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Effectiveness of ethyl acetate extract from *Aspergillus flavipes* AUMC 11390 culture filtrate on biological and physiological performance of the spiny bollworm, *Earias insulana,* (Boisd.) (Lepidoptera: Nolidae)

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Abstract

Background Spiny bollworm, *Earias insulana* is a serious cotton pest in Egypt. Besides the economic losses it caused, treatment with chemical insecticides has negative effects on human health and the environment, thus the development of a powerful safe control strategy rather than chemical pesticides is an international goal.

Results Ethyl acetate extract from *Aspergillus flavipes* AUMC 11390 culture filtrate has an insecticidal activity against *E. insulana* causing larval and pupal mortality of 58.33, and 15.59%, respectively, compared with controls, in addition, reduction in adult's emergency and deformation of emerged adults. The impact of fungal extract treatment extended to adult stages by diminishing the male and the female longevity, the number of produced eggs and the hatchability percent. Furthermore, *A. flavipes* AUMC 11390 ethyl acetate extract caused a strong disturbance on some insect enzymes including amylase, invertase, trehalase, GOT, GPT and acetylcholinesterase, alongside total lipid and total protein. Analysis of ethyl acetate fungal extract revealed the presence of one hydrocarbon 3-Eicosene and four long-chain alcohols namely hexadecanol, 1-hexadecanol, 1-octadecanol, and 1-pentadecanol which are known for their insecticidal activity.

Conclusion *A. flavipes* AUMC 11390 culture filtrate might represent a promising source for different important bioactive compounds that could be used as a potential biocontrol agent involved in *E. insulana* management strategies.

Keywords *Earias insulana*, *Aspergillus flavipes*, Long-chain alcohols, Insecticidal activity

1 Background

The spiny bollworm, *Earias insulana,* (Boisd.) (Lepidoptera: Nolidae) [1] is a profoundly severe cotton pest. In Egypt, about one million kantars of cotton can be lost due to their infection by this pest [1–3]. *E. insulana* has several alternative host plants okra, maize and other economic plants which play a significant role in the carrying-over of *Insulana* spp to cotton [4–6]. The larvae infect the early stages of plant growth, by entering near the terminal bud and then burrows down inside the main stem causing the death of the main stem growing point [7]. On older cotton plants, *E. insulana* feeds in buds, flowers and green bolls then tends to be an internal feeder and is found commonly in bolls [8].

In Egypt up till now, chemical techniques are used for controlling the spiny bollworm pest, but because of the

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indiscriminate and extensive usage of such pesticides, serious ecological hazards have been raised in livestock, human health and the environment, in addition to disruption of the biological control system and the emergence of strains resistant to many insecticides [9, 10]. All of these factors lead to the phase-out of many chemical agricultural agents from the Egyptian market such as fluometuron and chlorpyrifos (CPS) compounds. Therefore, discovering powerful, safe and novel bio-control agents as natural alternatives that work alone or in combination with conventional pesticides were imperative [11, 12].

For agriculture pest's control, several natural compounds extracted from fungal secondary metabolites are reported to have antifeedant and insecticidal properties [13, 14]. The greatest number of substances with insecticidal properties were found to be produced by soil fungi, mainly from the genera *Aspergillus* and *Penicillium* [15].

Species of the genus *Aspergillus* are a prolific resource for diverse biologically active compounds [16, 17]. About 100 new compounds were isolated from the resting structures of *Aspergillus* sp. A number of them had been patented as potential insecticidal molecules [15]. For instance, *A. niger* IHCS-4 metabolic extract has insecticidal activity against *Chrysomya chloropyga* larvae inducing 20 and 65% mortality at different concentrations within 24 h. Larval growth was inhibited at concentrations of 0.04 mg/g or higher, and the larval weight decreased over time [18]. Also, the long-chain alcohol extracted from *Trichoderma citrinoviride* ITEM 4484 culture has a repellent, antifeedant activity against *Rhopalosiphum padi* [19]. So, there is clearly a need to identify more new compounds which could be used as an alternative to conventional chemical pesticides. Thus, the objective of this work is to evaluate the insecticidal activity of EtOAc extract of *A. flavipes* AUMC 11390 culture filtrate against newly hatched larvae of *E. insulana* in addition to its influence on some biological and biochemical markers.

