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Original Research Article

Investigation of *Melissa officinalis* L. essential oil for antifungal activity against *Cladosporium carrionii*

ABSTRACT

Cladosporium carrionii is considered the most important pathogenic species of genus because of the numerous cases of disease which causes in the world. Due to its antifungal resistance, these fungal infections are difficult to treat. Given the broad biological activity displayed by natural products, essential oils obtained from plants are often investigated to determine their antimicrobial activity. Therefore, we identified components of *Melissa officinalis* L. essential oil, investigating *in vitro* antifungal activity against strains of *C. carrionii*. Identification of the chemical composition of the oil was performed by gas chromatography coupled to a mass spectrometer (GC-MS). The antifungal activity of *M. officinalis* L. essential oil was investigated against 08 strains of *C. carrionii* determining minimum inhibitory concentration (MIC), minimum fungicidal concentration (MFC), effects on mycelial growth, and conidial germination. The GC-MS results revealed 4 major components; geranial (52%), citral (38.90%), trans-β-caryophyllene (1.22%), and germacrene D (0.84%). *M. officinalis* L. essential oil inhibited the growth of all (100%) of the strains of *C. carrionii* tested. The MIC and MFC were established at 256 µg/mL. Inhibition of radial mycelial growth began at 128 µg/mL (MIC/2), and at both 2 X MIC and 4 X MIC the inhibition was complete. We also observed significant conidial germination inhibition at all concentrations when compared to the control ($P<0.05$). The inhibition increased with concentration so that at 2 X MIC complete (100%) conidia germination inhibition was observed. Finally, our study results point to *Melissa officinalis* L. essential oil as a potential antifungal agent against *C. carrionii*.

Keywords: Essential oil, Lamiaceae, Melissa officinalis L., Cladosporium carrionii

1. INTRODUCTION

The genus *Cladosporium* includes many species of opportunistic dematiaceous fungi, and contaminations can occur in diverse environments [1]. *C. carrionii,* being an agent of chromoblastomycosis, feo-hifomicoses, and allergies is considered the most important pathogenic species of the genus due to the numerous cases of disease reported around the world. Infections caused by *C. carrionii* are chronic, and appear as mycoses in the skin, subcutaneous tissues, and nails. They are difficult to treat due to long treatment periods, limited treatment options, resistance to common antifungal agents, and their greater prevalence among immunocompromised patients. All of these characteristics invite recurrences [2,3].

There exists a clear need for more, therapeutically effective antifungals. In this context, our attention has been focused on the antifungal activities of aromatic plants and their constituents because of their potential biological properties [4]. *Melissa officinalis* L. belongs to the Lamiaceae family, is an aromatic plant, and is popularly known as lemon balm, lemongrass, Melissa Roman, and Tea of France. Its leaves and flowers are used in the form of teas, for soothing in cases of anxiety and insomnia, as well as for medication

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against headaches, migraine, digestive problems, rheumatic pains, and to normalize gastrointestinal functions [5].

The essential oil of *Melissa officinalis* L. is widely used by the pharmaceutical, cosmetic, and food industries due to the antibacterial, antifungal, anxiolytic, antioxidant, and antitumor activities it possesses [6,7,8,9]. Due the antifungal properties of this essential oil, the aim of our study was to evaluate the essential oil of *Melissa officinalis* L. for antifungal activity against strains *of Cladosporium carrionii*.

2. MATERIAL AND METHODS

2.1 Chemical

Melissa officinalis L. essential oil was supplied by Quinari Ind. Com. Ltda., Ponta Grossa, Paraná, Brazil; fluconazole and amphotericin B were purchased from Sigma-Aldrich, Brazil. The drugs were dissolved in dimethyl sulfoxide (DMSO), and sterile distilled water was used to obtain solutions of 1024 µg/ml for each of these antifungals. The concentration of DMSO in the assays did not exceed 0.5 %.

