

CHEMICAL CHARACTERIZATION OF *Lippia alba* ESSENTIAL OIL: AN ALTERNATIVE TO CONTROL GREEN MOLDS

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Submitted: November 10, 2010; Returned to authors for corrections: January 21, 2011; Approved: May 16, 2011.

ABSTRACT

The essential oil of *Lippia alba* is reported as an antifungal against human pathogenic microorganisms but few articles report its use as an alternative to synthetic fungicides on green mould control. The objective of this study was to determine chemical characteristics of *L. alba* essential oil and its antifungal activity against green molds as an alternative to synthetic fungicides. Essential oil was extracted by Clevenger hydrodistillation, characterized by GC-MS analysis, and the structure of the main compounds confirmed by ¹H and ¹³C-NMR spectroscopy. Microdilution assays evaluated the essential oil minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC). Commercial fungicides Ketoconazole and Bifonazole were used as control. Essential oil yield is of 0.15% and the major components are neral (33.32%) and geranial (50.94%). The *L. alba* essential oil has MIC of 0.300-1.250 mg/mL and MFC of 0.600-1.250 mg/mL. Ketoconazole and Bifonazole show MIC ranging from 0.025-0.500 to 0.100-0.200 mg/mL, and MFC ranging from 0.250-0.100 to 0.200-0.250 mg/mL, respectively. *L. alba* essential oil is classified as citral type and the results indicate that it is a potential alternative to synthetic fungicides.

Key words: antifungal, essential oil, neral, geranial, *Lippia alba*.

INTRODUCTION

Lippia alba (Mill.) N.E. Br. ex Britton & P. Wilson (Verbenaceae) is an aromatic plant widely used all over South and Central America for different purposes. This family comprises over 175 genera and 2,800 species in Africa, Latin America (25) and India (6, 32). Several papers have presented ethnopharmacological studies dealing with *L. alba* as sedative,

antidepressant and analgesic properties (18). The essential oil of *L. alba* also has many applications such as stomachic, anti-spasmodic, digestive, anti-hemorrhoidal and anti-asthmatic (18). Different biological activities such as cytotoxic, antifungal, antibacterial, antiviral and anti-inflammatory, have been identified in essential oils or extracts of *L. alba* (5, 11, 19, 26, 37).

Mesa-Arango *et al.* (25) reported two chemotypes, citral

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and carvone but Tavares *et al.* (35) reported three chemotypes, citral, carvone and linalool for the same species. Shukla *et al.* (32), Tavares *et al.* (35) and Mesa-Arango *et al.* (25) stated that the main constituents of *L. alba* essential oil were geranial varying from 22.21% to 33.98% and neral varying from 14.20% to 25.82%. Although *L. alba* essential oil is well described, its chemotypes and constituents may vary according to the environment. Thus, it is important to determine the chemical characterization of *L. alba* essential oil in order to identify the components that have antifungal activity.

The antifungal activity of the essential oils of *L. alba* against human pathogenic fungi such as *Candida albicans*, *Candida guilliermondii*, *Candida parapsilosis*, *Candida neoformans*, *Trichophyton rubrum* and *Fonsecaea pedrosoi* has been previously demonstrated for the citral and myrcene-citral chemotypes (26). *L. alba* essential oil and two of its major components were evaluated for fungitoxicity and anti-aflatoxigenicity against *Aspergillus flavus* (32). The antifungal activity was evaluated against *C. parapsilosis*, *Candida krusei*, *A. flavus* and *Aspergillus fumigatus* strains (25). Antifungal screening was carried out also against *Saccharomyces cerevisiae*, *A. flavus*, *Aspergillus niger* and *C. albicans* (6).

