

Antifungal activity, yield, and composition of *Ocimum gratissimum* essential oil

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ABSTRACT. *Ocimum gratissimum* L. or clove basil, belongs to the Lamiaceae family, has various desirable uses and applications. Beyond its aromatic, seasoning, and medicinal applications, this plant also has antimicrobial activity. This study was aimed at assessing the antifungal activity, yield, and composition of the essential oil (EO) of *O. gratissimum*. The species was cultivated in garden beds with dystrophic red latosol soil type containing high organic-matter content. The EO was obtained by hydrodistillation of dried leaves in a modified Clevenger apparatus, followed by determination of its content. Chemical characterization was carried out by gas chromatography-mass spectrometry (GC-MS). Microbial activity was assessed using the broth microdilution method, by determining the minimum inhibitory concentration (MIC), in order to compare the antimicrobial effect of EO in 10 isolates-*Fusarium oxysporum* f. sp. *tracheiphilum* (CMM-0033), *F. oxysporum* f. sp. *cubense* (CMM-0813 and CMM-2819), *F. oxysporum* f. sp. *lycopersici* (CMM-1104), *F. solani* (CMM-3828),

Rhizoctonia solani (CMM-3274), and *Macrophomina phaseolina* (CMM-2715, CMM-3875, CMM-3615, and CMM-3650). The EO was a highly effective inhibitor of the studied phytopathogenic fungi, with MICs varying from 31.25 to 125 µg/mL. *F. oxysporum* f. sp. *lycopersici* and *R. solani* were the most sensitive; both were inhibited at an MIC of 31.25 µg/mL. The EO content in the plant extract was 0.18%. Thirty chemical compounds were detected via GC-MS, with linalool (32.9%) being the major compound followed by 1,8-cineole (21.9%), both oxygenated monoterpenes. It can be concluded that clove basil EO is a highly effective antifungal agent, and therefore, a potential alternative for the control of plant pathogenic diseases.

Key words: Antifungal agent; Basil; Linalool; Medicinal plants; Plant pathogens

INTRODUCTION

Phytopathogenic fungi can decrease crop yield as their reproductive structures allow them to spread across large cultivated areas very quickly. These structures are the spores disseminated by wind, soil, and water, which can infect a large number of plants. Unfavorable germination conditions cause spore latency for extended periods. After infection and growth, the fungi can produce enzymes and toxins that damage plant structures, causing metabolic imbalance, and consequently, a decreased yield or even total crop loss (Fonseca et al., 2015).

The use of chemical products to combat phytopathogenic microorganisms is very common in farming practices. However, some problems have been reported. Environmental contamination, increased production costs, and the pathogens' resistance to chemicals are the current challenges being faced. The problem of resistance has worsened with the need for higher doses of crop protection products. These products do not meet export criteria, thus affecting economic development in addition to their environmental and biological impacts (Joshi, 2013; Rizvi et al., 2014).

In order to minimize these impacts, the use of alternative natural products is being studied. Plant extracts and essential oils (EOs) have shown efficacy in the control of plant pathogens, whether resistant or not to common chemical products (Fonseca et al., 2015).

Ocimum gratissimum L. belongs to the Lamiaceae family and is commonly found in Africa, Asia, and South America. The *Ocimum* genus contains approximately 30 known species that are distributed across the warmest regions of the planet. This plant genus produces an EO that is valued by the perfumery, cosmetic, pharmaceutical, and food industries (Pandey et al., 2014). *O. gratissimum* is popularly known as clove basil, African basil, and in Hawaii as wild basil. It is characterized by a woody stem at the base and reaching up to 3 m in height. It has ovate leaves ranging from 5 to 13 cm in length and 3 to 9 cm in width, with petioles 1 to 6 cm in length. Its flowers are arranged in simple or branched bunches, ranging from 5 to 30 cm in length (Matasyoh et al., 2007).

The species is commonly used as a condiment in the food industry and as a medicinal plant, given its anti-inflammatory, analgesic, hepatoprotective, anti-mutagenic, antihypertensive, and anticarcinogenic effects. Its antinociceptive, intestinal motility antagonistic, antibacterial, and antifungal activities have been proven in previous study (Gontijo et al., 2014).

Its pharmacological properties are associated with its EO, which is concentrated mainly in the stem, leaves, and flowers, and is composed mainly of eugenol, linalool, camphor, and thymol (Matasyoh et al., 2007; Pandey et al., 2014). In addition to EO, the plant also produces saponins, flavonoids, tannins, and cardiac glycosides, as products of their secondary metabolism (Gontijo et al., 2014). Recent studies have demonstrated that clove basil EO has inhibitory effect on the growth of some fungi such as *Penicillium* and *Rhizoctonia* (Philippe et al., 2012; Sethi et al., 2013; Rizvi et al., 2014).