2.2 Analysis of the essential oil

To analyze constituents of the essential oil we used a coupled gas chromatography/mass spectrometer GC17-A (GC-MS) Shimadzu operated by electron impact. The mobile phase consisted of helium and was pumped at a flow rate of 1.6 ml/min, split 1:5. Chromatographic separation was performed using a DB-5 capillary column (30m x 0.25 mm, 0.25 µm). The column oven temperature was programmed to move from an initial temperature of 60 °C to 120 °C at 5 °C/min, and from 120 °C to 280 °C at 20 °C/min. The temperature of the injector and detector were respectively 260 and 280 °C. The total time was 20 minutes and the injection volume was 1.0 µl.

The identification of the essential oil constituents was performed by the computer system and a data processing (workstation) connected to the GC-MS. The system was equipped with a Wiley library database, 6th edition of the class 5000-1999, with 229.119 spectra.

2.3 Microorganisms

The tested *Cladosporium carrionii* strains (CL 03, LM 0212, LM 227, CQ 02, LM 01, CQ 03) were taken from the Microorganisms Collection of the Mycology Laboratory, at the Department of Pharmaceutical Sciences, Health Sciences Center, Federal University of Paraíba, Brazil. The strains (URM 5109, URM 2871) were taken from the Pernambuco (Brazil) Federal University, Biological Sciences Center - Mycology Department fungal collection. Stock cultures were maintained on Sabouraud Dextrose agar (SDA) slants, and stored in a refrigerator (4 °C).

Stock inoculate suspensions of the *C. carrionii* strains were prepared from 10-day old sabouraud dextrose agar (Difco Lab., USA) cultures grown at 28 °C. Fungal colonies were covered with 5 ml of sterile saline solution (0.9%), the surface was gently scraped with a sterile loop, and the saline solution with the fungal elements was transferred to a sterile tube. These suspensions were shaken for 2 minutes using a vortexer, and allowed to stand for 5 minutes to allow hyphal fragments to fall out of the suspensions so that the supernatant containing the conidia could be collected. Tubes containing the inocula were standardized to 0.5 McFarland scale (10^6 CFU/ml). Mold conidia were counted using a hemocytometer. The inocula of the conidial suspensions were adjusted using sterile NaCl 0.9 % to contain approximately 10^6 CFU/ml [10,11].

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2.4 Minimum inhibitory concentration (MIC)

Broth microdilution assays were used to determine the MICs of *Melissa officinalis* L. essential oil, fluconazole and amphotericin B. Sabouraud dextrose broth (SDB - Difco Lab., USA) was added to all the wells of 96-well plates. Two-fold serial dilutions of the three agents were prepared to obtain concentrations varying between 4 µg/ml and 1024 µg/ml. Finally, 10 µl aliquots of the inoculate suspension were added to the wells, and the plates were incubated at 28 ^{o}C for 5 days. Negative controls (without drugs) were used to confirm conidia viability and sensitivity controls (for DMSO) were also included in the studies. At 72 hours (and confirmed at 7 days) there were visual observations for fungal growth. The MIC was defined as the lowest concentration capable of visually inhibiting fungal growth by 100 %. The results were expressed as the arithmetic mean of three experiments [10,12].

2.5 Minimum fungicidal concentration (MFC)

To determine the MFC of *M. officinalis* essential oil and fluconazole, 10 µl samples from each well (with no fungal growth) were subcultured on SDA plates, the SDA plates were incubated at 28° C for 72 hours. The MFC was defined as the lowest concentration of essential oil cultivated in SDA that showed either no growth or fewer than three colonies, obtaining approximately 99.9 % or more of killing activity. Two independent experiments on different occasions were performed [13,14,15].

