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CHEMICAL CONSTITUENTS AND ANTIFUNGAL PROPERTIES OF ORGANIC EXTRACTS OF *Ocimum basilicum* AGAINST *Bipolaris* AND *Cochliobolus* spp.

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ABSTRACT

The main constituents of the ethyl acetate extract of *Ocimum basilicum* were methyl cinnamate (58.43%), camphor (6.14%) and 1,8-cineole (4.55%), while butylated hydroxytoluene (BHT) (14.10%), *trans*-caryophyllene (9.66%), phytol (9.61%), neophytadiene (9.61%) and methyl cinnamate (7.43%) were the major compounds in the methanol extract, when were analyzed by GC–MS. The ethyl acetate extract mainly consisted of aromatic oxygenated monoterpenes (58.43%), whereas sesquiterpene hydrocarbons (25.70%) were the characteristic constituents of the methanol extract. Both ethyl acetate and methanol extracts were evaluated for antifungal activity against *Bipolaris ellisii*, *B. hawaiensis*, *B. spicifera*, *Cochliobolus australiensis* and *C. cynodontis*. The ethyl acetate extract exhibited complete inhibition on the mycelial growth of all fungi except *C. australiensis* at 16 mg/ml followed by methanol extract on *B. hawaiensis*, *B. spicifera* and *C. cynodontis* at the same concentration. Spore germination and germ tube elongation were completely inhibited by ethyl acetate extract for *B. hawaiensis* with MIC values ranged from 16 to 32 mg/ml. The methanol extract showed weak inhibition on the conidial germination and germ tube length. These results suggest that these extracts are potential and promising antifungal agents for the control of plant and human fungal pathogens.

Keywords: Antifungal activity, *Bipolaris* sp., *Cochliobolus* sp., *Ocimum basilicum*, organic solvent extracts, GC–MS

INTRODUCTION

The fungus *Cochliobolus* is the teleomorph of *Bipolaris* and *Curvularia* which are economically important plant pathogens worldwide associated with over 60 host genera (Manamgoda *et al.,* 2011). *Bipolaris* is relatively common with approximately 100 described species (Crous *et al.,* 2004). *Bipolaris* and *Cochliobolus* reported to cause several plant diseases including southern leaf blight of maize, root rot and leaf spot in wheat, black kernel of rice, spot blotch in wheat and barley, eyespot and brown stripe in sugarcane (Borrás-Hidalgo *et al.,* 2005; Kumar *et al.,* 2007; Worapattamasri *et al.,* 2009). Furthermore, some species of *Bipolaris*, notably *B. australiensis*, *B. hawaiiensis* and *B. spicifera*, are the etiologic agent of several human diseases such as phaeohyphomycosis (Costa *et al.,* 1991), fungal sinusitis (Buzina *et al.,* 2003), keratitis (Saha and Das, 2005), meningitis (Latham, 2000), fungal peritonitis (Bava *et al.,* 2003) and disseminated infection (Kobayashi *et al.,* 2008).

The application of natural products such as plant-based essential oils and extracts has recently become a very attractive strategy of controlling both human and plant pathogenic microorganisms because of their antimicrobial properties, non-phytotoxicity and biodegradability. Basil (*Ocimum basilicum* L.), a member of the Lamiaceae family, is one of the most popular aromatic plants that has been used extensively in the food and medical industries with more than 150 species (Runyoro *et al.,* 2010). Basil is well known also as a plant of medicinal treatments for headaches, coughs, diarrhea, constipation, worms, warts and kidney malfunctions (Telci *et al.,* 2006). Both essential oil and extracts of *O. basilicum* has been known to possess biological activities such as antibacterial (Bozin *et al.,* 2006; Stefan *et al.,* 2013), antifungal properties (Oxenham *et al.,* 2005; Dambolena *et al.,* 2010) and antioxidants activities (Lee *et al.,* 2005; Kwee and Niemeyer, 2011).

Therefore, the objectives of the present study were (1) to examine the chemical composition of various organic extracts of *O. basilicum* by GC–MS and (2) to evaluate the antifungal activities of the ethyl acetate and methanol extracts against *B. ellisii*, *B. hawaiensis*, *B. spicifera*, *C. australiensis* and *C. cynodontis*, economically important plant and human fungal pathogens.

