Bioactivity Of Fungal Secondary Metabolites Against Some Economic Pests

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Abstract

Fungi have been shown to be a good source for lead molecules in drug discovery and development. However, many compounds derived from fungi were not evaluated for their bioactivity against economic pests. In this study, twelve fungal secondary metabolites (**1-12**) were evaluated for herbicidal activity against *Silybum marianum*, fungicidal activity against *Alternaria solani* and *Fusarium oxysporum*, insecticidal activity against *Culex pipiens* larvae, and antifeedant activity against *Spodoptera littoralis* larvae. Among the tested metabolites, *brefeldin* A (**6**) and 6-eopxy-4-hydroxy-3-methoxy-5-methyl-cyclohex-2-en-1-one (**11**) showed potent herbicidal activity against *S. marianum* with complete inhibition of seed germination at 500 mg/l. Compound **6** revealed an exceptional herbicidal activity as it caused complete inhibition of root growth and strong reduction in shoot growth ($I = 74.5\%$) and germination (10.0%) at 25 mg/l. In addition, anthracobic acid A (**7**) showed the highest antifungal activity against *F. oxysporum* ($EC_{50} = 8.21$ mg/l) and 19-(α -D-glucopyranosyloxy)isopimara-7,15-dien-3 β -ol (**2**) was the most potent compound against *A. solani* ($EC_{50} = 140.8$ mg/l). Furthermore, dehydroaustin (**9**), phomaxanthone A (**4**) and deacetylphomaxanthone A (**5**) displayed a potent toxicity against fourth larval instar of *C. pipiens* with LC_{50} values of 3.27, 57.03 and 63.5 mg/l, respectively. Among the tested metabolites, four compounds (**4**, **5**, **7** and **11**) showed antifeedant activity against third-instar larvae of *S. littoralis* at 1000 mg/l. The results presented in this study should be useful for developing *brefeldin* A (**6**) and dehydroaustin (**9**) as natural herbicide and insecticide, respectively.

Introduction

Fungi are a rich source of diverse natural compounds that have the potential for the discovery of new drugs and agrochemicals¹. It has been estimated that only 5% (around 70,000) of fungi total populations (1.5 million) have been explored². This confirms fungi as possible alternatives to plants for producing valuable natural products. Although the soil fungi are well-known as source of important antibiotics and drugs^{3,4} much effort has been devoted in last two decades to study the secondary metabolites from marine and endophytic fungi as new sources for drugs and agrochemicals^{5,6}.

Interest in microbial metabolites as pesticides arises to overcome the problems associated with the continuous use of synthetic pesticides, such as adverse effects of pesticide residues on human, non-target organisms and environment, and emergence of pest resistance⁷. In fact microbial metabolites have several advantages to be good candidates in pest control, such as structure diversity, wide-spectrum of bioactivity, new modes of action and biodegradability⁸. Several pesticides of microbial origin have been introduced into field of pest management include: avermectin, bialaphos, milbemycin, kasugamycin, blasticidin S, polyoxin, mildiomyacin, validamycin and tetranactin⁹. Therefore, it is highly important to investigate new fungal metabolites and examine their bioactivities against economic pests.

Silybum marianum (L.) Gaertn. (Asteraceae) is a dangerous weed in many parts of Middle East, Africa, Australia, and North and South America¹⁰. In Egypt, it is considered as an invasive weed that spread

through wheat fields in both new reclaimed lands and desert areas. *Alternaria solani* Sorauer, 1896, the causal agent of early blight, is a major threat to global potato and tomato production^{11,12}. *Fusarium oxysporum* is a worldwide soil-borne phytopathogen causing common vascular wilt or root rot in many plants of Solanaceae, particularly tomato and potato^{13,14}. *Culex pipiens* L. (Diptera: Culicidae) is the most widespread mosquito species in Egypt¹⁵. This insect is able to transmit several pathogens, such as West Nile virus, *Bancroftian filariasis* and Rift Valley Fever virus¹⁶. *Spodoptera littoralis* (Boisduval), is one the most destructive lepidopterous insects to numerous economic crops. It causes a huge loss in crop yields in tropical and subtropical zones^{17,18}.

In our continuous efforts to search for new natural products with possible application in pest management, twelve fungal secondary metabolites (**1-12**) were examined for their effects on different groups of pests. The herbicidal effect (against *S. marianum*), antifungal activity (against *A. solani* and *F. oxysporum*), toxicity (against *C. pipiens*), and antifeedant activity (against *S. littoralis* larvae) were tested to explore their potential for the management of these economic pests.

