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Pupation inhibition and larvicidal activity of tyrosinase on *Culex pipiens* third-instar larvae

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Abstract

Background: Mosquitoes are considered to be the main vector of a variety of diseases in both humans and domesticated animals. The development of insecticide resistance and the limitations of traditional insecticide-based strategies have resulted in significant efforts to develop eco-friendly, alternative methods.

Main body: In this study, nine fungi species were screened to produce tyrosinase enzyme. This was done in order to evaluate its inhibitory activity against *Culex pipiens* third-instar larvae. The extracellular tyrosinase was produced by five strains. *Aspergillus tamarii* NRC3 was found to possess the highest tyrosinase activity and was therefore used in this study. Some factors were studied to enhance the production of the enzyme. The enzyme was partially purified using ammonium sulfate at a 70% saturation, giving 1.861 purification fold. The toxicity on *Culex pipiens* varied, depending upon the tyrosinase concentration and the period of exposure. In addition, notable histological effects were seen in the midgut region.

Conclusions: A concentration of 80% on third-stage larvae showed 90% inhibition in the formation of pupae at 72 h post-treatment. In addition, a significant cellular microvillus disruption was seen in the midgut region at 24 h post-treatment.

Keywords: *Aspergillus tamarii* NRC3, Inhibition, Larvae, Mosquito, Tyrosinase

Background

Culex pipiens (*Cx. pipiens*) is the main vector of a variety of diseases in humans and domesticated animals such as sheep, goat, cattle and dog. A serious consequence of mosquito bites may be transmission of serious diseases and viruses such as dengue virus, Zika, malaria, and West Nile fever, which can lead to meningitis, encephalitis, and microcephaly (El Sadawy et al. 2018). Vector control based on larval management of this mosquito species is most practicable where vector-borne diseases at low transmission levels and their breeding sites are limited in number and are relatively permanent (WHO 2005). *Cx. pipiens* larvae breed in different kinds of water body, including polluted water associated with home industries

(Zayed et al. 2015). Controlling the vector has mainly focused on the use of organophosphorus insecticides, insect growth regulators, and bacterial larvicides (WHO 2006). Nevertheless, the indiscriminate use and increasing reports of the development of resistance to these insecticides in the vector population have led to the subsequent failure of control efforts (Zaim and Guillet 2002). The occurrence of such difficulties has been accompanied by a growing interest in the use of new and safe bioinsecticides with new mode of action (El Sadawy et al. 2018). Many species of free-living microorganisms produce bioactive compounds such as lytic enzymes. This reflects their importance in the biological control of insects (Hussain et al. 2002). One of these enzymes is protease—produced from streptomycetes—which has been suggested as an insecticidal agent (Harrison and Bonning 2010). It catalyzes the hydrolytic reaction, which brings about the breakdown of protein molecules to amino acids and peptides (Tunga et al. 2003). Another lytic enzyme is

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tyrosinase, which is a copper metalloprotein that is ubiquitously dispersed in nature (Claus and Decker 2006). Tyrosinase is both a monooxygenase and a bifunctional enzyme that catalyzes the O-hydroxylation and subsequent oxidation of O-phenols and monophenols to quinines (Claus and Decker 2006). Due to properties of oxidative stress, tyrosinase has biotechnological applications for both protein-associated phenolic groups and small phenolic compounds. As a result, tyrosinase can be applied in contaminated soils and in the detoxification of phenol-containing wastewater (Martorell et al. 2012). In addition, tyrosinase plays a significant role in the production of L-3,4-dihydroxyphenylalanine (L-DOPA), which is a recommended treatment of Parkinson's disease (Franciscon et al. 2012). Recently, as an alternative to the more expensive synthetic anthelmintics, the in vitro effects of tyrosinase—produced from *Streptomyces* spp. isolated from Egyptian soil—could offer a promising bio-control agent against *Toxocara vitulorum* (Shalaby et al. 2020).

