Journal of Food Research; Vol. 2, No. 5; 2013
ISSN 1927-0887 E-ISSN 1927-0895
Published by Canadian Center of Science and Education

Identification and Quantification of the Major Fungitoxic Components of the Brazilian Basil (*Ocimum basilicum* L.) Essential Oil

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Received: December 29, 2011 Accepted: August 17, 2013 Online Published: September 6, 2013

doi:10.5539/jfr.v2n5p124 URL: http://dx.doi.org/10.5539/jfr.v2n5p124

This research was financed by the Brazilian govt. agencies CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), CNPq (Conselho Nacional de Pesquisa) and FAPEMIG (Fundação de Amparo à Pesquisa do Estado de Minas Gerais)

Abstract

Hydrodistillation of Brazilian basil (*Ocimum basilicum* L.) yielded 0.4% of essential oil (EO), with activity against eight important postharvest deteriorating fungi. The crude EO completely inhibited the radial growth of the fungi at a concentration of 0.1%. Thirty-two components were identified in crude EO by Kováts retention index, mass spectrometry and standards. Preparative TLC-bioautography of the crude EO presented only one band with an antifungal activity greater than that of crude EO with the following chemical composition (%): 1,8-cineole (6.2), linalool (36.3), camphor (9.7), α-terpineol (3.8), methyl chavicol (9.1) and eugenol (34.9). Based on these results, these compounds were considered to be the major fungitoxic components. This is the first study in the literature that presents data obtained simultaneously on the activity of crude basil EO against eight important post-harvest deteriorating fungi, its unequivocal chemical identification and quantification (area %).

Keywords: basil, *Ocimum basilicum*, antifungal activity, fungitoxic components, essential oil

1. Introduction

Synthetic compounds presently used to manage fungi pose a challenge to control food deterioration due to their high toxicity. Hence, alternate control measures are needed and essential oils (EOs) from edible plants appear to be promising (Pawar & Thaker, 2006, 2007; Jardim et al., 2008; Tripathi et al., 2008).

Basil (*Ocimum basilicum* L.) is an annual aromatic herb cultivated in several regions worldwide, being one of the most popular aromatic plants used in food seasoning. Although the basil EO chemical composition has been reported (Oxenham et al., 2005; De Masi et al., 2006; Tchoumbougnang et al., 2006; Gobbo-Neto & Lopes, 2007; Politeo et al., 2007; Shatar et al., 2007; Chalchat & Özcan, 2008; Hussain et al., 2008; Zheljazkov et al., 2008) with its antifungal properties (Basílico & Basílico, 1999; Anthony et al., 2004; Oxenham et al., 2005; Soković & Griensven, 2006; Bozin et al., 2006; Atanda et al., 2007; Herath & Abeywickrama, 2008; Hussain et al., 2008), compounds (antifungals) responsible for the activity have not been isolated. Identification of the major antifungal compounds may lead to the discovery new antifungal compounds.

This study was conducted to assess the activities of crude Brazilian basil (*O. basilicum*) EO and its bioactive fraction (BAF) against the following eight postharvest deteriorating fungi: *Aspergillus flavus*, *A. glaucus*, *A. niger*, *A. ochraceous*, *Colletotrichum gloesporioides*, *C. musae*, *Fusarium oxysporum* and *F. semitectum*. In addition, the chemical compositions of the crude EO, its BAF were unequivocally determined by Kováts retention index (RI), a mass spectrometry database and standards. Components of the crude EO and the BAF were quantified by gas chromatography (GC) (relative area %). Both data (chemical composition and antifungal) were obtained simultaneously with fresh basil EO.