Results

Isolation, structure elucidation of isolated secondary metabolites. Twelve secondary metabolites (**1-12**) have been isolated from different fungal strains (Table 1) using different chromatographic techniques, including silica gel and ODS columns, PTLC and HPLC. Among the isolated metabolites, nine compounds (**1, 2, 4-7** and **10-12**) were isolated from endophytic fungi and three compounds (**3, 8, and 9**) were isolated from soil fungi. The chemical structure (Fig. 1) of the isolated compounds was elucidated on the basis of their spectroscopic data of ultraviolet (UV), infrared (IR), high resolution mass spectroscopy (HRMS) and nuclear magnetic resonance (NMR). The isolated compounds belong to ten different chemical groups of natural products (Table 1). Among the isolated metabolites, four compounds (**1, 2, 7** and **11**) have been isolated in our laboratory for the first time (new compounds) and eight compounds (**3-6, 8-10** and **12**) have been isolated and characterized as known metabolites by comparing their spectral data with those reported in the literature.

Table 1
Names, chemical class and source of fungal secondary metabolites

Name	Chemical group	Fungus (fungal origin)	Reference
Nodulisporone B (1)	Phenylisobenzofuranones	<i>Nodulisporium</i> sp. SH-1 (<i>Xylaria polymorpha</i>)	[19] Hayasaka et al. 2011
19-(α -D-glucopyranosyloxy)isopimara-7,15-dien-3 β -ol (2)	Isopimarane diterpenoids	<i>Paraconiothyrium</i> sp. MY-42 (unidentified plant)	[20] Shiono et al. 2011
Pencolide (3)	Maleimides	<i>Penicillium sclerotiorum</i> (Soil)	[21] Lucas et al. 2007
Phomaxanthone A (4)	Dimeric xanthonenes	<i>Phomopsis</i> sp. (<i>Rhizophora mucronata</i>)	[22] Elsaesser et al. 2005
Deacetylphomaxanthone A (5)	Dimeric xanthonenes	<i>Phomopsis longicolla</i> (<i>Sonneratia caseolaris</i>)	[23] Ronsberg et al. 2013
Brefeldin A (6)	Macrolides	<i>Penicillium brefeldianum</i> (<i>Pinellia ternata</i>)	[24] Hutchinson et al. 1983
Anthracobic acid A (7)	Polyketides	<i>Anthracobia</i> sp. (unidentified plant)	[25] Shiono 2006
Fasciculol C (8)	Lanostane triterpenoids	<i>Neamatoloma fasciculare</i> (Mushroom)	[26] Ikeda et al. 1977; [27] Kim et al. 2013
Dehydroaustin (9)	Meroterpenoids	<i>Penicillium brasilianum</i> (Soil)	[28] Schürmann et al. 2010
Pyrrocidine A (10)	Alkaloids	<i>Acremonium zeae</i> (<i>Zea mays</i>)	[29] Wicklow and Poling 2009

Name	Chemical group	Fungus (fungal origin)	Reference
6-Eopxy-4-hydroxy-3-methoxy-5-methyl-cyclohex-2-en-1-one (11)	Cyclohexenones	Xylariaceous endophytic fungus (YUA 026) (unidentified plant)	[30] Shiono et al. 2005
Secalonic acid A (12)	Dimeric xanthones	<i>Claviceps purpurea</i> (<i>Secale cereale</i>)	[31] Masters and Bräse, 2002

Effect of fungal metabolites on germination and seedling growth of *S. marianum*. Effect of fungal secondary metabolites (**1-4** and **6-12**) on germination, root and shoot growth of *S. marianum* at concentration of 500 mg/l is shown in Table 2. Among the eleven tested metabolites, compounds **3, 4, 6, 8, 9, 10** and **11** caused reduction in seed germination after 12 days of treatment. Compounds **6** and **11** induced complete inhibition of seed germination at this concentration. In addition, seven compounds (**2, 3, 4, 6, 8, 11** and **12**) revealed significant inhibition in root growth compared with control treatment. Alongside, compounds **6** and **11** which showed complete inhibition of germination, compounds **3, 4** and **12** strongly inhibited root growth with 87.5, 80.4 and 74.8% inhibition, respectively. Conversely, compounds **1** and **10** significantly increased the root growth. Similarly, compounds **3, 4, 8** and **12**, showed the greatest reduction in shoot growth. The results revealed that the tested compounds caused higher inhibitory effects on root and shoot growth than on seed germination. Also, some of the tested compounds had greater inhibitory effects on root growth than on shoot growth.