2 Methods

2.1 The spiny bollworm cultures

Newly hatched larvae of spiny bollworm, *E. insulana* were obtained, and the insect was reared for several generations without any insecticide treatments and kept at 26 ± 2 °C with 75 ± 5% RH on a semi-artificial diet as described by [20].

2.2 Fungal isolate

The *Aspergillus flavipes* AUMC 11390 isolate used in this study was purchased from the Mycological Center, Assuite University, Egypt. A partial sequence of small subunit ribosomal RNA gene was submitted to the Gene Bank NCBI with accession No. MZ066617.1. The fungal inoculum was transferred to potato dextrose agar (PDA) media and incubated in total darkness at 25 ± 2 °C for 5–7 days. Subcultures were maintained on PDA at 4 °C for subsequent studies.

2.3 Preparation of fungal metabolic extracts

Two plugs of actively growing *A. flavipes* AUMC 11390 cultures (5–7 days) were inoculated into a 250-ml Erlenmeyer conical flask containing sterile 50 ml of PDA media. The cultures were incubated at 28 °C under stationary conditions. After 15 days of incubation, the liquid culture was filtrated through sterile filter paper (Whatman®1, England) and the filtrate was extracted with the organic solvent, ethyl acetate. Liquid–liquid extraction with ethyl acetate (1% v/v) was carried out 3 times [21]. An equal volume of ethyl acetate was thoroughly mixed with filtrate for 10 min. The two clear immiscible layers were obtained, and the upper layer of ethyl acetate was separated with a separation funnel. The ethyl acetate extract was concentrated by evaporation at 40 °C with a rotary vacuum evaporator. The yield was weighted and stored as stock in a glass vial in the freezer for further studies [22]. Organic solvent extract ethyl acetate was dissolved in 0.5 ml dimethyl sulfoxide (DMSO) and added to the distilled water to obtain final tested concentrations, while the control treatment containing (0.5 ml/l) DMSO mixed with distilled water (positive control). Another negative control treatment was applied by using water only [23].

2.4 Effect of fungal metabolites on some biological parameters

The activity of the recovered fungal extract has been evaluated against 1st instar larvae of *E. insulana* by mixing 2 ml of fungal extract with 4 g of the artificial diet in a 9-cm Petri dish. After 30 min, a group of 20 1st instar larvae were transferred immediately after hatching using a fine brush to each treated Petri dish. Treated Petri dishes were covered by a fine and soft paper below the glass cover to prevent larvae escape. The diet of control was mixed with DMSO (positive control) and water (negative control). Each treatment was replicated three times. All treatments were incubated at the constant conditions of 26 ± 1 °C and 70 ± 5% RH. After 24 h. of exposure and feeding, dead and alive larvae were counted. The mortality percentage was calculated after correlation with Abbott's formula [24].

The remained alive larvae of each treatment were transferred individually to glass tubes (2×7.5 cm) containing about 4 g of an untreated control diet and covered with a piece of absorbent cotton and held under the same conditions as mentioned above. Larvae were examined daily

to record larval duration and pupation percentage. After pupation, the pupae were transferred individually to other clean tubes and incubated until moth emergency. Pupal duration, adult emergence percentage, sex ratio (as females) and deformed adults were calculated.

Twenty pairs of emerged moths from each treatment were sexed (male and female) and caged in pairs under the previously mentioned rearing conditions. A piece of cotton wool previously soaked in 10% sugar solution was hung inside glass jars near its upper opening for moth feeding and changed by a new one every 2 days. The upper openings of jars were covered by muslin cloth followed by a tightly secured paper with rubber bands. Each jar was examined daily to record pre-ovipositional, and ovipositional periods, the number of deposited eggs, post-ovipositional period and the longevity of males and females. The deposited eggs were collected daily from strips of muslin cloth then transferred to a convenient glass jar and incubated at the same conditions to record hatchability percentages.

2.5 Biochemical studies

Different biochemical markers have been determined in order to understand the mode of action of the extracted fungal compounds and to ensure their efficacy against *E. insulana* larvae. Control samples were DMSO and water. Twenty post-treated larvae were transferred after 24 h into clean jars and left to starve for 4 h. The starved larvae were homogenized in distilled water (1 g larva/ml) using a teflon homogenizer surrounded with a jacket of crushed ice for 3 min. The homogenate larvae were centrifuged at 3500 rpm for 10 min. at 5 °C. The supernatant was immediately assayed to determine enzymes activity.

