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Antifungal activity of *Gallesia integrifolia* fruit essential oil

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ABSTRACT

Gallesia integrifolia (Phytolaccaceae) is native to Brazil and has a strong alliaceous odor. The objective of this study was to identify the chemical composition of *G. integrifolia* fruit essential oil and evaluate fungicidal activity against the main food-borne diseases and food spoilage fungi. The essential oil was extracted by hydrodistillation and identified by GC–MS. From 35 identified compounds, 68% belonged to the organosulfur class. The major compounds were dimethyl trisulfide (15.49%), 2,8-dithianonane (52.63%) and lenthionine (14.69%). The utilized fungi were *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus ochraceus*, *Aspergillus versicolor*, *Penicillium funiculosum*, *Penicillium ochrochloron*, *Penicillium verrucosum* var. *cyclopium*, and *Trichoderma viride*. Minimal fungicidal concentration for the essential oil varied from 0.02 to 0.18 mg/mL and bifonazole and ketoconazole controls ranged from 0.20 to 3.50 mg/mL. The lower concentration of the essential oil was able to control *P. ochrochloron*, *A. fumigatus*, *A. versicolor*, *A. ochraceus* and *T. viride*. This study shows a high fungicidal activity of *G. integrifolia* fruit essential oil and can support future applications by reducing the use of synthetic fungicides.

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Introduction

Gallesia integrifolia (Spreng.) Harms and its synonymies Crateva gorarema Vell., *Gallesia gorazema* (Vell. Conc.) Moquin, *Gallesia gorazema* (Vell.) Moq., *Gallesia ovata* O. C. Schmidt, *Gallesia scorododendrum* Casar., *Thouinia integrifolia* Spreng. and *G. integrifolia* var. ovata (O.C. Schmidt) Nowicke belong to Phytolaccaceae family.[1] This tree is popularly known as pau d'alho (common name in Portuguese) and is native to Brazil, from the states of Ceará to Paraná.[2,3] In popular medicine, the bark of this species is utilized to prepare teas for the flu, coughing, pneumonia, vermin, gonorrhea, prostate tumors and rheumatism.[4] However, despite the ethnopharmacological use, there are no studies on the chemical composition and antifungal activity of *G. integrifolia* fruit essential oil.

Microorganisms such as the genera *Aspergillus*, *Fusarium*, and *Penicillium* are responsible for food poisoning and food-borne infections that can also deteriorate foods and increase the cost of agricultural production, and health care.[5,6] In addition, the genera *Trichoderma*, *Aspergillus* and *Penicillium*, known as green molds, occur on mushroom production when the composting is not correctly prepared and/or does not become selective enough.[7]

There are still few studies on fungal resistance to chemical products, but Arendrup[8] describes that the global prevalence of azole resistance in *Aspergillus* is estimated to be around 3–6%. In addition, the resistance in *Aspergillus* spp seems to be related to the use of agricultural azoles for crop protection.[9,10] Besides the resistance of these microorganisms, the indiscriminate use of fungicides in the production of foods can damage human and animal health.[11,12] These chemical compounds can be toxic and their residues can have carcinogenic and teratogenic side effects.[13] Thus, the search for new antimicrobial molecules are of interest for public health as well as for the maintenance and broadening of food product and an alternative to reduce microbial resistance.[14–17]

Therefore, the present study aimed to evaluate the chemical composition and the fungicidal activity of *G. integrifolia* fruit essential oil against the main food-borne diseases and food spoilage fungi.

Materials and methods

Essential oil

Fresh fruits of *G. integrifolia* were collected in the month of June, 2015 in the morning, at the coordinates of S23°46′16″ and WO53°19′38″ and altitude of 442 m. The fruit essential oil was obtained by hydrodistillation technique in a modified Clevenger equipment for 2 h and stored at −20 °C.

Chemical identification

Chemical identification of the essential oil occurred by using a gas chromatographer coupled to a mass spectrometer (GC–MS; Agilent 19091J-433). An HP-5MS UI 5% analytical column (30 m × 0.25 mm × 0.25 μm) was utilized, with an initial temperature of 60 °C, and kept for 3 min; then, a ramp of 5 °C/min and the temperature was increased to 300 °C and kept for 10 min and, finally, to 310 °C with a ramp of 10 °C/min for 10 min. Helium was utilized as the carrier gas at the linear speed of 1 mL/min until 300 °C and pressure release of 56 kPa. The injector temperature was 300 °C; the injection volume was 2 μL; the injection was in split mode (20:1). The transfer line was kept at 285 °C and the ionization source and quadrupole at 230 °C and 150 °C, respectively. The EM detection system was utilized in "scan" mode, in the range of mass/load ratio (*m*/*z*) of 40–550 with 3-min solvent delay. The compounds were identified by comparing their mass spectra with the ones from NIST 11.0 libraries, and comparing their retention indices (RI) obtained by a homologous series of n-alkane standards (C7–C28).[18]

