016a). Most EOs and their compounds destroy the integrity of fungal cell membranes, resulted in the outflow of intracellular components and cell death (Kalily et al. 2016; Zhang et al. 2016b; Zhou et al. 2017; Souza et al. 2020; Yan et al. 2020).

Research on EOs as biological agents for protecting wood and prolonging their application life and as an alternative to chemical wood preservatives is becoming increasingly a necessity. For this reason, this

study examined the antifungal activity of 37 EOs against wood-decay fungi. We also analyzed the chemical composition of EOs with the strongest antifungal activity by gas chromatography-mass spectrometry. In addition, we evaluated the antifungal activity of the major active constituents in the selected EOs and elucidated the relationship between the active constituents and their antifungal properties. Finally, we investigated the changes in plasma membrane permeability of *T. hirsuta* and *L. sulphureus* caused by selected EOs.

2. Materials And Methods

2.1. Wood decay fungi

Trametes hirsuta (CFCC 84683) and *Laetiporus sulphureus* (CFCC 86368) were procured from China Forestry Culture Collection Center.

2.2. Essential oils and chemicals

The antifungal activities of 37 EOs against wood rot fungi in vitro were screened (Table 1). *Litsea cubeba* and *Verbena officinalis* were procured from Rihua Chemical Co. Ltd. (Guangzhou, China). The other EOs was purchased from Huien International Business Co. Ltd. (Shanghai, China). Geraniol, neral, citral, and geranial were purchased from Sigma-Aldrich (China).

Table 1
List of plant essential oils tested for wood-decay fungi

oil	source of plant	family name	part	origin
Chamomile	*Anthemis nobilis*	Compositae	Flower	France
Wormwood	*Artemisia argyi*	Compositae	Leaves	China
Cypress	*Cupressus sempervirens*	Cupressaceae	Leaves	France
Juniper berry	*Juniperus communis*	Cupressaceae	Fruit	France
Palmarosa	*Cymbopogon martini*	Gramineae	Leaves	Brazil
Citronella	*Cymbopogon winterianus*	Gramineae	Leaves	Java
Vetiver	*Vetiveria zizanoides*	Gramineae	Root	India
Lavender	*Lavandula angustifolia*	Lamiaceae	Leaves	France
Melissa	*Melissa officinalis*	Lamiaceae	Leaves	France
Peppermint	*Mentha arvensis*	Lamiaceae	Leaves	America
Basil	*Ocimum basilicum*	Lamiaceae	Leaves	Italy
Marjoram	*Origanum majorana*	Lamiaceae	Flower	Egypt
Patchouli	*Pogostemon cablin*	Lamiaceae	Leaves	Malaysia
Rosemary	*Rosmarinus officinalis*	Lamiaceae	Leaves	Morocco
Clary sage	*Salvia sclarea*	Lamiaceae	Leaves	Russia
Litsea	*Litsea cubeba*	Lauraceae	Fruit	China
Ravensara	*Ravensara aromatic*	Lauraceae	Leaves	Madagascar
Eucalypyus	*Eucalyptus globulus*	Myrtaceae	Leaves	Australia
Tea tree	*Melaleuca alternifolia*	Myrtaceae	Leaves	Australia
Cajeput	*Melaleuca leucadendra*	Myrtaceae	Leaves	Australia
Niaouli	*Melaleuca viridiflora*	Myrtaceae	Leaves	Australia
Cedarwood	*Cedrus atlantica*	Pinaceae	Bark	America
Lignum cedrium	*Cedrus deodara*	Pinaceae	Heart wood	America
Black pepper	*Piper nigrum*	Piperaceae	Fruit	India
Bergamot	*Citrus aurantium bergamia*	Rutaceae	Peel	Italy
Neroli	*Citrus aurantium amara*	Rutaceae	Flower	Egypt
Orange	*Citrus aurantium dulcis*	Rutaceae	Peel	Italy
Grapefruit	*Citrus grandis*	Rutaceae	Peel	Italy

oil	source of plant	family name	part	origin
Lemon	*Citrus limon*	Rutaceae	Peel	Italy
Mandarin	*Citrus reticulate*	Rutaceae	Peel	Italy
Dill Seed	*Anethum graveolens*	Umbelliferae	Seed	China
Coriander	*Coriandrum sativum*	Umbelliferae	Fruit	China
Caraway	*Carum carvi*	Umbelliferae	Seed	China
Fennel	*Foeniculum vulgre*	Umbelliferae	Seeds	Hungary
Chuanqiong	*Ligusticum chuanxiong*	Umbelliferae	Root	China
Verbena	*Verbena officinalis*	Verbenaceaex	Leaves	Spain
Ginger	*Zingiber officinale*	Zingiberaceae	Stem	China

2.3. GC-MS

The chemical analysis of EOs compounds was determined by GC-MS using an Agilent 6890A/5975C, equipped with a HP-5 capillary column. Analytical conditions were as follows: The GC oven temperature was set at 50°C for 10 min, and raised to 280°C at 10°C/min; He was the carrier gas at 1.0 ml/min; the injection of 1.0 µl; and split ratio of 1:50. The chemical components were identified by NIST mass spectrometry Library (NIST 11.0) and retention index (RI). The relative indices were determined in relation to the series of n-alkanes, with respect to those reported in the literature (Adams, 2007).

2.4. Antifungal assay

The antifungal activity of EOs and active compounds were examined using an in vitro assay by Xie et al. (2017a). Briefly, 25–400 µg/ml of EOs or their major constituent were added to 20 ml sterilize PDA medium and poured into in 9 cm petri dishes, then inoculation mycelial disc (5 mm) were pace in the center of each dish and incubated at 26 ± 1°C for 5–7 days. Three replicates were done for each dose. When the mycelia reached the edge of control plates (only distilled water), antifungal indices were calculated.

