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ORIGINAL ARTICLE

Using the secondary metabolites of some fungi and wild plants as natural pesticides to control cotton mealybug, *Phenacoccus solenopsis* (Hemiptera: Pseudococcidae)

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Abstract

As alternatives to chemical insecticides, entomopathogenic fungi or wild plants and their secondary metabolites are being used. These biocontrol agents are significant because of their biodegradability, specificity, eco-friendliness, and utility as agents to reduce insecticide resistance. In this study five ethyl acetate extracts of locally isolated fungal strains (*Talaromyces atroroseus, Fusarium chlamydosporum*, *Talaromyces stipitatus*, *Trichoderma lixii, Beauveria bassiana*) as well as alkaloid extract of *Haloxylon salicornicum* were extracted and investigated as biocontrol agents against cotton mealybug *Phenacoccus solenopsis*. The results indicated that all extracts had toxic effects against *P. solenopsis* except the extract of *T. stipitatus*. The LC_{50} values and toxicity index indicated that the alkaloid extract of *H. salicornicum* was the most toxic one (26 ppm) after 72 hours of treatment followed by the extracts of *F. chlamydosporum* (77 ppm), then *B. bassiana* (84 ppm) and *T. lixii* (118 ppm). On the other hand, there were significant changes in tested insect enzyme activities (amylase, lipase, glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), and acetyl choline esterase (AchE) as well as total proteins and lipids in the insects treated with the alkaloid extract of *H. salicornicum*, and ethyl acetate extracts of *F. chlamydosporum* and *B. bassiana* after 24 hours of treatment compared to the control. GC/MS analyses of fungal extracts indicated that there were some bioactive compounds like hexadecanoic acid, octadecanoic acid, and tetradecanoic acid. In addition, the anabasine compound was found as a major constituent of the alkaloid extract of *H. salicornicum* and identified by 1H NMR and GC/MS analysis. In conclusion, according to this study, it was recommended that the alkaloid extract of *H. salicornicum* and the ethyl acetate extracts of *F. chlamydosporum, B. bassiana,* and *T. lixii* be used as alternatives to chemical insecticides for controlling the cotton mealybug *P. solenopsis*.

Keywords: biopesticides, entomopathogenic fungi, *Haloxylon salicornicum*, insect enzyme activity, *Phenacoccus solenopsis*

Introduction

The cotton mealybug, *Phenacoccus solenopsis* Tinsley (Hemiptera: Pseudooccidae), is a piercing sucking insect that attacks about 183 plant species that belong to 52 plant families (Hodgson *et al.* 2008; Khuhro *et al.* 2012; Ata 2019). Arif *et al.* (2012) mentioned that in Egypt, the cotton mealybug *P. solenopsis* is considered to be a harmful polyphagous insect pest that attacks cotton, okra, vegetables, and ornamental plants. Additionally, the insect can cause stunting and distortion in the plant because it secretes honeydew, which encourages the growth of associated molds as well as decreases photosynthesis. Farmers mainly use insecti-

cides for the control of insect pests, including mealybugs. Nevertheless, due to a lack of end-user protection and low public understanding of pesticide hazards, the use of pesticides is frequently abusive and inept, leading to serious human health issues, insect resistance, and environmental degradation (Nadeem *et al.* 2014). On the other hand, Kairo *et al.* (1997) mentioned that a biological control strategy is safer for farmers and has a higher success rate in controlling mealybugs.

As alternatives to chemical insecticides, bioactive substances of biological origin from entomopathogens and wild plants are being investigated. These biopesticides are significant because of their biodegradability, specificity, eco-friendliness, and utility as weapons to combat insecticide resistance. In order to replace traditional chemical pesticides in pest management programs, several researchers are interested in using natural compounds that have been extracted from microorganisms or wild plants (Ravindran *et al.* 2018). *Halexylon salicornicum* (the old scientific name was *Hammad elegans*) is a wild plant that belongs to the family Chenopdiaceae and contains mainly alkaloids such as anabasine, betine, piperidine, aldotripiperideine, haloxine, haloxyline A and B (Ferheen *et al.* 2005). Anabasine has insecticidal activity against lepidopterous larvae (*Pieris rapae*) at low concentrations, as mentioned by Zammit *et al.* (2014). Anabasine sulfate was widely used as a botanical insecticide in the former Soviet Union until the 1970s (Robert 2010).

The fungus *Talaromyces atroroseus* is primarily isolated from soil, fruits, and indoor environments (Frisvad *et al.* 2013). Pyripyropene A compound was reported as a secondary metabolite extracted from *Talaromyces* cultures which displayed high activity against *Myzus persicae* (Chaiyosang *et al.* 2021) at a concentration of 1.25 ppm in both laboratory and applications on cabbage plants. Also, Horikoshi *et al.* 2018 reported that pyripyropene A has high toxic effects against *Aphis gossypii*, *P. comstocki*, *Bemisia tabaci* and *Trialeurodes vaporariorum*. The insecticidal activity of extracts, fractions and pure compounds extracted from *Fusarium* sp. culture, was evaluated against *Spodoptera frugiperda* Smith and *Ceratitis capitata* Wiedemann. In addition, in a field experiment Mantzoukas *et al.* (2022) investigated seven species of *Fusarium* sp. as bioinsecticides for controlling the cotton bollworm *Helicoverpa armigera* (Hübner). They found that all tested species had insecticidal effects against *H. armigera* larvae. In another study by Vivekanandhan *et al.* (2018), the toxicity of *F. oxysporum* extract was evaluated against larvae and pupae of three mosquito species: *Anopheles stephensi*, *Aedes aegypti*, and *Culex quinquefaciatus*. The results indicated a strong effect at low concentrations especially when *F. oxysporum* extract was mixed with temephos. Two species of *Trichoderma* and their secondary metabolites were tested against nematode, *Meloidogyne incognita* (Jegathambigai *et al.* 2011). They prevented the hatching of *M. incognita* eggs and the growth of the second juveniles. Also, an ethyl acetate extract of *T. harzianum* was examined against *A. gossypii* and *S. littoralis* as an insecticide by Abdullah (2019). He found that the LC_{50} values of *T. harzianum* extract against *A. gossypii* and *S. littoralis* were 389 and 3238 ppm, respectively.