MATERIALS AND METHODS

Plant material

The leaves of *O. basilicum* L. were collected from Mansoura University campus in Mansoura city (latitude 31° 3′ 0″ N, longitude 31° 23′ 0″ E, temperature 24-30°C, loam soil), which located in the north of Egypt. The taxonomic identification of plant materials was confirmed by a taxonomist from Botany Department, Faculty of Agriculture, Mansoura University.

Preparation of organic solvent extracts

The air-dried leaves of *O. basilicum* L. were pulverized into powdered form. The powder (100 g) was extracted with ethyl acetate and methanol separately at room temperature. After 48 h, extracts were filtrated through Whatman No.1 filter paper, and then solvents were evaporated by vacuum rotary evaporator. Residues were re-extracted twice with the same solvents. The extraction process yielded in ethyl acetate (19.2 g) and methanol (18.8 g) extracts.

Gas chromatography–mass spectrometry (GC-MS) analysis

The chemical composition of organic solvent extracts of *O. basilicum*, was performed using Agilent 6890 gas chromatograph equipped with an Agilent mass spectrometric detector, with a direct capillary interface and fused silica capillary column PAS–5MS (30 m × 0.32 mm × 0.25 µm film thickness). The column oven temperature was initially held at 40°C and then increased by 8°C /min to 280°C. The injector and detector (MS transfer line) temperatures were kept at 250 and 280°C, respectively. Helium was used as a carrier gas at a constant flow rate of 1 ml/min. The solvent delay was 3 min and diluted samples of 1 µl were injected manually 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

quadrupole temperatures were set at 230 and 150°C, respectively. The components were identified by comparison of their retention times and mass spectra with those of WILEY and NIST 05 mass spectral database (NIST, 2014).

***Bipolaris* and *Cochliobolus* isolates**

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

Effect of organic extracts on mycelial growth of fungal pathogens

Organic solvent extracts were dissolved in dimethyl sulfoxide (DMSO) and added to the Petri dishes (90 mm diameter) containing PDA at 40-45°C to obtain final concentrations 2, 4, 8 and 16 mg/ml for ethyl acetate and methanol extracts. PDA plates containing DMSO without organic solvent extracts were used as a control. Mycelial discs (5 mm diameter) were removed from previous cultures of all the fungal isolates and placed in the center of the plates. Plates were incubated at 25±2°C until the growth in the control reaches the edge of the plates. Mean of growth measurements were calculated from three replicates for each treatment and the experiment was repeated twice. The percentage of growth inhibition by treatment was calculated using the following equation:

$$\textbf{Inhibition of mycelial growth (\%)} = [(C - T) / C] \times 100$$

where C and T are the mycelial growth (mm) in the control and treated plates, respectively.

Effect of organic extracts on conidial germination and germ tube elongation of *Bipolaris* isolates

Conidia were harvested from actively growing culture (9-11 day old) on PDA by applied distilled sterile water onto the culture plates and gently scraping the plate surface with a glass rod to facilitate the release of conidia. The number of conidia in the suspension was adjusted to 1×10^6 conidia/ ml using a haemocytometer slide. A 20 µl aliquots of spore suspension drops were spread onto the surface of PDA medium supplemented with different concentrations of organic solvent extracts (2, 4, 8 and 16 mg/ml) dissolved in dimethyl sulfoxide (DMSO). PDA plates containing DMSO only were used as a control. After 24 h of incubation at 25±2°C, at least 100 spores in each replicate (three replicates per treatment) were observed microscopically to determine germination rate and germ tube length. The spore was considered germinated when the length of the germ tube equaled or exceeded the spore diameter. The experiment was performed two times. The percent inhibition was calculated according to Abbott's formula:

$$\textbf{Inhibition of spore germination (\%)} = [(Gc - Gt) /Gc)] \times 100$$

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

Determination of minimum inhibitory concentration (MIC)

Different concentrations of organic solvent extracts (2, 4, 8, 16, 32, 64 and 128 mg/ml) dissolved in DMSO, were incorporated in PDB medium (potato dextrose broth). Aliquots of 10 µl spore suspension (1×10^6 spores/ml) of each *Bipolaris* 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. The lowest concentration that did not permit any visible fungal growth was defined as the MIC. The experiment was repeated two times.

Statistical analysis

All data were subjected to one-way analysis of variance (ANOVA) and the significance of the treatments was determined using Tukey's HSD test ($P < 0.05$). The data were analyzed using SAS (version 9.1, SAS Institute, Cary, NC, USA).