2.6 Effects on mycelial growth

The strains URM 5109 and CQ 03 were chosen for use in this test. Inhibition of *C. carrionii* fungal mycelia growth was determined using the poisoned substrate technique (dilution in solid medium), by daily measuring of the radial mycelial growth on SDA adding essential oil in an amount adjusted to provide a final concentration similar to the MIC/2, MIC, 2 X MIC, and 4 X MIC previously found. For this, 2 mm plugs taken from a 10-day-old mold culture cultivated on SDA slants at 28° C were placed at the center of the sterile SDA Petri dishes containing essential oil. At different intervals (0, 2, 4, 6, 8, 10, 12, and 14 days) of incubation at 28° C, the mold's radial mycelial growth was measured (mm) with calipers. The controls in this assay were the mold's radial growth on SDA without adding essential oil, or fluconazole at (MIC/2, MIC, 2 X MIC and 4 X MIC). Each assay was performed twice and the results were expressed as the average of the two repetitions [16,17,18].

2.7 Effects on conidial germination

Melissa officinalis essential oil and fluconazole were tested to evaluate their effects on the germination of *C. carrionii* conidia. Strains URM 5109 and CQ 03 were chosen for use in this test, along with negative controls. 500 µl aliquots of the drugs at different concentrations (MIC/2, MIC and 2 X MIC) were added to sterile tubes, mixed with 500 µl of fungal suspension, and then immediately incubated at 28° C. Samples were taken at 48 h to assess the number of germinated and un-germinated conidia through the use of a hemocytometer from which the percentage of germinated conidia was calculated. The test was performed in triplicate [19].

2.8 Data analysis

The results were expressed in mean ± SE. Statistical analyses were performed with the student *t*–test, and Fisher's exact test, $P < 0.05$ was considered significant.

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3. RESULTS

The chemical components of the essential oil, the percentages of each constituent, and the retention indices are summarized in Table 1.

Table 1 - Components of *M. officinalis* essential oil.

Peaks	Retention time (min)	Compound	Retention index	% in the formulation
1	12.73	Citral	1240	38.90
2	13.28	Geranial	1270	52.00
3	15.68	Trans β-cariofileno	1418	1.22
4	16.51	Germacrene D	1480	0.84
5	-	Other Compound	-	7.04

As can be seen, four constituents were identified, representing 92.9 % of the oil. The majority phytoconstituent was geranial at (52 %), followed by citral at (38.90 %), trans-β-caryophyllene at (1.22 %), and germacrene D at (0.84%).

In the biological activity study, *M. officinalis* essential oil inhibited the growth of all (100 %) of the *C. carrionii* strains tested. The MIC and MFC were established at 256 µg/ml. Fluconazole (positive control) exerted inhibitory effects against all fungi tested, inhibiting 100 % of the strains at a concentration of 128 µg/ml. Amphotericin B showed an MIC of greater than 1024 µg/ml. During the comparative analysis of MIC and MFC we observed that the essential oil showed similar MFC and MIC values for all strains. Fluconazole showed an MFC value of (512 µg/ml), four times higher than the MIC value for the strains tested. DMSO did not inhibit the growth of the controls, and all strains were found to grow in the absence of the drugs, confirming the viability of the fungi.

Based on the MIC and MFC values found, two *C. carrionii* strains (URM 5109 and CQ 03) were employed to explore the effects of *M. officinalis* essential oil and fluconazole on the kinetics of mycelial growth and conidial germination. The effect of the drugs at MIC/2, MIC, 2 X MIC, and 4 X MIC on mycelial growth was determined by measuring the *C. carrionii* mycelia diameter (mm) in order to observe the effect of different concentrations of the products over time (Fig. 1, 2).