Therefore, this study aimed at assessing the microbial activity, yield, and composition of the EO of open-field cultivated clove basil.

MATERIAL AND METHODS

Experimental procedure and soil analysis

The clove basil plants (voucher specimen deposited in the Paranaense University Educational Herbarium; No. 1900) cultivated on a 30-m² garden bed located in the Medicinal Herbarium of the Educational Faculty of Dois Vizinhos (FAED) - UNISEP (União de Ensino do Sudoeste do Paraná, Paraná State - Brazil), at coordinates 25°46'31.04"S and 53°02'56.78"WO.

The seeds were purchased from a commercial retail vendor. They were disinfected with 70% alcohol solution for 15 min. Then, they were placed in boxes containing substrate for seedlings and cultivated up to 15 cm in height for transplantation into the garden bed.

Soil samples (\pm 500 g) from the Medicinal Herbarium were collected at 0-10 cm depth from five different points of the bed and submitted for analysis. The samples were placed in plastic bags and stored in a refrigerator (4°C) until the lab analysis, which was performed in duplicate.

A composite soil sample was sent to the Solo Fértil laboratory. All analyses followed the standards recommended by CELA (State Commission of Agronomic Analysis Laboratories in Paraná State).

Essential oil

The clove basil was harvested in July 2015, during the morning hours of 7-8 am. The fresh aerial parts of the clove basil plants were subjected to hydrodistillation in a modified Clevenger apparatus for 3 h. Extracts were obtained in triplicate for each treatment. The EO was removed from the device with hexane, filtered with anhydrous Na₂SO₄, stored in amber flasks, and refrigerated in open flasks until total evaporation of the hexane. Then, each extract was weighed to calculate the EO content (weight/weight %).

Chemical identification of essential oil via gas chromatography-mass spectrometry analysis

The EO analysis was carried out in a gas chromatographic system (Agilent 7890 B) (Agilent Technologies, Santa Clara, CA, USA), coupled to a mass spectrometer (Agilent 5977 A) equipped with an Agilent HP-5MS capillary column (30 m x 0.250 mm x 0.25 μ m). The following conditions were used: injector temperature of 250°C, injection volume 1 μ L at a ratio of 1:2 (split mode), and initial column temperature of 40°C and heated gradually to 300°C at

a rate of 8°C/min. The carrier gas (helium) flow was set at 1 mL/min. The temperatures of the transfer line, ion source, and quadrupole were 250°, 230°, and 150°C, respectively. The mass spectra were obtained in a range of 40-500 (m/z) provided through scan mode with solvent delay time of 3 min, and the compounds were identified based on comparison of their retention indices (RI) obtained using various n-alkanes (C8-C25), according to Adams (2012).

Fungal isolates and determination of the minimum inhibitory concentration

The minimal inhibitory concentrations (MIC) of EO were determined for the following isolates: *Fusarium oxysporum* f. sp. *tracheiphilum* (CMM-0033), *F. oxysporum* f. sp. *cubense* (CMM-0813 and CMM-2819), *F. oxysporum* f. sp. *lycopersici* (CMM-1104), *F. solani* (CMM-3828), *Rhizoctonia solani* (CMM-3274), and *Macrophomina phaseolina* (CMM-2715, CMM-3875, CMM-3615, and CMM-3650). All isolates were provided from culture collection of pathogenic fungi at Federal Rural University of Pernambuco (UFRPe), Brazil. A 40,000 µg/mL EO solution diluted with 2% polysorbate 80 in Sabouraud broth with 2% glucose was prepared to treat the isolates. The culture medium (100 µL) was distributed into wells of a 96 microdilution plate, and then 200 µL EO solution was added to the second well. Following homogenization, the solution was transferred to the third well, and so on until the tenth well. Thus, the final concentrations obtained after serial dilution were: 40, 20, 10, 5, 2.5, 1.25, 0.625, and 0.312 mg/mL. A microbial suspension was prepared in saline with turbidity equivalent to the tube 0.5 of the McFarland scale (1×10^6 UFC-Unit forming colony/mL). Next, the 1:100 fungal suspension was diluted with Sabouraud broth to obtain 1×10^4 UFC/mL as inoculums. Similarly, the 1:50 fungal suspension was diluted 1:20 in Mueller-Hinton broth modified for fungi to yield 1×10^5 UFC/mL as inoculums. The suspensions (100 µL) were inoculated in triplicate to each well containing the EO concentrations. The control for sterility was in well number 1. The toxicity control was 2% polysorbate 80 in culture medium in well number 11. The growth control was in well number 12 where the microbial suspension was inoculated into the culture medium. Microplates were incubated at 37°C for 48 h under aerobic conditions. The lowest concentration of EO causing complete inhibition of growth (CLSI, 2008) was reported. The same procedure was performed with fractions of the EO, using an initial solution of 20 mg/mL. A solution of commercial fungicide ketoconazole (Zorkapharma™) was used as positive control (1-4000 mg/mL) (Linde et al., 2016).