2. Method

2.1 Plant Material and EO Extraction

Shrubs were harvested in Viçosa, Minas Gerais, Brazil and identified by visually comparing freshly collected leaves with existing (registration VIC no 31879) herbalized leaves at the herbarium of Universidade Federal de Viçosa. Immediately after collection, the aerial parts were transported to the laboratory and the EO was extracted. Portions (100 g) of the leaves were mixed with water (1:10 w/v) for hydrodistillation. The distillate (800 ml) was extracted twice with dichloromethane (5:1 v/v). The combined organic phases were dried with anhydrous sodium sulfate and filtered. Dichloromethane was evaporated in a rotary evaporator at 30 ºC under reduced pressure; the EO was weighed, stored at 4 ºC in sealed ampoules, and used within a few days.

2.2 Standards, Solvents, Materials, Reagents, and TLC Plates

Standards (1,8-cineole, linalool, camphor, α-terpineol, methyl chavicol and eugenol) were obtained from Sigma (St. Louis, MO). All organic solvents were of analytical grade (Vetec Química Fina, Rio de Janeiro, RJ, Brazil) and distilled before use. Pre-coated analytical (2 x 20 cm) and preparative thin-layer chromatography (TLC) silica gel plates (20 x 20 cm, 1-mm thick, 60 GF254) were purchased from Merck (Rio de Janeiro, Brazil). Disposable Petri dishes (60 x 15 mm and 90 x 15 mm) and potato-dextrose agar (PDA) were obtained from Prolab (Rio de Janeiro, Brazil).

2.3 Identification and Quantification of the Compounds in the Crude EO

Tentative identification of compounds in the crude EO was based on RI, GC-MS database (Adams, 2007), and by visual comparison of the mass spectra of the crude EO peaks with those stored in the database. RIs were determined on a gas chromatograph (Shimadzu, Kyoto, Japan, model GC 17A) with a flame ionization detector-FID, an auto sampler and a workstation. Gas chromatography-mass spectrometry (GC-MS) data were obtained on a gas chromatograph-mass spectrometer (Shimadzu, Kyoto, Japan, model QP 5000 software program-Classs-5000, Version 1.2) fitted with an auto sampler, workstation and a database (Wiley 229) with about 350,000 entries. Tentative identification was based on the best similarity index (SI), provided by the system software and RIs. Final identification was conducted by comparing the retention time (t_R) and relative retention time (rt_R) (relative to 1,8-cineole) with standards (1,8-cineole, linalool, camphor, *α*-terpineol, methyl chavicol and eugenol). Percentage of each compound was obtained by dividing the area of the peak of interest with that of the total peak area of the identified compounds and multiplying by 100. In addition, confirmation was based on spiking the crude EO with each of the six standards individually. Final identification was conducted on a Varian 8400 chromatograph with an auto sampler and a flame ionization detector. All analyses were conducted on fused silica capillary columns (30 m x 0.25 mm id.; 0.25 μm film thickness) coated with the DB-5 stationary phase (Supelco, Bellefonte, PA). For GC, the following conditions were used: oven temperature was programmed from 60 ºC (1 min hold) to 240 ºC (9 min hold) at a rate of 3 ºC /min; the injector and the detector were maintained at 240 and 250 ºC, respectively. Nitrogen was used as the carrier gas at a flow of 1.3-ml/min. For RI data, a mixture of linear saturated hydrocarbons was co-injected with EOs into the gas chromatograph. RIs were calculated from the following formula: $RI=100y+100(z-y) \times t_{R(x)} - t_{R(y)} / t_{R(z)} - t_{R(y)}$, where y and z are the carbon numbers of the hydrocarbons eluting before and after a peak of interest, respectively; $t_{R(x)}$ is the retention time of the peak of interest; and $t_{R(y)}$, $t_{R(z)}$ are the retention times of the hydrocarbons eluting before and after a peak of interest, respectively.

For GC-MS analysis, the conditions used were as described for GC analysis with the transfer line maintained at 250 ºC with helium as the carrier gas at a flow of 1.3-ml/min. The mass spectrometer was scanned from *m/z* 40 to 350 in the electron impact mode (70 eV). To obtain representative data, the mass spectra over the entire peaks (~10 scans) were grouped and subtracted from the grouped mass spectra of the region closest (before or after) where no compound eluted (~50 scans). Only compounds with SIs of 90%, or over, were considered as positive identifications. A 1-μl aliquot of the EOs dissolved in hexane (10%) was injected in the split mode (split ratio of 20:1) with the split vent being closed for 30 sec.