Various organisms such as bacteria, mushrooms, fungi and plants have been used for the production of tyrosinase enzymes on a commercial scale (Allouche et al. 2004). The enzyme production being studied in both batch and fed-batch cultivations, and phytase genes from these organisms were later expressed in *Escherichia coli*. For the in vitro production of extracellular tyrosinase, the *Streptomyces* spp. (isolated from soil) were used (Sambasiva et al. 2013). In addition, different fungi have been investigated for the isolation of tyrosinase, such as *Agaricus bisporus* (Strothkamp et al. 1976), *Amanita muscaria* (Mueller et al. 1996), *Aspergillus oryzae* (Nakamura et al. 2000), *Lentinula edodes* (Kanda et al. 1996), *Lentinula boryana* (de Faria et al. 2007), *Neurospora crassa* (Lerch 1983), and *Pycnoporus sanguineus* (Halaouli et al. 2005). Tyrosinase was also produced through the cultivation of the filamentous *Trichoderma reesei* at a concentration of approximately 1 g/L (Caf et al. 2012). In this study, nine fungi species (*Aspergillus flavus*, *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus tamaris* NRC3, *Chaetomium cervicicola*, *Fusarium equiseti*, *Fusarium oxysporum*, *Penicillium minioluteum*, and *Trichoderma harzianum*) were screened for tyrosinase activity. The species that yielded the most enzymatic activity was selected and assessed as a larvicidal agent against *Cx. pipiens*, with reference to its histological effects on exposed larvae.

Methods

Organisms

The following nine fungi species were obtained from the Department of Microbial Chemistry, National Research Centre, Egypt: *Aspergillus flavus* (*As. flavus*), *As. niger*, *As. oryzae*, *As. tamaris* NRC3, *Chaetomium cervicicola*,

Fusarium equiseti, *Fusarium oxysporum*, *Penicillium minioluteum*, and *Trichoderma harzianum*.

Screening of extracellular tyrosinase from different fungi

The selected fungi were screened for tyrosinase production. This was assessed using a tyrosine agar medium (agar 20 g, beef extract 3 g, L-tyrosine 5 g, peptone 5 g, and pH 7.0 (Shraddha et al. 2011). The brown color around the colony indicated the presence of the tyrosinase enzyme.

Culture medium and cultivation processes of tyrosinase

Tyrosinase production was carried out in 250-ml flasks. Each flask contains 25 ml basal medium (Sharma et al. 2006); then, this was sterilized by autoclaving at 121 °C. The flasks were inoculated with a loop of the potent isolate and then incubated on a rotary shaker at 37 °C (Innova 4080, New Brunswick Scientific Co., NJ, USA) and 200 rpm for the cultivation processes. The fungal growth, final pH, tyrosinase activity, total dry weight and protein content were determined at the end of each experiment.

Determination of cell dry weight for all fungi species

Cell dry weight was estimated according to Singh et al. (2012).

Enzyme extraction

After complete fermentation, the broth was centrifuged at 10,000 rpm for 15 min at 4 °C to remove the mycelium. Ammonium sulfate powder was added slowly to the supernatant with continuous stirring in an ice bath, until 75% saturation was achieved. The mixture was kept at 4 °C overnight, followed by centrifugation at 10,000 rpm in a cooling centrifuge. The supernatant was discarded, and the precipitate was dissolved in 0.2 M Tris-HCl buffer (pH 7.5), with continuous mixing using a magnetic stirrer. The suspended precipitate was then checked for enzymatic activity and total protein content for 20 min (Dolashki et al. 2009). The dialysis membrane was cut and pretreated in boiling water for 60 min and stored in 0.2 M Tris-HCl buffer (pH 7.5). The membrane was suspended overnight at 4 °C in a glass beaker containing 0.2 M Tris-HCl buffer (pH 7.5). The dialysis sample was checked for tyrosinase activity and total protein content (Bradford 1976).

Tyrosinase activity determination

An aliquot of the enzyme solution was added to a 0.2 M Tris-HCl buffer (pH 7.5) containing L-DOPA and 1-mM L-tyrosine. The suitable concentration of the enzyme was assayed, and the absorbance of dopachrome was monitored by measuring at 475 nm. Dopachrome “colored

intermediate” is an intermediate of melanin biosynthesis that is made from O-quinones by nonenzymatic oxidation. One international unit of tyrosinase activity is defined as the amount of enzyme required to oxidize 1 μmol of L-tyrosine to dopachrome per minute under the above conditions (Danial and Al-Bishri 2018).

Inhibitory activity of tyrosinase against *Culex pipiens* larvae

A local strain was reared and obtained from the department of mosquito research at the Medical Insect Research Institute, Al Agouzah, Giza Governorate, Egypt. Larvae of the third instar of *Cx. pipiens* (Diptera: Culicidae) were selected as the target insect for the enzyme assay due to its lifespan length to shed to pupal stage. Experiments were carried out at 27 ± 0.8 °C. The larvae were placed in plastic dishes (30 × 20 × 10 cm) with 1500-ml distilled water. The larvae were fed equal parts dried yeast, biscuits, and dried milk powder, according to the method proposed by Hafez (2000).