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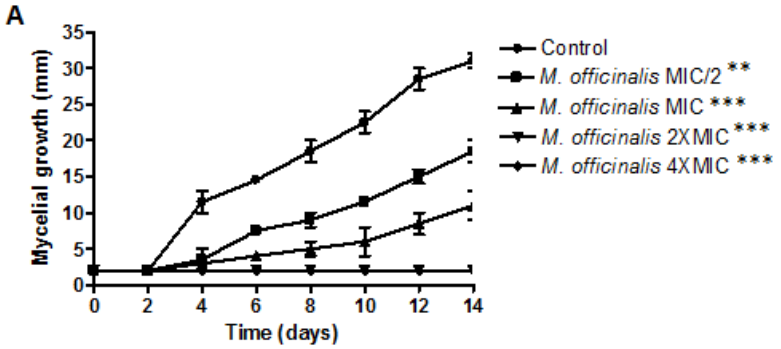


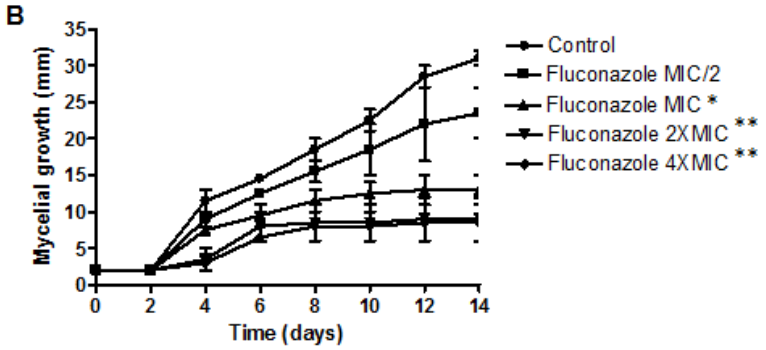
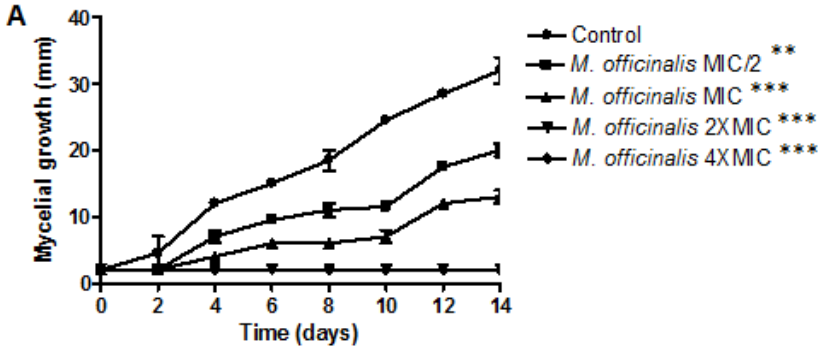


Fig. 1. Radial mycelial growth produced by *Cladosporium carrionii* URM 5109 in the absence (control) and presence *M. officinalis* essential oil **(A)** (MIC/2: 128 µg/ml; MIC: 256 µg/ml; 2 X MIC: 512 µg/ml; 4 X MIC: 1024 µg/ml) and fluconazole **(B)** (MIC/2: 64 µg/ml; MIC: 128 µg/ml; 2 X MIC: 256 µg/ml; 4 X MIC: 512 µg/ml). *$P < 0.05$; **$P < 0.005$, ***$P < 0.0001$ compared to control.



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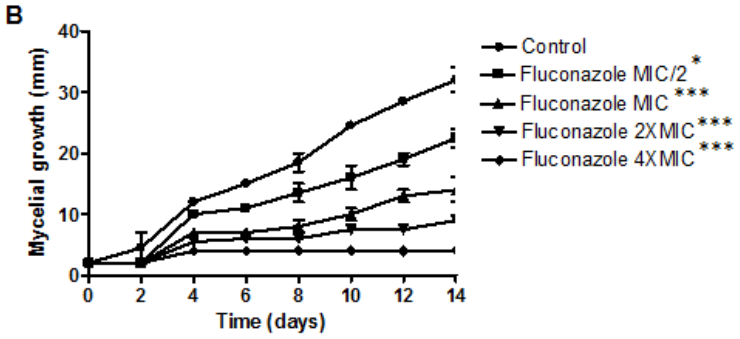
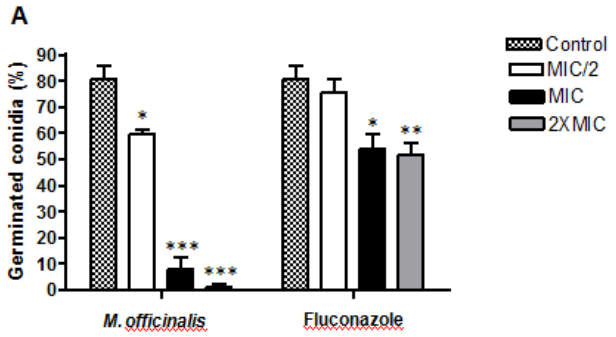