Although many studies reported the antifungal activity of *L. alba* essential oils against human pathogenic microorganisms, few articles were about *Fusarium*, *Aspergillus* and *Penicillium* genera (34). These fungi are well known as causal agents of food-borne diseases and food spoilage, which increase the costs of food production and health care in the world (24). In addition, the genera *Trichoderma*, *Aspergillus* and *Penicillium*, known as green moulds, occur on mushroom production when the composting is not correctly prepared and/or does not become selective enough (15). Those fungi can spread very rapidly competing for carbohydrates in the substrate at the time of spawning, or in the casing layer, and some of them produce toxins that can damage mushroom tissue

(15). The genus *Trichoderma* is the most common contaminant on mushroom cultivations and facilities causing enormous economic losses around the world (9). Many sanitary procedures were adopted in mushroom farms to control *Trichoderma* sp and other green molds (15) and also on spawn production or spawning procedures where synthetic fungicides are used on mushroom cultivation. Although the fungicides that are commonly used in cultivation are very effective and inexpensive, it has been suggested that they leave residual toxicity that may cause side effects, including carcinogenesis and teratogenesis (20, 23, 33). Most of these synthetic fungicides have been restricted in several countries since the early 1960's.

Despite of the potential use of essential oils on fungus control, there are no reports about the essential oil toxic effects on basidiomycete development or the use on mushroom production. Thus, new studies should be done to evaluate the viability of spraying essential oil solutions on the surface of casing layers where green molds are very common. Also, microbicidal essential oils are generally considered less harmful than synthetic chemicals and are being used on other organic cultures (28). An additional advantage of essential oils is their volatile nature, which implies on low or no residues after treatment and low environmental impact.

Based on alternatives for organic production with natural substances to control undesirable fungi, the objective of this study was to determine the chemical characteristics of *L. alba* essential oil and its antifungal activity against green molds as an alternative to synthetic fungicides.

MATERIALS AND METHODS

General

The major equipment used was gas chromatography-mass spectrometry (GC-MS) 7890 (Agilent-Technologies, California, USA), nuclear magnetic resonance (NMR) spectrometer Varian GEMINI 2000 and Clevenger-type hydro

distillation apparatus. All other chemicals (analytical grade) Glamočlija, J. *et al.*

study were from Merck (Darmstadt, Germany), unless stated otherwise. Malt agar and Sabourad maltose agar were from the Institute of Immunology and Virology, Torlak (Belgrade, Serbia); Ketoconazole (Galenika, a.d. Belgrade, Serbia) and Bifonazole (Srbolek, Belgrade, Serbia).

Plant Material

Leaves of *L. alba* were collected in the morning at nine o'clock from March to May in 2009, in the Medicinal Plant Garden at Paranaense University-Campus of Umuarama - Brazil. Dirt was removed with tap water and stored in polyethylene plastic bags at -20 °C.

Extraction of the essential oil

Defrosted leaves (100 g) were transferred to a Clevenger apparatus for hydrodistillation for one hour. The essential oil extracted was stored at -20 °C and the yield was determined by the essential oil volume obtained in 100 g of leaves, expressed in percentage of volume per mass (mL/100 g of leaves x 100).

Analysis of essential oil

GC-MS analysis: the GC-MS analysis was performed using an Agilent 7890 gas chromatograph coupled to an Agilent 5975 C mass selective detector (MSD) in the positive ion electron impact (EI) mode. The separation was achieved using an HP-5MS fused silica capillary column, 30 m x 0.25 mm i.d., 0.25 µm film thickness. GC oven temperature was programmed from 60 °C to 285 °C at a rate of 4.3 °C/min. Helium was used as the carrier gas; inlet pressure was 25 kPa; linear velocity: 1 mL/min at 210 °C. Injector temperature: 250 °C and injection mode: split 1:50. MS scan conditions: source temperature, 200 °C; interface temperature, 250 °C; E energy, 70 eV; mass scan range, 40-350 amu.