Amylase, invertase and trehalase enzymes activities were estimated spectrophotometrically [25]. The enzyme glutamic pyruvic transaminase (GPT) and glutamic oxaloacetic transaminase (GOT) activities were determined according to [26]. The total soluble protein in the total homogenate of larvae of spiny bollworm was carried colorimetrically according to [27] while total lipids were determined by the method of [28].

2.6 GC–MS/MS analysis of fungal ethyl acetate extract

The active insecticidal compounds from the potent fungal isolate were detected by a Thermo Scientific, Trace GC Ultra/ISQ Single Quadrupole MS, TG-5MS-fused silica capillary column (30 m, 0.251 mm, 0.1 mm film thickness). For GC/MS detection, ionization was elected in the electron impact mode (EI) at 70 eV, helium gas was used as the carrier gas at a constant flow rate of 1 ml/min. The injector and detector temperature were set at 280 °C. The oven temperature was programmed at an initial temperature of 50 °C (hold 2 min) to 150 °C at an increasing rate of 7 °C/min. The chemical identity of the bioactive compounds in the extracts was determined reliant on a comparison of their mass spectra and retention time referencing MASS BANK EU and WILEY libraries, in addition to fragmentation pattern of the mass spectral data with those reported in the literature. The name, molecular weight and structure of the components of the test material were ascertained.

2.7 Statistical analysis

All the experiments were conducted with three biological replicates. The obtained results of mortality and biological parameters were subjected to one way ANOVA, Tukey HSD were determined using Costat software program [29].

3 Results

3.1 Efficacy of *A. flavipes* AUMC 11390 ethyl acetate extract on some biological parameters of newly hatched *E. insulana*

The effectiveness of *A. flavipes* AUMC 11390 ethyl acetate extract toward the 1st instar larvae of spiny bollworms was investigated by examining its effect on some insect's biological parameters and their larvicidal activity. The extract of *A. flavipes* AUMC 11390 had no significant

Table 1 The effect of *A. flavipes* AUMC 11390 ethyl acetate extract on different parameters of larvae of spiny bollworm *E. insulana*

Treatment	Larval duration	Larval mortality %	Pupation %	Pupal weight	Pupal mortality %	Adult emergence %	Deformed adult %
Fungal extract	15.33	58.33a	41.67b	0.6020b	15.59a	73.57b	10.83
DMSO control	15.33	1.67b	98.33a	0.7555a	0b	100a	0
Water control	15.00	0b	100a	0.7731a	0b	100a	0
P	Ns	0.0000***	0.0000***	0.0000***	0.0002***	0.0072**	ns
$LSD_{0.05}$	1.2851	9.4182	9.4184	0.0237	4.5201	14.8967	11.83

The same letter in the same column means not significant at $P_{0.05}$

LSD The least significant difference

*slightly significant,**moderatesignificant, ***high Significant

effect on larvae duration compared to negative and positive control samples as presented in Table 1. Both negative and positive controls exhibited the same value of 15.33 days (larval duration) which was nearly the same as the fungal extract. The fungal extract exhibited a noticeable insecticidal activity against *E. insulana* larvae at 58.33% compared to 1.67% and 0% with DMSO and water control, respectively. Such insecticidal effect extended to pupa as well, it caused about 15.59% pupa mortality, in contrast to 0% mortality in both controls. Pupation percent also affected greatly with treatment, as it reduced upon treatment to 41.67%, while the DMSO and water control display 98.33 and 100%, respectively. Obviously, data in Table 1 indicated that the fungal metabolites haven't affected only the pupation percent but also the pupal weight as the treated pupa showed about 0.6 g lower weight compared to both control samples 0.75 and 0.77 g. Significant retardation was recorded in adults that emerged from treated larvae by 28% beside 10.83% deformation in emerged moths appeared as deformed, twisted wings to moths with incomplete or very short wings compared to normal emerged moths from control samples.

