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Isolation, Characterization and Insecticidal Activity of Methylene Chloride Extract of *Cladosporium cladosporioides* Secondary Metabolites against *Aphis gossypii* (Glov.)

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ABSTRACT

The cotton aphid, *Aphis gossypii* Glover is a serious plant pests causing serious damage to the cultivated crops. Entomopathogenic fungi were early used for controlling aphids. *Cladosporium cladosporioides* is one of the promising candidates fungus can be used as a microorganism or can be used as a source of toxins against insect pests. Present research studied the chemical constituents of methylene chloride extracts of *C. cladosporioides*. It revealed 17 volatile compounds when analyzed by GC/MS technique. Also, two major compounds were isolated and identified as Pentadecanoic acid methyl ester (Compound 1) and Lupeol (Compound 2). Furthermore, the insecticidal activity of methylene chloride extracts of *C. cladosporioides* was tested against both adults and nymphs of cotton aphid, *A. gossypii*. It showed the most effective against nymphs of LC_{50} of 212.2319 ppm, LC_{90} of 1407.5663 ppm and toxicity index of 100%, while, it showed LC_{50} of 276.75 ppm, LC_{90} of 1902.15 ppm and toxicity index of 76.69% against adult stage.

Keywords: *Cladosporium cladosporioide*, Secondary metabolites, methylene chloride extracts, insecticidal activity and *Aphis gossypii*.

INTRODUCTION

The cotton aphid, *A. gossypii* Glover has a great economic importance due to its very wide host range and its serious damage to the cultivated crops. Cotton aphids suck nutrients from the plant and they secrete honeydew stimulating the growth of sooty mold hindering the photosynthetic capacity. Moreover, cotton aphid effectively transmits polyviruses, such as cucumber mosaic virus, watermelon mosaic virus 2 and zucchini yellow mosaic virus (Capinera, 2005).

The synthetic chemical insecticide of insecticides accompanied by harmful impact on human health, environment and non-target organisms. So, the need to replace these traditional insecticides by another secure alternatives is inevitable. One of the most important alternatives is biological pesticides which depend on living microorganisms and/or their toxins. Entomopathogenic fungi were among the first organisms used for this aim. They have a good epizootic characteristics and over dependence on the environmental factors. Moreover, they do not have to be ingested by the insect host but can invade the host upon contact with the insect cuticle (Boucias *et al*., 1988).

Species of the *Cladosporium* genus were used in the biological control of plant-insect pests like aphids and whiteflies which had been developing resistance to chemical insecticides. *C. cladosporioide* was naturally associated with cowpea *Aphis crassivora* (Ibrahim, 2012) and cabbage aphid, *Brevicoryne brassicae* L. (Ibrahim, 2017) and showed high virulence against aphid populations. Many bioactive compounds were isolated from *C. cladosporiodes*. Sakagami *et al*. (1995) isolated Cladosporol, which showed antitumor activity in mice that had gastric cancer from the culture filtrate of *C. cladosporioide*. Also, some pentacyclic composts of cytokines and tyrosine kinase inhibitory properties, was isolated from *C. cladosporioides* (Wrigley *et al*., 2001).

Therefore, the present study aimed to characterization and isolation of the chemical constituents of *C. cladosporioides* methylene chloride extract and evaluate the effect of it in controlling *A. gossypii*.

MATERIALS AND METHODS

Entomopathogenic Fungi

The culture of *C. cladosporioides* was obtained from Dr. Heba, Y. E. Ibrahim as pure strain. This strain was isolated from *A. craccivora* and was identified by Assiut Univ. Mycological Center (AUMC), Egypt.

The Tested Insect Pest

Aphis gossypii strain was obtained from the farm of faculty of Agriculture, Mansoura University, and has been known to be free from any insecticides. Aphid strain was reared on okra (2-3 weeks old) planted in small pots (15 cm3) and kept under plastic greenhouse conditions of 25± 5˚C, 70±7 RH and 10:14 hrs L:D. Aphid individuals were transferred weekly from old plants to new ones to avoid overcrowding.