2.4 Test-Fungi

The fungal isolates used in this study are deposited in the mycological collection of the Department of Phytopathology of the Universidade Federal de Viçosa, Viçosa, MG, Brazil. *A. flavus* Link (Moniliales), strain AFM 4, *A. glaucus* Teleomorph *Eurotium* (Wiggers) Link, strain AGM 9, *A. niger* van Tieghem (Moniliales), strain ANM 6, *A. ochraceous* Wilhelm (Moniliales), strain AOM 18, were isolated from maize seed. *C. gloesporioides* (Penz.) Sacc. (Melanoconiales), strain CGP 14, *C. musae* (Berk. Curt.) Arx (Melanoconiales), strain CMB 6, were isolated from papaya and banana, respectively. *F. oxysporum* Schlecht. Fr. (Moniliales),

strain FSB 7, *F. semitectum* Wollenw (Moniliales), strain FOB 11, were isolated from bean root seed, respectively.

2.5 Antifungal Activity of the Crude EO

Antifungal activities were tested on PDA, using poison food assay (Dhingra & Sinclair, 1995). A uniform dispersion was obtained by dissolving the test material in methanol (1:1 v/v), followed by adding cool molten PDA to obtain EO concentrations of 0.1, 0.09, 0.08, 0.075 and 0.05%. Ten-ml of the medium were poured into 9-cm culture plates. The medium in each plate was spot-seeded with the conidia (10^3/ml) of each fungus. Colony diameter was measured on the 6th day after incubation at 25 ºC. All tests were performed in triplicate. Percent of mycelia growth inhibition was calculated according to the equation: Inhibition (%) = dc - dt/dc x 100, where dc and dt are mycelia growth diameters (mm) in the control and treated samples. The data were analyzed by ANOVA and the means compared by the Tukey test (p = 0.05). The control consisted of PDA containing an equivalent amount of methanol with the EO.

2.6 Isolation of the BAF and Determination of Its Chemical Composition and Antifungal Activities

TLC-bioautography (Rahalison et al., 1991; Wedge & Nagle, 2000) was used to obtain the BAF of the crude EO. Ninety milligrams of the crude basil EO were placed on each of the nine preparative pre-coated silica gel TLC plates and eluted with dichloromethane: hexane (8:2) as solvent. After elution, the plates were air dried, and sprayed with 50 ml of molten PDA containing 100 mg/l streptomycin sulfate and conidia (10^3/ml) of the fungi separately. This was followed by covering the tray with a plastic film and incubation for 6 days at 25 ºC. The blank was prepared in exactly the same way without the EO. The region of the TLC plates without fungal growth (bioactive fraction) was delineated with a pencil, scrapped and extracted twice by shaking for two hours with 200 ml of dichloromethane. The combined extracts were filtered, dried over anhydrous sodium sulfate, and evaporated under a reduced pressure to obtain a residue which was weighed.

The antifungal activity and the chemical composition of the BAF were determined as described for the crude EO.

2.7 Statistical Analysis

Percent radial growth inhibition reported is the average of three replications. The data were analyzed by ANOVA and the results statistically evaluated by the Tukey test (P = 0.05).

3. Results

3.1 Essential Oil Yield

Hydrodistillation of the *O. basilicum* leaves yielded 0.4% EO (fresh weight basis) (Table 1).

3.2 Chemical Composition of the Crude EO and Its Antifungal Activity

The crude EO presented the following percentage composition of the six major compounds (elution order) corresponding to 81.4% of the total chromatographic peak area: 1,8-cineole (16.6), linalool (28.0), camphor (10.7), eugenol (18.4), germacrene D (4.6) and *α*-murolol (3.1) (Tables 1 and 2, Figure 1A).