However, data in (Table 2) illustrated the female sex ratio, the pre-oviposition time and the post-oviposition time were non-significantly different from the control samples, with significant retardation on both male and female longevity to 13.86 and 13.73 days compared to negative and positive control. As a consequence, A highly significant difference in the number of eggs per female moth of *E. insulana* was observed. The egg number was reduced to 165.77 eggs compared to 219.09 and 233.53 eggs for DMSO and water control, respectively. Moreover, the hatchability percentage reduced to 71.46% compared to 90.70 and 92.2 for DMSO and water control, respectively.

3.2 Biochemical analysis of newly hatched E. insulana larvae in response to A. flavipes AUMC 11390 ethyl acetate extract

The data in (Fig. 1) illustrate biochemical activities of newly hatched *E. insulana* larvae that assessed after 24 h post-treatment with 2 ml of *A. flavipes* AUMC 11390 ethyl acetate extract. Overall, an obvious disturbance in *E. insulana* larvae proteins, lipids and enzymatic activities were recorded as responsive to treatment with fungal extract compared to DMSO and water controls. A strong decrease in the total lipids was recorded upon treatment with 2 ml of fungal extract. Apparently, the tested extracts decreased the total lipids of *E. insulana* larvae by about 25% compared to both controls, while a slight reduction in total protein was observed. The activity of GPT was increased by 59% in response to fungal extracts after 24 h of treatment, in contrast, GOT was decreased by 50% compared to their water control. A slight reduction in the activity of acetylcholine esterase was observed by about 4% after 24 h of treatment compared to water controls.

3.3 Chemical characterization of the bioactive compounds from A. flavipes AUMC 11390

The chemical identity of the active compounds from the ethyl acetate extract of *A. flavipes* AUMC 11390 was subjected to GC–MS/MS analysis. From the GC–MS/MS chromatogram of ethyl acetate extract of *A. flavipes* AUMC 11390 (Fig. 2), one hydrocarbon compound was resolved named 3-Eicosene with the arbitrary putative area 11.14, and four long-chain alcohols namely hexadecanol, 1-hexadecanol, 1-octadecanol, and 1-pentadecanol with the arbitrary putative area 9.86, 28.89, 26.26, and 2.92%, respectively. However, there were some minor unknown compounds or impurities.

Table 2 The effect of *A. flavipes* AUMC 11390 ethyl acetate extract on different parameters of adult stage of spiny bollworm *E. Insulana*

Treatment	♀ sex ratio%	Pre-ovi (days)	Oviposition (days)	Post-ovi (days)	♀ longevity (days)	♂ longevity (days)	Egg no.	Hatchability%
Fungal extract	50	2.2467	4.58	7.03b	13.86b	13.37b	165.77b	71.46b
DMSO control	50	2.1433	5.26	9.04a	16.54a	16.05a	219.09a	90.70a
Water control	50	2.4733	5.59	9.04a	17.1a	16.46a	233.53a	92.02a
P	Ns	ns	Ns	0.0182*	0.0008***	0.0002***	0.0008***	0.0008***
$LSD_{0.05}$	11.5348	0.3093	1.1059	1.4153	1.1054	0.8032	22.7072	5.0268