Table 2
Effect secondary metabolites isolated from endophytic fungi on germination and seedling growth of *Silybum marianum* after 12 days of sowing at 500 mg/l^a

Compound	Germination ^b (% ± SE)	Root		Shoot	
		Length (cm) (Mean ± SE)	I ^c (%)	Length (cm) (Mean ± SE)	I (%)
Control	86.7±3.34ab	11.39±0.22bc	0.0	2.16±0.06abc	0.0
Nodulisporone B (1)	86.7±3.34ab	14.32±0.14a	28.2	2.33±0.13ab	7.87
19-(α-D-glucopyranosyloxy)isopimara-7,15-dien-3β-ol (2)	86.7±3.34ab	5.47±0.23d	52.0	1.86±0.24bc	13.9
Pencolide (3)	76.7±3.34b	1.42±0.06g	87.5	1.33±0.17d	38.4
Phomaxanthone A (4)	80.0±5.78b	2.23±0.25f	80.4	1.30±0.10d	39.8
Brefeldin A (6)	0.0±0.0c	0.0±0.0h	100.0	0.0±0.0e	100.0
Anthracobic acid A (7)	96.71±3.34a	11.93±0.11b	4.74	2.33±0.25ab	7.87
Fasciculol C (8)	83.3±3.34ab	5.05±0.42d	55.7	1.35±0.09d	37.5
Dehydroaustin (9)	80.0±5.78b	11.12±0.17c	2.37	2.53±0.06a	17.1
Pyrrocidine A (10)	83.3±6.67ab	13.82±0.17a	21.3	1.80±0.26c	16.7
6-Eopxy-4-hydroxy-3-methoxy-5-methyl-cyclohex-2-en-1-one (11)	0.0±0.0c	0.0±0.0h	100.0	0.0±0.0e	100.0
Secalonic acid A (12)	90.0±5.78ab	2.87±0.11e	74.8	1.31±0.16d	39.4
df	11	11		11	
F	63.74	801.70		29.30	
P	0.00	0.00		0.00	
^a Values are means ± SE of three replicates with 10 seeds in each one.					
^b Values followed by different letters are significantly different at 0.05 probability level.					
^c I = Inhibition.					

The previously presented results showed that compounds **6** and **11** caused complete inhibition of seed germination at 500 mg/l. Therefore, these two compounds were further evaluated at lower concentrations (25, 50, 100, 250 and 500 mg/l). Compound **6** caused complete inhibition of root growth at all tested concentrations (Table 3). Likewise, it induced complete inhibition of germination and shoot growth at all tested concentrations except 25 mg/l. This compound showed strong inhibition of germination (10.0%)

and shoot growth (I = 74.5%) at 25 mg/l. In addition, compound **11** induced significant reduction in seed germination at all of the tested concentrations. Also, it caused strong inhibition of root and shoot growth at 100 and 250 mg/l.

Table 3

Effect of brefeldin A and 6-eopxy-4-hydroxy-3-methoxy-5-methyl-cyclohex-2-en-1-one on germination and seedling growth of *Silybum marianum* after 12 days of sowing at different concentrations^a

Compound	Conc. (mg/l)	Germination (% ± SE) ^b	Root		Shoot	
			Length (cm) (Mean ± SE)	I ^c (%)	Length (cm) (Mean ± SE)	I ^c (%)
Control	0.0	75.0±2.89a	6.33±0.49a	0.0	1.96±0.21a	0.0
Brefeldin A (6)	25	10.0±5.78d	0.0±0.0d	100.0	0.50±0.28c	74.5
	50	0.0±0.0d	0.0±0.0d	100.0	0.0±0.0d	100.0
	100	0.0±0.0d	0.0±0.0d	100.0	0.0±0.0d	100.0
	250	0.0±0.0d	0.0±0.0d	100.0	0.0±0.0d	100.0
	500	0.0±0.0d	0.0±0.0d	100.0	0.0±0.0d	100.0
6-Eopxy-4-hydroxy-3-methoxy-5-methyl-cyclohex-2-en-1-one (11)	25	60.0±5.78b	6.27±0.40a	0.95	1.63±0.03a	16.8
	50	56.7±3.33b	4.50±0.17b	28.9	1.87±0.20a	4.59
	100	43.3±3.33c	0.83±0.27c	86.9	1.20±0.17b	38.8
	250	6.7±3.33d	0.02±0.02d	99.7	0.17±0.17cd	91.3
	500	0.0±0.0d	0.0±0.0d	100.0	0.0±0.0d	100.0
df		10	10		10	
F		88.34	139.35		32.99	
P		0.00	0.00		0.00	
^a Values are means ± SE of three replicates with 10 seeds in each one.						
^b Values followed by different letters are significantly different at 0.05 probability level.						
^c I = Inhibition.						

