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Chemical Composition and Fungicidal Effects of *Ocimum basilicum* Essential Oil on *Bipolaris* and *Cochliobolus* Species

E. A. Elsherbiny[1*], A. Y. El-Khateeb[2], and N. A. Azzaz[3]

ABSTRACT

The fungus *Cochliobolus* is the teleomorph of *Bipolaris* and *Curvularia* which are economically important plant pathogens worldwide. Several species of *Bipolaris* are well documented human pathogens. The aim of the present study was to evaluate the efficacy of the essential oil of *Ocimum basilicum* against some *Bipolaris* and *Cochliobolus* species. Sixteen compounds, representing 95.4% of the chemical components of the essential oil of *Ocimum basilicum*, were identified by Gas Chromatography–Mass Spectrometry (GC-MS). The main compounds were estragole (55.95%), 1,8-Cineole (10.56%), methyl eugenol (10.09%) and linalool (5.57%). Aromatic oxygenated monoterpenes (57.42%) were the dominant constituents of the essential oil followed by oxygenated monoterpenes (16.13%) and sesquiterpene hydrocarbons (6.9%). The essential oil exhibited a complete inhibition of the growth of *Bipolaris ellisii, Bipolaris hawaiensis*, *Bipolaris spicifera, Cochliobolus australiensis* and *Cochliobolus cynodontis* at 80 mg/mL and fungicidal effect on *Cochliobolus australiensis* only at the same concentration after six and twelve days of exposure. Spore germination and germ tube elongation of *B. hawaiensis* were completely inhibited by the essential oil (at 40 mg/mL) and *B. spicifera* (at 80 mg/mL) with minimum inhibitory concentration (MIC) values ranging from 40 to 160 mg/mL. These results suggest that the essential oil of *Ocimum basilicum* is a potential and promising antifungal tool for controlling plant and human fungal pathogens.

Keywords: GC–MS, Antifungal tools, Plant pathogens

INTRODUCTION

The genus *Cochliobolus* and its asexual states *Bipolaris* and *Curvularia* are worldwide pathogens of economically important crops and grasses associated with over 60 host genera (Manamgoda *et al.,* 2011). *Bipolaris* is dematiaceous filamentous fungi and relatively common with more than 100 species described (Crous *et al.,* 2004). The teleomorphic form, *Cochliobolus*, is extremely rare in nature and, thus, the anamorphic form, *Bipolaris*, causes infection in the fields (Worapattamasri *et al.,* 2009). Some examples of severe diseases caused by *Cochliobolus* and *Bipolaris* are black kernel of rice, root rot and leaf spot of wheat, eyespot and brown stripe of sugarcane, and southern leaf blight of maize (Borrás-Hidalgo *et al.,* 2005; Kumar *et al.,* 2007). Furthermore, several species of *Bipolaris* have been reported to cause several human diseases, including fungal sinusitis, hay fever, asthma, keratitis, lung mass and subcutaneous lesions, central nervous system infection and disseminated infection (Washburn *et al.,* 1988; Fothergill, 1996; Buzina *et al.,* 2003; Saha

[1] Plant Pathology Department, Faculty of Agriculture, Mansoura University, Mansoura 35516, Egypt.
[*] Corresponding author; e-mail address: sherbiny44@hotmail.com
[2] Agricultural Chemistry Department, Faculty of Agriculture, Mansoura University, Mansoura 35516, Egypt.
[3] Agricultural Chemistry Department, Faculty of Agriculture, Damietta University, Damietta, Egypt.

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and Das, 2005; Kobayashi *et al.,* 2008; Revankar and Sutton, 2010).

The *Ocimum* is the largest genus of the Lamiaceae family with more than 150 species native to the tropical and subtropical regions of Africa, Asia, and South America (Sajjadi, 2006). Among these species, basil, *Ocimum basilicum* is considered as the major essential oil crop and is cultivated commercially in different regions all over the world (Runyoro *et al.,* 2010). All parts of basil plants were used in folk medicine for treatment of cold, coughs, as a sedative, and for eliminating toxins (Sharafati-Chaleshtori *et al.,* 2015). Also, basil is used in making flavoring and perfume (Telci *et al.,* 2006).

Therefore, the present study was performed to examine the chemical composition of the essential oil of *O. basilicum* by GC–MS and to evaluate the potential antifungal activities of the essential oil against *B. ellisii*, *B. hawaiensis*, *B. spicifera*, *C. australiensis,* and *C. cynodontis*, fungal pathogens of economically important plants and humans.