2.5 Membrane integrity determination

2.5.1 Effect of EOs on fungal membrane integrity with propidium iodide (PI) dyeing

Membrane integrity of *T. hirsuta* and *L. sulphureus* was determined following Yan et al. (2020) method with a confocal laser scanning microscope (CLSM). The fungi were incubated for 24 h in PDB containing the four EOs and its major constituents (1 µl/ml), respectively, and then collected mycelia and stained with PI (1 µg/ml) for 30 min at 28°C in dark. After staining, the mycelia were washed three times with the phosphate buffered saline (PBS, PH 7.4) to remove residual dye. Then use CLSM (Zeiss LSM880,

Germany) to observe PI with excitation/emission wavelengths 561 nm/591 to 635 nm. Each experiment was repeated three times.

2.5.2 Effect of EOs on leakage of fungal nucleic acid and protein

Fungal nucleic acid and protein leakage were measured according to the methods of Shao et al. (2013) with slight modifications. Fungi were incubated for 24 h in PDB with four EOs or their major constituent (1 µl/ml), then supernatant was used for nucleic acid and protein leakage determination, which was quantified using NanoDrop ONE (Thermo SCIENTIFIC). The experiment was performed in three replicates each.

2.5.3 Effect of EOs on fungal soluble sugar content

Soluble sugar content was measured according to the anthrone-sulfuric acid method by Smith et al. (1985), with slight modifications. 800 µl of filtrates and 100 µl of anthrone-ethyl acetate were mixed, then 1 ml of H_2SO_4 was added and incubated at 95°C for 10 min, and cooled down at room temperature. Finally, the absorbance was recorded at 620 nm.

2.6. Statistical analyses

The experiments were performed in three replicates, and the experimental results were obtained from mean ± SD. The data of inhibition rate was analyzed using one-way ANOVA by Duncan's test ($p < 0.05$).

3. Results

3.1. Antifungal activity of the EOs

The antifungal activity of 37 plant EOs against two wood-decay fungi (Table 2), of these, 4 EOs, litsea (*Litsea cubeba*), melissa (*Melissa officinalis*), palmarosa (*Cymbopogon martini*), and verbena (*Verbena officinalis*), achieved 100% inhibition of *T. hirsuta* and *L. sulphureus* at 400 µg/ml. The litsea and verbena EOs showed 100% antifungal activity when the concentration was decreased to 200 µg/ml (Fig. 1A-D).

Table 2
Antifungal activities of essential oils against white-rot fungs *T. hirsuta* and brown-rot fungs *L. sulphureus*

Plant species	Inhibition (%, mean ± SD)	
	T. hirsuta	*L. sulphureus*
Chamomile	62.2 ± 1.3 bc	77.0 ± 1.6 b
Wormwood	4.1 ± 2.0 jk	8.1 ± 1.0 jk
Cypress	21.5 ± 1.5 fghij	23.3 ± 1.3 fghij
Juniper berry	25.6 ± 1.3 fghi	26.3 ± 0.4 fghij
Palmarosa	**100 a**	**100 a**
Citronella	51.5 ± 1.6 cd	75.2 ± 2.6 b
Vetiver	76.7 ± 2.8 b	83.0 ± 1.3 ab
Lavender	0 k	21.1 ± 2.3 ghij
Melissa	**100 a**	**100 a**
Peppermint	23.0 ± 2.0 fghij	42.2 ± 3.4 def
Basil	64.8 ± 1.3 bc	100 a
Marjoram	28.5 ± 8.1 efghi	19.6 ± 0.7 ghij
Patchouli	68.9 ± 2.3 bc	83.3 ± 2.3 ab
Rosemary	23.0 ± 2.0 fghij	34.4 ± 1.3 efg
Clary sage	26.3 ± 1.6 fghi	23.7 ± 1.6 fghij
Litsea	**100 a**	**100 a**
Ravensara	38.1 ± 1.0 def	18.1 ± 1.6 ghijk
Eucalypyus	14.1 ± 2.3 hijk	0 k
Tea tree	15.2 ± 1.6 ghijk	10.0 ± 2.2 jk
Cajeput	15.2 ± 1.6 ghijk	18.1 ± 3.5 ghijk
Niaouli	25.9 ± 0.7 fghi	26.3 ± 2.6 fghij
Cedarwood	34.4 ± 1.3 defgh	37.4 ± 1.6 defg
Lignum cedrium	26.7 ± 1.7 fghi	13.0 ± 1.6 hijk
Black pepper	26.7 ± 1.3 fghi	53.7 ± 3.2 cde
Bergamot	0 k	26.0 ± 1.6 fghij

400 μg/ml was treated; Means within a column followed by the same letters are not significantly different ($P < 0.05$, Duncan's test).

Plant species	Inhibition (%, mean ± SD)	
	T. hirsuta	*L. sulphureus*
Neroli	17.0 ± 2.0 fghijk	31.9 ± 1.6 fgh
Orange	0 k	11.9 ± 2.3 ijk
Grapefruit	0 k	0 k
Lemon	0 k	18.1 ± 1.3 ghijk
Mandarin	13.0 ± 1.3 ijk	7.8 ± 0.6 jk
Dill Seed	29.3 ± 2.7 efghi	30.0 ± 2.3 fghi
Coriander	49.6 ± 1.3 cde	53.7 ± 3.2 cde
Caraway	60.7 ± 1.3 bc	100 a
Fennel	55.6 ± 1.7 bcd	71.1 ± 1.9 bc
Chuanqiong	35.9 ± 2.1 defg	54.4 ± 1.3 cd
Verbena	**100 a**	**100 a**
Ginger	3.7 ± 1.3 jk	34.8 ± 2.9 efg

400 μg/ml was treated; Means within a column followed by the same letters are not significantly different ($P < 0.05$, Duncan's test).