The aim of this study was to evaluate the insecticidal activities of the extracted secondary metabolites from some indigenous fungal isolates and the wild plant, *H. salicornicum,* against the cotton mealybug, *P. solenopsis.*

Materials and Methods

Isolation and identification of fungal strains

Agriculture soil samples (100 gm · sample^{-1}) were collected from different fields in Dakahliah governorate and then transported to the laboratory at the Plant Protection Research Institute, Mansoura branch, Agriculture Research Center, Egypt. Ten grams of the soil sample were added to 100 ml of sterilized water, stirred for 5 minutes, and then allowed to precipitate. Sterilized potato dextrose agar (PDA) plates were prepared and inoculated with 0.1 ml of soil solution, then incubated at 25°C. for 7 days. All the fungal colonies that appeared were purified on another PDA plate and incubated at 25°C. for 7 days. All fungal isolates were identified based mainly on the shape and color of the colony on the PDA plate, mycelium, and spores on glass slides under a light microscope. The fungal isolates were primarily tested to select the best isolates for use as biopesticides against cotton mealybug. The best fungal isolates were identified again to confirm identification with a molecular method using PCR with the primers ITS1/ITS4 (5/TCCGTAGGGTGAACCTGCGG/3) / (5/TCCTCCGCTTATTGATATGC/3) (White *et al.* 1990). *B. bassiana* (Bb) strain, obtained from a previous study (Abdullah 2019), was used as a reference strain.

Extraction and identification of secondary metabolites from fungal cultures

Fungal isolates were grown on potato dextrose broth (PDB) medium and incubated at 25°C for 21 days in a shaking incubator. After the incubation period, the fungal broth culture was filtered with muslin cloth. The supernatant volume was measured and mixed well with ethyl acetate solvent (1 supernatant: 0.5 ethyl acetate) in a separating funnel, then left for 2 hours until well separated. The upper layer was collected and kept in a conical flask. Also, ethyl acetate was added to

the lower layer, mixed well, and left for another hour for separation. The upper layer was collected and kept in the same conical flask. It may be necessary to repeat these steps many times. At the end, ethyl acetate extracts were concentrated by the rotary system. The crude extracts were weighed and refrigerated (Abdullah 2019). The crude extracts of fungal strains were analyzed by gas chromatography-mass spectrometry (GC-MS) for identification of their components in the GC-MS unit at the National Research Center, Dokki, Giza, Egypt. The ethyl acetate extract of *B. bassiana* broth culture and its GC-MS analysis were obtained from the previous study of Abdullah (2019), as shown in Table 1.

The wild plant

Haloxylon salicornicum Rimth Saltbush was collected from the Kilo 100, International Coastal Road, Alexandria-Morsa Matrouh, in April 2020. Prof. Dr. Ibrahim Mashaly of the Botany Department, Faculty of Science, Mansoura University, identified the plant.

Extraction of alkaloid extract from *Haloxylon salicornicum*

The air dried whole plant (1 kg) was extracted by soaking at room temperature in methanol (2 l × 5). Under lower pressure, the extract was concentrated to 0.5 l. Then the extract was acidified (pH 2) by adding 2 l of distilled water and HCl, left for 24 hours, and extracted with petroleum ether followed by chloroform by using a separating funnel. After that, the extract was basified (pH 12) by adding ammonium hydroxide and extracted after 24 hours by chloroform (alkaloid extract, 4.6 gm). Alkaloid extract (3.0 gm) was separated by column chromatography on aluminum oxide with eluent system petroleum ether/ethyl acetate till 50 : 50, then chloroform (100%). After that methanol was added gradually to produce four fractions. Fraction II at eluent system chloroform/methanol (95 : 5) gave compound 1 where it was purified using PTLC (silica gel, chloroform/methanol, 9 : 1, R_f 0.44, 12 mg). GC/MS afforded compound (1) (Rt = 18.00, 85.50%).

Table 1. Gas chromatography-mass spectrum analysis of ethyl acetate extract of *B. bassiana* broth culture (Abdullah 2019)

No.	Rt [min]	Compounds	Molecular Formula	Molecular Weight	Peak area [%]
1	12.367	4-Hydroxy-10-methyl-3,4,7,8,9,10-hexahydro-2H-oxecin-2-one	$C_{10}H_{16}O_3$	184	1.20
2	13.264	2-Carboxymethyl-3-n-hexylmaleic acid anhydride	$C_{12}H_{16}O_5$	240	0.97
3	14.740	1-Hexadecanol	$C_{16}H_{34}O$	242	1.29
4	14.912	4,5-Diethyl-2,3-dimethyl-2,3-dihydrofuran #	$C_{10}H_{18}O$	154	1.32
5	15.730	4,7,7-Trimethyl-3,9-dioxatricyclo[6.1.0.0(2,4)]nonan-5-one	$C_{10}H_{14}O_3$	182	1.77
6	18.854	Methyl 11-(3-pentyl-2-oxiranyl) undecanoate, trans-	$C_{19}H_{36}O_3$	312	1.84
7	20.483	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	14.96
8	20.710	7,9-Di-tert-butyl-1-oxaspiro[4.5]deca-6,9-diene-2,8-dione	$C_{17}H_{24}O_3$	276	1.29
9	21.608	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	21.14
10	22.069	cis-10-Heptadecenoic acid, methyl ester	$C_{18}H_{34}O_2$	282	1.50
11	22.992	cis-10-Heptadecenoic acid	$C_{17}H_{32}O_2$	268	0.23
12	23.145	10,18-Bisnorabieta-8,11,13-triene	$C_{18}H_{26}$	242	1.97
13	23.410	1-Hexadecanol, 2-methyl-	$C_{17}H_{36}O$	256	1.71
14	23.594	9,12-Octadecadienoic acid, methyl ester, (E,E)-	$C_{19}H_{34}O_2$	294	11.98
15	23.693	11-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	296	1.94
16	24.123	Tetradecanoic acid, 12-methyl-, methyl ester	$C_{16}H_{32}O_2$	256	12.25
17	24.652	Oleic Acid	$C_{18}H_{34}O_2$	282	2.86
18	25.162	Octadecanoic acid	$C_{18}H_{36}O_2$	284	1.50
19	25.359	Erucic acid	$C_{22}H_{42}O_2$	338	0.88
20	25.451	Ethanol, 2-(octadecyloxy)-	$C_{20}H_{42}O_2$	314	1.07
21	27.191	Heptacosane	$C_{27}H_{56}$	380	1.94
22	27.425	Z-5-Methyl-6-heneicosen-11-one	$C_{22}H_{42}O$	322	1.10
23	29.995	17-Pentatriacontene	$C_{35}H_{70}$	490	1.25
24	31.108	Diisooctyl phthalate	$C_{24}H_{38}O_4$	390	2.01
25	31.421	Thiocarbamic acid, N,N-dimethyl, S-1,3-diphenyl-2-butenyl ester	$C_{19}H_{21}NOS$	311	1.03