RESULTS

Chemical composition of organic solvent extracts

GC–MS analyses of the ethyl acetate extract of *O. basilicum* led to the identification of 27 different components, representing 85.71% of the total extract. The identified compounds are listed in Table 1 according to their elution order on a PAS–5MS capillary column. The main compound was methyl cinnamate (58.43%). Camphor (6.14%) and 1,8-cineole (4.55%) were also found to be the minor components of this extract. Aromatic oxygenated monoterpenes (58.43%) were the characteristic constituents of the ethyl acetate extract of *O. basilicum* followed by oxygenated monoterpenes (14.28%) and sesquiterpene hydrocarbons (4.46%).

In the methanol extract of *O. basilicum*, 19 compounds were identified, representing 80.97% of the total extract (Table 1). The major components detected in this extract were butylated hydroxytoluene (BHT) (14.10%), *trans*-caryophyllene (9.66%), Phytol (9.61%), neophytadiene (9.61%), methyl cinnamate (7.43%) and α-amorphene (6.58%). Analyzed methanol extract of *O. basilicum* mainly consisted of sesquiterpene hydrocarbons (25.70%) followed by oxygenated sesquiterpenes (16.37%) and monoterpene hydrocarbons were absent.

Effect on mycelial growth of *Bipolaris* and *Cochliobolus* isolates

The ethyl acetate extract of *O. basilicum* exhibited a complete mycelial growth inhibition against *B. ellisii*, *B. hawaiensis*, *B. spicifera* and *C. cynodontis* at 16 mg/ml, followed by methanol extract which caused 100% inhibition on the mycelial growth of *B. hawaiensis*, *B. spicifera* and *C. cynodontis* at the same concentration (Fig. 1). These results indicate that the inhibition of mycelial growth increased with increasing concentrations of organic extracts for all isolates tested.

Table 1. Chemical composition of ethyl acetate and methanol extracts of *Ocimum basilicum*

Rt[a]	Compound[b]	Peak area (%)[c]		Molecular formula
		EAE	ME	
7.25	1,8-Cineole	4.55	2.55	$C_{10}H_{18}O$
7.96	*cis*-Sabinene hydroxide	0.47	–	$C_{10}H_{18}O$
8.55	Linalool	1.66	–	$C_{10}H_{18}O$
9.47	Camphor	6.14	3.27	$C_{10}H_{16}O$
10.07	Terpinen-4-ol	1.10	–	$C_{10}H_{18}O$
10.31	α-Terpineol	0.22	–	$C_{10}H_{18}O$
10.43	Myrtenol	0.14	–	$C_{10}H_{16}O$
13.76	Methyl cinnamate	58.43	10.21	$C_{10}H_{10}O_2$
14.35	*trans*-Caryophyllene	1.82	9.66	$C_{15}H_{24}$
14.48	β-Cubebene	0.21	1.14	$C_{15}H_{24}$
14.59	α-Guaiene	0.12	0.82	$C_{15}H_{24}$
14.89	α-Caryophyllene	–	1.29	$C_{15}H_{24}$
15.01	epi-Bicyclosesquiphellandrene	0.15	1.46	$C_{15}H_{24}$
15.31	Germacrene D	0.62	3.92	$C_{15}H_{24}$
15.71	Butylated hydroxytoluene (BHT)	1.28	14.10	$C_{15}H_{24}O$
15.80	α-Amorphene	1.01	6.58	$C_{15}H_{24}$
15.92	*cis*-Calamenene	0.16	0.83	$C_{15}H_{22}$
16.05	β-Gurjunene (calarene)	0.05	–	$C_{15}H_{24}$
16.79	β-Spathulenol	0.32	–	$C_{15}H_{24}O$
16.89	Caryophyllene oxide	0.40	–	$C_{15}H_{24}O$
17.32	α -Cubebene	0.32	–	$C_{15}H_{24}$
17.68	α-Cadinol	1.98	2.27	$C_{15}H_{26}O$
21.29	Hexadecanoic acid, methyl ester	0.87	1.30	$C_{17}H_{34}O_2$
23.41	Linolenic acid, methyl ester	0.20	0.41	$C_{19}H_{32}O_2$
23.54	Phytol	1.62	9.61	$C_{20}H_{40}O$
23.86	Linolenic acid, ethyl ester	0.33	–	$C_{20}H_{34}O_2$
24.70	Neophytadiene	1.02	9.61	$C_{20}H_{38}$
30.80	Supraene	–	1.94	$C_{30}H_{50}$
34.43	Eicosane	0.52	–	$C_{20}H_{42}$
	Total identified	85.71	80.97	
	Oxygenated monoterpenes	14.28	5.82	
	Aromatic oxygenated monoterpenes	58.43	10.21	
	Sesquiterpene hydrocarbons	4.46	25.70	
	Oxygenated sesquiterpenes	3.98	16.37	
	Diterpenes	1.62	9.61	
	Other constituents	2.94	13.26	