Identification of compounds: a library search and mass spectral deconvolution and extraction were performed using Automated Mass Spectral Deconvolution and Identification

dimethylsulfoxide (DMSO), ethanol, and methanol used in this *L. alba* essential oil to control green molds

System (NIST AMDIS) software version 2.4, using retention index (RI) calibration data analysis parameters with 'strong' level and 7% penalty for compounds without an RI. The RI was experimentally determined using the standard method (1, 36) involving retention times of *n*-alkanes, injected after the essential oil under the same chromatographic conditions. The search was performed against our own library containing 4951 spectra. The percentage (relative) of the identified compounds was computed from GC peak area.

NMR spectroscopy: ¹H and ¹³C NMR spectra were recorded on Varian GEMINI 2000 spectrometer (200 MHz for ¹H; 50 MHz, ¹³C) in 5 mm standard tubes. Chemical shifts are given on the δ scale relative to TMS as internal standard.

Antifungal activity

For the bioassays, seven fungal strains were used: *Aspergillus ochraceus* (ATCC 12066), *Aspergillus versicolor* (ATCC 11730), *A. niger* (ATCC 6275), *A. fumigatus* (ATCC 9142), *Penicillium ochrochloron* (ATCC 9112), *Penicillium funiculosum* (ATCC 10509) and *Trichoderma viride* (IAM 5061). All of the tested organisms were from the Mycological Laboratory, Department of Plant Physiology, Institute of Biological Research „Siniša Stanković“, Belgrade, Serbia. The micromycetes were maintained on malt agar (MA) and Sabouraud agar (SBA), stored at 4 °C and subcultured once a month (7).

Microdilution method: in order to investigate antifungal activity of the isolated essential oil a modified microdilution technique was used (12, 17). The fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% Tween 80 (v/v). The fungal cell suspension was adjusted with sterile saline to a concentration of 1.0 x 10⁶ in a final volume of 100 µL per well. The inocula were stored at 4 °C for further use. Dilutions of the inocula were cultured

on solid MA for fungi to verify the absence of contamination and the validity of the inoculum.

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microtitre plates. The investigated essential oil was added in broth malt medium with inoculum. The microplates were incubated for five days at 25 °C for fungi. The lowest concentrations without visible growth under optical microscope were defined as MIC.

The minimum fungicidal concentration (MFC) was determined by serial subcultivation of 2 µL into microtitre plates containing 100 µL of broth per well and further incubation for 72 h at 25 °C. The lowest concentration with no visible growth under optical microscope was defined as MFC indicating 99.5% killing of the original inoculum. Each experiment was done in triplicate. Ketoconazole and Bifonazole were used as positive controls (1 mg/mL).

RESULTS AND DISCUSSION

L. alba essential oil yield, in this work, was of 0.15% but Shukla *et al.* (32) reported yield of 0.08%, and Castro *et al.* (10) reported yield ranging from 0.15 to 0.61% where higher yields were from leaves collected in the summer. *L. alba* leaves represent 80% of the total plant mass with an annual production of five tons per hectare in Brazil. Although few agronomical studies were done on the genetic improvement of this plant or on the essential oil yield (2), it is still a potential plant for studies on diversification of farm production. Such issues are valid worldwide, but they are especially true in areas in which cultivation is constrained by environmental and economic factors that often reduce rural areas to marginal conditions. Thus, new culture opportunities have become an important topic in agricultural research.

Minimum inhibitory concentration (MIC) determination was performed by a serial dilution technique using 96-well

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The chemical analysis of *L. alba* essential oil showed that geranial (50.94%) and neral (33.32%) are the main components. 97.69% of the total essential oil was identified (Table 1) and could be classified as a citral chemotype based on their chemical constituents. Monoterpenes neral and geranial were the main constituents of the citral chemotype (25). For *L. alba*, two chemotypes, citral and carvone, are reported by Mesa-Arango *et al.* (25) and three chemotypes, citral, carvone and linalool, are reported by Tavares *et al.* (35). For the same plant species, essential oil composition and yield can vary according to harvesting seasons, part of the plant and geographical cultivation location (8). For instance in general, essential oils from leaves harvested during or immediately after flowering possess stronger antimicrobial activity (8). Also, different essential oil chemical composition has been found among seeds and immature leaves of coriander (*Coriandrum sativum* L.) (8). For *L. alba*, genetic factors seems to be the principal responsible for variations on chemical constituents more than environmental ones (35). Shukla *et al.* (32) reported that the main constituents of *L. alba* essential oil were geranial (22.21%) and neral (14.20%), Tavares *et al.* (35) reported that they were geranial (33.98%) and neral (25.82%) and Mesa-Arango *et al.* (25) reported that they were geranial (30.5%) and neral (23.6%). The quantity of geranial and neral was more concentrated in our study, 50.94% and 33.32%, respectively (Table 1), than in the ones reported in previously cited literature.