The same letter in the same column means not significant at $P_{0.05}$

LSD The least significant difference

*slightly significant,**moderatesignificant, ***high Significant

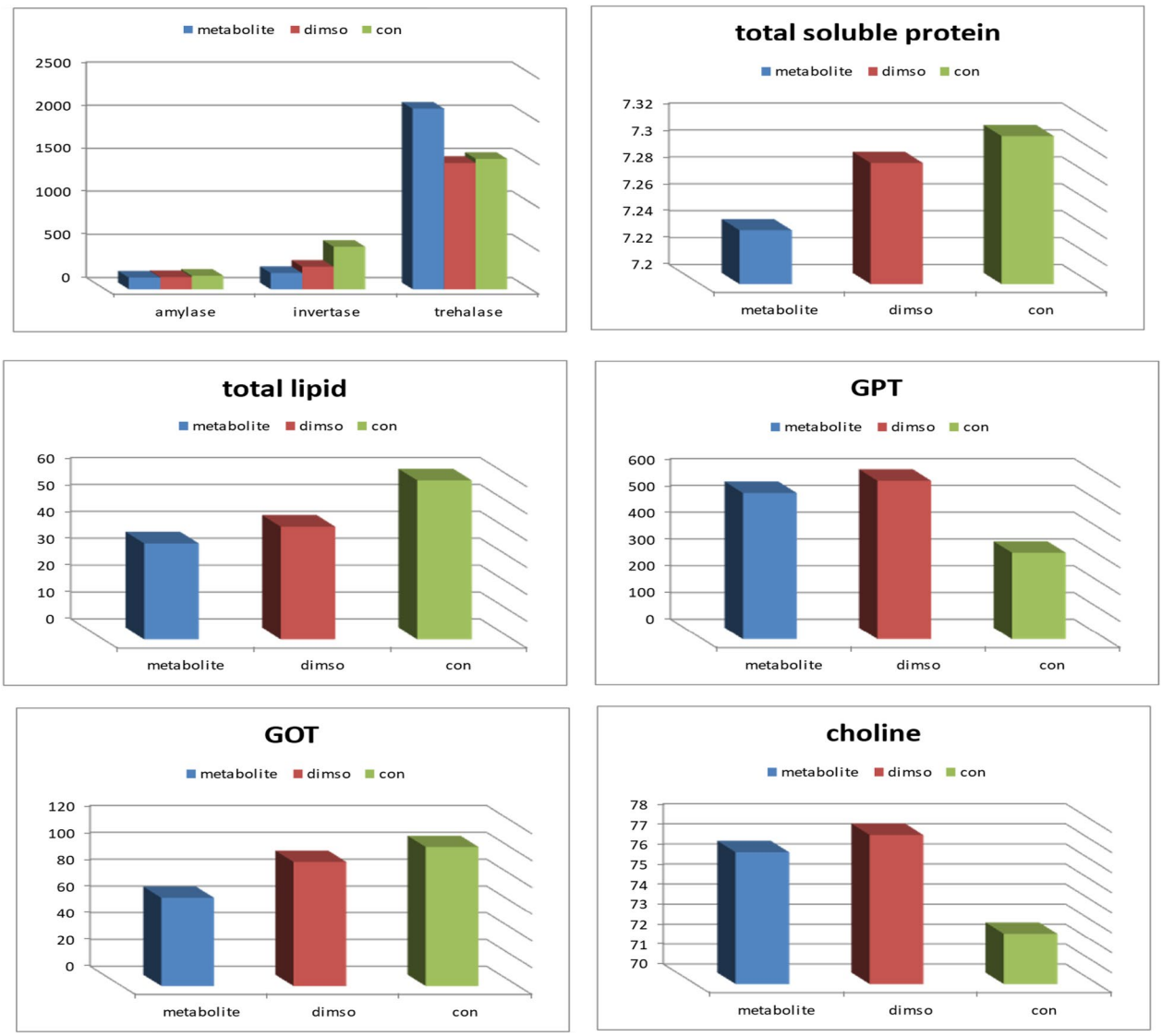

Fig. 1 The effect of *A. flavipes* AUMC 11390 ethyl acetate extract on physiological activities of spiny bollworm *E. Insulana*. The graph shows the mean values of various physiological activities (amylase, invertase, trehalase, total soluble protein, total lipid, GPT, GOT, and choline metabolite) in spiny bollworms treated with the extract compared to the control group

4 Discussion

The biopesticides compromise one of the most promising alternatives for pest's control. Fungi could serve as a source for novel biologically active compounds that may afford alternative tools to control pests and pathogens [30, 31]. Filamentous fungi have long been known as producers of biologically active compounds, such as alkaloids, terpenoids, phenolics which could be useful for several purposes including the development of novel agrochemicals [32, 33]. *Aspergillus* sp. could serve as reservoirs of biologically active compounds with insecticidal activity. *A. flavipes* AUMC 11390 ethyl acetate extract showed promising insecticidal activity toward *E. insulana* larvae. The ethyl acetate extract of *A. fumigatus* JRJ111048 has insecticidal activity against newly hatched larvae of *Spodoptra litura* at the concentration of 20 µg/ml and caused a reduction in larval growth, the reduction of the pupae weight and malformation of the adults. Similarly, ethyl acetate and dichloromethane extract of *A. nidulans* caused accumulative mortality of 40.7 and 55.6%, respectively, after 18 days of post-treatments. Additionally, the ethyl acetate extract of *Alternaria alternata* caused a significant insecticidal effect against *S. litura* [34, 35], which may be attributed to anti-feeding behavior or gustatory repellency or impairment in the food assimilation after feeding on a treated diet [36].