The antifungal activity of 4 EOs against two wood-decay fungi was given in Table 3. The IC_{50} values of litsea, verbena, palmarosa, and melissa on *T. hirsuta* were 72.3, 79.8, 154.1, and 156.3 μg/ml, respectively. In addition, their IC_{50} values of against *L. sulphureus* were 40.2, 59.0, 142.0, and 143.2 μg/ml (Table 3), respectively.

Table 3
IC_{50} values (μg/ml) of the essential oils and major component against wood-rot fungus *T. hirsuta* and *L. sulphureus*

Essential oils	*T. hirsuta*		*L. sulphureus*	
	IC_{50} (CI_{95})[a]	χ^{2b}	IC_{50} (CI_{95})	χ^2
litsea	72.3 (60.1–87.3)	3.549	40.2 (28.7–51.6)	2.914
melissa	156.3 (127.3-196.5)	9.953	143.2 (116.8-178.7)	6.856
palmarosa	154.1 (126.0-192.5)	8.704	142.0 (117.2-174.2)	5.597
verbena	79.8 (67.1–95.3)	3.471	59.0 (46.5–73.6)	5.656
geranial	56.6 (44.8–70.0)	3.954	33.3 (21.5–44.0)	2.554
geraniol	99.2 (77.4-128.2)	5.728	64.0 (50.4–80.0)	8.840
neral	66.3 (53.6–81.6)	5.077	40.6 (29.0-52.1)	2.885
citral	57.7 (45.3–71.9)	5.062	40.0 (28.6–51.2)	3.013

[a] Value in μg/ml and CI_{95}-95% confidence intervals, compounds activity is considered significantly different when the 95% CI fail to overlap.

[b] Pearson χ^2statistic with P values indicating goodness-of-fit for data to the expected probit response mode

3.2 Chemical compositions of the EOs

The chemical compositions of litsea, melissa, palmarosa, and verbena EOs were shown in Table 4. The major constituents of litsea oil were geranial (32.12%), neral (29.43%), limonene (16.99%), linalool (2.18%), and myrcene (2.17%). The main components in melissa oil were geraniol (31.0%), followed by citronellal (20.84%), citronellol (12.87%), elemol (6.22%), and β-elemene (3.99%). Geraniol (77.42%) was the most abundant, followed by geranyl acetate (12.29%), linalool (3.36%), caryophyllene (2.07%), and nerol (2.02%), in palmarosa oil. The rich component in verbena oil was citral (42.57%), followed by neral (37.32%), geranyl acetate (3.54%), geraniol (3.41%), and linalool (1.37%).

Table 4
Chemical composition of the 4 essential oils assayed for fungicidal activity

No	Components	RI[a]	LIT RI[b]	litsea	melissa	palmarosa	verbena
1	α-Pinene	939	932	1.97	3.78	0.59	1.10
2	Camphene	954	946	0.47	1.11	3.36	0.22
3	β-Pinene	979	974	1.35	1.09	2.02	0.96
4	Myrcene	991	988	2.17	20.84	**77.42**	1.37
5	3-Carene	1013	1008	16.99	12.87	0.58	0.43
6	Limonene	1027	1024	0.56	0.33	1.23	0.55
7	β-Phellandrene	1028	1025	2.18	**31.00**	12.29	0.52
8	1,8-Cineole	1034	1026	1.74	3.46	0.44	**37.32**
9	β-Ocimene	1038	1032	0.68	0.81	2.07	3.41
10	Linalool	1097	1095	1.03	3.88	100	**42.57**
11	Isopulegol	1141	1144	0.69	1.66	0.59	3.54
12	Citronellal	1154	1148	1.58	3.99	97.34	0.13
13	Terpinen-4-ol	1177	1174	29.43	1.03	2.07	0.77
14	α-Terpineol	1191	1186	0.90	0.50		0.10
15	Nerol	1228	1227	0.20	2.28		0.18
16	Citronellol	1233	1223	**32.12**	1.32		0.29
17	Pulegone	1235	1233	0.25	3.84		93.46
18	Neral	1240	1235	0.23	6.22		1.32
19	Geraniol	1250	1249	1.11	100		90.8
20	Anethole	1254	1254	0.11	3.78		1.05
21	Geranial	1272	1264	0.25	77.05		0.29
22	Citronellyl formate	1277	1271	96.01	12.96		
23	Citral	1316	1302	23.51	6.22		
24	α-Cubebene	1345	1345	70.55			
25	Geranyl acetate	1352	1350	1.70			

[a] RI, linear retention indices on HP-5MS column, experimentally determined using homologue series of n-alkanes.

[b] Relative retention indices taken from Adams.

No	Components	RI[a]	LIT RI[b]	litsea	melissa	palmarosa	verbena
26	Eugenol	1356	1355				
27	Neryl acetate	1365	1359				
28	β-Elemene	1391	1389				
29	Caryophyllene	1419	1417				
30	α-Humulene	1455	1452				
31	α-Amorphene	1473	1483				
32	γ-Muurolene	1480	1476				
33	Germacrene D	1485	1484				
34	α-Muurolene	1500	1500				
35	δ-Cadinene	1523	1522				
36	Elemol	1549	1548				
37	Caryophyllene oxide	1587	1582				
	Total identified (%)						
	Monoterpene hydrocarbons						
	Oxygenated monoterpenes						
	Sesquiterpene hydrocarbons						
	Oxygenated sesquiterpenes						

[a] RI, linear retention indices on HP-5MS column, experimentally determined using homologue series of n-alkanes.