RESULTS AND DISCUSSION

Characteristics of growth field and clove basil essential oil

The soil where the clove basil plants were cultivated was classified as dystrophic haplustox (red latosol) (LVd19 - Oxisol) (EMBRAPA - Empresa Brasileira de Pesquisa Agropecuária, 2013) and its chemical characteristics are described in Table 1.

It was observed that the assessed parameters diverged from the reference values (Table 1). The amount of organic matter, represented by the carbon rate, exceeded the reference standard. This was explained by Freitas et al. (2015) who affirmed that preparing and cultivating soil for crops cause mineralization of the organic matter owing to the accumulation of plant debris. In addition, the authors associate an increased cation exchange capacity (CEC) with an increased accumulation of organic matter, which explains the results found in this study.

Table 1. Chemical properties of the soil used in the experimental area.

	pH (CaCl ₂)	P mg/dm ³	C g/dm ³	Al ³⁺	H ⁺ +Al ³⁺	Ca ²⁺	Mg ²⁺	K ⁺	SB	CEC	V %
				Cmol _c /dm ³							
Soil ^a	6.47	16.42	17.53	0.00	2.36	7.50	3.63	0.26	11.38	13.74	82.83
Reference ^b	3.8-6.6	16-24	0.8-15.9	-	0.6-5.0	0.3-7.2	0.3-3.3	0.1-0.7	-	2.2-12.5	-

^aMethods: C: dichromate/colorimetric; P and K extracted using Mehlich-I; and Ca, Mg, and Al extracted using 1 M KCl. ^bSource: Sambatti et al. (2003). CEC: cation exchange capacity; SB: sum of bases; V: base saturation.

In this study, the EO content in the extract of fresh leaves of clove basil plants was $0.18 \pm 0.04\%$. Fokou et al. (2014) found similar EO content (0.19%) in extracts of clove basil plants cultivated in Cameroon. Aguiar et al. (2015) obtained 0.45% EO yield, when the plants were cultivated in the Crato region of Ceará, Brazil.

Borges et al. (2012) compared the EO yield from fresh and dry leaves of clove basil plants. The fresh leaves yielded 0.13% EO content, similar to this study. The dry leaves produced a higher 1.02% yield. Therefore, it is important to indicate the extraction method, plant age, and moisture content in order to explain the variable EO content in different studies (Ade-Ademilua et al., 2013; Lermen et al., 2015; Urcoviche et al., 2015; Linde et al., 2016).

In a comparative study carried out in Nigeria, Ade-Ademilua et al. (2013) compared different methods of cultivation and determined EO contents of the clove basil plants. The plants were cultivated in shade or sunlight, and with or without water stress. Significant differences were found among the EO yields. Plants cultivated in sunlight and subjected to water stress produced 0.2% EO. In contrast, plants cultivated in shade but also subjected to water stress produced 1% EO. This result indicates that growing conditions are factors that directly influence the EO content of plants.

In the present study, 30 chemical compounds were identified in the clove basil EO (Table 2). Oxygenated monoterpenes (72.4%), with linalool (32.9%) and 1,8 cineole (21.9%) as the major compounds, were the most predominant compounds identified. In another study by Kpoviessi et al. (2014), they reported 32.96-35.43% of oxygenated monoterpenes in the EO of *O. gratissimum* cultivated in Benin, which is only half the quantity found in this study. Adjou et al. (2012) identified 31 chemical compounds in the clove basil EO extracted from fresh leaves. The gas chromatography-mass spectrometry (GC-MS) analysis also indicated different groups of terpenes. The major chemical compounds found in their study were thymol (26%), followed by γ -terpinene (20%) and p-cymene (17.6%).

Stefan et al. (2013) analyzed the chemical composition of EO from three plants of the *Ocimum* genus cultivated in Romania. Linalool was also the major compound found in the EO of *O. gratissimum*.