Of the remaining area, 15.8% was made up by twenty-six constituents with relative concentration of less than 3% and the remaining compounds (2.8% of the area) could not be identified by the mass spectral database. Of the thirty-two compounds (97.2% of the total chromatographic peak area) tentatively identified, the presence of six compounds (1,8-cineole, linalool, camphor, *α*-terpineol, methyl chavicol and eugenol), corresponding to 77.5% of the total chromatographic peak area, was confirmed by commercially available standards.

Sensitivity to the crude EO varied with the fungal species. The crude EO completely inhibited the radial growth of all the eight fungi at a concentration of 0.1%. At other concentrations, the activity depended on the fungus. For instance, at a concentration of 0.09%, inhibition of *A. flavus*, *A. glaucus*, *A. niger, A. ocrhaceous, C. gloesporioides*, *C. musae*, *F. oxysporum, F. semitectum* was 72, 90, 78, 78, 100, 96, 95 and 95%, respectively. At concentrations of 0.80, 0.075 and 0.05%, the inhibition decreased consistently for all fungi.

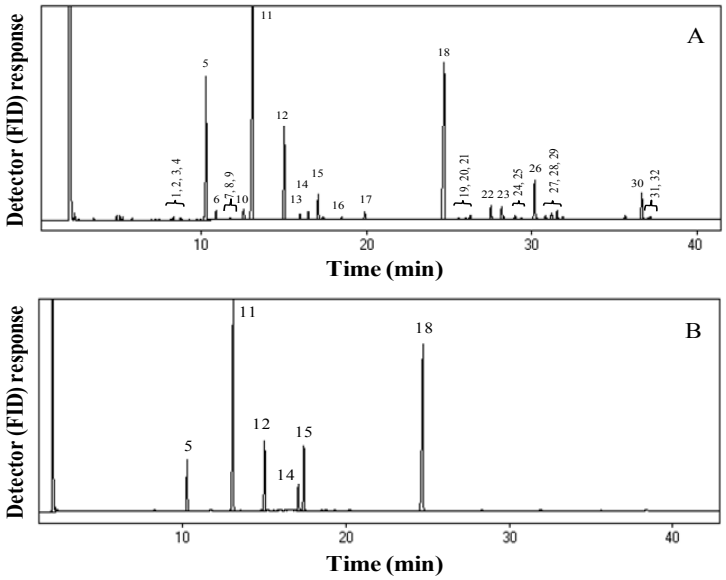

Figure 1. Gas chromatograms obtained on analysis of crude EO (A) and the bioactive fraction (B) of the Brazilian *Ocimum basilicum* essential oil. Chromatographic conditions were: oven temperature programmed from 60 ºC (1 min hold) to 240 ºC (9 min hold) at 3 ^{o}C min^{-1}; N_2 carrier gas (1.3 ml min^{-1}), temperatures of the injector and the detector 220 and 240 ºC, respectively, with a fused silica capillary column coated with the DB-5 stationary phase. Peaks 1-32 identified in the chromatograms refer to the compounds listed in Table 2

Table 1. Percentage yields and compositions of the major components of basil essential oil (EO) reported from some countries

Country	Yield (%)	Composition (%) of the major components	Reference/Comment
Serbia Montenegro	0.37	Chavicol (45.8) and linalool (24.2)	Bozin et al., 2006
Italy	Not reported	Linalool (33.5 to 63.6) for all cultivars, except one with 6.2% and eugenol (0.3 to 17.9)	De Masi et al., 2006
Several countries from Africa	0.02-1.3	Linalool (46.0), limonene (17.1), 1,8-cineole (8.5), terpin-4-ol (3.6) (Cameroon)	Tchoumbougnang et al., 2006
Brazil (northeastern state of Ceara)	3.0	Linalool (42.5), methyl chavicol (33.1) and 1,8-cineole (11.0)	Trevisan et al., 2006
Brazil (northeastern state of Sergipe)	Not reported	Linalool (69.3), eugenol (10.8) and *α-trans*-bergamotene (7.7)	Almeida et al., 2007
Brazil (southeastern state of Minas Gerais)	Not reported	Linalool (47.0), 1,8-cineole (15.0) and camphor (9.5)	Santoro et al., 2007
Turkey	1.0	Methyl chavicol (52.6), limonene (13.6), *exo*-fenchyle acetate (11.0), fenchone (5.7), *α*-phellendrene (4.1) and *p*-cymene (2.3)	Chalchat & Özcan, 2008
Pakistan	0.5-0.8	Linalool (56.7–60.6%), *epi*-α-cadinol (8.6–12.4%), α-bergamotene (7.4–9.2%) and γ-cadinene (3.2–5.4%)	Hussain et al., 2008
Brazil (southeastern state of Minas Gerais)	0.4	1,8-cineole (16.6), linalool (28), camphor (10.7) and eugenol (18.4)	Results of this study with the presence of all the four major components confirmed by standards