[a]Rt, retention time (min).
[b]Compounds are listed in the order of their elution.
[c]EAE, ethyl acetate extract; ME, methanol extract.

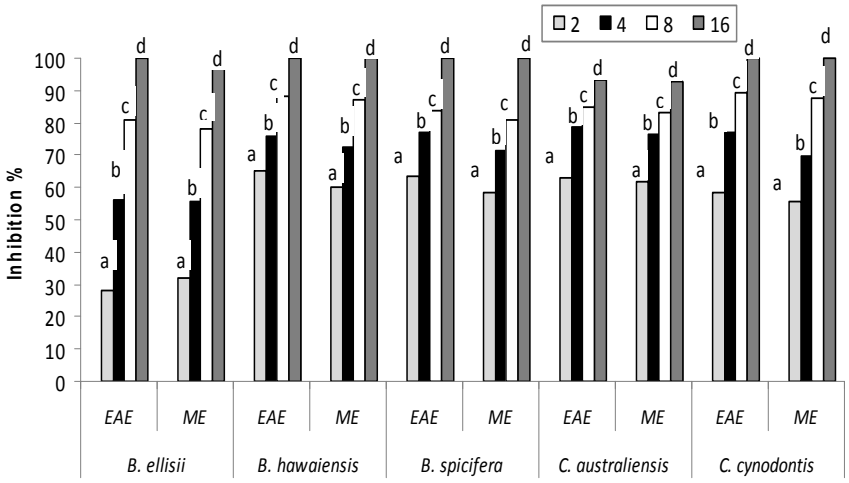

Fig. 1. Effect of *Ocimum basilicum* extracts on mycelial growth of *Bipolaris* and *Cochliobolus* isolates. Bars, for each concentration, with different letters represent values that are significantly different according to Tukey's HSD test at $P < 0.05$. EAE: ethyl acetate extract; ME: methanol extract.

Effect on conidial germination and germ tube elongation of *Bipolaris* isolates

A 100% inhibition of fungal spore germination and germ tube length was observed in *B. hawaiensis* at 16 mg/ml of ethyl acetate extract (Fig. 2 and Table 2).

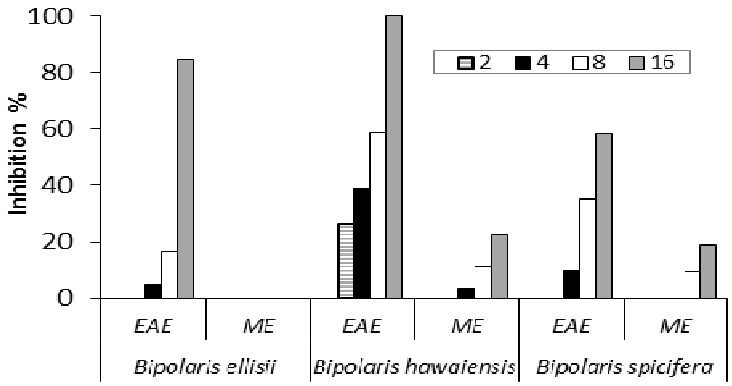

Fig. 2. Effect of *Ocimum basilicum* extracts on conidial germination of *Bipolaris* isolates at 2, 4, 8 and 16 mg/ml. EAE: ethyl acetate extract; ME: methanol extract

Also, this extract exhibited a potent inhibitory effect on the conidial germination (84.7% inhibition) and germ tube elongation of *B. ellisii* and moderate inhibition on the spore germination of *B. spicifera* by 58.3% at the same concentration. The methanol extract caused a weak reduction in the conidial germination of *B. hawaiensis* and *B. spicifera* by 22.7 and 19% at 16 mg/ml, respectively, and the effect on germ tube elongation was similar to those exhibited against conidial germination (Fig. 2 and Table 2).