Rt – retention time

Characterization of anabasine 1

Anabasine 1 is a pale yellow oil (12 mg). ^{1}H NMR (500 MHz) in ($CDCl_3$), 8.55 (brs, H-2), 7.28 (m, H-4), 7.75 (t, J = 6.9 Hz, H-5), 8.52 (s, H-6), 3.68 (d, J = 9.9 Hz, H-2'). GC/MS, m/z (rel. int.): 162 (49%) $[C_{10}H_{14}N]^{+\cdot}$, 133 (70%) $[C_9H_{11}N]^+$, 119 (58%) $[C_8H_9N]^+$, 105 (75%) $[C_7H_7N]^+$, 92 (20%) $[C_6H_6N]^+$, 84 (100%) $[C_5H_{10}N]^+$.

The insect

The adult stage of cotton mealybug, *P. solenopsis* was obtained from the Department of Scale Insects and Mealybug, Plant Protection Research Institute, Mansoura branch, Agriculture Research Center, Egypt. The collected individuals were reared under laboratory conditions at 30 ± 2°C, 65 ± 5 RH and 13 : 11 (L : D) photoperiod on potato tubers.

Bioassay test

The adult stage of *P. solenopsis* was used to evaluate the efficiency of six extracts (five ethyl acetate extracts of fungal cultures and one alkaloid extract of a wild plant) using spray bioassay. Four serial concentrations were prepared from each extract (100, 200, 400, 800 ppm). Hibiscus leaves were cleaned and placed on Petri dishes (10 cm in diameter). Twenty individuals of *P. solenopsis* were transferred to each Petri dish. Two ml of each concentration were sprayed on each Petri dish and left to dry before being covered. A mixture of water and solvent was used to spray the control treatment. Each treatment was repeated four times. The dead insects in each treatment were recorded 1 and 3 days after treatment.

Measuring the insect enzyme activity

The median lethal concentration (LC_{50}) of each extract was calculated according to Finney (1971). The same method of the previous bioassay test was repeated by using only the LC_{50} value of each extract. After 1 day of treatment, the live individuals of *P. solenopsis* were collected and weighed in Eppendorf tubes. These Eppendorf tubes were kept in a freezer until measuring the enzyme activity. The enzyme activities were measured in the Biochemical Analysis Unit, Plant Protection Research Institute, Agriculture Research Center, Dokki, Giza, Egypt. Five enzyme activities were measured: amylase, lipase, glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), and acetyl choline esterase (AchE), as well as total protein and total lipid.

Statistical analysis

In the bioassay experiment, the mortality percentage was corrected by Abbot's formula (Abbott 1925). The median lethal concentration (LC_{50}) and toxicity index were calculated to compare treatments according to Finney (1971) and Sun (1950). In the estimation experiment of insect enzyme activities, the data were subjected to analysis of variance (ANOVA). The least significant difference (LSD) and Duncan's multiple at $p \leq 0.05$ were calculated by SAS software (SAS 1997) to compare the treatments.

Results

Identification of fungal strains

Four fungal isolates were selected from sixteen isolates based on their efficiency as biopesticides in the primary test against cotton mealybug. The *B. bassiana* (Bb) strain was obtained from the previous study of Abdullah (2019). The unknown fungal isolates were cultured on PDA plates and primarily identified in the Plant Pathogen Research Institute, Agriculture Research Center, Giza, Egypt. These isolates were sent to Macrogen Company (South Korean public biotechnology company) in Seoul, South Korea for molecular identification based on ITS gene. Table 2 shows the molecular identification of four fungal isolates.

Identification of extracted secondary metabolites from fungal cultures using GC-MS

In the current investigation, a GC-MS system was used to analyze crude extracts of fungal cultures in order to identify some of their constituent parts. Figure 1 shows the chromatograms of GC-MS of ethyl acetate extracts of fungal broth cultures (F1 – *T. atroroseus*,

Table 2. Identification of fungal isolates by molecular identification using ITS gene

Isolate code	Scientific name	Accession number in GenBank	Percent identity [%]
F1	*Talaromyces atroroseus* DTO390-I4 (Ascomycete fungi)	MN788119	99.8
F2	*Fusarium chlamydosporum* RPF2 (Ascomycete fungi)	OP038630	100
F3	*Talaromyces stipitatus* T1D1 (Ascomycete fungi)	MN518410	100
F4	*Trichoderma lixii* CI11 (Ascomycete fungi)	MW805725	95.29