MATERIALS AND METHODS

Plant Material

The leaves of *O. basilicum* were collected in Mansoura, Egypt (latitude 31° 3′ 0″ N, longitude 31° 23′ 0″ E, temperature 24-30 °C, loam soil) from plants growing in the Mansoura University campus in July 2013. The taxonomic identification of plants was confirmed at the Botany Department, Faculty of Agriculture, Mansoura University, where a voucher specimen has been deposited.

Extraction of the Essential Oil

The air-dried leaves (1.5 kg) of *O. basilicum* were subjected to hydrodistillation using a Clevenger-type apparatus for 4 hours. The oily layer obtained on top of the aqueous distillate was separated and dried with anhydrous sodium sulfate (Na_2SO_4). The extracted essential oil was kept in sealed air-tight glass vials and covered with aluminum foil at 4 °C until used for GC–MS analysis and biological activity tests. The yield of the essential oil was 24.25% (v/w).

Gas Chromatography–Mass Spectrometry (GC-MS) Analysis

Analysis of the essential oil was performed using Agilent 6890 gas chromatography equipped with an Agilent mass spectrometric detector, with a PAS-5MS fused silica capillary column (30 m × 0.32 mm × 0.25 µm film thickness). The oven temperature was initially 40 °C, increased at a rate of 8 °C /min to 280 °C. The injection port temperature was 250 °C and the detector temperature was 280 °C. The carrier gas was helium, at a ratio of 1 mL/min. Diluted sample of 1 µL was manually injected in the splitless mode. EI mass spectra were collected at 70 eV ionization voltages over the range of m/z 50-500. The electron multiplier voltage was 1250 V. The ion source and quadruple temperatures were set at 230 and 150 °C, respectively. The components of essential oil were identified tentatively by comparing their relative retention times and mass spectra with those of WILEY and NIST 05 mass spectral database.

Fungal Isolates

B. ellisii CBS 193.62, *B. hawaiensis* AUMC 1120, *B. spicifera* AUMC 459, *C. australiensis* AUMC 1384, and *C. cynodontis* AUMC 2393 were obtained from Assiut University Mycological Centre (AUMC), Egypt and Centraalbureau voor Schimmelcultures (CBS), Utrecht, Netherlands. Cultures of fungal species were maintained on potato dextrose agar (PDA) slants and stored at 4 °C.

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Effects of the Essential Oil of Basil on Fungal Growth

Contact Phase Effect

The essential oil was dissolved in dimethyl sulfoxide (DMSO)-Tween 80 (1% v/v) and added to the Petri dishes (90 mm diameter) containing PDA at 40-45 °C to obtain final concentrations of 10, 20, 40, and 80 mg/mL. PDA plates containing DMSO-Tween 80 (1% v/v) only served as a control. A disc of 5 mm in diameter of each fungal strain was placed in the center of the petri dishes. The plates were incubated at 25±2 °C until the growth in the control plates reached the edges of the plates. All treatments were performed in triplicate and the experiment was repeated twice. The relative growth inhibition of the treatments compared to the control was calculated by percentage, using the following formula:

Inhibition % = [1 - (mycelial growth of treatment / mycelial growth of control)] × 100

Fungistatic and Fungicidal Effects

To examine a difference between fungistatic or fungicidal effects of the essential oil on the fungal isolates, an agar discs of fungal isolates, which failed to grow, was transferred onto fresh PDA media without essential oil to assess their viability after three, six, and twelve days of exposure and grown at 25±2 °C for 7 days. Activity of each treatment of the essential oil was considered fungicidal if the fungus did not grow, or fungistatic if the fungus growth began again. Mean of growth values were calculated from three replicates for each treatment as described before as well as the inhibition percentage of mycelial growth. The experiment was conducted twice.

Effects of the Essential Oil on Conidial Germination and Germ Tube Elongation

Spore suspension (1×10^6 spores/mL) of each *Bipolaris* sp. isolate was prepared from actively growing 10 d old culture on PDA in distilled sterile water. A 20 µL aliquots of spore suspension drops were spread onto the surface of PDA medium supplemented with different concentrations of essential oil (10, 20, 40, and 80 mg/mL) dissolved in dimethyl sulfoxide (DMSO)-Tween 80 (1% v/v). PDA plates, containing DMSO-Tween 80 (1% v/v) only, were used as a control. After 24 h of incubation at 25±2 °C, at least 100 spores in each replicate were observed microscopically to determine germination rate and germ tube length. Germination was defined as the point at which the germ tube length equaled or exceeded the spore diameter. The percent inhibition was calculated as follows:

Inhibition % = [(Gc – Gt) /Gc)] × 100

Where, Gc and Gt represent the mean number of germinated conidia in the control and treated plates, respectively.