Table 2. Effect of *Ocimum basilicum* extracts on germ tube elongation of *Bipolaris* isolates

mg/ml	Germ tube elongation (µm)					
	B. ellisii		*B. hawaiensis*		*B. spicifera*	
	EAE	ME	EAE	ME	EAE	ME
0	ND*	ND	ND	ND	ND	ND
2	60.7 a	128.7 a	33.7 a	112 a	66.7 a	99.0 a
4	53.6 b	118.3 b	21.7 b	98.6 b	45.7 b	87.6 b
8	27.6 c	98.0 c	15.7 c	90.6 c	24.7 c	64.6 c
16	14.7 d	77.6 d	0.0 d	21.3 d	11.7 d	33.0 d

*** ND, not detected; the germ tubes were very long and entwined each other so they could not be measured. Values in the same column followed by different letters are significantly different according to Tukey's HSD test at P < 0.05. EAE: ethyl acetate extract; ME: methanol extract.**

Minimum inhibitory concentration (MIC)

According to the results in Table 3, the ethyl acetate extract showed significant antifungal effect as minimum inhibitory concentrations against all plant pathogens tested with MIC values of 16 mg/ml against *B. hawaiensis* and 32 mg/ml against *B. ellisii* and *B. spicifera*. The methanol extract displayed antifungal activity against *B. hawaiensis* and *B. spicifera* with MIC value of 64 mg/ml.

Table 3. Minimum inhibitory concentrations of organic solvent extracts of *Ocimum basilicum* against *Bipolaris* isolates

Fungal isolates	MIC (mg/ml)	
	EAE	ME
Bipolaris ellisii	32	128
Bipolaris hawaiensis	16	64
Bipolaris spicifera	32	64

EAE, ethyl acetate extract; ME, methanol extract; PEE.

DISCUSSION

In the present study, compounds identified in appreciable amounts in the ethyl acetate and methanol extracts included methyl cinnamate, butylated hydroxytoluene (BHT), 1,8- cineole, α-cadinol, *trans*-caryophyllene and phytol. However, the two extracts differed in some of the components present in one of the extracts in appreciable amounts were absent in the other extract. For example, camphor and linalool, present in the ethyl acetate

extract by 6.14 and 1.66% respectively, were absent in the methanol extract, while supraene, which was identified in the methanol extract by 1.94%, was absent in the ethyl acetate extract. Dev *et al.* (2011) studied the chemical composition of two types of ethyl acetate extract of *O. basilicum*, EA-1 (extracted in Soxhlet apparatus with ethyl acetate) and EA-2, (subjected with methanol in Soxhlet apparatus and then extracted with ethyl acetate). 1,2-dimethoxy-4-(2-propenyl) benzene (53.06%) and 2-pentanone (18.06%) were the major compounds in EA-1, while 1, 2-benzene dicarboxylic acid (49.44%), 1, 2, 3, 4-tetramethyl benzene (9.9%) and eugenol (7.72%) were found to be the major components in EA-2. Kocić-Tanackov *et al.* (2011) identified 38 components in the basil extract, commercially available (ETOL, Celje, Slovenia). The major components were estragol or methyl chavicol (86.72%), *trans*-α-bergamotene (2.91%) and eucalyptol (2.67%). As mentioned above there was a great variations in the chemical composition of organic solvent extracts of *O. basilicum*, these variability depends on many factors including climate conditions, growing place, soil characteristics, harvesting period, season, plant age, plant part and different chemotypes.

Previous studies have demonstrated that organic extracts of *O. basilicum* usually have higher antifungal and antibacterial activities, but the effect on the mycelial growth of *Bipolaris* and *Cochliobolus* species have yet to be reported. For instance, Adiguzel *et al.* (2005) reported that the hexane, methanol and ethanol extracts of *O. basilicum* showed a high antibacterial activity, but none of these extracts have antifungal activities. Kocić-Tanackov *et al.* (2011) reported that the basil extract, commercially available (ETOL, Celje, Slovenia) completely inhibited the mycelial growth of *Fusarium* spp. at the concentration of 1.5% (v/v). Also, the ethanol, methanol and water extracts of the stem bark of *Ocimum basilicum* exhibited high antibacterial activity against 11 bacteria and appreciable activity against *Candida albicans* (Issazadeh *et al.,* 2012). Similarly, the fraction of the methanol extract of *O. basilicum* was found to be able to suppress *Bacillus subtilis*, *Escherichia coli*, and *Vibrio cholera* (Saha *et al.,* 2013). According to Vlase *et al.* (2014) the ethanolic extract of *O. basilicum* showed a moderate antibacterial activity against *Staphylococcus aureus* and low antibacterial effect on *Listeria monocytogenes*, *Escherichia coli* and *Salmonella typhimurium*.