Antifungal activity of fungal metabolites against *F. oxysporum* and *A. solani*. The inhibitory effect of the tested compounds on the mycelial growth of *A. solani* and *F. oxysporum* at 500 mg/l is shown in Table 4.

The results indicated that the mycelial growth inhibition was compound and fungus dependent. Compounds **2**, **4** and **7** exhibited the highest antifungal activity against *F. oxysporum* with mycelial growth inhibition 90.97, 69.21 and 77.55%, respectively, at 500 mg/l. Based on EC₅₀ values, compound **7** (EC₅₀ = 8.21 mg/l) was the most active one, followed by compound **2** (EC₅₀ = 72.8 mg/l) and compound **4** (EC₅₀ = 193.5 mg/l). In the case of *A. solani*, compounds **2** and **3** showed the highest antifungal activity with mycelial growth inhibition 87.10 and 79.51%, respectively, at 500 mg/l. Furthermore, compound **2** (EC₅₀ = 140.8 mg/l) was more active than compound **3** (EC₅₀ = 341.4 mg/l) against *A. solani*.

Table 4
Antifungal activity of fungal metabolites against *Fusarium oxysporum* and *Alternaria solani*

Compound	Mycelial growth inhibition (%±SE) at concentration (500 mg/l)*	
	<i>Fusarium oxysporum</i>	<i>Alternaria solani</i>
Nodulisporone B (1)	15.05±2.00g	13.44±4.93e
19-(α-D-glucopyranosyloxy)isopimara-7,15-dien-3β-ol (2)	90.97±1.20a (EC ₅₀ = 72.8)**	87.10±5.59a (EC ₅₀ = 140.8)
Pencolide (3)	16.90±3.56g	79.51±4.18b (EC ₅₀ = 341.4)
Phomaxanthone A (4)	69.21±2.80c (EC ₅₀ = 193.5)	35.78±1.62d
Brefeldin A (6)	6.02±0.80h	46.60±4.02c
Anthracobic acid A (7)	77.55±1.45b (EC ₅₀ = 8.21)	36.76±5.90d
Fasciculol C (8)	9.26±4.01h	13.44±2.02e
Dehydroaustin (9)	0.0±0.0i	5.10±1.25f
Pyrrocidine A (10)	38.43±0.80e	11.02±2.03ef
6-Eopxy-4-hydroxy-3-methoxy-5-methyl-cyclohex-2-en-1-one (11)	51.83±3.53d	37.58±0.99d
Secalonic acid A (12)	32.87±3.83f	50.75±0.65c
F	446.49	177.14
Df	10	10
P	0.001	0.001
*Values followed by the same letter are not significantly different (<i>P</i> = 0.05)		
** The concentration causing 50% mycelial growth inhibition.		

Larvicidal activity of fungal metabolites against *C. pipiens*. Toxicity of twelve fungal metabolites against the fourth larval instar of *C. pipiens* at concentrations of 100, 250 and 500 mg/l is presented in Table 5. Four compounds (4, 5, 8 and 9) caused complete mortality (100%) of larvae at 500 mg/l, while three compounds (4, 5 and 9) caused complete mortality of larvae at 250 mg/l. At 100 mg/l, compound 9 was the only compound that caused complete mortality of larvae. Also, compounds 4 and 5 induced strong

larval toxicity at this concentration with 95.0 and 90.0% mortality, respectively. Comparative toxicity results (Table 6) indicated that compound **9** revealed the highest larval toxicity (LC_{50} = 3.27 mg/l), followed by compound **4** (LC_{50} = 57.03 mg/l) and compound **5** (LC_{50} = 63.5 mg/l).