[b] Relative retention indices taken from Adams.

3.3 Antifungal activity of the major constituents

To further research the relationship between EOs and it major constituents and antifungal activity, geranial, geraniol, neral, and citral, which were the major constituents of the litsea, melissa, palmarosa, and verbena EO, respectively, were selected for this study. Figure 2 showed geranial, geraniol, neral, and citral completely inhibited *T. hirsuta* growth at 400 μg/ml. When the concentration was decreased to 200 μg/ml, geranial, neral, and citral caused complete inhibition (Fig. 2). When the concentration of the constituent was 200 μg/ml, geranial, geraniol, neral, and citral completely inhibited the growth of *L. sulphureus* (Fig. 2).

As shown in Table 3, geranial and citral exhibited the best antifungal activities against *T. hirsuta*, with IC_{50} values of 56.6 and 57.7 μg/ml, respectively. In addition, the IC_{50} values of geranial, citral, neral, and

geraniol against *L. sulphureus* were 33.3, 40.0, 40.6, and 64.0 µg/ml, respectively (Table 3).

3.4 Membrane integrity of *T. hirsuta* and *L. sulphureus* exposed to 4 EOs and their major constituents

PI can detect the membrane permeability, which enters the damaged plasma membranes and combines with nucleic acids to produce red fluorescence. PI staining was used to determine whether four EOs (litsea, melissa, palmarosa, and verbena) and their major constituents (geranial, geraniol, neral, and citral, respectively) led to damage of membrane permeability in *T. hirsuta* and *L. sulphureus*. Four EOs and their major constituents were used to treat mycelium and stained by PI, however, the control mycelium were not stained (Fig. 3A, B), indicating a considerable destruction in membrane integrity.

To confirm that the four tested EOs and their major constituents could disrupt the cell membrane integrity of *T. hirsuta* and *L. sulphureus*, the leakage of intracellular content was determined. The results are represented in Fig. 4, after being treated with the four EOs and major constituents (1 µl/ml) for 24 h, the nucleotides, proteins, and soluble sugars in suspensions of *T. hirsuta* and *L. sulphureus* were released in a superior significantly quantity than that in the control mycelia. Exposure to litsea, melissa, palmarosa, and verbena EOs, the release of nucleotides, proteins, and soluble sugars significantly increased (Fig. 4A-C), with litsea exhibiting the strongest impact. Similar results were obtained for the major constituents, geranial, geraniol, neral, and citral for the leakage of cellular components (Fig. 4A-C), with geranial exhibiting the strongest impact. In addition, the four EOs and their major constituents caused leakage of nucleotides, proteins, and soluble sugars in *L. sulphureus* than *T. hirsuta*.

4. Discussion

It is generally known that the fungicidal, insecticidal, and nematocidal activities of plant EOs can be attributed to various compounds, notably alcohols, aldehydes, phenol, terpenes, and terpenoids (Boulogne et al. 2012; Tak and Isman 2017; Benelli et al. 2018; Liu et al. 2019; Gong and Ren 2020). In previous studies, the chemical analysis of litsea, melissa, palmarosa, and verbena has been reported (Seo et al. 2009; De Martino et al. 2011; Si et al. 2012; Kakaraparthi et al. 2015; Rehman et al. 2017; Khalili et al. 2018; Kittler et al. 2018a,b; Pouyanfar et al. 2018). Comparisons between previous results revealed differences in the ratios of major and minor constituents. Xie et al. (2012) and Kakaraparthi et al. (2015) found that the chemical composition of EOs differ widely with genotype, cultivation and production conditions, environment factors, and extraction methods.

In this study, litsea, melissa, palmarosa, and verbena EOs had excellent antifungal activity against wood-rotting fungi, which had not been reported previously. In our previous study we had showed that, *C. citratus*, *C. zeylanicum*, and *O. vulgare* EOs had good antifungal activity against *T. hirsuta* (IC_{50} = 79.1–96.9 µg/ml) and *L. sulphureus* (IC_{50} = 36.9–69.2 µg/ml) (Xie et al. 2017a). Similarly, *Cryptomeria japonica* heartwood EO (IC_{50}, 39 µg/ml) and *C. japonica* sapwood (IC_{50}, 94 µg/ml) exhibited to have far more strong antifungal effect against *L. sulphureus* (Cheng et al. 2005). In another investigation, Wang et al. (2005) and Cheng et al. (2006) demonstrated that the growth was completely inhibited by 200 µg/ml

C. osmophloeum EO against *L. sulphureus*. On the other hand, the extract of *C. konishii* (IC_{50}, 62 μg/ml) showed excellent antifungal activity (Cheng et al. 2012). These results demonstrated that litsea, melissa, palmarosa, and verbena EOs have excellent antifungal activity.

To evaluate the relationship between the main constituents and antifungal activity, 4 major constituents of these 4 EOs were selected and tested their antifungal activity. In our study, geranial, geraniol, neral, and citral, which were the main constituents of litsea, melissa, palmarosa, and verbena EOs, respectively, exhibited excellent antifungal activity. Similar results showed that eugenol is the major agent responsible for the strong antifungal activity of clove oil (Cheng et al. 2008; Komala et al. 2012; Matan et al. 2014; Xie et al. 2017a). In addition, our previous studies showed that six EOs exhibited antifungal activity, which contributed to their major constituent (Xie et al. 2017a). Similarly, in previous studies, *Carum capticum* oil exhibited strong toxicity contributed by thymol (Singh et al. 2004; Park et al. 2007). These results demonstrate that EOs have excellent antifungal activity contributed by their major constituents.