Minimum Inhibitory Concentration (MIC)

An appropriate quantity of essential oil was dissolved in DMSO-Tween 80 (1% v/v) and incorporated in PDB (potato dextrose broth) to produce the final concentrations of 10, 20, 40, 80, and 160 mg/mL. A 10 µL of spore suspension (1×10^6 spores/mL) of each *Bipolaris* sp. isolate was inoculated in the test tubes in PDB medium and incubated at 25±2 °C. The control tubes containing PDB medium were inoculated with fungal spore suspension and DMSO-Tween 80 (1% v/v). The lowest essential oil concentration that did not permit any visible fungal growth was defined as the MIC.

Statistical Analysis

All data were statistically evaluated with analysis of variance (one-way ANOVA) procedures of SAS (version 9.1, SAS Institute, Cary, NC, USA). Treatments means were compared using Tukey's HSD test ($P < 0.05$).

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RESULTS

Chemical Composition of the Essential Oil of Basil

GC–MS analysis of the essential oil led to the identification of 16 different compounds, representing 95.4% of the total oil. The identified components with their percentages, retention times, and molecular formulas are listed in Table 1. Estragole or methyl chavicol (55.95%) was the main constituent of the essential oil of basil leaves. 1,8-Cineole (10.56%) was the second major constituent detected in the oil followed by methyl eugenol (10.09%). Linalool (5.57%) and *(Z,E)*-α-farnesene (4.45%) were also identified in the oil. The essential oil of basil contains a complex mixture consisting of mainly aromatic oxygenated monoterpenes (57.42%) and oxygenated monoterpenes (16.13%).

Effect on Mycelial Growth of *Bipolaris* sp. and *Cochliobolus S*p. Isolates

The essential oil of basil had a significant activity and inhibited the mycelial growth of all isolates in a dose dependent manner (Figure 1). The essential oil exhibited a complete inhibition of mycelial growth of all strains at 80 mg/mL. *B. ellisii* was found to be the most sensitive to the essential oil (Figure 1). After growth inhibition of *Bipolaris* sp. and *Cochliobolus* sp. isolates had been established with essential oil, the mycelial discs were transferred onto PDA medium without the essential oil. Essential oil of basil caused irreversible inhibition e.g., fungicidal effect on *C. australiensis* only, at the concentration of 80 mg/mL, after six and twelve days of exposure.

Table 1. Chemical composition of essential oil of *Ocimum basilicum*.

No.	Rt [a]	Compound [b]	Peak area (%)	Molecular formula
1	5.44	α-Pinene	0.39	$C_{10}H_{16}$
2	6.26	β-Pinene	0.27	$C_{10}H_{16}$
3	7.43	1,8-Cineole	10.56	$C_{10}H_{18}O$
4	8.97	Linalool	5.57	$C_{10}H_{18}O$
5	11.21	Estragole (methyl chavicol)	55.95	$C_{10}H_{12}O$
6	11.80	Carvone	0.78	$C_{10}H_{14}O$
7	13.43	Eugenol	0.37	$C_{10}H_{12}O_2$
8	13.95	Methyl cinnamate	0.32	$C_{10}H_{10}O_2$
9	14.39	Methyl eugenol	10.09	$C_{11}H_{14}O_2$
10	14.81	(Z,E)-α-Farnesene	4.45	$C_{15}H_{24}$
11	15.53	Longiborneol	0.33	$C_{15}H_{26}O$
12	16.06	α-Amorphene	0.50	$C_{15}H_{24}$
13	16.26	γ-Muurolene	0.48	$C_{15}H_{24}$
14	17.12	β-Caryophyllene oxide	0.42	$C_{15}H_{24}O$
15	18.01	α-Selinene	1.47	$C_{15}H_{24}$
16	20.21	Octadecane	3.45	$C_{18}H_{38}$
		Total identified	95.40	
		Monoterpene hydrocarbons	0.66	
		Oxygenated monoterpenes	16.13	
		Aromatic oxygenated monoterpenes	57.42	
		Sesquiterpene hydrocarbons	6.90	
		Oxygenated sesquiterpenes	0.75	
		Other constituents	13.54	

[a] Rt, retention time (min). [b] Compounds are listed in the order of their elution.