Table 5

Mortality percentages of *Culex pipiens* fourth instar larvae after 24 h of treatment with secondary metabolites isolated from endophytic fungi at different concentrations

Compound	Mortality (%) after 24h		
	100 mg/l	250 mg/l	500 mg/l
Control	0.0	0.0	0.0
Nodulisporone B (1)	0.0	0.0	0.0
19-(α -D-glucopyranosyloxy) isopimara-7,15-dien-3 β -ol (2)	0.0	_a	-
Pencolide (3)	0.0	0.0	0.0
Phomaxanthone A (4)	95.0	100.0	100.0
Deacetylphomaxanthone A (5)	90.0	100.0	100.0
Brefeldin A (6)	0.0	0.0	46.7
Anthracobic acid A (7)	0.0	0.0	10.0
Fasciculol C (8)	0.0	6.25	100.0
Dehydroaustin (9)	100	100.0	100.0
Pyrrocidine A (10)	0.0	0.0	15.0
6-Eopxy-4-hydroxy-3-methoxy-5-methyl-cyclohex-2-en-1-one (11)	0.0	0.0	38.9
Secalonic acid A (12)	0.0	0.0	0.0
a = Not tested.			

Table 6
Comparative toxicity of fungal metabolites against *Culex pipiens* fourth instar larvae after 24h of exposure

Compound	LC ₅₀ ^a (mg/l)	95% confidence limits (mg/l)		Slope ± SE	Intercept ± SE	(χ ²) ^b	P ^c
		Lower	Upper				
Phomaxanthone A (4)	57.03	52.62	61.39	6.75±0.81	11.85±1.47	0.001	0.978
Deacetylphomaxanthone A (5)	63.50	58.75	68.41	6.50±0.72	11.73±1.31	0.006	0.941
Dehydroaustin (9)	3.27	0.38	6.53	1.11±0.30	0.57±0.40	0.181	0.671
^a The concentration causing 50% mortality.							
^b Chi square value.							
^c Probability value.							

Antifeedant activity of fungal metabolites against *S. littoralis*. The antifeedant activity of fungal metabolites against the 3rd larval instar larvae *S. littoralis* is shown in Table 7. The results indicated that compounds **4**, **5**, **7** and **11** were active antifeedants at 1000 mg/l (corresponding to a concentration of ca. 20 µg/leaf.cm²), while the other compounds had no antifeedant activity at this concentration.

Table 7
Antifeedant activity of fungal metabolites against fourth instar larvae of *Spodoptera littoralis* at 1000 mg/l

Compound	Antifeedant activity
Nodulisporone B (1)	-
19-(α-D-glucopyranosyloxy)isopimara-7,15-dien-3β-ol (2)	-
Pencolide (3)	-
Phomaxanthone A (4)	+
Deacetylphomaxanthone A (5)	+
Brefeldin A (6)	-
Anthracobic acid A (7)	+
Fasciculol C (8)	-
Dehydroaustin (9)	-
Pyrrocidine A (10)	-
6-eopxy-4-hydroxy-3-methoxy-5-methyl-cyclohex-2-en-1-one (11)	+

Discussion

New pest management strategies are based on decrease the use of synthetic pesticides and increase the use of natural alternatives. Therefore, introducing new natural products with low risk on human and environment, and new modes of action is highly needed in the field pest management nowadays³². In this regard, microorganisms are a rich source for natural compounds with successful use in pest management^{33,34}. In this study, the herbicidal, fungicidal, insecticidal and antifeedant activities of twelve secondary metabolites isolated from fungi were investigated to explore their possible use in pest control programs.

The results showed that most of the tested compounds reduced seed germination and seedling growth of *S. marianum* at concentration of 500 mg/l. Among the eleven tested metabolites, compounds **6** and **11** revealed excellent herbicidal activity with complete inhibition of germination and seedling growth at this concentration. Moreover, compound **6** showed strong herbicidal activity at 25 mg/l. Based on the obtained results, this compound is among the most effective natural herbicides reported so far. In our previous study, compounds **6** and **11** were also the most potent inhibitors of seed germination, and growth of root and shoot of *Echinochloa crus-galli* at concentration of 2mM³⁵. Also, compound **6** was found to be more effective than compound **11** against *E. crus-galli*. The herbicidal activity of compound **11** was further supported by the studies of Shiono and Murayama³⁶ who stated that this compound inhibited the root growth of lettuce by 46% at concentration of 50 mg/l. In addition, Compound **6** has been described to inhibit wheat germination and the root growth of *Allium cepa*³⁷.