The structure-activity relationships (SARs) of EO monoterpenoids against fungi have been well studied (Zhang et al. 2016a; Xie et al. 2017a, b). The SAR of monoterpenoids and antifungal activity against decay fungi was investigated by Xie et al. (2017a). They found that aldehyde compounds (cinnamaldehyde and citral) generally had more antifungal activity to wood-decay fungi than alcohols (citronellol). In our study, aldehyde compounds (neral, citral, and geranial) exhibited stronger antifungal activity than the alcohol compound (geraniol). Similarly, Zhang et al. (2016a) reported that the aldehyde compound (citral) demonstrated higher activity than alcohol compounds (β-citronellol, geraniol, and 3, 7-dimethyl-1-octanol) against wood-decay fungi. These results demonstrated that α, β-unsaturated carbonyl compounds (neral, citral, and geranial) had a stronger active antifungal effect. Moreover, previous studies reported that aldehydes exhibited the strongest antitermitic activity (Xie et al. 2014). In another investigation, α, β-unsaturated carbonyl compounds were important in insecticidal, fungicidal, and nematocidal activities (Kim et al. 2008; Lee et al. 2008; Seo et al. 2009). This might indicate that the double bond at the α, β position in carbonyl compounds enhances insecticidal, fungicidal, and nematocidal activities.

In this study, the litsea, melissa, palmarosa, and verbena EOs and their major constituents (geranial, geraniol, neral, and citral) had excellent antifungal activity against the two tested fungi. In addition, some researchers have found that these oils and their major constituents have excellent termiticidal properties. Seo et al. (2009) reported that litsea EO had fumigant antitermitic activity against the Japanese termite (*Reticulitermes speratus*). Similarly, the EO from palmarosa exhibited good antitermitic activity against *Nasutitermes corniger* (Lima et al. 2013). In our previous study, citral and geraniol exhibited excellent antitermitic activity (Xie et al. 2014). Similarly, geranial, geraniol, and neral also have been demonstrated excellent termiticidal activity (Seo et al. 2009). Therefore, litsea, melissa, palmarosa, and verbena EOs and their major constituents, geranial, geraniol, neral, and citral, respectively, have promising potential as eco-friendly preservatives.

Cell membrane permeability is critical to the survival of fungal cells, where the damage of membrane could lead to the outflow of intracellular constituents to result in their death (Zhou et al. 2017; Souza et al. 2020; Yan et al. 2020). In this study, the effects of litsea, melissa, palmarosa, and verbena EOs on the integrity of mycelium membranes of *T. hirsuta* and *L. sulphureus* were observed by confocal microscopy. The PI staining results demonstrated that litsea, melissa, palmarosa, and verbena EOs disrupted the membrane integrity, with litsea having the highest effect. Similar results have been previously reported for *Fusarium solani* conidia treated with *Aniba canelilla* and *Aniba parviflora* EOs, indicating damage to the conidia's cytoplasmic membrane (Souza et al. 2020). Yan et al. (2020) also demonstrated that *Mentha spicata*, *M. piperita*, and *Thymus vulgaris* (CT carvacrol and CT thymol) EOs inhibited *Rhizopus stolonifer* growth by destroying the permeability of the cell membrane. In general, EO had an effective antifungal activity attributed to its major constituent (Xie et al. 2017a). This study found that geranial, geraniol, neral, and citral disrupted the cell membrane integrity of *T. hirsuta* and *L. sulphureus* hyphae. Similarly, Tian et al. (2015), Yun and Lee (2017), and Li et al. (2018) reported that ethyl p-coumarate, perillaldehyde, and silymarin had high antifungal activity, which can be attributed to their destruction of the permeability of the fungal plasma membrane. Kalily et al. (2016) demonstrated that linaool destroyed the permeability of the cell membrane, resulting in leakage of intracellular components and cell death. Zhang et al. (2016b) and Zhou et al. (2017) also reported that carvacrol, cinnamaldehyde, and eugenol could disrupt the plasma membrane of *Escherichia coli* and *R. stolonifera*, inducing the intracellular contents leakage.

In this study, litsea, melissa, palmarosa, and verbena EOs and their major constituents, geranial, geraniol neral, and citral, respectively, resulted in the leakage of cytoplasmic contents of nuclei acids, proteins, and sugars increased significantly, indicating the breakdown of plasma membrane structures and function. This is consistent with PI staining results. Similarly, Souza et al. (2020) and Yan et al. (2020) showed that *A. canelilla*, *A. parviflora*, and *T. vulgaris* EOs damaged the plasma membrane of *R. stolonifer* and *F. solani* and resulted in the leakage of intercellular electrolyte. Zhou et al. (2017) also demonstrated that carvacrol and eugenol could result in the damage of membrane permeability, causing the outflow of cytoplasm, nucleic acid, and protein content of *R. stolonifer*.

5. Conclusion

The present work reported the antifungal properties and mechanism of the essential oils from Litsea (*L. cubeba*), Melissa (*M. officinalis*), Palmarosa (*C. martini*) and Verbena (*V. officinalis*) and their major active constituents. The 4 EOs and their major active constituents significantly inhibited mycelial growth of *T. hirsuta* and *L. sulphureus* through disrupting plasma membrane integrity, and resulting in leakage of nucleic acid, protein, and soluble sugar. The essential oils of litsea, melissa, palmarosa and verbena and their major compounds have potential as environmental-friendly fungicides.

Declarations

Ethical approval

Not applicable.

Consent to participate

Not applicable.