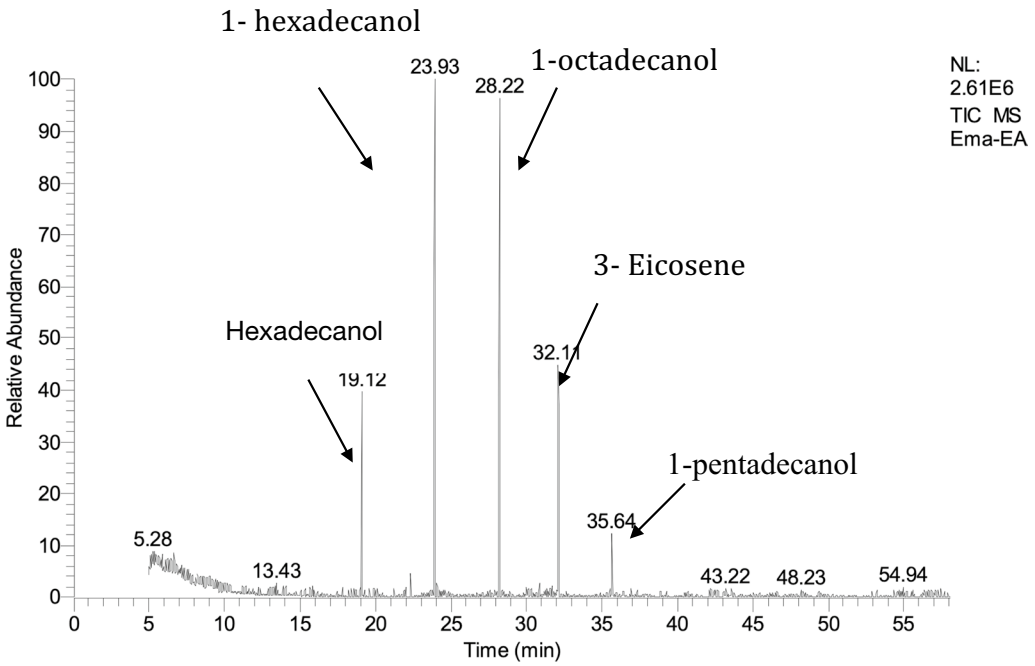

Fig. 2 Gas chromatography-mass spectrometry (GC–MS) analysis of ethyl acetate extract from *A. flavipes* AUMC 11390. The chromatograph shows the different compounds detected by GC–MS that analysis the compounds involves separating and identifying individual components in a complex mixture based on their unique retention time and mass spectral characteristics. The extract contains a mixture of fatty acids, phenolics, terpenoids, and other compounds, some of which have known biological activities

A. flavipes AUMC 11390 ethyl acetate extract hasn't affected the larval duration of *E. insulana* 15 days for treatment and both controls but has a significant reduction on pupation percent 41.67 and adult emergency 73.57 compared with controls that have not affected, while the adult deformation percent was 10.83%, compared with 0% in the two controls. Recent studies have demonstrated the detrimental effects of ethyl acetate extract of *A. nidulans* which significantly prolongs the total larval duration of *S. littoralis* from 8 to 18 days post-treatment, also affected the total development period of immature stages besides strong deformation in adults by about 25.1% was observed on the individuals descending from larvae, comparing to positive and negative controls [36]. Likewise, the ethyl acetate extract of *S. strictum* caused 10% larvae deformation in 2nd larval instar of *S. littoralis,* while, the pupation ratio was reduced by about 68.3% and reduction of pupal duration with pupal deformation percent by 36.5% [22].

The ethyl acetate extract *A. flavipes* AUMC 11390 caused a significant difference on retardation of both male and female longevity resulted in a significant difference in the number of eggs per female in addition to a reduction of hatchability. It is worthy to mention that *S. strictum* ethyl acetate extract caused a significant decline in the number of eggs per female moth of *S. littoralis*. Consistently, the fertility of lepidopterous adults depends on the concentration of amino acids in the larval haemolymph and the size of the larval fat body [37]. Thus, the larval haemolymph was considered a major source of egg protein.