Larvicidal bioassays

To assess the inhibitory activity of the tyrosinase enzyme on the development of *Cx. pipiens* third-instar larvae, the enzyme was adjusted to four concentrations (20%, 40%, 60%, and 80% (v/v), whereas control replicates were treated with distilled water only. In addition, larval mortality was recorded daily for 96 h post-treatment. Five replicates were performed (n=5) in each concentration as recommended by the WHO (2005). Briefly, 10 larvae were placed in each well of a standard, sterile 12-well tissue culture test plate (Nunclon Delta surface, Thermo Fischer Scientific, Denmark) with 2-ml of each enzyme concentration or dH₂O for the negative control larval group. Each bioassay was run for 96 h. An absence of response by larvae to gentle prodding with a needle was considered as dead, according to Brown et al. (1998). According to Abbott’s formula (Abbott 1925), the larval mortality (%) was calculated for each concentration. Each concentration was replicated five times for every experiment tested. The following formula was used to estimate the reduction percentage of larval development induced by the tested enzyme:

$$\text{Inhibitory activity} = 100 - (\% \text{ of } A \times 100 / \% \text{ of } B)$$

where *A* denotes treated larvae developed into pupa; *B* control larvae developed into pupa.

Histopathological and immunohistochemical observations of third-instar *Culex pipiens* larvae exposed to tyrosinase

The digestive system was performed for histopathological assessment by using treated (24 h post-treatment) and control larvae. The specimens were fixed in 10% neutral buffered formalin and decalcified for 18 days in

10% formic acid. A rotary microtome (Leica-RM2245, Germany) was used to cut thick parts (4-μm), which were then stained with hematoxylin and eosin (Liu et al. 2019). Images were taken using a digital microscopic mounted camera (OMX1200C, Nikon, Japan) to observe the stained parts under light microscopy. Also, the immunohistochemical assay for treated and control third-instar larvae was fixed with 10% neutral formaldehyde and processed routinely for paraffin embedding according to Abdeltawab et al. (Abdeltawab et al. 2019).

Statistical analysis

The pupation, emergence and mortality results of the third-instar larvae were statistically analyzed by ANOVA followed by Duncan test using the SPSS computing program (IBM SPSS statistics 16 software were used for data analysis). All experiments performed were repeated five times. Results were given as mean ± SE, and statistical significance was defined as *p* < 0. 05.

Results

Screening of extracellular tyrosinase (qualitative studies)

All nine fungal species (*Asp. flavus*, *Asp. niger*, *Asp. oryzae*, *Asp. Tamaris* NRC3, *Chaetomium cervicicola*, *Fusarium equiseti*, *Fusarium oxysporum*, *Penicillium minioluteum*, and *Trichoderma harzianum*) were screened for extracellular tyrosinase production. The results are summarized in Table 1. Extracellular tyrosinase was produced by six species of which *As. Tamaris* NRC3 possessed the highest brown color around colony (highest tyrosinase activity) and was therefore used in the following procedures.

Table 1 Screening of extracellular tyrosinase production of the nine fungi species tested (qualitative test)

Fungi spp.	Tyrosinase
<i>Aspergillus flavus</i>	++
<i>Aspergillus niger</i>	-
<i>Aspergillus oryzae</i>	+++
<i>Aspergillus tamaris</i> NRC3	+++++
<i>Chaetomium cervicicola</i>	-
<i>Fusarium equiseti</i>	-
<i>Fusarium oxysporum</i>	+++
<i>Penicillium minioluteum</i>	-
<i>Trichoderma harzianum</i>	+

- = no production, no color around colony
 ++ = weak production light brown color
 +++ = medium production; brown color
 ++++ = high production, dark brown color

Quantitative production of extracellular tyrosinase by *Aspergillus tamaris* NRC3

The quantification of extracellular tyrosinase activities in *As. tamaris* NRC3 showed that extracellular tyrosinase activity was observed during the seven days of incubation. The extracellular tyrosinase activity on the fourth day of incubation was 480.2 U/mL, and specific activity was 307 U/mg protein.

Effect of L-tyrosine concentration on tyrosinase production

Different concentrations of L-tyrosine ranged from 0.0% to 1.8% (w/v) were used to enhance the production of tyrosinase. As presented in Table 2, the maximum tyrosinase production was attained in the presence of a L-tyrosine concentration of 1.2% (w/v).

Partial purification of tyrosinase by ammonium sulfate saturation

The partial purification of tyrosinase using 50 ml of the enzyme is corresponding to a yield of 62.052% (Table 3).