Table 2. Percent composition of the crude essential oil (EO) of the Brazilian basil (*Ocimum basilicum*) and the bioactive fraction (BAF) based on Kováts retention index (RI), mass spectrometry database and a visual comparison of the mass spectra of the sample peaks with those stored in the mass spectral database along with authentic standards

Peak no. (Figure 1)	Retention index (RI)	Compounds	Composition (%)	
			Crude EO	BAF
1	977	Sabinene	0.1	-.
2	979	*β*-Pinene	0.2	-
3	981	Octen-3-ol	0.3	-
4	992	Myrcene	0.2	-
5	1035	1,8-Cineole*	16.6*	6.2
6	1051	(*E*)-*β*-Ocimene	1.0	-
7	1063	*γ*-Terpinene	0.1	-
8	1070	*Cis*-sabinene hydrate	0.1	-
9	1072	Octanol	0.2	-
10	1089	Fenchone	1.2	-
11	1100	Linalool*	28.0*	36.3
12	1151	Camphor*	10.7*	9.7
13	1174	Terpin-4-ol	0.6	-
14	1186	*α*-Terpineol*	0.9*	3.8
15	1198	Methyl chavicol*	2.9*	9.1
16	1259	Chavicol	0.9	-
17	1291	Isobornyl acetate	0.1	-
18	1365	Eugenol*	18.4*	34.9
19	1386	*α*-Copaene	0.2	-
20	1395	*β*-Bourbonene	0.2	-
21	1402	*β*-Elemene	0.5	-
22	1429	(*E*)-Caryophyllene	1.6	-
23	1444	*α*-*Trans*-Bergamotene	1.5	-
24	1447	*α*-Guaiene	0.3	-
25	1462	*α*-Humulene	0.5	-
26	1485	Germacrene D	4.6	-
27	1498	Bicyclogermacrene	0.4	-
28	1516	*γ*-Cadinene	1.0	-
29	1526	*Δ*-Cadinene	0.3	-
30	1645	*α*-Murolol	3.1	-
31	1648	(*Z*)-Methyl jasmonate	0.3	-
32	1658	Bulnesol	0.3	-
Total identified			97.2 (77.5*)	100.0

*Confirmed by standards.

3.3 Chemical Composition of the BAF and Its Antifungal Activity

The BAF isolated from the crude EO presented the following % composition, corresponding to 100% of the total chromatographic peak area: 1,8-cineole (6.2), linalool (36.3), camphor (9.7), *α*-terpineol (3.8), methyl chavicol (9.1) and eugenol (34.9) (Figure 1B).

The sensitivity to the purified EO fraction varied with the fungal species. The crude EO completely inhibited the radial growth of all the eight fungi at concentrations of 0.1 and 0.09%. However, at other concentrations, the activity varied with concentration. For instance, at a concentration of 0.080%, percentage inhibition was almost complete for all the fungi studied.