Antifungal activity

For the antifungal bioassays, eight fungi were used: *Aspergillus fumigatus* Fresenius (ATCC 1022), *Aspergillus niger* van Tieghem (ATCC 6275), *Aspergillus ochraceus* Wilhelm (ATCC 12066), *Aspergillus versicolor* (Vuillemin) Tiraboschi (ATCC 11730), *Penicillium funiculosum* Thom (ATCC 8725), *Penicillium ochrochloron* Biourge (ATCC 9112), *Penicillium verrucosum* var. *cyclopium* (Westling) Samson, Stolk & Hadlok (food isolate), and *Trichoderma viride* Pers. (IAM 5061). Microorganisms were obtained from the Mycological Laboratory, Institute for Biological Research 'Siniša Stanković', University of Belgrade, Serbia. Fungi were kept on malt extract agar (20 g/L) and the cultures stored at 4 °C and subcultured once a month.[19] In order to investigate the antifungal activity of the compounds, a modified microdilution technique was used.[20,21] The fungal spores were washed from the surface of agar plates with a sterile 0.85% saline solution containing 0.10% polysorbate-80 (v/v). The spore suspension was adjusted with sterile saline solution to a concentration of 1×10^5 in a final volume of 100 μL per well. The inocula were stored at 4 °C for further use. Dilutions of inocula were culture on solid malt agar to verify the absence of contamination and to check the validity of each inoculum. Minimum inhibitory concentration (MIC) determinations were performed by a serial dilution technique using 96-well microtiter plates. The investigated compounds were dissolved in 5% dimethyl sulfoxide (DMSO) solution containing 0.1% polysorbate-80 (v/v) (1 mg/mL) and added in broth malt extract medium with inoculum. The microplates were incubated in a rotary shaker (160 rpm) for 72 h at 28 °C. The lowest concentrations without visible growth under the microscope light were defined as MIC. The minimum fungicidal concentration (MFC) was determined by serial subcultivation of 2 μL of tested compounds dissolved in culture medium and inoculated for 72 h onto microtiter plates containing 100 μL broth per well and with further incubation for 72 h at 28 °C. The lowest concentration with no visible growth was defined as MFC indicating 99.5% killing of the original inoculum. A solution of 5% DMSO was used as a negative control. Commercial fungicides bifonazole (Srbolek, Belgrade, Serbia) and ketoconazole (Zorkapharma, Šabac, Serbia) were used as positive controls (1–3500 μg/mL). All experiments were performed in duplicate and repeated three times.

Statistical analysis

All the tests were carried out in triplicate. The results were expressed as arithmetic mean values ± standard deviation, and analyzed by one-way analysis of variance (ANOVA) followed by Tukey HSD (honest significant difference) test with $\alpha = 0.05$ to determine whether there is a statistically significant difference among the results. The analysis was carried out by Statistical Package for the Social Sciences (SPSS) version 18.0.

Results

Chemical identification

Essential oil chemical compounds, 34 out of 35, obtained by GC–MS, were identified (Table 1). The major compounds were dimethyl trisulfide (15.49%), 2,8-dithianonane (52.63%) and lenthionine (14.69%). The mass spectra obtained for these major compounds are shown in Figs. 1–3, respectively.

Antifungal activity

The fungistatic activity (MIC) for the essential oil ranged from 0.01 to 0.09 mg/mL, and positive bifonazole and ketoconazole controls varied from 0.10 to 2.50 mg/mL (Fig. 4). MIC values for the essential oil were all lower ($p \leq 0.05$) than the positive controls (Fig. 4). The fungicidal activity (MFC) for the essential oil varied from 0.02 to 0.18 mg/mL and bifonazole and ketoconazole controls ranged from 0.20 to 3.50 mg/mL (Fig. 5). MFC values for the essential oil were all lower ($p \leq 0.05$) than the positive controls (Fig. 5). In general, the essential oil concentration was from 1.4 to 10.0 times lower than bifonazole and from 2.8 to 175 times lower than ketoconazole, both with fungicidal effect (Figs. 4 and 5). Specifically, *P. ochrochloron* needs a concentration of the controls bifonazole or ketoconazole 12.5 or 175 times, respectively, higher than the essential oil to obtain the same fungicidal activity. The essential oil concentration was from 22 to 25 times lower than ketoconazole control against *A. fumigatus*, *A. versicolor*, *A. ochraceus* and *T. viride* (Fig. 4). These results make evident that the essential oil of *G. integrifolia* fruits have excellent performance in fungistatic and fungicidal control of several fungi.