Based on our results of chemical composition of organic extracts of *O. basilicum*, it is possible to conclude that the antifungal activity of these extracts is apparently related to their higher percentage of aromatic oxygenated monoterpenes (58.43%) in the ethyl acetate extract and sesquiterpene hydrocarbons (25.70%) in the methanol extract. In addition, the antifungal activity could be provoked by the major compounds of the plant extracts or due to a synergistic effect between the major compounds and the minor ones (Carovic-Stanko *et al.,* 2010).

Several studies have shown the effect of compounds of plant extracts acting as natural fungicides. However, just a few studies present the action mechanism of these compounds on the microbial cell. For instance, the compounds of monoterpenes increase the permeability of the plasma membrane and inhibit the respiration on mitochondrial membrane of fungi (Cox *et al.,* 2000; Imelouane *et al.,* 2009). Also, these components at low

concentrations lead to lipid peroxidation in fungi and increases ergosterol biosynthesis (Lucini *et al.,* 2006). Additionally, the toxic effects of phenolic compounds include enzyme inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups or through more nonspecific interactions with the proteins (Cowan, 1999). Cabral *et al.* (2013) suggested that some hydrophobic compounds present in the plant extracts could change the permeability of the microbial membranes for cations (H+ and K+), and so could cause a change in the flow of protons, modifying cell pH and affecting chemical composition of the cells and their activity. These compounds can also inhibit the activity of protective enzymes and sequentially inhibit one or more biochemical pathways (Xing *et al.,* 2012).

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التركيب الكيميائى والخصائص المضادة للفطريات لبعض المستخلصات العضوية لنبات الريحان ضد فطريات *Bipolaris* و *Cochliobolus*

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بالتحليل الكيميائى لمستخلص الإيثيل استات لأوراق نبات الريحان باستخدام جهاز GC–MS وجد أن المركبات الرئيسية فى هذا المستخلص كانت methyl cinnamate (58.43%) و camphor (6.14%) و 1,8-cineole (4.55%) بينما كانت مركبات butylated hydroxytoluene (BHT) (14.10%) و *trans*-caryophyllene (9.66%) و phytol (9.61%) و neophytadiene (9.61%) و methyl cinnamate (7.43%) هى المركبات الرئيسية فى المستخلص الميثانولى لأوراق الريحان. وكانت مجموعة aromatic oxygenated monoterpenes هى المجموعة الكيميائية الأساسية فى مستخلص الإيثيل استات بنسبة 58.43% بينما كانت مجموعة sesquiterpene hydrocarbons هى الأكثر وجوداً فى المستخلص الميثانولى بنسبة 25.70%.

وتم اختبار كل من مستخلص الإيثيل استات والمستخلص الميثانولى لأوراق نبات الريحان على النمو الميسليومى وانبات الجراثيم وطول انبوبة إنبات جراثيم الفطريات الآتية *Bipolaris ellisii* و *B. hawaiensis* و *B. spicifera* و *Cochliobolus australiensis* و *C. cynodontis*. وأظهرت الدراسة أن مستخلص الإيثيل استات قد أعطى تثبيطاً كاملاً بنسبة ١٠٠% على النمو الميسليومى لجميع الفطريات المختبرة فيما عدا فطر *C. australiensis* وذلك عند تركيز 16 mg/ml وكذلك أعطى المستخلص الميثانولى تثبيطاً كاملاً بنسبة ١٠٠% على النمو الميسليومى لفطريات *B. hawaiensis* و *B. spicifera* و *C. cynodontis* وذلك عند نفس التركيز. كما أعطى مستخلص الإيثيل استات تثبيطاً كاملاً بنسبة ١٠٠% على انبات الجراثيم وطول انبوبة إنبات جراثيم فطر *B. hawaiensis* مع قيم MIC تتراوح بين 16 mg/ml و 32 mg/ml للفطريات المختبرة, بينما أظهر المستخلص الميثانولى تأثيراً ضعيفاً على كل من انبات الجراثيم وطول انبوبة إنبات جراثيم الفطريات المختبرة.