Cultivation of the Tested Fungus

The fungal strain of *C. cladosporioides* was cultivated on autoclaved Sabouraud Dextrose yeast extract Agar (SDYA) [10 g/L peptone, 40 g/L dextrose, 10 g/L yeast extract and 20 g/L agar] and incubated at 25± 2˚C and 80±5% RH. small pieces of mycelial mats of 7-days old culture were transferred to 1000 ml bottles for autoclave containing 700 ml of (SDY) medium [i.e. the same medium components without agar]. Then, they incubated under 25± 2˚C, and 12:12 hrs L: D for 21 days. The culture fluid filtrated through two layers of whatman No. 1 filter paper.

Extraction of the Fungal Metabolites

Filtrate broth of *C. cladosporioides* was extracted with methylene chloride using separating funnel, then, filtered over anhydrous Na_2SO_4 and was dried by using fan to remove any traces of solvents and to obtain the final residues.

The residues were collected. A sample of it was analyzed by GC/MS technique for characterization and identification of its volatile components. The residue was subjected to column chromatography (CC) packed with silica gel as absorbent. Then, the collected similar sub-fractions were subjected to thin layer chromatography (TLC) using different solvent systems. Bands on (TLC) sheets were marked under ultra violet (UV) light 254 and 365 nm and/or detected by spraying with p-anisaldhyde – sulfuric acid reagent (Wagner *et al*., 1984).

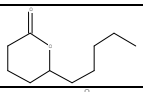
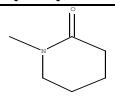
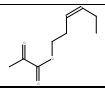
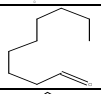
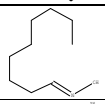
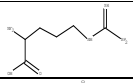
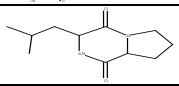
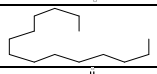
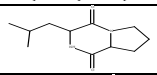
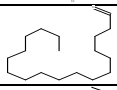
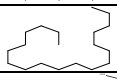
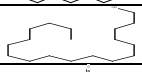
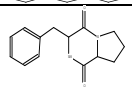
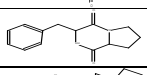
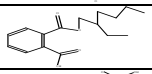
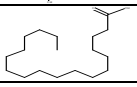
Efficiency of the fungal metabolite extracts against *A. gossypii*

Methylene chloride extract of the fungal secondary metabolites was formulated as emulsion in distilled water containing 0.3% Tween 80. Diluted series were prepared and were tested immediately after preparation. Ten individuals of the same age were transferred to a Petri-dish containing okra leaf to be considered as one replicate. Each concentration had 3 replicates and another three replicates sprayed only with water and 0.3% aqueous Tween 80 to be considered as control. Nymphs of two days old, were treated with the extract emulsion. All treatments were maintained under laboratory conditions of 25 ± 2°C,75 ± 5 % RH. Mortality percentages were recorded daily for a week and were corrected by Abbott's formula (1925) and analyzed for determination of 50 & 90% mortalities and slope values according to Finney (1971). In addition, the toxicity indexes were calculated by using Sun's equation (1950).

RESULTS AND DISCUSSION

The GC/MS chromatogram showed seventeen peaks corresponding to seventeen compounds which were characterized by comparing their mass spectra with those of their analogous reported by Wiley, NIST and Pfleger libraries. The obtained results were reported in Table 1.