In order to confirm the structure of the main compounds, the essential oil was studied by ¹H and ¹³C-NMR spectroscopy. The essential oil ¹H NMR spectrum (Figure 1) gave resonances for a mixture of two isomers as indicated by the disparity in

single hydrogen peaks. Signals due to aldehyde protons were detected as two doublets at δ 9.99 (J=8.0 Hz) and 9.90 (J=8.0 Hz). From the NMR integration of those peaks, the ratio between the two components was determined to be 1:0.67. The presence of neral and geranial is supported by ^{13}C NMR data (Table 2), according to Ragasa *et al.* (30).

L. alba essential oil has antifungal activity with MIC in a range of 0.300-1.250 mg/mL, and MFC in a range of 0.600-1.250 mg/mL (Table 3). The commercial preparation of fungicidal agent, Ketoconazole, showed MIC in a range of 0.025-0.500 mg/mL and MFC in a range of 0.250-0.100 mg/mL. Bifonazole showed lower antifungal activity with a MIC of 0.100-0.200 mg/mL and MFC of 0.200-0.250 mg/mL (Table 3).

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Table 1. The chemical composition of essential oil of *Lippia alba*.

| Compound | RI | % |
|----------------------------|------|-------|
| 1-Oct-en-3-ol | 968 | 0.45 |
| 2-methyl-1-Hepten-6-one | 979 | 0.37 |
| Myrcene | 984 | 0.50 |
| Linalol | 1090 | 0.49 |
| β -Pinene oxide | 1142 | 0.18 |
| (Z)-Isocitral | 1158 | 0.23 |
| Rosefuran epoxide | 1170 | 0.19 |
| Neral | 1244 | 33.32 |
| Piperitone | 1252 | 0.22 |
| Geranial | 1277 | 50.94 |
| <i>p</i> -Menth-1-en-7-al | 1281 | 0.71 |
| Nd | 1360 | 0.72 |
| α -Copaene | 1372 | 0.24 |
| β -Elemene | 1389 | 0.27 |
| (<i>E</i>)-Caryophyllene | 1417 | 3.07 |
| α -Humulene | 1450 | 0.21 |
| Germacrene D | 1479 | 0.14 |
| Aciphyllene | 1504 | 0.53 |
| Cubebol | 1513 | 0.31 |
| δ -Cadinene | 1521 | 0.20 |
| Nd | 1531 | 0.12 |
| (<i>E</i>)-Nerolidol | 1561 | 0.52 |
| Caryophyllene oxide | 1580 | 2.17 |
| Allo-aromadendrene epoxide | 1648 | 2.43 |
| Nd | 1660 | 0.43 |
| Nd | 2107 | 0.24 |
| Nd | 2237 | 0.15 |
| Nd | 2326 | 0.13 |
| Total | | 97.69 |

Legend: RI: retention index; Nd: not determined.