Fig. 2. Radial mycelial growth produced by *Cladosporium carrionii* CQ 03 in the absence (control) and presence *M. officinalis* essential oil **(A)** (MIC/2: 128 µg/ml; MIC: 256 µg/ml; 2 X MIC: 512 µg/ml; 4 X MIC: 1024 µg/ml) and fluconazole **(B)** (MIC/2: 64 µg/ml; MIC: 128 µg/ml; 2 X MIC: 256 µg/ml; 4 X MIC: 512 µg/ml). *$P < 0.05$; **$P < 0.005$, ***$P < 0.0001$ compared to control.

As seen in Figure 1A, all tested concentrations of *Melissa officinalis* essential oil (especially at MIC, 2 X MIC and 4 X MIC), inhibited the mycelial growth of *C. carrionii* URM 5109 ($P < 0.0001$) as compared with the control (mycelia diameter being 100 %). Similar effects were noted with CQ 03 in that the essential oil effectively inhibited the mycelial growth (Fig. 2A). Strain controls showed a constant rate of mycelial growth over the time evaluated, indicating good antifungal effect for the essential oil tested. The fluconazole test on *C. carrionii* URM 5109 showed significant inhibition of mycelial growth from the MIC (128 µg/mL) ($P < 0.05$) (Fig. 1B). With the CQ 03 strain at the CIM/2 (64 µg/mL), inhibition of mycelial growth was already significant compared to the control ($P < 0.05$) (Fig. 2B). However, in the presence of fluconazole, we observed a decrease in mycelial growth, and no fungicidal effect, (even in higher concentrations) as was observed with the essential oil.



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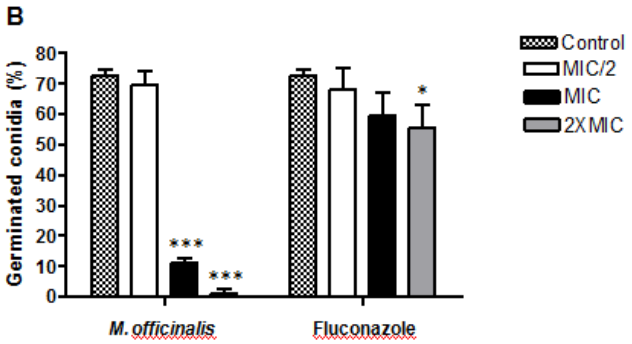

Fig. 3. Percentage of conidial germination of *Cladosporium carrionii* URM 5109 (A) and CQ 03 (B) in the absence (control) and presence of essential oil of *M. officinalis* (MIC/2: 128 µg/ml; MIC: 256 µg/ml; 2 X MIC: 512 µg/ml) and fluconazole (MIC/2: 64 µg/ml; MIC: 128 µg/ml; 2 X MIC: 256 µg/ml). *$P < 0.05$; **$P < 0.005$, ***$P < 0.0001$ compared to control.