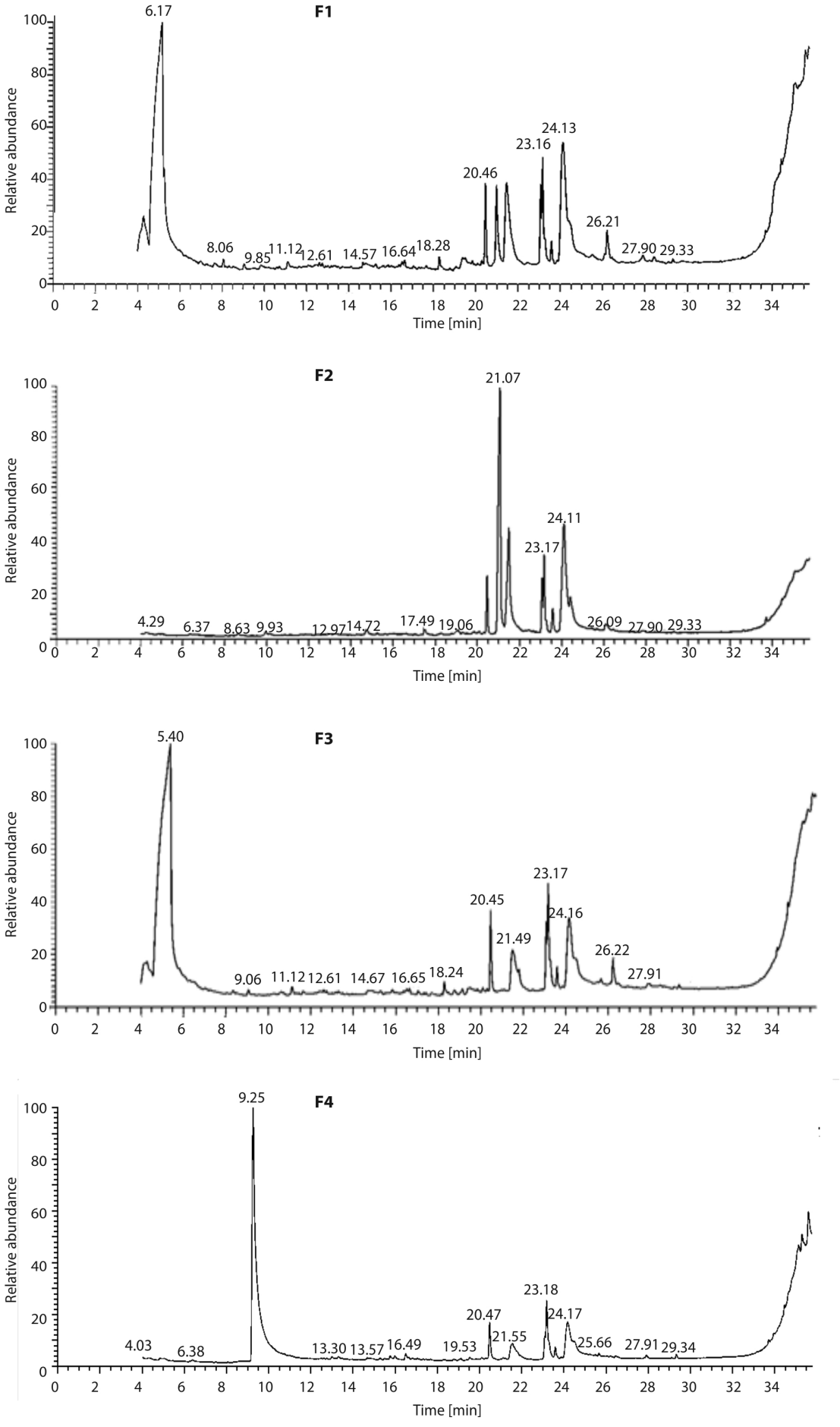

Fig. 1. GC-MS chromatograms of the ethyl acetate extracts of potato dextrose broth medium of fungal isolates (F1 – *Talaromyces atroroseus,* F2 – *Fusarium chlamydosporum,* F3 – *Talaromyces stipitatus,* and F4 – *Trichoderma lixii*)

F2 – *F. chlamydosporum*, F3 – *T. stipitatus,* and F4 – *T. lixii*). Each GC-MS chromatogram illustrated the peak area and retention times (Rt) of each chemical compound in four fungal strain extracts.

The chemical compounds that were identified from ethyl acetate extracts of four fungal strains are listed in Tables 3, 4, 5, and 6. In the case of F1 – *T. atroroseus* extract, detected constituents included 16 compounds (Table 3). The major compounds were DL-aepfelsaeure (10.92%), 3,5-dimethyl-hexane-1,3,4-triol (17.18%), n-hexadecanoic acid (10.71%) and 9,12-octadecadienoic acid (Z,Z)- (15.71), as shown in Table 3. There were 13 detected compounds in F2 – *F. chlamydosporum* extract but the major compounds were 5a-ethoxy-9a-methyl-3,4,5a,6,7,8,9a,10-octahydropyrano[4,3-b] chromene-1,9-dione (39%), n-hexadecanoic acid (12.08%) and oleic Acid (16.86%) as shown in Table 4. In addition, there were 15 compounds in the extract of F3 – *T. stipitatus*' culture, while four compounds in the extract had a higher percentage of peak area: D,L-hydroxybutadioic acid (12.95%), hexane-1,3,4-triol, 3,5-dimethyl- (28.70%), 11-octadecenoic acid, methyl ester,(Z)- (12.48%) and oleic Acid (11.98%) as shown in Table 5. Also, the detected constituents in F4 – *T. lixii* extract were 8 compounds (Table 6). The major compounds were 2-methoxy-4-vinylphenol (54.65%), hexadecanoic acid, methyl ester (7.13%), 9-octadecanoic acid (Z)-, methyl ester (15.11%) and 2,3-dihydroxypropyl elaidate (10.97%).

Identification of compounds in (*Haloxylon salicornicum*)

Compound 1 was isolated as yellow oil (12 mg) which became orange with Dragendorffs' reagent on TLC which indicated that it is an alkaloid compound. ^{1}H NMR spectrum (Table 7) revealed the presence of pyridine ring δ_H 8.55 (brs, H-2), δ_H 7.28 (m, H-4), δ_H 7.75 (t, J = 6.9 Hz, H-5) and δ_H 8.52 (s, H-6). On the other hand, a signal was detected at δ_H 3.68 (d, J = 9.9 Hz, H-2') for H-2' of piperidine ring that attached to pyridine ring. Also, 2H-6' were seen at δ_H 3.21 and δ_H 3.48 ppm. Mass spectrum indicated that the molecular ion peak was at m/z 162 as $[M]^{+\cdot}$ and base peak at m/z 84 for piperidine ring. As shown in Figure 2, the fragment ion peaks at m/z 133, 119, 105 and 92 due to fragment ions $[C_9H_{11}N]^+$, $[C_8H_9N]^+$, $[C_7H_7N]^+$ and $[C_6H_6N]^+$, respectively, were in agreement with anabasine (Ali 2004).