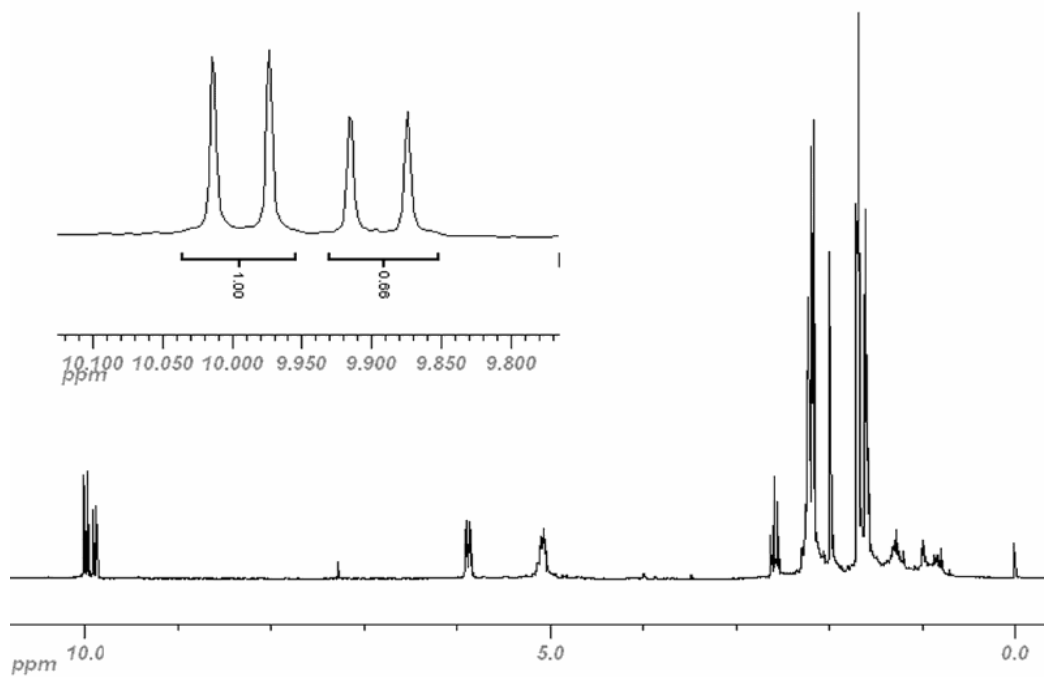
Table 2. 200 MHz ^1H NMR and 50 MHz ^{13}C NMR spectral data of neral and geranial from *Lippia alba* essential oil.

| Position | Neral | Geranial |
|----------|-------|----------|
|----------|-------|----------|

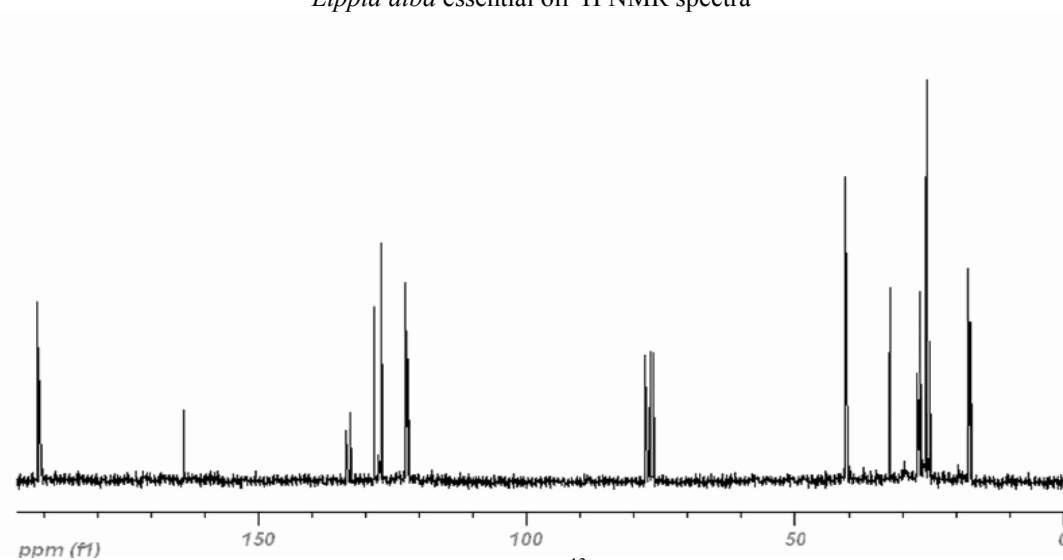
| | δ_C | δ_H mult. (J Hz) | δ_C | δ_H mult. (J Hz) |
|----|------------|-------------------------|------------|-------------------------|
| 1 | 191.50 | 9.99 d (8.0 Hz) | 190.63 | 9.90 (8.0 Hz) |
| 2 | 127.17 | 5.88 d (8.0 Hz) | 128.73 | 5.88 d (8.0 Hz) |
| 3 | 163.98 | - | 163.98 | - |
| 4 | 40.38 | 2.20 | 32.34 | 2.59 |
| 5 | 25.53 | 2.17 | 27.82 | 2.17 |
| 6 | 122.44 | 5.16 | 122.15 | 5.10 |
| 7 | 132.96 | - | 133.44 | - |
| 8 | 25.34 | 1.68 | 25.34 | 1.68 |
| 9 | 17.40 | 1.61 | 17.40 | 1.61 |
| 10 | 17.27 | 1.99 | 24.77 | 1.99 |