Consent to publish

All authors whose names appear on the submission approved the version to be published and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Authors Contributions

Yongjian Xie, Xi Yang, Hui Han, Zhilin Zhang and Dayu Zhang carried out the experimental stages, manuscript preparation, and statistical analysis.

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Competing interests

The authors declare no competing interests.

Availability of data and materials

Not applicable.

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Figures

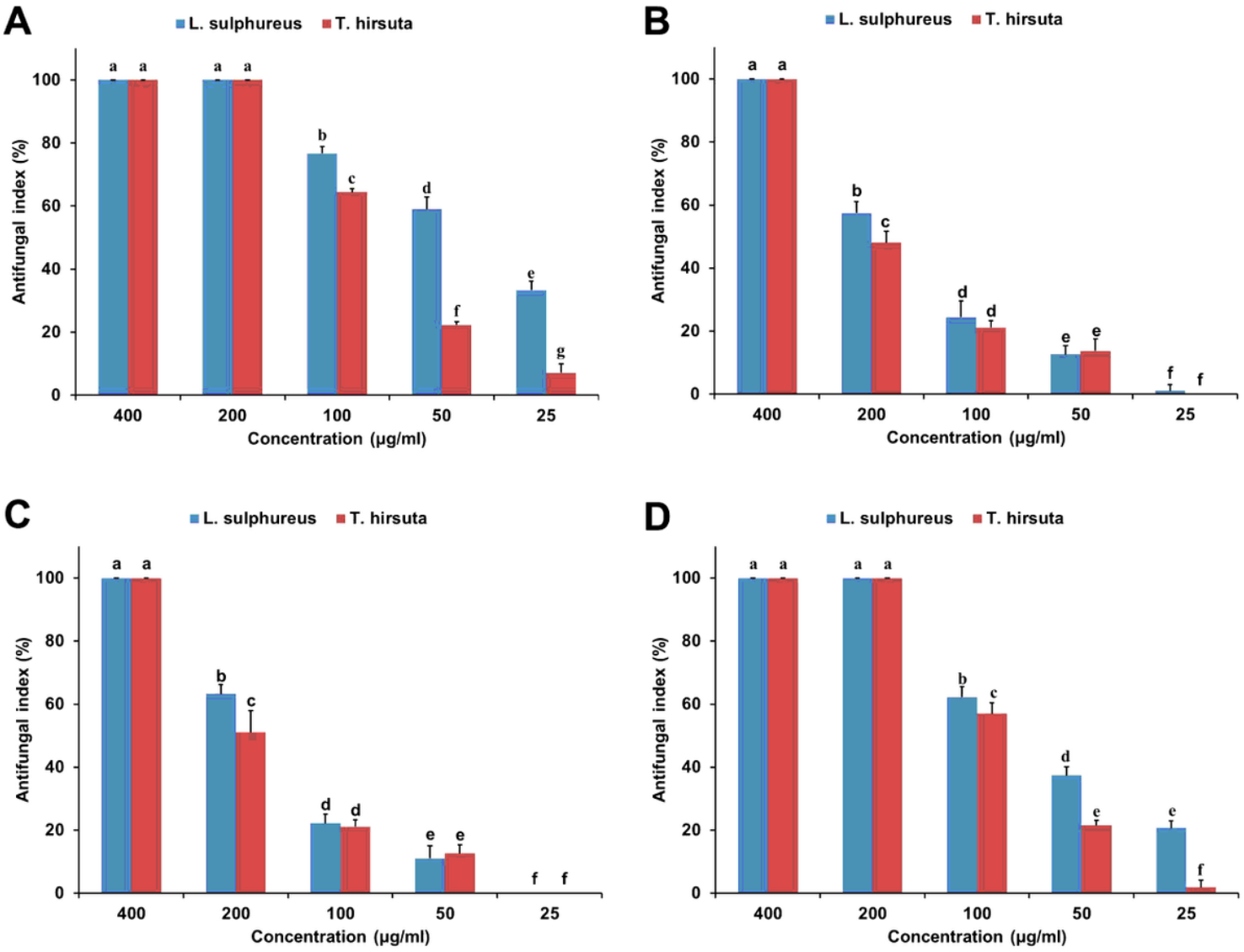

Figure 1

Antifungal activity of the selected essential oils (A: litsea; B: melissa; C: palmarosa; D: verbena) against wood-rot fungus T. hirsuta and L. sulphureus. Each experiment was performed × 3 and the data averaged (n = 3). Numbers followed by different letters (a-g) are significantly different at level of $P < 0.05$ according to Duncan's test.