Table 1. The GC/MS analysis of CH_2C_{12} fraction of *C. cladosporioides*

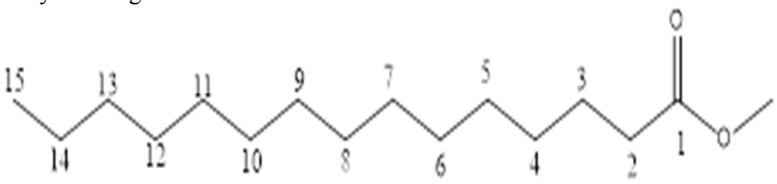
S.N.	Compound No.	R.T.	Area%	M.F.	Mol. Wt.	Structure
1	5-Decanolide(1)	10.72	16.99	$C_{10}H_{18}O_2$	170	
2	2-Piperidinone methyl (2)	19.47	0.78	$C_6H_{11}NO$	113	
3	3Z-Hexenyl pyruvate (3)	21.37	0.41	$C_9H_{14}O_3$	170	
4	Nonanal (4)	21.53	0.36	$C_9H_{18}O$	142	
5	Undecane (5)	22.42	0.72	$C_{11}H_{24}$	156	
6	Nonanal oxime (6)	22.83	1.67	$C_9H_{19}NO$	157	
7	L-Arginine = Arginine (S)-form (7)	23.65	2.26	$C_6H_{14}N_4O_2$	174	
8	Cyclo(leucylprolyl) (3S,8aS)-form(8)	25.49	3.67	$C_{11}H_{18}N_2O_2$	210	
9	Tetradecane (9)	26.90	0.83	$C_{14}H_{30}$	198	
10	Cyclo(leucylprolyl) (3R,8aR)-form(10)	27.67	18.65	$C_{11}H_{18}N_2O_2$	210	
11	Octadecanal (11)	29.52	20.41	$C_{18}H_{36}O$	268	
12	Nonadecane (12)	30.66	0.87	$C_{19}H_{40}$	268	
13	1-Octadecanol (13)	32.30	4.3	$C_{18}H_{38}O$	270	
14	Cyclo(phenylalanylprolyl) (3S,8aS)-form(14)	34.6	0.34	$C_{14}H_{16}N_2O_2$	244	
15	Cyclo(phenylalanylprolyl) (3R,8aR)-form(15)	35.33	9.42	$C_{14}H_{16}N_2O_2$	244	
16	Mono(2-ethylhexyl) phthalate (16)	38.01	0.76	$C_{16}H_{22}O_4$	278	
17	Octadecanoic acid(17)	42.82	0.40	$C_{18}H_{36}O_2$	284	

The two diketopiperazines, compounds 8, 10, 14 and 15 were early isolated from *Cladosporium. phlei* as stimulatory compounds. Compound 8&10 stimulated the pathogenic fungus to produce phleichrome which had antimicrobial and antifungal activities. (Seto *et al*. 2005). Also, these compounds were characterized by (Ibrahim 2012) in the methylene chloride fraction of *C. cladosporioides* using GC/MS technique.

The methylene chloride fraction was chromatographed over silica gel column and the major compounds were isolated by TLC. There were two major compounds were isolated as secondary metabolites of *C. cladosporioides*:

Compound (18):

Compound (18) was further purified by preparative layer chromatography using silica gel and the solvent system pet. ether, ether/ ethyl acetate (9.2 : 0.8), where compounds (18) was obtained as a white solid residue with Rf = 0.26 gave a violet color upon spraying with p-anisaldehyde-sulphuric acid reagent. A quick identification of (18) was proved by investigation of its 1H NMR spectrum as a fatty acid ester based on a collection of signals between 2.19 and 0.89 ppm which suggested the presence of methyl and methylene groups (Table 2). A very important singlet signal at 3.58 ppm (3H, s) was suggestive of the presence of the methoxy group of a methyl ester, in addition to the characteristic signals of α and β methylene protons to the ester group which appeared at 2.19 ppm (2H, t, J= 7.5 Hz, H-2) and 1.58 ppm (2H, m, H-3), the terminal methyl group appeared at 0.89 ppm (3H, t, J= 6.7 Hz). The mass spectrum (EI-MS) of compound (18) showed a molecular ion peak at m/z 256 corresponding to C16H32O2. The spectrum also showed an ion peak at m/z 241 (15%) due to expulsion of a methyl group. Another ion peak appeared at m/z 227 (42%) due to loss of [C2H5]+. Ion peaks appeared at m/z 129 (24%) and 73 (81%) corresponding to [C7H13O2]+ and [C3H5O2]+ respectively. The base peak which appeared at m/z 55 is due to $[C4H7]^+$. All the previous spectral data supported that compound (18) which was isolated from methylene chloride fraction is Pentadecanoic acid methyl ester (18).