Insecticidal activity of tested extracts against cotton mealybug

Mortality percentages of treated cotton mealybug and toxic effects of tested extracts are listed in Table 8. The results indicated that all tested extracts had high toxic effects against cotton mealybug except the extract of F3 (*T. stipitatus*). The extracts of F2 (*F. chlamydosporum*), Bb (*B. bassiana*) and Rimth (*H. salicornicum*) caused

Table 3. The compounds identified by gas chromatography – mass spectrum of ethyl acetate extract of *Talaromyces atroroseus* broth culture (F1)

No.	Rt [min]	Compound	Molecular formula	Molecular weight	Peak area [%]
1	4.28	Benzeneacetic acid, 2-octyl ester	$C_{16}H_{24}O_2$	248	9.66
2	4.73	DL-Aepfelsaeure	$C_4H_6O_5$	134	10.92
3	5.18	3,5-Dimethyl-hexane-1,3,4-triol	$C_8H_{18}O_3$	162	17.18
4	8.07	Tetradecanoic acid, 2-hydroxy-	$C_{14}H_{28}O_3$	244	0.64
5	18.28	Tetradecanoic acid, 12-methyl-, methyl ester	$C_{16}H_{32}O_2$	256	1.37
6	19.38	Pentadecanoic acid	$C_{15}H_{30}O_2$	242	1.05
7	20.46	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	5.97
8	20.98	Widdrol epoxide	$C_{15}H_{26}O_2$	238	6.02
9	21.46	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	10.71
10	23.07	8,11-Octadecadienoic acid, methyl ester	$C_{19}H_{34}O_2$	294	5.39
11	23.16	9-Octadecenoic acid, methyl ester,(E)-	$C_{19}H_{36}O_2$	296	8.23
12	23.27	10-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	296	1.49
13	23.58	Octadecanoic acid, methyl ester	$C_{19}H_{38}O_2$	298	1.63
14	24.09	9,12-Octadecadienoic acid (Z,Z)-	$C_{18}H_{32}O_2$	280	15.71
15	24.50	Octadecenoic acid	$C_{18}H_{36}O_2$	284	1.07
16	26.21	7,9-Dihydroxy-6,9α-dimethyl-3-methylenedecahydroazuleno[4,5-b]furan-2(3H)-one	$C_{15}H_{22}O_4$	266	2.37

Rt – retention time

Table 4. The compounds identified by gas chromatography – mass spectrum of ethyl acetate extract of *Fusarium chlamydosporum* culture (F2)

No.	Rt [min]	Compound	Molecular formula	Molecular weight	Peak area [%]
1	9.92	α-ylangene	$C_{15}H_{24}$	204	0.44
2	14.72	1,2-Benzenedicarboxylic acid diethyl ester	$C_{12}H_{14}O_4$	222	0.81
3	17.49	Widdrol epoxide	$C_{15}H_{26}O_2$	238	1.00
4	20.46	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	6.54
5	21.07	5a-Methoxy-9a-methyl-3,4,5a,6,7, 8,9a,10-octahydropyrano[4,3-b]chromene-1,9-dione	$C_{14}H_{18}O_5$	266	39.00
6	21.50	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	12.08
7	23.07	9,12-Octadecadienoic acid (Z,Z)-methyl ester	$C_{19}H_{34}O_2$	294	5.99
8	23.17	Oleic acid, methyl ester	$C_{19}H_{36}O_2$	296	9.06
9	23.26	10-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	296	0.99
10	23.58	Methyl octadecanoate	$C_{19}H_{38}O_2$	298	3.03
11	24.10	Oleic Acid	$C_{18}H_{34}O_2$	282	16.86
12	24.42	Octadecanoic acid	$C_{18}H_{36}O_2$	284	2.23
13	26.08	Spiculesporic acid	$C_{17}H_{28}O_6$	328	1.16

Rt – retention time

Table 5. The compounds identified by gas chromatography – mass spectrum of ethyl acetate extract of *Talaromyces stipitatus* broth culture (F3)

No.	Rt [min]	Compound	Molecular formula	Molecular weight	Peak area [%]
1	4.13	Benzene acetaldehyde	C_8H_8O	120	3.45
2	4.28	Amphetamine	$C_9H_{13}N$	135	1.34
3	4.84	D,L-Hydroxybutadioic acid	$C_4H_6O_5$	134	12.95
4	4.97	Hexane-1,3,4-triol, 3,5-dimethyl-	$C_8H_{18}O_3$	162	28.70
5	11.11	N-Phenethyl-2-methylbutylidenimine	$C_{13}H_{19}N$	189	0.83
6	18.28	Tetradecanoic acid, 12-methyl-,methyl ester	$C_{16}H_{32}O_2$	256	1.26
7	19.14	Versalide	$C_{18}H_{26}O$	258	0.73
8	20.46	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	7.36
9	21.47	Hexadecanoic acid	$C_{16}H_{32}O_2$	256	5.77
10	21.80	Hexadecanoic acid, 2,3- dihydroxypropyl ester	$C_{19}H_{38}O_4$	330	0.69
11	23.07	9,12-Octadecadienoic acid, methyl ester, (E,E)-	$C_{19}H_{34}O_2$	294	5.65
12	23.17	11-Octadecenoic acid, methyl ester,(Z)-	$C_{19}H_{36}O_2$	296	12.48
13	23.59	Octadecanoic acid, methyl ester	$C_{19}H_{38}O_2$	298	1.96
14	24.15	Oleic Acid	$C_{18}H_{34}O_2$	282	11.98
15	26.21	Pyrrolidine,1-(1-oxo-7,10-hexadecadienyl)-	$C_{20}H_{35}NO$	305	2.57

Rt – retention time

the highest mortality percentages of cotton mealybug. The mortality percentages of insects treated with the three extracts (F2, Bb, and Rimth) reached 77 and 89%, 91 and 98% and 93 and 98% after 1 and 3 days of treatment, respectively. Also, the LC_{50} and toxicity index values indicated that the previous extracts were more toxic than other extracts. The extract of Rimth plant had the highest toxicity (LC_{50} = 26 ppm) followed by F2 extract (LC_{50} = 77ppm), then Bb extract LC_{50} = 84 ppm) after 3 days of treatment as shown in Table 8 and Figure 3. The slope values and toxicity lines indicated that there was high homogeneity in the tested individuals of *P. solenopsis* towards the effect of tested extracts.