Recent studies have demonstrated the detrimental effects of *A. alternata* toxin on the reproduction of the rose aphid, *Mcrosiphum rosivorum* [37]. Consistently. Penicinoline, an alkaloid isolated from *Penicillium* sp. showed strong insecticidal activity against the sucking pest *Aphis gossypii* Glover [38]. Moreover, adverse effects of *Nigrospora* sp. on the survival and development of *S. litura* have been demonstrated [39]. Significant reduction in fecundity and prolonged life cycle of *A. gossypii* were achieved by *Coniothyrium* sp. and *Nigrospora* sp. that were isolated from chilli [40]. Decreased percent of adult emergence, longevity and fecundity were also recorded when *S. litura* larvae fed on the diet supplemented with ethyl acetate extract of *A. flavus.*

Insect enzymes have a great role in insect defence mechanisms and protection from the negative impact of the surrounding environment. Moreover, these enzymes can mediate the metabolism of hormones, pheromones and other biologically active substances. So, the changes in enzymes activities are reflected not only in insect resistance to insecticides but also in their capacity to

adapt to their host plant as well as in metamorphosis and development. Thus, malfunctioning and mortality might be resulted from enzymes disturbance caused by the fungal extract. For instance, the methanolic extracts from the mycelia and spores of *C. cladosporioide*s and *P. lilacinum* have caused an increase in the total carbohydrate content of *A. gossypii* adults. While the total protein content was significantly decreased by both fungal extracts. Findings also showed that *Beauveria bassiana* secondary metabolite increases total esterase and glutathione S-transferase activities in the hemolymph of treated adults of *Eurygaster integriceps*, and had an adverse effect on AChE activity of adults by decreasing its activity [41]. [42, 43] reported that *Galleria mellonella*, suffering from mycoses demonstrates a decreasing sensitivity to deltamethrin and malathion because of increasing levels of detoxifying enzyme activities due to body intoxication and tissue damage. *A. flavus* produce pyripyropene A from the group of mero terpenoids which is considered as an inhibitor of the acyl-CoA-cholesterol-acyltransferase, and has strong insecticidal properties [44].

Thus, the potential insecticidal activity of ethyl acetate extract of *A. flavipes* AUMC 11390 against *E. insulana* larvae could be due to the presence of different long-chain alcohols namely hexadecanol, 1-octadecanol, and 1-pentadecanol that have been reported by its repellent activity. Similarly, *Trichoderma citrinoviride* ITEM 4484 metabolites produced different long-chain alcohols that can modify aphid preferences and might be useful for aphid control [19]. The long chain of alcohols compromises apart of sexual pheromones of various insects. Fatty alcohol 1-octadecanol has been already used as a growth inhibitor in pest control approaches. Also, the fungal long-chain alcohols showed antifeedant activity against pea aphids (*Acyrthosiphon pisum*) as a potential biocontrol strategy [45].

So, there is clearly a need to identify more new compounds which could be used as alternative to conventional chemical pesticides. Thus, the objective of this work is to evaluate the insecticidal activity of secondary metabolites extracted from *A. flavipes* AUMC 11390 against newly hatched larvae of *E. insulana* in addition to its influence on some biological and biochemical markers.

5 Conclusion

Despite the fact that the chemical method remains common in practice due to the relative ease of production and application, higher biological efficiency and stability, there is a clear trend toward an increase in the number of registered insecticides of natural origin. Fungi and their metabolites including long-chain alcohols which are a source of new insecticidal molecules with very low acute toxicity to humans, furthermore some of them have already been used to develop commercial insecticides against pest arthropods.

Abbreviations

ARC	Agriculture Research Center
RH	Relative humidity
PDA	Potato dextrose agar
GPT	Glutamic pyruvic transaminase
GOT	Glutamic oxaloacetic transaminase
DMSO	Dimethyl sulfoxide
rpm	Rotates per minute
GC–MS	Gas chromatography–mass spectrometry
ANOVA	Analysis of variance

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Author contributions

EMA: performed the ethyl acetate extract of tested fungi. EMG: performed physiological experiments. RHMH: performed the rearing of the spiny bollworm and detected toxic effects of the fungal extract, all authors read and approved the final manuscript.

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All data and materials are available if requested.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

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Competing interests

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