Table 1 – Chemical composition of *Gallesia integrifolia* fruit essential oil.

Peak	[c]Compounds	[a]RI_{cal}	Area (%)	IM
1	Disulfide dimethyl	808	0.89	a,b,c
2	2,4-Dithiapentane	892	0.04	a,b,c
3	Camphene	938	t	a,b,c
4	Myrcene	938	t	a,b,c
5	2-Carene	939	t	a,b,c
6	α-Terpinene	939	t	a,b,c
7	Limonene	939	t	a,b,c
8	Methyl (methylsulfinyl)methyl sulfide (FAMSO)	974	0.84	a,b,c
9	1,2,4-Trithiolane	1094	0.11	a,b,c
10	Dimethyl trisulfide	1136	15.49	a,b,c
11	2,3,5-Trithiahexane	1174	0.28	a,b,c
12	Butane,1,4-bis(methylthio)	1202	0.10	a,b,c
13	Trithiomethoxymethane	1219	0.15	a,b,c
14	Thiophene,2-[(methylthio)ethynyl]	1263	0.35	a,b,c
15	1,2,4,5-Tetrathiane	1367	5.66	a,b,c
16	α-Ionone	1432	t	a,b,c
17	Dimehtyl tetrasulfide	1479	0.14	a,b,c
18	β-Ionone	1492	t	a,b,c
19	5,6-Dihydro-2,4,6-trimethyl-4H-1,3,5-dithiazine	1506	0.66	a,b,c
20	2,8-Dithianonane	1540	52.63	a,b,c
21	1-Oxa-4,7-dithiononane	1559	0.61	a,b,c
22	Trimethylsilyl methansulfonate	1618	0.08	a,b,c
23	3,5-Dithiahexanol-5,5-dioxide	1634	0.10	a,b,c
24	2,3,5,6-tetrathiaheptane	1718	0.12	a,b,c
25	L-Methionine, ethyl ester	1761	0.10	a,b,c
26	Disulfide, bis(2-sulfhydryl ethyl)	1780	0.14	a,b,c
27	Lenthionine	1780	14.69	a,b,c
28	Ethanol, 2-octhylthio	1792	0.11	a,b,c
29	n.i.	1797	0.09	a,b,c
30	Hexathiepane	1916	5.53	a,b,c
31	N-Ethyl-1,3-dithioisoindole	2027	0.10	a,b,c
32	Phytol	2121	t	a,b,c
33	5-Methyl-2-phenylindole	2176	0.56	a,b,c
34	Propane,1,1′-thiobis[3-(methylthio)]	2194	0.18	a,b,c
35	11,13-Dihydroxy-tetradec-5-ynoic acid, methyl ester	2325	0.23	a,b,c
	Total identified compounds		99.98	

[a] RI_{cal} = identification based on retention index (RI) using a homologous series of n-alkanes C7–C28 in an Agilent HP-5MS UI column.
[b] identification based on the comparison of mass spectra using Nist 11.0 libraries.
[c] Compounds listed in order of elution in HP-5MS UI column; n.i., non-identified compounds; t, traces. IM = Methods of Identification.

Discussion

G. integrifolia fruit essential oil presented fungicidal activity in much lower concentrations than bifonazole and ketoconazole controls. The fungicidal activity can be related to compounds found in the essential oil. Out of 35 identified compounds, 68% belong to the organosulfur class which, according to Kyung and Lee[22] and Dewick,[23] is synthetized in vegetal tissues from sulfur amino acids such as methionine and cysteine. The presence of sulfur increases the fungicidal activity of the compounds that protect plants.[24–27] According to Avato et al.,[28] the antimicrobial potential of organosulfur is also related to the presence of disulfide links of these molecules.