Effect of tested extracts on insect enzyme activities and total proteins and lipids

The changes in enzyme activities and total proteins and lipids in the cotton mealybug were investigated to

Table 6. The compounds identified by gas chromatography – mass spectrum of ethyl acetate extract of *Trichoderma lixii* broth culture (F4)

No.	Rt [min]	Compound	Molecular formula	Molecular weight	Peak area [%]
1	9.25	2-Methoxy-4-vinylphenol	$C_9H_{10}O_2$	150	54.64
2	16.49	9-Hexadecenoic acid	$C_{16}H_{30}O_2$	254	1.55
3	20.47	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	7.13
4	21.50	Hexadecanoic acid	$C_{16}H_{32}O_2$	256	4.86
5	23.09	9,12-Octadecadienoic acid, (Z,Z)-methyl ester	$C_{19}H_{34}O_2$	294	3.49
6	23.18	9-Octadecanoic acid (Z)-, methyl ester	$C_{19}H_{36}O_2$	296	15.11
7	23.59	Octadecanoic acid, methyl ester	$C_{19}H_{38}O_2$	298	2.25
8	24.16	2,3-Dihydroxypropyl elaidate	$C_{21}H_{40}O_4$	356	10.97

Rt – retention time

Table7. ^{1}HNMR data for compound 1 in $CDCl_3$ (δ) [ppm] (Multiplicity, *J* [Hz])

Position	(δ_H) compound 1
2	8.55 (brs)
4	7.28 (m)
5	7.75(t, *J* = 6.9 Hz, 1H)
6	8.52 (brs)
2′	3.68 (d, *J* = 9.9 Hz, 1H)
3′	2.78 (m), 1.91 (m)
4′	1.61 (m), 1.52 (m)
5′	1.80 (m), 1.69 (m)
6′	(m), 3.21 (m)

predict the possible mode of action of extracts and to know the insect reaction and resistance to the tested extracts. These changes were estimated in insects treated with the highest effective extracts (F2, Bb and Rimth) and compared to the control treatment. Total protein and lipids in haemolymph, two digestive enzymes (lipase and amylase) and the detoxifying enzyme (acetylcholine esterase) as well as transmission enzymes GOT and GPT were determined. The results showed significant changes in total proteins and total lipids in insects treated with *F. chlamydosporum* (F2) compared to the control. Total protein increased (6.4 mg · gm^{-1} body weight) but total lipids decreased (2.77 mg · gm^{-1} body weight) compared to the control. On the other hand, no significant changes in total protein and lipids were found in insects treated with *B. bassiana* (Bb) and *H. salicornicum*, as shown in Table 9. The results illustrated that there was a significant increase of estimated digestive enzyme (lipase and amylase) activities compared to the control. The lipase enzyme reached its highest activity in the insects treated with an extract of *F. chlamydosporum* F2 (12.28 U · L^{-1}), followed by an extract of *H. salicornicum* Rimth (7.90 U · L^{-1}). The highest activity of the amylase enzyme was found in the insects treated with an extract of *B. bassiana* Bb

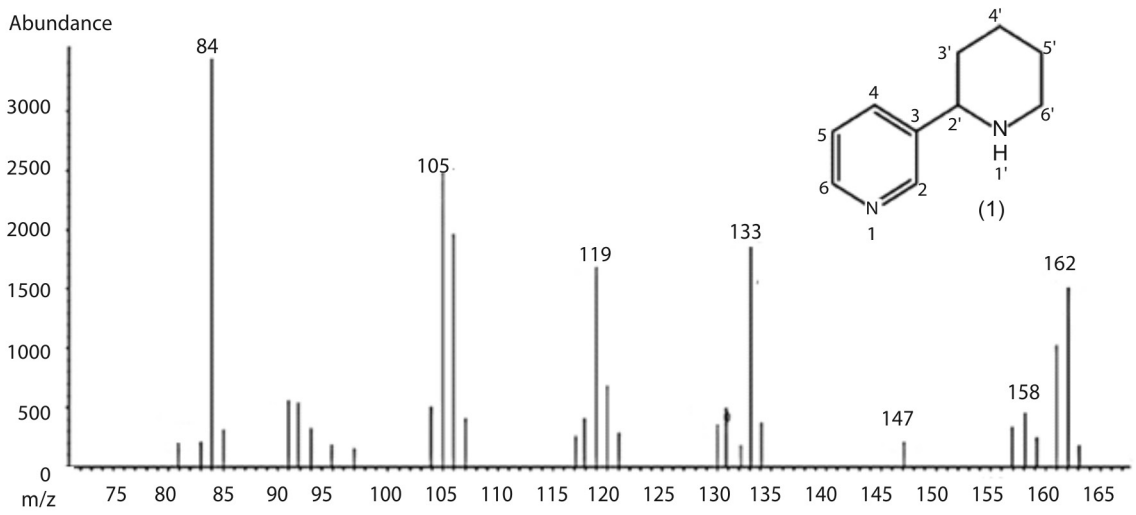

Fig. 2. Mass spectrum of compound 1

Table 8. Insecticidal activity of secondary metabolites of wild plant and fungal strains against cotton mealybug under laboratory conditions