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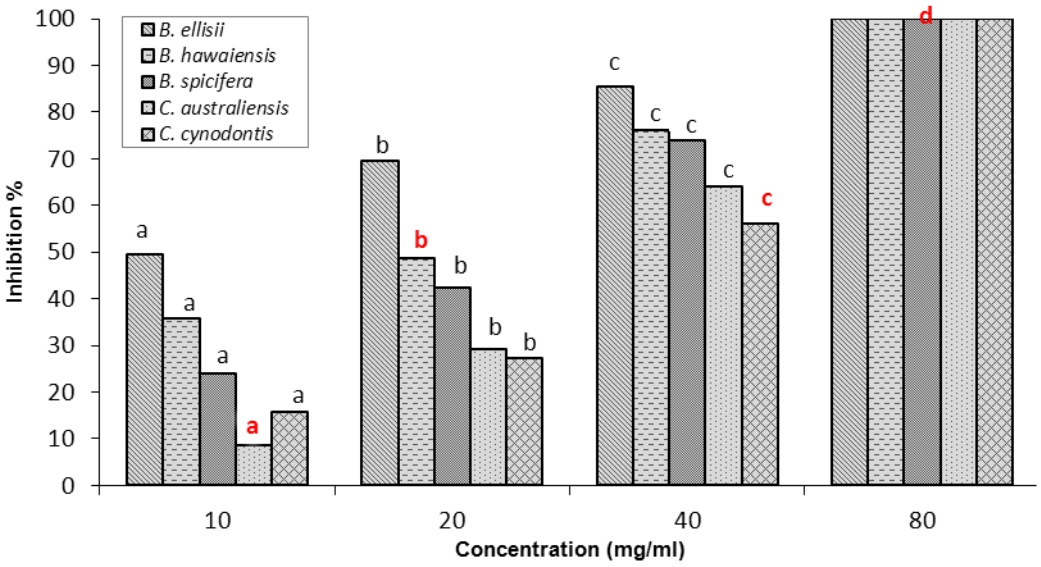

Figure 1. Effect of different concentrations of *Ocimum Basilicum* oil on mycelial growth of *Bipolaris* sp. and *Cochliobolus* sp. isolates. Bars, for each fungus, with different letters represent values that are significantly different according to Tukey's HSD test at $P < 0.05$.

Effect on Conidial Germination and Germ Tube Elongation of *Bipolaris* Sp. Isolates

There was a significant inhibition of fungal spore germination and germ tube length by different concentrations of the essential oil of basil after 24 h of incubation (Figure 2, Table 2). Complete inhibition of conidial germination and germ tube elongation by essential oil was observed for *B. hawaiensis* at 40 mg/mL and for *B. spicifera* at 80 mg/mL. Strong inhibition of

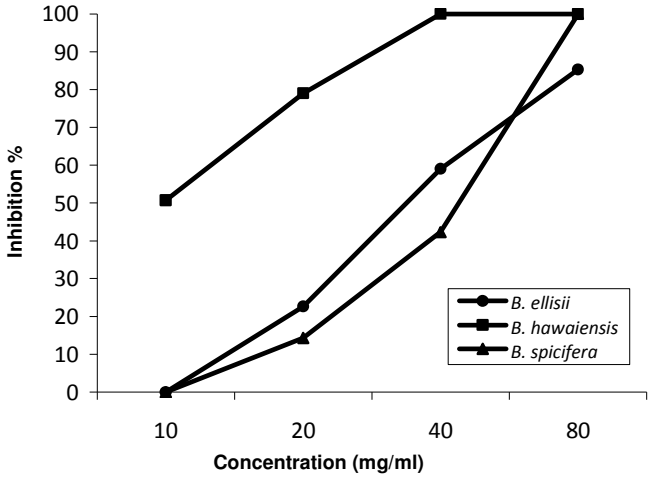

Figure 2. Effect of different concentrations of *Ocimum basilicum* oil on conidial germination of *Bipolaris* sp. isolates.

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Table 2. Effect of different concentrations of *Ocimum basilicum* oil on germ tube elongation of *Bipolaris* sp. isolates.