4. Discussion

Yields and percentage compositions of the major components of basil EO from several countries are reported in Table 1. As it can be noted, the EO yields obtained in this study were different from that reported in studies in other countries, including from Brazil. EO yield (0.4%) obtained in this study was much lower than the 3% yield reported in one Brazilian study (Trevisan et al., 2006) while yields were not reported in the other two studies in Brazil (Almeida et al., 2007; Santoro et al., 2007). Variations in EO yield and composition were expected as they are affected by several factors such as genotype, agro climatic conditions, solar irradiance level, etc. (Gobbo-Neto & Lopes, 2007; Hussain et al., 2008; Chang et al., 2008).

A typical gas chromatogram obtained on analysis of the crude EO and the BAF is presented in Figure 1. The chemical composition was quite different from that reported from other countries (Tables 1 and 2), including Brazil (Trevisan et al., 2006; Almeida et al., 2007; Santoro et al., 2007). In all the Brazilian studies, except ours, identification of the EO components was not confirmed by standards and, hence, not reliable. It is interesting to note that all the compounds identified tentatively in this study were confirmed by standards. This is the first study in the literature where the crude EO was fractionated, identified and quantified.

Chemical compositions of the basil EOs reported in the literature were based only on RI and on a mass spectral database without standards (Herath & Abeywickrama, 2008). However, standards were used for confirmation in a few studies (Oxenham et al., 2005; Zheljazkov et al., 2008). While identification based on RI and mass spectral databases is very widely used for volatiles (Adams, 2007), it is well known that it is tentative and must be confirmed by standards.

In our study, we obtained a good antifungal activity against eight post-harvest fungi, with activity being shown for the first time in a few cases. Antifungal activity of basil EO has been reported by other authors. Complete inhibition of *A. ochraceous* was reported at a concentration of 0.1% (Basílico & Basílico, 1999). Basil EO and several of its pure components (methyl chavicol, linalol, eugenol and caryophyllene) presented activity against *Botrytis fabae* and *Uromyces fabae* (Oxenham et al., 2005). An emulsion spray (0.16%) of the Indian basil was effective in controlling crown rot in banana (Herath & Abeywickrama, 2008). Basil EO showed an inhibitory effect on *A. niger* (Pawar & Thaker, 2006), *F. oxysporum* (Pawar & Thaker, 2007), *A. flavus and A. ochraceous* (Soliman & Badeaa, 2002). However, the chemical composition of the EO was not reported in these studies. *O. basilicum* oil containing linalool (69.3%) showed activity against *Verticillium fungicola* and *Trichoderma harzianum* at concentrations varying between 1.5-2.0% (Soković & Griensven, 2006). On the other hand, basil EO containing methyl chavicol (45.8%) and linalool (24.2%) as the major constituents showed a weak antimicrobial activity (Bozin et al., 2006). *A. niger*, *Mucor mucedo*, *F. solani*, *Botryodiplodia theobromae*, *Rhizopus solani* were affected by linalool (Hussain et al., 2008). Sensitivity of *A. flavus* and *A. ochraceous* to basil EO is of particular interest since they produce potentially carcinogenic mycotoxins in grain and feed they colonize. This EO can be a good candidate for use in such products, being considered safe for human consumption when appropriately used (Fandohan et al., 2008).

Major fungitoxics of the basil EO have not been isolated nor characterized. In this study, it was possible to quickly identify, for the first time, the major fungitoxics in the BAF using TLC-bioautography. In this technique, a crude EO is separated by conventional preparative TLC and then sprayed with a fungal suspension of interest followed by incubation for several days. “Clear zones” on the sprayed TLC plates, which suggest presence of antifungals, are scrapped from the plate, extracted by an organic solvent and identified. Despite being a convenient technique offering a great potential, TLC-bioautography is little used. Through this technique we have identified and quantified the following major fungitoxics in the crude EO: 1,8-cineole, linalool, camphor, α-terpineol, methyl chavicol, and eugenol. However, the participation of other compounds cannot be excluded.

5. Conclusion

This is the first study in the literature where the chemical composition of the Brazilian basil EO was unequivocally determined simultaneously with antifungal activity and characterization of the BAF. The information generated in this study will be useful for further studies in our laboratory as well as to laboratories worldwide.

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