Treat.	Conc.	Insecticidal activity after 24 hours of treatment						Insecticidal activity after 72 hours of treatment					
		Mort. %	LC_{50} [ppm]	confidence limit [ppm]		slope ± S.E.	toxicity index [%]	Mort. %	LC_{50} [ppm]	confidence limit [ppm]		slope ± S.E.	toxicity index [%]
				lower	upper					lower	upper		
F1	100	10	591	491	757	1.91 ± 0.22	15	33	190	148	231	1.53 ± 0.20	14
	200	14						52					
	400	35						69					
	800	63						83					
F2	100	37	195	140	250	1.18 ± 0.19	45	56	77	38	112	1.18 ± 0.21	34
	200	50						68					
	400	64						80					
	800	77						89					
F3	100	7	3813	1669	36432	1.02 ± 0.25	2	16	1060	705	2352	1.09 ± 0.20	2
	200	8						18					
	400	13						29					
	800	27						48					
F4	100	13	360	312	412	2.18 ± 0.22	24	46	118	84	149	1.59 ± 0.21	22
	200	26						63					
	400	53						80					
	800	79						91					
Bb	100	30	172	142	201	2.07 ± 0.22	51	56	84	59	106	2.14 ± 0.28	31
	200	57						79					
	400	78						93					
	800	91						98					
Rimth	100	53	88	56	117	1.56 ± 0.22	100	76	26	6	50	1.28 ± 0.24	100
	200	71						88					
	400	85						95					
	800	93						98					

Treat. – treatments, Conc. – concentration, Mort.% – Mortality percentage, F1 – *Talaromyces atroroseus*; F2 – *Fusarium chlamydosporum*, F3 – *Talaromyces stipitatus*, F4 – *Trichoderma lixii*, Bb – *Beauveria bassiana*, Rimth – *Haloxylon salicornicum*

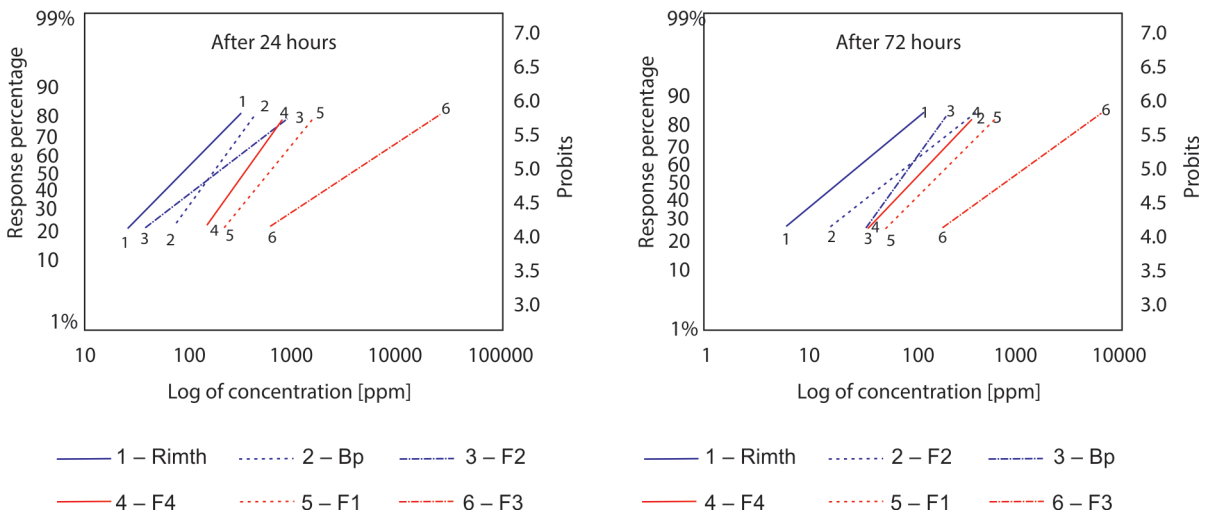

Fig. 3. Log Dose-Probit's Lines of treated cotton mealybug with secondary metabolites of wild plant and fungal strains under laboratory conditions

(3.19 U · L^{-1}), followed by an extract of *H. salicornicum* Rimth(2.89 U · L^{-1}).

On the other hand, some detoxifying enzyme activities were determined in the insects treated with extracts of F2, Bb and Rimth. The results in Table 10 showed a significant increase in acetylcholine esterase activity in the insects treated with all tested extracts compared to the control. But there were no significant changes in glutamic oxaloacetic transaminase (GOT) activity in the insects treated with all tested extracts except for the extract of F2, where it caused a significant increase reaching 25 U · L^{-1}. Also, there were significant increases of glutamic pyruvic transaminase (GPT) in the insects treated with extracts of F2 (11 U · L^{-1}) and Rimth (3 U · L^{-1}).

Discussion

In the present study, four fungal strains were isolated and identified to use their secondary metabolites as biocontrol agents against cotton mealybug. The efficiency of entomopathogens is affected by climatic conditions. Since the growth and reproduction of entomopathogens are affected by temperature, humidity and ultraviolet radiation, it is better to use their secondary metabolites in pest control rather than microorganisms. In several studies the secondary metabolites of entomopathogens have been extracted and used to control many insect pests (Bandani *et al.* 2000; Molnar *et al.* 2010). Also, the bioactive substances of biological origin extracted from entomopathogens and wild plants are significant because of their biodegradability, specificity, eco-friendliness, and utility as weapons to combat insecticide resistance. In order to replace traditional chemical pesticides in pest management programs, several researchers have become interested in using natural compounds extracted from microorganisms or wild plants (Ravindran *et al.* 2018). Furthermore, in this study, alkaloid extract of wild plant *H. salicornicum* and the secondary metabolites of *B. bassiana* were used in controlling cotton mealybug, *P. solenopsis.*

The secondary metabolites of isolated fungal strains and wild plant in this work were identified using GC-MS. The results indicated that many compounds can be used as bioinsecticides such as hexadecanoic acid, tetradecanoic acid, octadecanoic acid and anabasine. The previous studies mentioned that the

Table 9. Effect of secondary metabolites of wild plant and fungal strains on total proteins, total lipids, lipase and amylase activity in the adult stage of cotton mealybug