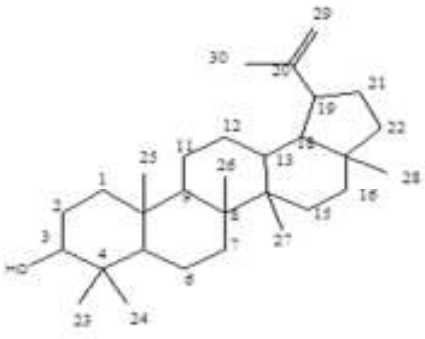
Pentadecanoic acid methyl ester

Table 2. 1H-NMR of compound (18) (CD3OD)

H atom	δ value, ppm	Multiplicity (J, Hz)
2	2.19	2H, t, J= 7.5 Hz
3	1.58	2H, m
4-14	1.28-1.31	22H, m
15	0.89	3H, t, J= 6.7 Hz
-OCH3	3.58	3H, s

Compound 19:

It was isolated as crystals, Rf = 0.84. 1H NMR spectrum (Table 3) revealed the presence of six angular methyl protons signals at δ 0.754, δ 0.780, δ 0.821, δ 0.935, 0.960 and δ 1.021 ppm (each singlet (s) for H-24, H-28, H-25, H-27, H-23 and H-26 respectively. The side chain signals appeared at δ 1.672 (s), 4.558 and 4.677 ppm for methyl group and two hydrogen proton of H-29. H-3 was represented by a double of doublet (dd) signal at 3.175 ppm.



Compound (19)

Table 3. 1H NMR of compound (19)

H atom	Δ value, ppm	Multiplicity(J,Hz)
3	3.175	dd, J=6.15
23	0.960	3H, s
24	0.754	3H, s
25	0.821	3H, s
26	1.021	3H, s
27	0.935	3H, s
28	0.780	3H, s
29	4.558, 4.677	2H, s,s
30	1.672	3H, s

The mass spectrum revealed the presence of M^+ ion at m/z 426(77%) corresponding to C30H50O. Also, the spectrum showed an ion peak at m/z 411 (16%) due to expulsion of methyl group, 393(4%) due to expulsion of methyl group and one molecule of H2O, 218 (47%) due to presence of [C16H26]+. The base peak was found at 55 (100%).

All the previous spectral data supported that compound (2) is Lupeol.

Compound 19 was isolated by (Ibrahim 2012) from methylene chloride fraction of *C. cladosporioides* broth after its characterization of it by GC/MS technique. This compound showed growth-disrupting effects against some insect pests in some plants, for example in *Senecio heritieri* (González-Coloma *et al*. 1999). Also, it has the potentiality as antibacterial, antioxidant, antitumor, cancer preventive, immunostimulant, chemo preventive, lipoxygenase inhibitor and pesticide.

Efficiency of Methylene Chloride Extract of *C. cladosporioides* metabolite against *A. gossypii*

Data presented in Table 4, showed that cumulative mortality percent of adults increased with increasing concentrations of the tested extract. Also, a fast activity against aphids was cleared after 24 hours of application.

Cladosporium cladosporioides methylene chloride extract showed cumulative mortality of cotton aphid adults ranged between 20.00- 53.33% at first day post treatment (Fig. 1). The maximum mortality percentages were obtained at 5th day post treatment to range between 50.00-93.33% at different tested concentrations. Cotton aphid treated with *C. cladosporioides* methylene chloride extract shrunk, lost its weight and turned to black color.

Aphid nymphs showed more susceptibility for *C. cladosporioides* methylene chloride extract than adults.

Data in Fig. 2 showed that *C. cladosporioides* methylene chloride extract revealed cumulative mortality of cotton aphid nymphs ranged between 26.67- 40.00% at first day post treatment. The mortality percentages increased to range between 46.67-96.67% at the 3rd day post treatment. The maximum mortality percentages were recorded at 5th day post treatment to range between 56.67-96.67% at different tested concentrations.

Also, data cleared that *C. cladosporioides* methylene chloride extract was most effective against nymphs showing LC_{50} of 212.2319 ppm, LC_{90} of 1407.5663 ppm and toxicity index of 100%, while, it showed LC_{50} of 276.75 ppm, LC_{90} of 1902.15 ppm and toxicity index of 76.69% for adults.

Table 4. Efficiency of *C. cladosporioides* methylene chloride extract against adults and nymphs of cotton aphid, *A. gossypii* under laboratory conditions of 25 ± 1 C0, 75 ± 7% RH.