Comparing the inhibitory effects of tested compounds on the seed germination with seedling growth of *S. marianum* indicated that the tested compounds were more effective against seedling growth than germination. These findings are in agreement with previous studies on many natural compounds in which the seedling growth were more sensitive than germination^{38–40}. Also, the results of current study revealed that the tested compounds were more potent inhibitors to root than to shoot with few exceptions. The higher sensitivity of roots could be due to the roots are the first to expose and absorb tested compounds from growing media⁴¹. Similar observations were reported on the inhibitory effects of other natural compounds on seedling growth^{42–44}.

Antifungal activity experiments showed that compounds (**2**, **4** and **7**) and compounds (**2** and **3**) exhibited pronounced inhibitory effect on the mycelial growth of *F. oxysporum* and *A. solani*, respectively. To the best of our knowledge, this is the first report on the antifungal properties of isolated secondary metabolites on plant pathogenic fungi. However, compound **6** has been reported to possess antifungal activity against *Aspergillus niger*, *Candida albicans*, and *Trichophyton rubrum*⁴⁵. Also, da Silva et al.⁴⁶ found that compound **12** had antifungal potential against *C. albicans* and *C. tropicalis* (MIC = 125 mg/l). On the other hand, some of the tested compounds have been described to have antibacterial activity against plant pathogenic bacteria. For example, compounds **3** and **11** inhibited the growth of *Rhizobium radiobacter*, *Ralstonia solanacearum*, *Pectobacterium carotovorum* subsp. *carotovorum* and

Pseudomonas syringae pv. *syringae* with MIC values ranged between 125 and 500 mg/l. Furthermore, compounds **2** and **4** inhibited the growth of *R. solanacearum* and *P. carotovorum* subsp. *carotovorum*, respectively, with MIC value of 125 mg/l³⁵.

The results of larvicidal bioassays against *C. pipiens* demonstrated that among the tested metabolites, compounds **9**, **4** and **5** had a promising larvicidal activity. In particular, compound **9** which displayed the highest toxicity with LC₅₀ = 3.27 mg/l. Based on our results, this compound is among the most toxic natural compounds reported so far against *C. pipiens* or other mosquito species. This finding is supported by previous study of Geris et al.⁴⁷ who found that compound **9** was highly toxic against third instar larvae of *Aedes aegypti* with LC₅₀ value of 2.9 mg/l. Compound **9** was more toxic to *C. pipiens* larvae than thymol (LC₅₀ = 37.95 mg/l), carvacrol (LC₅₀ = 44.38 mg/l), cinnamaldehyde (LC₅₀ = 58.97 mg/l), eugenol (LC₅₀ = 86.22 mg/l) and cuminaldehyde (LC₅₀ = 38.94 mg/l)^{48,49}. Similarly, compound **9** showed higher toxicity than curcumin (LC₅₀ = 19.07 mg/l) isolated from *Curcuma longa*⁵⁰ (Sagnou et al. 2012). Moreover, this compound showed similar toxicity to caulerpin (LC₅₀ = 1.99 mg/l) and caulerpinic acid (LC₅₀ = 4.89 mg/l) isolated from *Caulerpa racemosa*⁵¹.

The results indicated that the tested compounds had weak antifeedant activity with only compounds **4**, **5**, **7** and **11** being active antifeedants against *S. littoralis* at 1000 mg/l. Similar findings were concluded by Li et al.⁵² who examined the antifeedant activity of thirty-nine fungal metabolites isolated from *Aspergillus fumigatus* LN-4 against larvae of *Mythimna separata* Walker and found that only two compounds (fumitremorgin B and verruculogen) were active antifeedants.

The results of the current study indicate that the tested compounds showed high selectivity against the examined pests. For example, compounds **6** and **11** showed promising herbicidal activity against *S. marianum* while had no antifungal or insecticidal activity. Likewise, compounds **5** and **9** possessed potent insecticidal activity against *C. pipiens* but were not active against tested weed or fungi. Finally, compounds **2** and **7** had remarkable antifungal activity, while they showed no insecticidal or herbicidal activity. This selectivity is crucial for the development of these compounds as new bio-pesticides.