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Lippia alba essential oil ^1H NMR spectra



Lippia alba essential oil ^{13}C NMR spectra

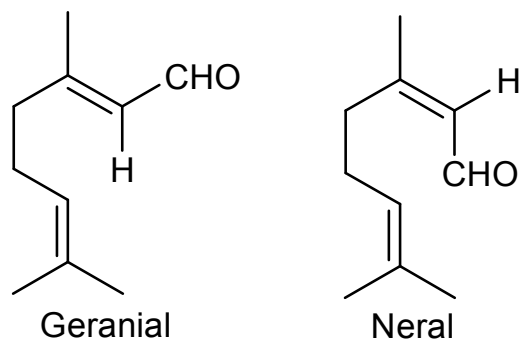


Figure 1. *Lippia alba* essential oil ^1H NMR and ^{13}C NMR spectra. Glamočlija, J. *et al.*

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Table 3. Minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) of *Lippia alba* essential oil.

| Fungi | | Essential oil (mg/mL) | Ketoconazole (mg/mL) | Bifonazole (mg/mL) |
|---------------------------------|-----|-----------------------|----------------------|--------------------|
| <i>Aspergillus ochraceus</i> | MIC | 0.300±0.057 | 0.025±0.150 | 0.100±0.030 |
| | MFC | 0.600±0.057 | 0.100±0.030 | 0.200±0.050 |
| <i>Aspergillus versicolor</i> | MIC | 0.300±0.057 | 0.100±0.060 | 0.100±0.030 |
| | MFC | 0.600±0.100 | 0.025±0.020 | 0.200±0.050 |
| <i>Aspergillus niger</i> | MIC | 0.600±0.060 | 0.025±0.057 | 0.150±0.010 |
| | MFC | 0.600±0.057 | 0.025±0.010 | 0.200±0.057 |
| <i>Aspergillus fumigatus</i> | MIC | 0.300±0.100 | 0.025±0.057 | 0.150±0.030 |
| | MFC | 1.250±0.230 | 0.050±0.010 | 0.200±0.100 |
| <i>Penicillium ochrochloron</i> | MIC | 0.600±0.057 | 0.010±0.080 | 0.150±0.020 |
| | MFC | 1.250±0.057 | 0.025±0.010 | 0.200±0.100 |
| <i>Penicillium funiculosum</i> | MIC | 1.250±0.000 | 0.025±0.010 | 0.200±0.030 |
| | MFC | 1.250±0.057 | 0.050±0.057 | 0.250±0.030 |
| <i>Trichoderma viride</i> | MIC | 0.600±0.057 | 0.050±0.010 | 0.200±0.050 |
| | MFC | 1.250±0.230 | 0.100±0.030 | 0.250±0.057 |

The results are expressed as mean ± standard deviation.