Treatment	Conc. (ppm)	Mortality %at indicated day after treatment.				LC50 and confidence limits at 95%		LC90 and confidence limits at 95%		Slope ± SE	X2	Toxicity Index
		1st day	3rd day	5th day	7th day							
Adults	250	20.00	46.67	50.00	50.00	276.75		1902.15		1.5309 ± 0.3985	0.6673	76.69
	500	33.33	56.67	63.33	63.33							
	1000	43.33	70.00	76.67	76.67	106.636	419.37	1148.95	7140.5			
	2000	53.33	90.00	93.33	93.33							
Nymphs	250	26.67	46.67	56.67	56.67	212.2319		1407.5663		1.5598 ± 0.3907	0.3518	100
	500	30.00	66.67	70.00	70.00							
	1000	36.67	76.67	83.33	83.33	67.9939	338.919	923.5143	3650.1183			
	2000	40.00	96.67	96.67	96.67							

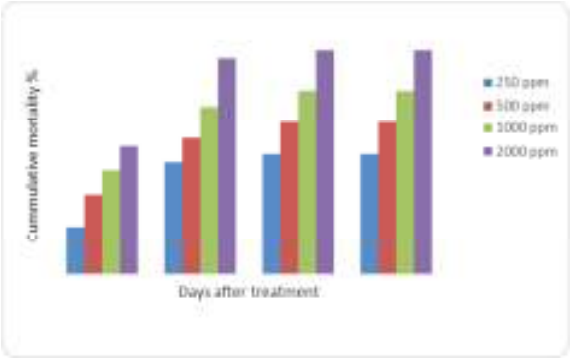


Fig. 1. The cumulative mortality % of methylene chloride extract of *C. cladosporioides* against adults of *A. gossypii*.

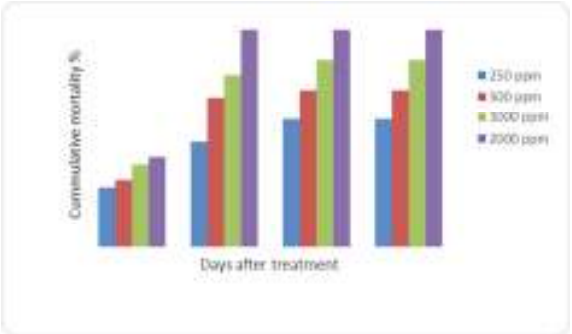


Fig. 2. The cumulative mortality % of methylene chloride extract of *C. cladosporioides* against nymphs of *A. gossypii*

From all previous data, *C. cladosporioides* is a promising microbial control agent can be used as a microorganism or used as toxins production for application as insecticides from nature origin.

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فصل وتعريف واختبار الابادة الحشرية لمستخلص كلوريد الميثيلين لمنتجات الأيض الثانوية لفطر *Cladosporium cladosporioides* على حشرة مَن القطن

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تعتبر حشرة مَن القطن آفة خطيرة تسبب أضرارا فادحة للمحاصيل الزراعية وقد تم استخدام الفطريات الممرضة للحشرات لمكافحة المن، ومن أهم هذه الفطريات الواعدة فطر *C. cladosporioides* الذي يستخدم كعامل من عوامل المكافحة الميكروبية أو يستخدم لاستخلاص سموماً يمكن استخدامها كمبيدات أمنة. وفي هذا البحث تم دراسة خلاصة كلوريد الميثيلين لمنتجات الأيض الثانوية لفطر *C. cladosporioides* وذلك بتحليلها عن طريق تقنية كروماتوجرافيا الغاز/طيف الكتلة وقد أسفر عن تعريف 17 مركب متطاير، أيضاً تم فصل مركبين أساسيين في هذا المستخلص وهما Pentadecanoic acid methyl ester و مركب Lupeol أيضاً تم دراسة الابادة الحشرية كلوريد الميثيلين لمنتجات الأيض الثانوية لفطر *C. cladosporioides* وأظهرت النتائج أعلى كفاءة ضد حوريات المن ثم يليها الأطوار البالغة حيث كانت قيم التركيز النصف مميت لمستخلص كلوريد الميثيلين لنواتج أيض هذا الفطر على الأطوار البالغة 276.75 ppm بكفاءة ابادية 76.69% بينما كانت قيم التركيز النصف مميت لمستخلص كلوريد الميثيلين لنواتج أيض هذا الفطر على الحوريات 212.2319 ppm بكفاءة ابادية 100%.