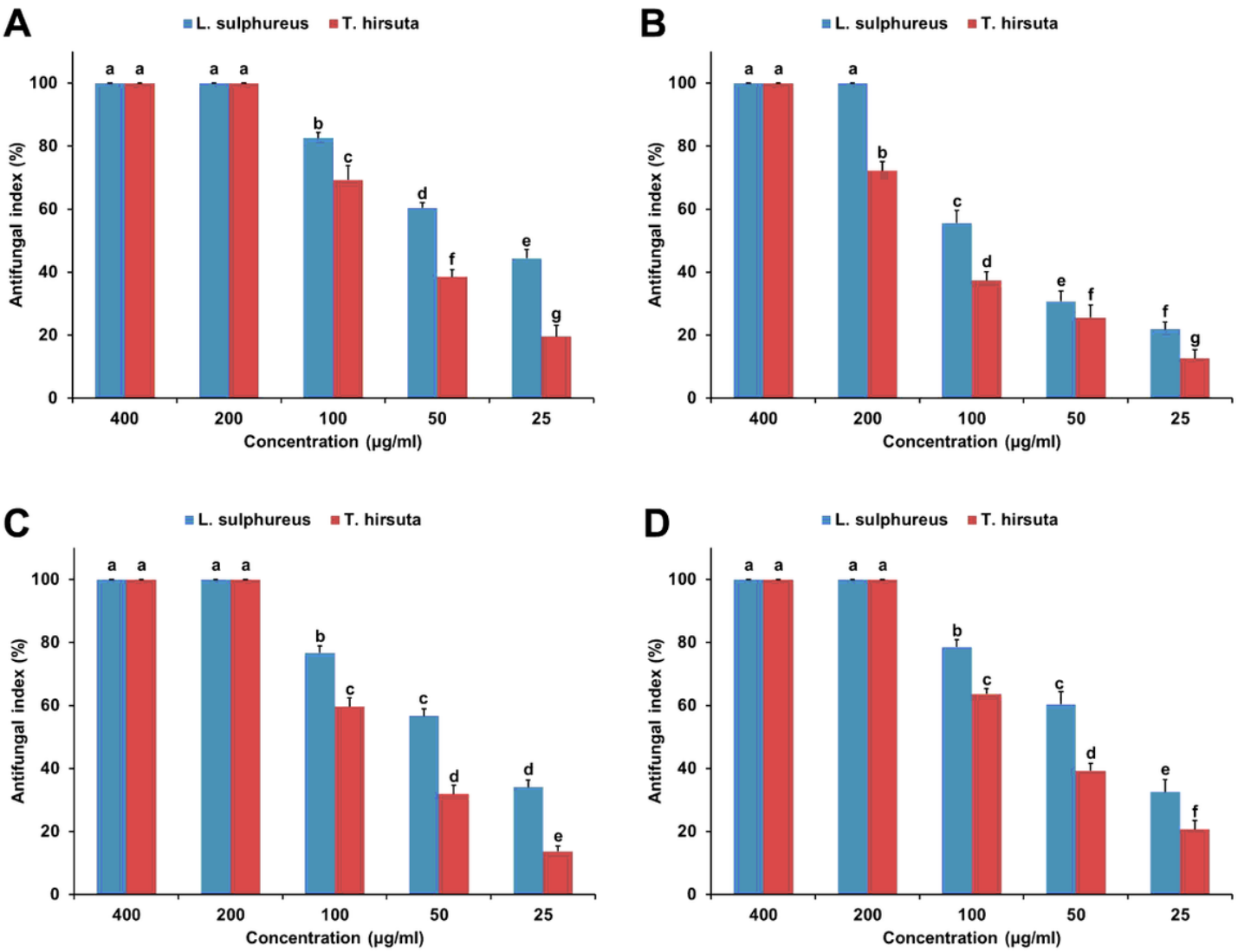

Figure 2

Antifungal activity of major component of the selected essential oils (A: geranial; B: geraniol; C: neral; D: citral) against wood-rot fungus T. hirsuta and L. sulphureus. Each experiment was performed × 3 and the data averaged (n = 3). Numbers followed by different letters (a-g) are significantly different at level of $P < 0.05$ according to Duncan's test.