L. alba essential oil for antifungal screening was performed *in vitro* by disc diffusion method against *S. cerevisiae*, *A. flavus*, *A. niger* and *C. albicans* and showed mild to moderate activity (6). Essential oil of *L. alba* and two of its components (geranial and neral) are highly effective against production of aflatoxin B1 by *A. flavus* (32), presenting high activity against gram-positive bacteria with MIC of 0.300-0.630 mg/mL, mainly *Staphylococcus aureus* and *Pseudomonas aeruginosa* (4). In our study, it is clear that geranial and neral are the main components of *L. alba* essential oil being geranial the prime component. Shukla *et al.* (32) reported that geranial from *L. alba* caused 100% inhibition

growth on 13 fungi among 17 tested; whereas, at the same concentration, the essential oil and neral component presented 100% of inhibition just on 9 and 2 fungal species, respectively. Thus, geranial seems to be the main fungicidal component of *L. alba* essential oil. The high geranial concentration of *L. alba* essential oil in our work may explain the good antifungal results and it is an alternative to synthetic fungicides such as Ketoconazole and Bifonazole on health, food and agriculture.

The development of applications for *L. alba* essential oil is still not well-explored. Rao *et al.* (31) reported antifungal activity against sugarcane pathogens and Lee *et al.* (21) and Park *et al.* (27) reported bioactivity of geranial and neral

against phytopathogens and dermatophytes, but no reports were found about *L. alba* essential oil against *T. viride*. The results of the present study on the effectiveness of *L. alba* essential oil against micromycetal food poisoning, fungus, plant, animal and human pathogens showed excellent antifungal activity. The essential oil MIC described in the literature for *T. viride* was 1.4 µg/mL for *Nepeta ratanjensis* (16), 25 µL/mL for *Salvia sclarea* (13) and 0.42 µL/mL for *Lippia gracilis* (3). The results of our study show that *L. alba* essential oil is a Glamočlija, J. *et al.*

however, it is suggested that Prochloraz and Benomyl may cause side effects (33). In addition, in many countries there is no effective control of those substances, making it difficult to detect misuse of those substances. *L. alba* essential oil may be used as a potential alternative to synthetic fungicides mainly on organic cultivation such as mushroom cultivation or encapsulated with cyclodextrins in food biofilms according to Linde *et al.* (22). However, further studies are required to develop strategies for application on health, food and agriculture.

CONCLUSIONS

It was concluded that *L. alba* essential oil is classified as citral type according to the presence of main components, neral (33.32%) and geranial (50.94%). The essential oil of *L. alba* presents antifungal activity with MIC of 0.300-1.250 mg/mL and MFC of 0.600-1.250 mg/mL. The commercial fungicides Ketoconazole and Bifonazole show MIC in a range of 0.025-0.500 and 0.100-0.200 mg/mL and MFC in a range of 0.250-0.100 and 0.200-0.250 mg/mL, respectively. Geranial seems to be the main fungicidal component of *L. alba* essential oil. *L. alba* essential oil is a potential alternative to synthetic fungicides. However, further studies are required to develop strategies for application on health, food and agriculture.

ACKNOWLEDGEMENTS

fungicidal and fungistatic alternative to control *T. viride*. Geels *et al.* (15) reported that green moulds (*Trichoderma*, *Aspergillus* and *Penicillium* species) can occur on compost and/or casing layer causing mushroom production losses. In order to avoid green moulds, Benomyl (Benlate[®]), among other fungicides that have a broad spectrum systemic fungicide, is used against a large number of important fungal pathogens on *Agaricus bisporus* cultivation (14, 29). Prochloraz (Sporgon[®]) is another commonly used fungicide in mushroom cultivation; *L. alba* essential oil to control green molds

The authors are grateful to the Ministry of Science and Technological Development of the Republic of Serbia for the financial support, Pr. n. 173032 and 172053, and to the Paranaense University.

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