Effect of tyrosinase on *Culex pipiens* third-instar larvae

Table 4 and Fig. 1 present the results of the inhibition of the emergence bioassay of *Cx. pipiens* third-instar larvae to the tyrosinase enzyme. Concentrations of 20%,

40%, 60%, and 80% of the tyrosinase enzyme were evaluated, showing at 96 h post-treatment mortality values (mean \pm SE) that were $2 \pm 2\%$, $12 \pm 2\%$, $22 \pm 8\%$, and $18 \pm 3\%$, respectively. Concentrations of 80% on third-stage larvae showed $90 \pm 8\%$ inhibition of the pupation at 72 h post-treatment (Fig. 1). However, pupation inhibition when third-instar mosquito larvae were treated with 20% resulted 70% (Fig. 1) when compared with the control untreated larvae, which showed a 100% pupation at 48 h. In the present study, with the increase of concentration treatments, larval viability decreased. Pupae formation was observed at concentrations of 40% and 20%.

Histopathological and immunohistochemical observations of *Culex pipiens* third-instar larvae after treatment with tyrosinase at 80% concentration

The histological composition of epithelial midgut cells of untreated larvae appeared to be normal. The posterior midgut was characterized by tall epithelial cells (The entire lateral plasma membrane displayed normal intercellular dark cells, a well-developed brush border, normal nuclei, and a typical adherent basement membrane, as seen in the control sections in Fig. 2a). At 24 h post-treatment, the anterior midgut lysis advanced through swollen, and nuclear degeneration (Fig. 2b). However, the posterior midgut showed high disruption of the dark cells, nuclear lysis, microvilli degeneration, and local detachment from the basal lamina. Most of epithelial cells degenerated and became vacuolated after 48 h from treatment. In addition, a large vacuole of various sizes with broken membranes were observed on the apical side of the epithelial cells and significant cellular microvillus disruption were seen (Fig. 2c).

The immunohistochemical results showed that the untreated third-instar larvae displayed negative Caspase-3 expression (Fig. 3a). The third-instar larvae exposed to tyrosinase enzyme for 24 h exhibited very mild apoptotic marker expression (Fig. 3b). Meanwhile, after 48 h from the treatment, the third-instar larvae displayed a substantially higher level of apoptotic marker expression (Fig. 3c).

Table 2 Effect of tested concentrations of L-tyrosine on the production of tyrosinase

L-tyrosine concentration % (W/V)	Mean enzyme activity (U/mL) \pm SE	SD	Mean specific activity (U/mg protein) \pm SE	SD
0	34.6 \pm 0.000 a	0.000	115.3 \pm 0.000 a	0.000
0.3	250.4 \pm 0.003 b	0.005	313.0 \pm 0.006 b	0.01
0.6	311 \pm 0.000 c	0.000	345.5 \pm 0.000 c	0.000
0.9	425.8 \pm 0.0006 d	0.001	387.0 \pm 0.000 d	0.000
1.2	690 \pm 0.05 e	0.1	575.0 \pm 0.04 e	0.08
1.5	671.6 \pm 0.2 f	0.35	373.1 \pm 0.01 f	0.2
1.8	673 \pm 0.03 g	0.06	249.2 \pm 0.003 g	0.005
p value	0.000		0.000	

W/V Different concentration of tyrosine used as w/v in medium containing 10 g/l casein, 0.5 g/l K_2HPO_4 , (pH 6.5), 50-ml basal medium, 0.25 g/l $MgSO_4 \cdot 7H_2O$. SE Standard error of mean values. Values with different letters (a, b, c, d...) are significantly different ($p < 0.05$) within group, SD Standard deviation

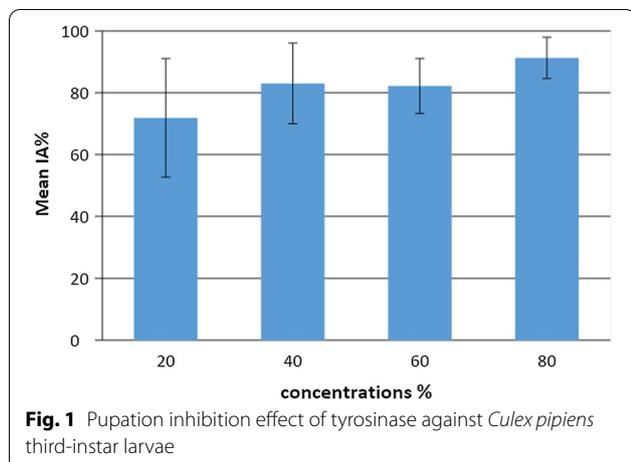
Table 3 Partial purification of tyrosinase by ammonium sulfate