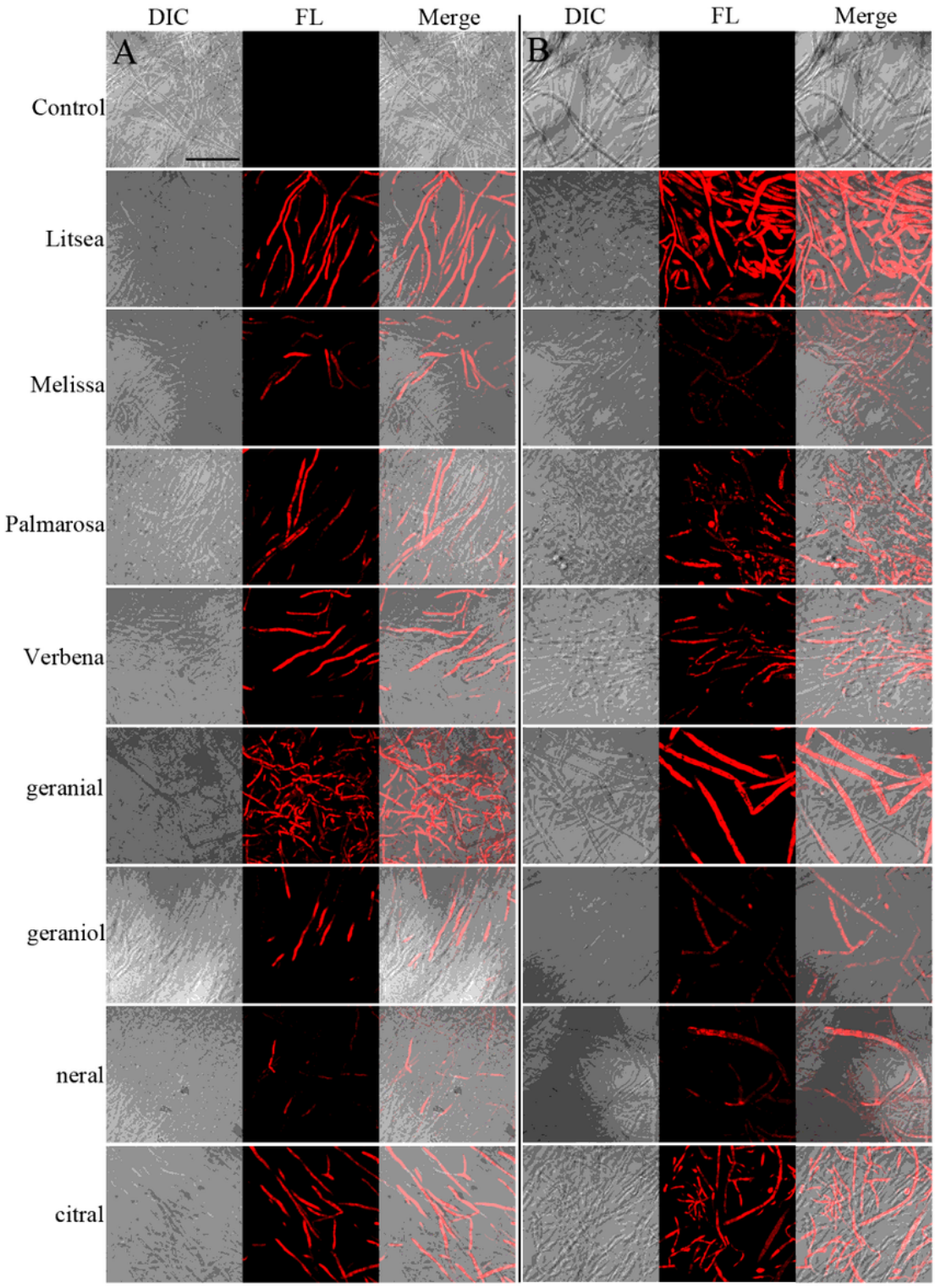

Figure 3

Confocal laser scanning microscopy images of T. hirsuta (A) and L. sulphureus (B) mycelium membrane integrity exposed to 4 essential oils and their major components at 1 μl/ml. DIC: differential interference contrast images without fluorescence. FL: red fluorescence images with propidiumiodide (PI) combined with nucleic acid. Bar = 50.0 μm.

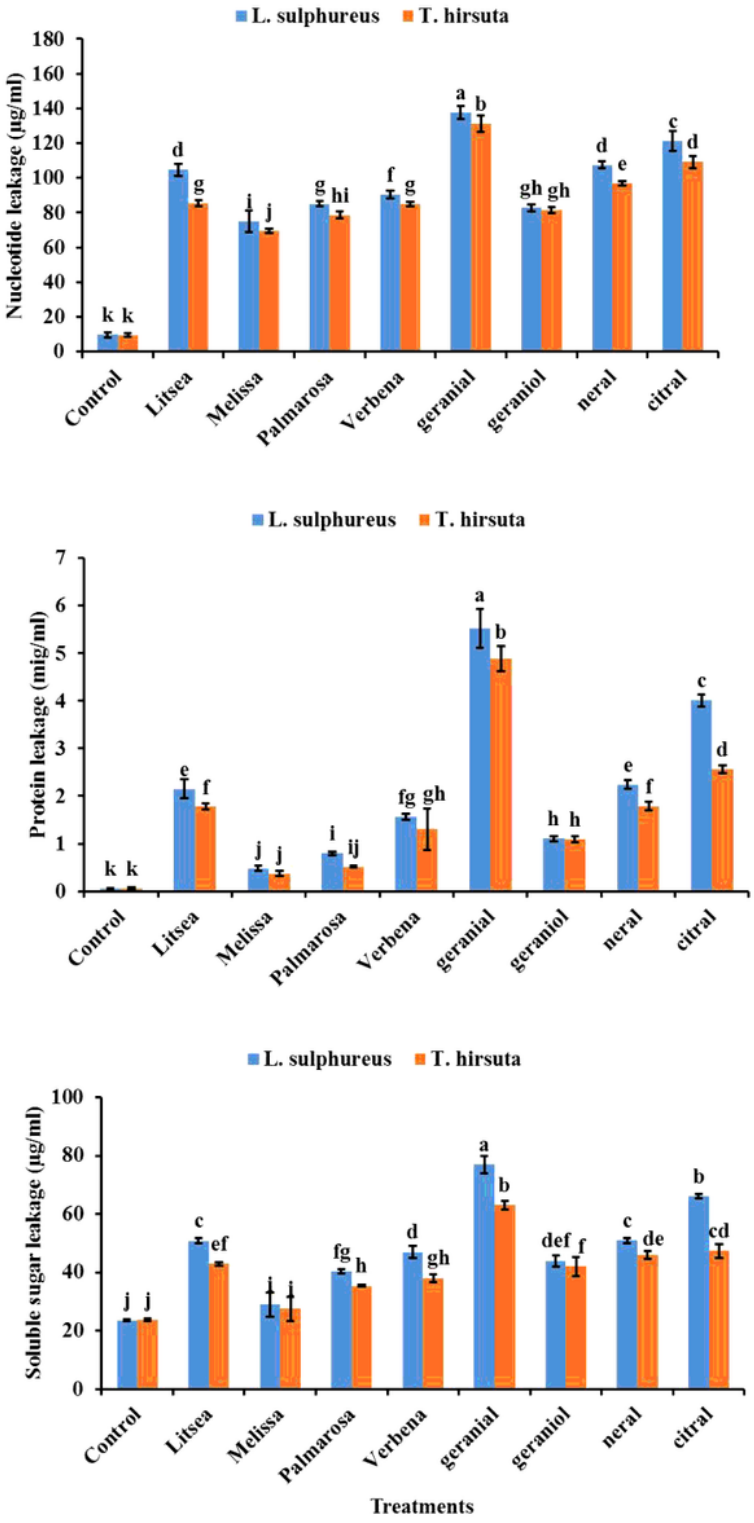

Figure 4

Effects of 4 essential oils and their major components at 1 μl/ml on leakage of nucleic acid (A), protein (B), and soluble sugar (C) of T. hirsuta and L. sulphureus mycelium. Each value is the mean for three replicates, and means with different letters are significantly different based on Duncan's ($P < 0.05$).