Another factor that can affect the antimicrobial activity is molecule polarity. Yin and Cheng[29] reported that among lipophilic organosulfurs [diallyl sulfide (CH_2=$CHCH_2SCH_2CH$=CH_2) and diallyl disulfide (CH_2=$CHCH_2SSCH_2CH$=CH_2)], and hydrophilic organosulfurs ($CH_3CH_2SCH_2CH(NH_2)$ COOH) and n-acetylcysteine ($HSCH_2CH(NHCOCH_3)COOH$)], most antimicrobial activity was obtained for diallyl sulfide, a lipophilic organosulfur with disulfide links. Thus, the presence of sulfur compounds in

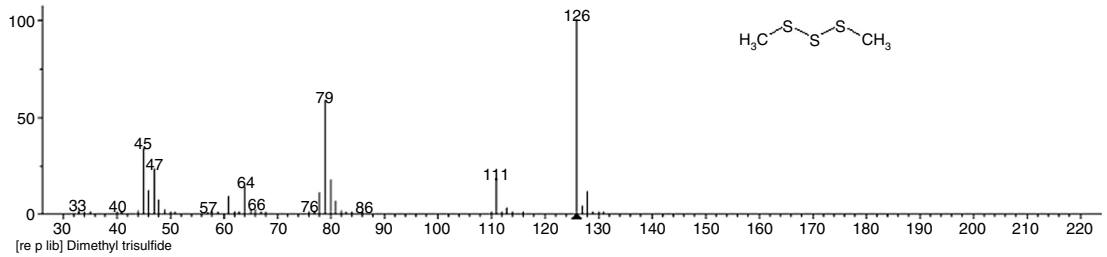

Fig. 1 – Mass spectrum of dimethyl trisulfide (*m/z* = 126) found in *Gallesia integrifolia* fruit essential oil obtained by GC–MS.

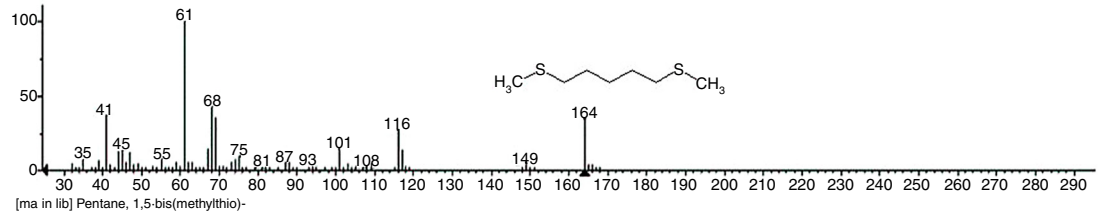

Fig. 2 – Mass spectrum of 2,8-dithianonane (*m/z* = 164) found in *Gallesia integrifolia* fruit essential oil obtained by GC–MS.

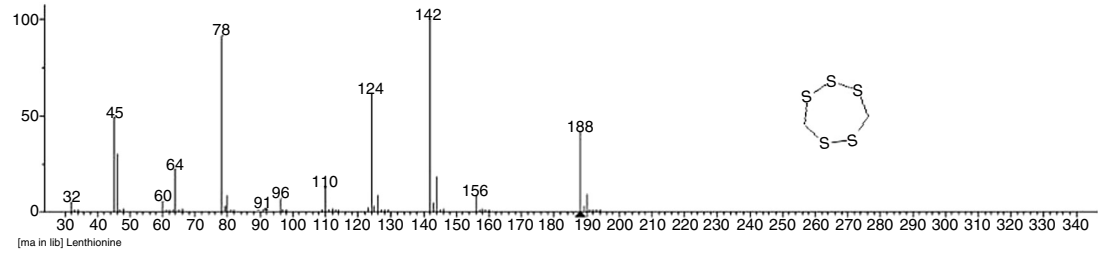

Fig. 3 – Mass spectrum of lenthionine (*m/z* = 188) found in *Gallesia integrifolia* fruit essential oil obtained by GC–MS.