Treatments	Total proteins [mg · gm^{-1} body weight] ± SE	Total lipids [mg · gm^{-1} body weight] ± SE	Lipase activity [U · L^{-1}] ± SE	Amylase activity [U · L^{-1}] ± SE
Control	4.0 b ± 0.58	3.55 a ± 0.10	4.85 d ± 0.050	0.90 d ± 0.05
F2	6.4 a ± 0.58	2.77 c ± 0.10	12.28 a ± 0.005	1.78 c ± 0.05
Bb	4.0 b ± 0.58	3.44 a ± 0.10	5.46 c ± 0.050	3.19 a ± 0.005
Rimth	4.0 b ± 0.58	3.10 b ± 0.01	7.90 b ± 0.050	2.89 b ± 0.05
LSD (5%)	1.88	0.18	0.089	0.089

F2 – *Fusarium chlamydosporum*, Bb – *Beauveria bassiana,* Rimth – *Haloxylon salicornicum*

Table 10. Effect of secondary metabolites of wild plant and fungal strains on glutamic oxaloacetic transaminase, glutamic pyruvic transaminase, and acetyl choline esterase activity in the adult stage of cotton mealybug

Treatments	Glutamic oxaloacetic transaminase [U · L^{-1}] ± SE	Glutamic pyruvic transaminase [U · L^{-1}] ± SE	Acetyl choline esterase [(µg AchBr · min · gm^{-1} body weight] ± SE
Control	2 b ± 0.58	1 c ± 0.06	369 c ± 25.7
F2	25 a ± 0.58	11 a ± 0.5	625 a ± 5.8
Bb	2 b ± 0.58	1 c ± 0.06	500 b ± 20.7
Rimth	2 b ± 0.58	3 b ± 0.06	459 b ± 37.5
LSD (5%)	2.3	0.96	88

F2 – *Fusarium chlamydosporum*, Bb – *Beauveria bassiana,* Rimth – *Haloxylon salicornicum*

extracts of fungal cultures or wild plants which contain n-hexadecanoic acid and hexadecanoic acid methyl ester, octadecadienoic acid methyl ester, tetradecanoic acid 12-methyl- methyl ester, oleic acid (Kumar *et al.* 2010; Sivakumar *et al.* 2011; Tulika and Mala 2017; Yokeswari *et al.* 2018; Abdullah 2019) and anabasine which, not like nicotine compounds (Glennon and Dukat 2000; Shao *et al.* 2007; Lisko *et al.* 2013; Slyn'ko *et al.* 2013; Zammit *et al.* 2014), have toxic effects against several insect pests. In this study, the data obtained from GC-MS analysis indicated that most tested extracts contain high percentages of these compounds as shown in Tables 3, 4, 5 and 6. Also, several studies used the tested fungal strains in this study as entomopathogenic fungi to control many insect pests: *Talaromyces* sp. (Panyasiri *et al.* 2007; Abdel Galil *et al.* 2019; Nicoletti and Becchimanzi 2022), *Fusarium* sp. (Vivekanandhan *et al.* 2018; Abdel Galil *et al.* 2019; Mantzoukas *et al.* 2022), *B. bassiana* (Valencia *et al.* 2011; Ritu *et al.* 2012; Abdullah 2019), and *Trichoderma* sp. (Khattab *et al.* 2018; Abdullah 2019; Caccavo *et al.* 2022).

In our study, insect enzyme activities and total proteins and lipids were determined in cotton mealybug treated with the effective extracts of fungal strains and alkaloid extracts of wild plants. The results indicated that there were significant changes in the activities of insect enzymes and the content of proteins and lipids in cotton mealybug. The increase of total protein in haemolymph may have happened according to the natural increase of detoxifying and hydrolytic protective enzymes that usually happen shortly after treatment (Assar *et al.* 2016). The soluble proteins in haemolymph are considered to be a reserve source of protein synthesis needed for development and growth of insects (Florkin and Jeanuiaux 1964). The detoxifying enzymes which help in the detoxification of toxicants from the insect body are synthesized from protein (Wilkinson 1976). The haemolymph lipid content assists in the development, metamorphosis, oogenesis and flight of insects (Downer and Matthews 1976). Hill and Izatt (1974) mentioned that lipid is more likely to be related directly to the lack of juvenile hormone. Amylases are necessary to digest carbohydrates as starch in plants or glycogen in insects for obtaining energy (Terra and Ferriera 2005). Several studies have mentioned that different changes happened in amylase enzyme activities in the insects treated with plant extracts (Shekari *et al.* 2008; Zibaee and Bandani 2010). Lipases are enzymes that hydrolyze the outer links of fat molecules and have been studied in insects. Previous studies reported that the enzyme activity significantly changes in insects due to the use of botanical insecticides (Senthil *et al.* 2006; Zibaee *et al.* 2008). Acetyl choline esterase enzyme is one of the detoxifying enzymes in insects and it is an important target for many insecticides in the central nervous system of insects (Nathan *et al.* 2008). Acetylcholine esterase is able to catalyze the hydrolysis of neurotransmitter, acetylcholine, in the nervous system of various organisms to terminate nerve impulses (Bourne *et al.* 2016). Glutamic-oxaloacetic transaminase (GOT/AST) and glutamic-pyruvic transaminase (GPT/ALT) are transaminase enzymes which can be affected by bioinsecticides (Ahmed 2020). Wigglesworth (2012) found a close relationship between protein synthesis and levels of transaminases in insects. Osman *et al.* (2015) mentioned that these enzymes may help in protein synthesis and are involved in the synthesis of amino acids during metamorphosis in insects.

Conclusions

According to this study, it is recommended to use the secondary metabolites of entomopathogenic fungi and wild plants as biocontrol agent alternatives to chemical insecticides. In this work, ethyl acetate extracts of *F. chlamydosporum*, *B. bassiana*, *T. lixii*, and an alkaloid extract of *H. salicornicum* led to a high toxic effect against *P. solenopsis*. As a result, they can be used as alternatives to chemical insecticides for controlling cotton mealybugs.

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