Concentration (mg/ml)	Germ tube elongation (µm)		
	Bipolaris ellisii	*Bipolaris hawaiensis*	*Bipolaris spicifera*
0	ND [a]	ND	ND
10	94.0 ± 1.2 a	15.3± 0.8 a	53.3 ± 0.8 a
20	61.7 ± 1.2 b	11.6 ± 0.3 b	19.7 ± 1.2 b
40	32.3 ± 1.3 c	0 ± 0 c	12.6 ± 0.9 c
80	18.7 ± 0.9 d	0 ± 0 c	0 ± 0 d

[a] ND, not detected; the germ tubes were very long and entwined each other so they could not be measured. Germ tube elongation was measured after 24 h of incubation at 25±2°C onto PDA. Mean values ± SE in the same column followed by a different letters are significantly different according to Tukey's HSD test at $P < 0.05$.

spore germination of *B. ellisii,* by 85.3% was also detected.

MIC of Essential Oil of Basil

The minimum inhibitory concentration (MIC) defined as the lowest concentration of the basil oil that resulted in complete growth inhibition of *B. hawaiensis*, *B. spicifera,* and *B. ellisii* were found to be 40, 80, and 160 mg/mL, respectively. *B. ellisii* displayed less susceptibility to the essential oil of basil.

DISCUSSION

Our GC-MS analysis revealed that estragole or methyl chavicol was the major compound in the basil oil. This is largely in agreement with other published results (Loapez *et al.,* 2005; Sajjadi, 2006; Chalchat and Ozcan, 2008; Carovic-Stanko *et al.,* 2010; Sienkiewicz *et al.,* 2013). Moreover, previous studies have reported that estragole (methyl chavicol) was the main component of the essential oil of other *Ocimum* species such as *O. selloi* (Paula *et al.,* 2003) and *O. sanctum* (Khan *et al.,* 2010). On the contrary, several researches showed that linalool was the major compound of the essential oil of basil growing in different regions of the world (Sokovic and Griensven, 2006; Hussain *et al.,* 2008; Carovic-Stanko *et al.,* 2010; Vieiraa *et al.,* 2014), whereas, 1,8-cineole (54.3%) was the major component of basil oil according to Runyoro *et al.* (2010). These great variations in the chemical composition of basil oil could be due to many factors including geographical area, climate conditions, soil characteristics, nutritional status of the plants, plant age, plant part, season, harvesting period, methods of extracted essential oil and different chemotypes.

The results of this study showed that the mycelial growth of all strains was completely inhibited by the essential oil of basil at higher concentration (80 mg/mL). Also, 100% inhibition of fungal spore germination was observed in all *Bipolaris* sp. Isolates, except *B. ellisii* (85.3% inhibition) at the same concentration of basil oil. These results are in agreement with those of Feng and Zheng (2007) who demonstrated the effect of five essential oils (thyme, sage, nutmeg, eucaptus and cassia) against mycelial growth, spore germination, and germ tube elongation of *Alternaria alternata*. A similar result was found by Soylu *et al*. (2010) who investigated the essential oils of origanum, lavender, and rosemary on the growth, conidial germination, and germ tube elongation of *Botrytis cinerea*, the causal agent of grey mould disease of tomato. To the best of our knowledge, there is no report found in the literature on the antifungal activity of the essential oil of basil against *Bipolaris* and *Cochliobolus* species. Nevertheless, several

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literature data showed that the basil oil have a stronger antifungal activity against different species of fungi such as *Penicillium islandicum* and *Aspergillus flavus* (Loapez *et al.,* 2005), *Botrytis fabae* and *Uromyces fabae* (Oxenham *et al.,* 2005), *Verticillium fungicola* and *Trichoderma harzianum* (Sokovic and Griensven, 2006), *Aspergillus parasiticus* CFR 223 and aflatoxins produced *in vitro* (Atanda *et al.,* 2007), *Aspergillus niger*, *Mucor mucedo*, *Fusarium solani*, *Botryodiplodia theobromae* and *Rhizopus solani* (Hussain *et al.*, 2008). It has also been reported that the basil oil has antibacterial activity on a number of Gram-negative bacteria (*Escherichia coli, Enterobacter cloacae, Yersinia enterocolitica, Salmonella choleraesuis, Klebsiella pneumoniae* and *Pseudomonas aeruginosa*) and Gram-positive bacteria (*Staphylococcus aureus, Bacillus cereus, Enterococcus faecalis, Streptococcus viridans* and *Listeria monocytogenes*) (Loapez *et al.,* 2005; Hussain *et al.,* 2008; Carovic-Stanko *et al.,* 2010; Stefan *et al.,* 2013).