The percentage of *C. carrionii* (URM 5109 and CQ 03) germinated conidia are recorded in Figure 3. The essential oil showed strong inhibitory effects on conidia germination with statistically significant results ($P < 0.05$) when compared to the controls at sub-inhibitory concentrations (MIC/2), with the effects most evident at MIC (256 µg/mL), and 2 x MIC (512 µg/mL). The fluconazole test with the URM 5109 strain (Figure 3A) showed significant inhibition on conidia germination at the MIC ($P < 0.05$). With the CQ 03 strain (Figure 3B), inhibition was statistically significant only at the 2 X MIC concentration as compared with the control ($P < 0.05$), so that the effect of the *M. officinalis* essential oil for the two strains was higher than the fluconazole.

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4. DISCUSSION

The chemical composition of *M. officinalis* essential oil is reported in several studies; citral is its major compound. Citral is composed as an isomeric mixture in which the trans-isomer is (2E)-3,7-dimetilocta-2,6-dienal, known as geranial or citral A, and the cis-isomer is (2Z)-3,7-dimetilocta-2,6-dienal, known as neral or citral B [20]. Geranial and neral are therefore isomeric acyclic monoterpene aldehydes, with a typical lemon aroma [21].

The findings of this study, concerning the chemical composition of *M. officinalis* essential oil corroborate the data obtained by other authors. Meftahizade et al. (2010) reported that the main constituents as citral (geranial and neral), citronellal, geraniol, beta-pinene, alpha-pinene, and beta–caryophyllene, all comprising 96% of the oil. Mimica-Dukin et al., (2004), the percentages of the main constituents were as follows: citral (neral + geranial, 39.9%), citronellal (13.7%), limonene (2.2%), geraniol (3.4%), α-caryophyllene (4.6%), caryophyllene oxide (1.7%) and germacrene D (2.4%).

According to the criteria proposed by [23], *M. officinalis* essential oil and fluconazole showed strong antifungal activity against *C. carrionii.* The MIC values of *M. officinalis* and fluconazole were lower than 500 µg/ml. Fluconazole was used as the positive control because it is one of the most commonly used antifungal drugs for the treatment of *Cladosporium* spp. infections [24]. *Melissa officinalis* essential oil has also been found to be active against yeast, and filamentous fungal species [25,26].

In this study, *Melissa officinalis* essential oil disrupted mycelia growth as demonstrated when testing the radial mycelial growth kinetics, which reflects the effect of different product concentrations over time. The fungi mycelium is (on the whole) the hyphae, and fungal filaments, or segments of filamentous mycelium. Like other filamentous fungi, the genus *Cladosporium* presents a simple non-sexual cell cycle, a mycelial colony forms via

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hyphal growth, and this results in linear stretching at its apex. In an infection, longitudinal growth of the hyphae facilitates penetration into the inner layers of the skin, while lateral growth can exacerbate the damage [27,28]. Given the importance of mycelial growth in the development of mycoses, the inhibition of *C. carrionii* mycelial growth caused by *M. officinalis* essential oil as observed in this study is a significant contribution to the search for new natural products with antifungal activity.

Conidia represent the most common mode of asexual reproduction; they play an important role in natural fungal propagation and are structurally resistant [29]. It is important to note that the conidia are not merely quiescent cells; a basal level of RNA and protein synthesis is required for survival of the spores [30]. Conidia are distributed in large quantities in the atmosphere, and some have the ability to cause disease in humans, animals, and plants. Among the various taxa, *Cladosporium* spp. is one of the most cosmopolitan of fungi. With generally high concentrations in the air, spores of *Cladosporium* spp. have been characterized as major allergens [31].

Given the importance of *C. carrionii* conidia, this study's findings are significant because *M. officinalis* essential oil (as well as fluconazole), inhibits *C. carrionii* conidial germination.

4. CONCLUSION

This study supports the view that *Melissa officinalis* essential oil is an important and promising antifungal product which contributes to the arsenal of products with known antifungal activity against *C. carrionii*. Future investigations will provide a deeper pharmacological understanding of this essential oil, a better understanding of its modes of action, its toxicity, and its possible therapeutic application.

CONSENT

All the authors declare that no consent was obtained for this study.

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