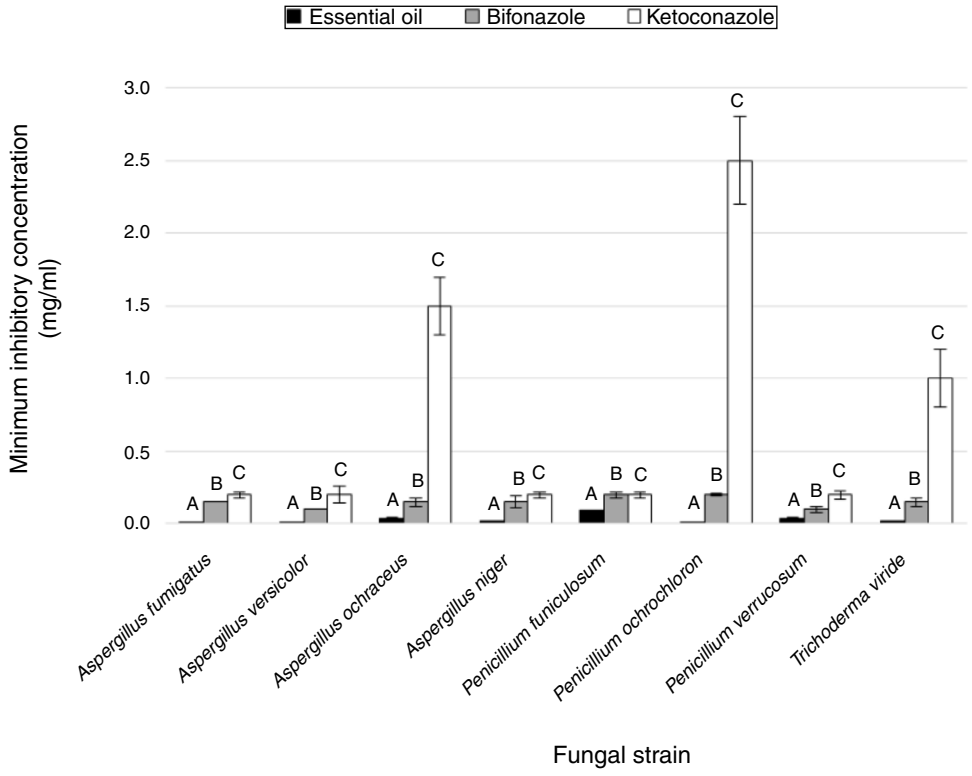

Fig. 4 – Minimum inhibitory concentration of *Gallesia integrifolia* fruit essential oil, bifonazole, and ketoconazole against fungal strains. Different letters above bars indicate statistically significant differences among treatments for each fungal strain according to Tukey test ($p \leq 0.05$).

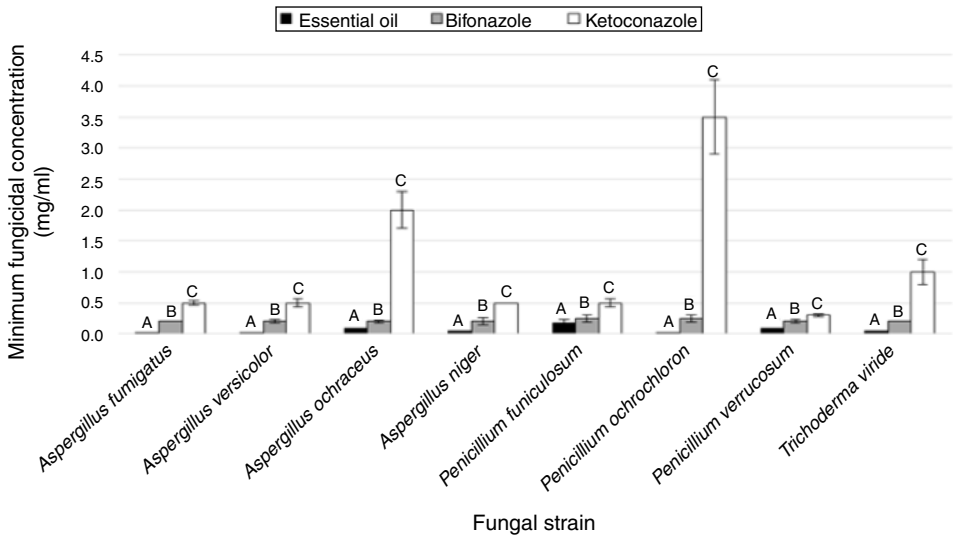

Fig. 5 – Minimum fungal concentration of *Gallesia integrifolia* fruit essential oil, bifonazole, and ketoconazole against fungal strains. Different letters above bars indicate statistically significant differences among treatments for each fungal strain according to Tukey test ($p \leq 0.05$).

essential oils with polysulfide bridges (–S_n–) can increase its apolarity and broaden its chemical affinity with the structure of microorganism cell wall and membrane consisting mainly of chitin and ergosterol.[30–33] This interaction with the membranes can promote membrane rupture and can unbalance microbial cell.[34] Li et al.[35] verified that cellular organelles such as vacuoles, mitochondria, and storage granules of *Candida albicans* were severely damaged after 2 h in 1.39 μg/mL garlic (*Allium sativum* L.) essential oil. For these authors, the results were consistent with the damages observed in *P. funiculosum* mycelia treated with garlic essential oil.[35] Dziri et al.[36] verified that garlic essential oil extracted by different methods consists of 84.3 to 98.9% of sulfuric compounds, and the major ones are diallyl trisulfide (37.3–45.9%), diallyl disulfide (17.5–35.6%) and methyl allyl trisulfide (7.7–10.4%). The polysulfur groups can also interact with amino acids and proteins acting as inhibitors of enzymatic reactions and protein synthesis. For Li et al.,[35] garlic essential oil changed the expression of a large number of genes in *C. albicans* after garlic oil treatment.