Linalool is an acyclic monoterpene tertiary alcohol, widely used in the cosmetic industry due to its fragrance properties. In addition to fragrance properties, studies have proven its biological activities as an antinociceptive, antileishmanial, and antimicrobial agent (Camargo and Vasconcelos, 2014).

Nguemtchouin et al. (2013) found 32 compounds in the EO extracted from fresh leaves of clove basil plants. In this case, the major compound was thymol (53.9%), followed by γ -terpinene (17.8%). The authors also reported five chemotypes recently identified in clove basil plants, which include eugenol, thymol, citral, linalool, and ethyl cinnamate as major compounds.

According to Kpoviessi et al. (2014), divergence can occur with respect to the chemical composition of EO, and can be associated with various factors, including the vegetative phase

Table 2. Chemical composition of the essential oil obtained from the leaves of *Ocimum gratissimum*.

Peak	Compounds	Area (%)	^b RI calculated	Methods of identification
1	α -Pinene	0.53	937	a,b
2	Camphene	0.46	952	a,b
3	Sabinene	0.60	979	a,b
4	β -Pinene	1.25	985	a,b
5	Myrcene	0.83	992	a,b
6	α -Terpinene	t	1019	a,b
7	Limonene	1.15	1031	a,b
8	1,8-Cineole	21.91	1034	a,b
9	<i>cis</i> -Sabinene hydrate	t	1069	a,b
10	Fenchone	1.34	1089	a,b
11	Linalool	32.95	1101	a,b
12	Camphor	11.97	1147	a,b
13	δ -Terpineole	t	1168	a,b
14	Terpineol 4	0.79	1188	a,b
15	α -Terpineole	3.36	1190	a,b
16	Eugenol	7.42	1359	a,b
17	α -Copaene	t	1376	a,b
18	β -Bourbenene	t	1384	a,b
19	β -Elemene	0.93	1391	a,b
20	<i>trans</i> -Caryophyllene	1.68	1418	a,b
21	α -Bergamotene	1.07	1436	a,b
22	α -Humulene	t	1453	a,b
23	Epi-bicyclosesquiphelandrene	t	1462	a,b
24	Germacrene D	4.76	1480	a,b
25	Bicyclogermacrene	t	1494	a,b
26	δ -Guaiene	t	1504	a,b
27	γ -Cadiene	1.05	1512	a,b
28	δ -Cadiene	t	1523	a,b
29	Cadina 1,4-diene	0.67	1613	a,b
30	α -Cadinol	5.18	1640	a,b
Compound groups (%)				
Monoterpene hydrocarbons		4.82		
Oxygenated monoterpenes		72.30		
Sesquiterpene hydrocarbons		10.16		
Oxygenated sesquiterpenes		5.18		
Phenylpropanoids		7.42		
Total identified compounds (%)		99.87		

Compounds listed in order of elution from HP-5 column. RI: Retention index; t: trace. ^aIdentification based on RI. ^bIdentification based on comparison of mass spectra.

of the plant. In their study, the major compounds of the clove basil EO were cymene, before flowering, and thymol, after flowering.

Edaphoclimatic factors as well as methods of obtaining EOs can directly influence the EO composition. It is known that the soil composition, both chemical and organic, is directly connected to the EO composition owing to the variability of macro- and micro-nutrients available to the plants. Factors such as climate, water availability, crop season, geographic location, and incidence of pathogens can influence the production of chemical compounds of EOs (Ade-Ademilua et al., 2013; Lermen et al., 2015; Urcoviche et al., 2015).

Minimum inhibitory concentration

The antifungal effect of the clove basil EO on the phytopathogenic fungi isolates was expressed in MICs (Table 3). These isolates were chosen due may cause substantially

decrease in yield on several crops, such as, beans, soybeans, tomato, banana, papaya, melon and etc. Some of OE presents antifungal traits, and maybe be used in agriculture, reducing agrochemicals applications, bring to the consumers food more sustainable and with more quality. The clove basil EO had an antifungal effect on all tested isolates; however, the MIC values varied. The antifungal effect was more potent on 2 of the 10 tested fungi, *F. oxysporum* f. sp. *lycopersici* (CMM-1104) and *R. solani* CMM-3274), and both were sensitive at 31.25 µg/mL MIC (Table 3).

Table 3. Minimum inhibitory concentration (MIC) of essential oil of *Ocimum gratissimum*.