In conclusion, this study described, for the first time, the herbicidal, antifungal, insecticidal and antifeedant activities of twelve fungal metabolites against the economic agricultural pests, *S. marianum*, *F. oxysporum*, *A. solani*, *C. pipiens* and *S. littoralis*. Among the tested metabolites, brefeldin A (**6**) and 6-eopxy-4-hydroxy-3-methoxy-5-methyl-cyclohex-2-en-1-one (**11**) revealed promising herbicidal activity against *S. marianum*, in particular, brefeldin A (**6**) which could be developed as a new natural herbicide. In addition, dehydroaustin (**9**) exhibited remarkable larvicidal activity against *C. pipiens* indicating its possible use in integrated management programs of mosquitoes.

Materials And Methods

Test organisms. Field biotype seeds of variegated thistle, *Silybum marianum* (L.) Gaertn. (Asteraceae), were collected from Faculty of Agriculture Farm, Alexandria (31°12'56.30"N, 29°57'18.97"E). The plant seeds were identified by Prof. FathAllah Zaitoon of the Plant Pathology Department, Faculty of Agriculture, Alexandria University. Voucher specimen (SM-1909) was deposited in Department of Chemistry and Technology, Faculty of Agriculture, Alexandria University. All local, national or international guidelines and legislation were adhered to in the production of this study. Uniform and undamaged seeds were used for the germination and seedling growth tests. Seeds were examined for their germination before experiments. The seed germination was 80% after 12 days of sowing. *Alternaria solani* Sorauer, 1896 (EMCC 756) and *Fusarium oxysporum* (Schlecht. Emend. Snyder & Hansen) (EMCC 137) were obtained from Microbiological Resource Centre, Ain Shams University, Cairo, Egypt. The fungal strains were maintained on potato dextrose agar medium (PDA: potato 200 g, dextrose 20 g and agar 15 g in 1 liter DW). *Culex pipiens* L. (Diptera: Culicidae) was reared in an insectary at 27±2°C and 75±5% R.H. at the Department of Applied Entomology and Zoology, Alexandria University, Egypt as described by Zahran and Abdelgaleil¹⁵. *Spodoptera littoralis* Boisduval (Lepidoptera: Noctuidae) was reared under laboratory conditions at 26±2°C and 70±5% RH. The larvae were fed on *Ricinus communis* L. (Euphorbiaceae) leaves as described by El-Defrawi et al.⁵³.

Fungal strains and isolation of secondary metabolites. Strains of different endophytic and soil fungi were isolated from different sources (Table 1), identified by BEX Co. Ltd., Japan, using a DNA analysis of the 18S rDNA regions and had been deposited at our laboratory in the Faculty of Agriculture of Yamagata University. After fermentation on unpolished rice media (unpolished rice 1000 g + 35 ml of 3.5% NaCl at 25°C for four weeks), the fungal growth media were extracted with MeOH. The MeOH extracts were concentrated and partitioned into *n*-hexane and EtOAc. The EtOAc extracts were subjected to silica gel column chromatography with mixtures of *n*-hexane–EtOAc (100:0 – 0:100, v/v), and mixture of EtOAc–MeOH (50:50 – 0:100, v/v). The rustling fractions were further chromatographed on ODS by eluting with H₂O and an increasing ratio of MeOH (100:0 to 0:100) or silica gel by eluting with a mixture of CHCl₃–MeOH (90:10, v/v). For HPLC analysis, a reversed phase HPLC (Semipreparative HPLC with Shimadzu pump and UV LC-10A detector (set at 210 nm) on a Mitysil ODS column (150 × 6.0 mm i.d.) at a flow rate of 2.0 mL/min, solvent systems, MeOH:H₂O, 60:40 – 80:20, v/v) and PTLC (EtOAc – *n*-hexane, 70:30 – 30:70, v/v) were used.