Another factor that may have affected antimicrobial activity of *G. integrifolia* essential oil can be related to the number of sulfur atoms found in the molecules. According to Kyung,[37] heterocyclic organosulfurs with 5 and 6 atoms of sulfur in the molecule were more effective than the microbial control when compared to heterocyclic ones with 4 sulfur atoms.

Therefore, the antifungal activity of *G. integrifolia* fruit essential oil is possibly due to the presence of organosulfur compounds. In addition, 29% of the fruit essential oil compounds presented disulfide links of lipophilic nature and/or heterocyclic chains. Among the major compounds, lenthionine (14.69%) presents heterocyclic chain with five sulfur atoms in its molecule, and it has already been isolated from the red algae *Chondria californica* and the edible mushroom shiitake (*Lentinula edodes*).[38] In studies done by Morita and Kobayashi,[39] the isolated compound lenthionine presented antimicrobial potential against several microorganisms such as the following fungi: *Glomerella cingulata* (MIC of 12.50 μg/mL), *Pyricularia oryzae* (MIC of 12.50 μg/mL), *C. albicans* (MIC of 6.25 μg/mL), *Trichophyton mentagrophytes* (MIC of 3.12 μg/mL), *Saccharomyces cerevisiae* (MIC of 6.25 μg/mL), *Cryptococcus neoformans* (MIC of 6.25 μg/mL) and *Trichophyton rubrum* (MIC of 3.12 μg/mL). Analyzing these results obtained for lenthionine in the reported study, we can consider that the presence of this organosulfur compound in the pau d'alho fruit essential oil influenced the antifungal activity of this study in which the obtained MIC values ranged from 10 to 90 μg/mL, representing a smaller concentration than the ones in the control (bifonazole and ketoconazole) (Fig. 4).

G. integrifolia fruit essential oil presented higher antifungal activity than the controls (bifonazole and ketoconazole) against all tested fungi. These microorganisms are related to several human diseases such as *A. fumigatus* which is the main etiologic agent of lung.[40] Several tested fungi promote agricultural losses, food deterioration, produce mycotoxins, and are found in several grains, and may cause damages during the storage with loss of food quality and germinating capacity.[41–43]

In the production of edible mushrooms, *T. viride* is one of the main worldwide contaminants causing economic losses and reducing the availability of this food.[14,44] In general, the fungal control occurs with synthetic fungicides that with time cause the development of resistance to pathogens, contaminate the environment and may cause carcinogenic effects.[45,46] An alternative to synthetic fungicides is the substitution for natural products that reduce the environmental contamination.[47,48] Geels et al.[7] reported that green molds such as *Trichoderma* genus can contaminate and cause mushroom production losses. Benomyl (Benlate®), among other fungicides, is broadly used in *Agaricus bisporus* mushroom cultivation[49,50] Prochloraz (Sporgon®) is another commonly used as fungicide in mushroom cultivation; however, it is suggested that Prochloraz and Benomyl may cause side effects.[45] Thus, *G. integrifolia* fruit essential oil can be an alternative to

control *T. viride* and other fungi that contaminate mushroom production.

In conclusion, the major compounds of *G. integrifolia* fruit essential oil are dimethyl trisulfide (15.49%), 2,8-dithianonane (52.63%) and lenthionine (14.69%). Fruit essential oil consists of 68% of organosulfur compounds mainly lenthionine, which is likely the responsible for its fungicidal activity. The essential oil has antifungal (fungistatic and fungicidal) activity against all evaluated fungi in much lower concentrations than the ones used in the controls (bifonazole and ketoconazole), mainly against *P. ochrochloron*, *A. fumigatus*, *A. versicolor*, *A. ochraceus* and *T. viride*. The essential oil from *G. integrifolia* fruit is a potential alternative to reduce the use of synthetic fungicides.

Conflicts of interest

The authors declare no conflicts of interest.

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