Phytopathogenic fungi	MIC (µg/mL)
<i>Fusarium oxysporum</i> f. sp. <i>tracheiphilum</i> (CMM-0033)*	62.5
<i>F. oxysporum</i> f. sp. <i>cubense</i> (CMM-0813)	62.5
<i>F. oxysporum</i> f. sp. <i>cubense</i> (CMM-2819)	62.5
<i>F. oxysporum</i> f. sp. <i>lycopersici</i> (CMM-1104)	31.25
<i>F. solani</i> (CMM-3828)	62.5
<i>Rhizoctonia solani</i> (CMM-3274)	31.25
<i>Macrophomina phaseolina</i> (CMM-2715)	125.0
<i>M. phaseolina</i> (CMM-3875)	125.0
<i>M. phaseolina</i> (CMM-3615)	62.5
<i>M. phaseolina</i> (CMM-3650)	62.5
Positive control - Ketoconazole	500.0

*CMM: Code used for culture collection of pathogenic fungi at Federal Rural University of Pernambuco (UFRPE).

This antifungal effect can be explained by the chemical composition of the EO. Monoterpenes and sesquiterpenes with aromatic rings and phenol groups are capable of forming hydrogen bonds with the active sites of target enzymes, and is the main cause for the antimicrobial effects of the EO. Other compounds such as alcohols, aldehydes, and esters also contribute to the antimicrobial activity (Bellelli et al., 2004). The two major compounds found in the clove basil EO, linalool and 1,8-cineole, have antimicrobial activity as reported earlier in the literature (Pandey et al., 2014). Linalool can cause protein denaturation and dehydration of vegetative cells, which causes the death of microorganisms in contact with the EO. This mechanism is comparable to that of chlorhexidine, an important antimicrobial agent (Camargo and Vasconcelos, 2014). Pattnaik et al. (1997) analyzed the antifungal action of oxygenated monoterpenes and found that linalool showed an inhibitory action on *Candida albicans* at 200 µg/mL MIC, while this yeast was resistant to 1,8-cineole for MIC values up to 5000 µg/mL. Adegoke et al. (2000) assessed the microbial activity of 1,8-cineole on *C. tropicalis* and found inhibitory action at 156 µg/mL MIC.

In this study, the antifungal effect of the EO can be considered very potent against all tested isolates, surpassing even that of the positive control ketoconazole at 500 µg/mL MIC (Table 3). According to Duarte et al. (2005), MIC values up to 500 µg/mL of the tested substance can be considered highly effective. Considering this reasoning, all phytopathogenic fungi tested can be considered highly susceptible to the EO of *O. gratissimum*. This finding is very important for further studies with EO of *O. gratissimum* that can lead to less use of chemical fungicides in agriculture, and produce foods with better quality and that are more sustainable.

Sethi et al. (2013) tested the antimicrobial activity of various EOs from the *Ocimum* genus, all cultivated in the Uttarakhand region, India, on *R. solani* and observed that MIC of EO extracted from *O. gratissimum* was 62.5 µg/mL, twice the value found in this study (31.25 µg/mL).

Philippe et al. (2012) tested the antifungal effect of the EO extracted from fresh

leaves of clove basil cultivated in Benin. The studied isolates were from the genera *Fusarium*, *Penicillium*, and *Aspergillus*. The authors observed total inhibition of all fungal isolates at 1000 µg/mL MIC, but with decreasing concentration, the effectiveness declined proportionally. For the lowest MIC (200 µg/mL), they observed that the fungal inhibition was less than 50%. The authors also determined the chemical composition of the oil and observed that the major compound was thymol (28.1%), followed by γ -terpinene (21.3%).

Joshi (2013) demonstrated that the MIC of the clove basil EO for *Aspergillus* and *Penicillium* was less than 1000 µg/mL. This result affirms the effectiveness of the EO as an inhibitor of the phytopathogenic fungi.

Rizvi et al. (2014) studied the chemical composition and the antifungal activity of the clove basil EO from India. The major compound found in the oil was eugenol (77.8%). The authors also assessed the antifungal effect of the EO on two species of *Penicillium* causing diseases in apple fruits, and observed that both species were inhibited at 500 µg/mL MIC. This also confirms your findings in the present study with 10 others phytopathogens fungal.

CONCLUSIONS

An effective antimicrobial activity on the 10 tested phytopathogens was observed. The MIC of the clove basil EO was equal to or less than 125 µg/mL for the tested isolates. Therefore, it can be concluded that clove basil EO is highly effective as an antifungal agent, showing great potential as an alternative for the control of diseases caused by phytopathogenic fungi.

The EO content of the clove basil leaves was found to be 0.18%. Thirty compounds of the EO were detected via GC-MS, with the predominance of oxygenated monoterpenes. The major chemical compound found in the oil was linalool, followed by 1,8-cineole.

Conflicts of interest

The authors declare no conflict of interest.

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