Germination and seedling growth inhibition bioassay. The effect of fungal secondary metabolites on seed germination, and root and shoot growth of *S. marianum* was evaluated using a bioassay method explained by Abdelgaleil et al.⁴². The tested compounds were dissolved first in DMSO and diluted with distilled water containing 0.02% of Triton-X 100 to give final concentration of 500 mg/l. Three replicates with 10 seeds in each one were prepared for each compound. The compound solutions (6 ml) were transferred to each Petri dish (9 cm) lined with filter paper (Whatman No. 2). Petri dishes were then placed in polyethylene bags which were expanded and closed to prevent moisture loss. In the control treatment, a solution of 0.5% of DMSO and 0.02% of Triton-X 100 in distilled water was added. All treatments were kept at 20±2 °C and a 12-h photoperiod for 12 days. Then, the number of germinated seeds and length of

root and shoot were taken. Growth inhibition (I %) of root and shoot lengths was calculated from this equation:

$$I (\%) = [1 - T/C] \times 100$$

where T and C are the root or shoot lengths (cm) in treatment and control. Moreover, two compounds (**6** and **11**) were further evaluated on germination and seedling growth at a series of concentrations of 25, 50, 100, 250 and 500 mg/l.

Antifungal assay. The antifungal activity of fungal secondary metabolites was tested against *A. solani* and *F. oxysporum* by using growth inhibitory procedure⁵⁴. Stock solutions (25000 mg/l) of compounds were prepared in acetone. One milliliter of the stock solutions was mixed with PDA medium (50 ml) at 40-45°C before pouring into the Petri dishes (9.0 cm diameter) to give a final concentration of 500 mg/l. All compounds were tested in triplicate. Control dishes were prepared by mixing acetone only with PDA. Mycelial felt discs (0.5 cm diameter) of fungi were aseptically transferred to the center of Petri dishes. All treatments were incubated at 26 °C until fungal growth in the control treatments completely covered the Petri dishes. Then, the diameters of colonies were measured in treatments and control, and mycelial growth inhibition was calculated by using the formula:

$$GI (\%) = [(DC-DT) / DC] \times 100$$

where DC and DT are average diameters (cm) of fungal colony of control and treatment, respectively. Furthermore, compounds **2**, **3**, **4** and **7** were tested at a series of concentrations of 50, 100, 250 and 500 mg/l. Values of EC₅₀ of compounds were determined by using probit analysis⁵⁵.

Larvicidal bioassay. The toxicity of fungal secondary metabolites was carried out against *C. pipiens* larvae following the recommended method of the World Health Organization⁵⁶ with few modifications. The tested compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted with distilled water containing 0.1% of Tween-20 to give three final concentrations (100, 250 and 500 mg/l). Water in control treatments was mixed with DMSO (0.5%) and Tween-20 (0.1%). Twenty *C. pipiens* larvae were separately put into 200-ml plastic cups containing 100 ml of the compound solutions. Each concentration was replicated three times. All treatments were kept under the same insect rearing conditions for 24 h. Then, the number of dead larvae was counted and mortality (%) was calculated. Three compounds (**4**, **5** and **9**) were further tested at concentrations of 10, 25, 50, 70 and 100 mg/l. Mortality data of these compounds were subjected to probit analysis and LC₅₀ values were estimated⁵⁵.

Antifeedant test. A choice leaf-disc method was used to evaluate the antifeedant activity of fungal secondary metabolites against 3rd larval instar of *S. littoralis*⁵⁷. All tested compounds were tested at one concentration (1000 mg/l). The leaf discs (12 mm diameter) of *Ricinus communis* were soaked for 2 s in acetone solutions of the test compounds. Acetone was allowed to evaporate, then, the treated and control discs were put alternatively in a Petri dish (9 cm diameter). Then, ten larvae were located in the center of

each Petri dish. Each compound was replicated three times. The eaten areas of leaf discs were visually evaluated. The test was ended when larvae had eaten 50% of one of the discs.

Statistical analysis. Significant differences among mean values of germination percentages, root and shoot lengths, and mycelial growth inhibition percentages were determined ($P = 0.05$) by using a one-way analysis of variance (ANOVA) followed by Tukey's HSD test. The LC_{50} and EC_{50} of compounds were estimated by probit analysis⁵⁵ using the SPSS 21.0 software (Statistical Package of Social Sciences Inc., USA).

Declarations

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Author contributions

Conceptualization- S.A.M.A. and Y.S. Investigation and data curation- S.A.M.A., M.M.G.S. and N.E.M.T. Formal analysis- S.A.M.A. and Y.S. Project administration and supervision S.A.M.A. and Y.S. Writing: original draft S.A.M.A. Writing: review and editing- S.A.M.A. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that they have no conflict of interest in the publication.

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Figures

Figure 1

Chemical structure of fungal metabolites.