Based on the results of chemical composition of the essential oil of basil, it is possible to conclude that the higher percentage of aromatic oxygenated monoterpenes (57.42%) could be responsible for higher antifungal activity. In this context, Carovic-Stanko *et al.* (2010) indicated that the antimicrobial activity could be induced by the major compounds of the essential oil or due to a synergistic effect between the major compounds and the minor ones. Results from different studies (Burt, 2004; Bajpai and Kang, 2012; Hyldgaard *et al.,* 2012) revealed that the bioactive compounds in the essential oils are oxygenated terpenoids (e.g., alcohols and phenolic terpenes), while some hydrocarbons also exhibit antimicrobial activities. A few studies pointed to the mechanisms of action of active compounds in essential oil on the microbial cell. For instance, the presences of monoterpene alcohols increase the permeability of the plasma membrane and inhibit the respiration on mitochondrial membrane of fungi (Cox *et al.,* 2000; Imelouane *et al.,* 2009), while Lucini *et al.* (2006) found that the presence of monoterpenes at low concentrations could increase the concentration of lipidic peroxides such as hydroxyl, alkoxyl and alkoperoxyl radicals and so bring about cell death. Conner and Beuchat (1984) suggested that the antimicrobial activity of the essential oils or their compounds could be the result of damage to enzymatic cell systems, including those associated with energy production and synthesis of structural compounds. Additionally, the oil of *O. basilicum* var. *purpurascens* (estragol chemotype) is suspected to be carcinogenic and genotoxic (Heberer *et al.* 2007).

In conclusion, the present results demonstrate that the essential oil of basil is an effective antifungal agent *in vitro* against mycelial growth, spore germination, and germ tube elongation of *B. ellisii*, *B. hawaiensis*, *B. spicifera*, *C. australiensis* and *C. cynodontis*. Therefore, these results concluded that the basil essential oil could be used as a natural source for possible applications in controlling fungal plant pathogens, alternative to synthetic fungicides and some medical industries.

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ترکیب شیمیایی واثرات قارچ کشی اسانس *Ocimum basilicum* روی گونه های *Bipolaris* و *Cochliobolus*

ا. ع. الشریینی، ا. ی. الخاطب، و ن. ع. عزاز

چکیده

قارچ *Cochliobolus* تلئومورف *Bipolaris* و *Curvularia* است که پاتوژن های گیاهی مهم اقتصادی در سراسر جهان هستند. همچنین، چندین گونه از *Bipolaris* از شمار پاتوژن های انسانی

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مستند شده می باشند. هدف پژوهش حاضرارزیابی موثر بودن اسانس گیاه ریحان (*Ocimum basilicum*) بر علیه بعضی گونه های *Bipolaris* و *Curvularia* بود. با تجزیه شیمیایی به وسیله کروماتوگرافی گازی-اسپکترومتری جرمی(GC-MS)، ۱۶ ماده که ۹۶٪ ترکیبات شیمیایی اسانس *Ocimum basilicum* را تشکیل می دادند شناسایی شد. مواد اصلی شناسایی شده عبارت بودند از:estragole (۵۵/۹۵٪)، 1,8-Cineole (۱۰/۵۶٪)، methyl eugenol (۱۰/۰۹٪)، وlinalool (۵/۵۷٪). ترکیب اصلی مواد سازنده اسانس ریحان مونو ترپن اکسیژن دارمعطر(۵۷/۴۲٪) بود و پس از آنها مونو ترپن اکسیژن دار(۱۶/۱۳٪) و هیدرو کربن های سزکویی ترپن (۶/۹٪) بودند. اسانس مزبوردر غلظت ۸۰ میلی گرم در میلی لیتر خاصیت بازدارندگی کامل رشد *Bipolaris ellisii*، *Bipolaris hawaiensis*، *Bipolaris spicifera*، *Cochliobolus australiensis*، و*Cochliobolus cynodontis* را نشان داد ولی خاصیت ضد قارچی آن فقط در همان غلظت و بعد از ۶ و ۱۲ روزروی *Ocimum basilicum* موثر بود. اسانس مزبوراز جوانه زنی هاگ و ازدیاد (دراز شدن) لوله تندش مربوط به *B. hawaiensis* (در غلظت ۴۰ میلی گرم در میلی لیتر) و در مورد *B. spicifera* (در غلظت ۸۰ میلی گرم در میلی لیتر) جلوگیری کرد و مقادیر حد اقل غلظت بازدارنده (MIC) بین ۴۰ تا ۱۶۰میلی گرم در میلی لیتربود. این نتایج چنین نشان می دهد که اسانس ریحان (*Ocimum basilicum*) قارچ کشی مستعد و امید بخش برای کنترل پاتوژن های قارچی گیاهان و انسان است.

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