Purification stage	Total volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (U/mg protein)	Purification fold	Yield %
Crude extract	500	0.33	809	2451	1.0	100
Precipitation at 70% saturation	150	0.21	630	3000	1.224	69.38
Dialysis	50	0.11	502	4563	1.861	62.052

Table 4 The effect of tyrosinase on *Culex pipiens* third-instar larvae

		Tyrosinase concentrations %											
		Mean percentages ± SEs											
Interval (hours)	Control	20			40			60			80		
		Pupation (%)	Emergence	Dead larvae	P	E	DL	P	E	DL	P	E	DL
6	6 ± 4a	0	0	0	4 ± 4a	0	0	2 ± 2a	0	0	2 ± 2a	0	0
12	40 ± 6.3b	0	0	0	6 ± 4a	0	0	2 ± 2a	0	0	4 ± 2a	0	0
24	86 ± 2.4c	0	0	0	16 ± 4a	0	2 ± 2	2 ± 2a	0	0	8 ± 4ab	0	0
48	100d	0	0	0	22 ± 8a	10 ± 0b	2 ± 2	8 ± 3.7ab	0	4 ± 4	16 ± 5ab	0	0
72	-	100	0	0	24 ± 9a	22 ± 5.8c	2 ± 2	8 ± 3.7ab	0	12 ± 2	16 ± 5ab	0	0
96	-	-	-	-	1.8	14.6	2 ± 2	14 ± 4b	0	12 ± 2	22 ± 8b	0	0
F	166.6	-	-	-	Ns	<0.001	-	2.5	-	-	2.5	-	-
p-value	<0.001	-	-	-				0.05	-	-	0.06	-	-

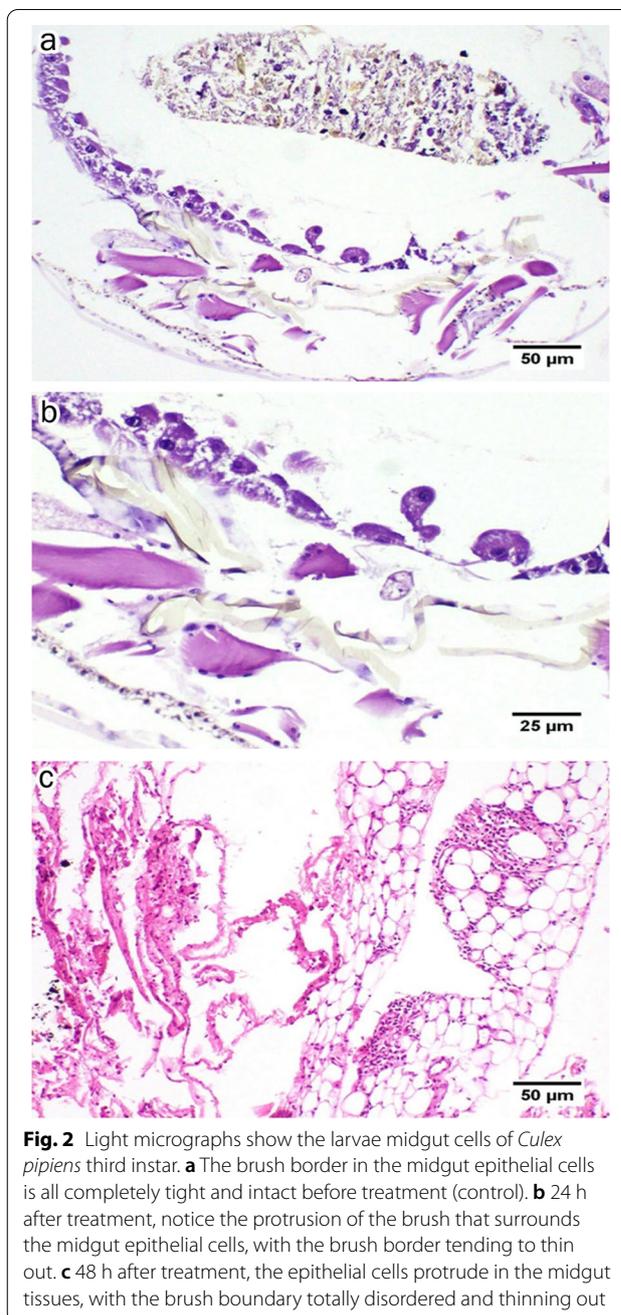
SE Standard error of mean values. Values with different letters (a, b, c, d) are significantly different ($p < 0.05$) within hours for each concentration (based on the nonoverlapping confidence limits). Ms: nonsignificant. R = 5, N = 10, ΣN = 50; P pupation, E emergence, DL dead larvae



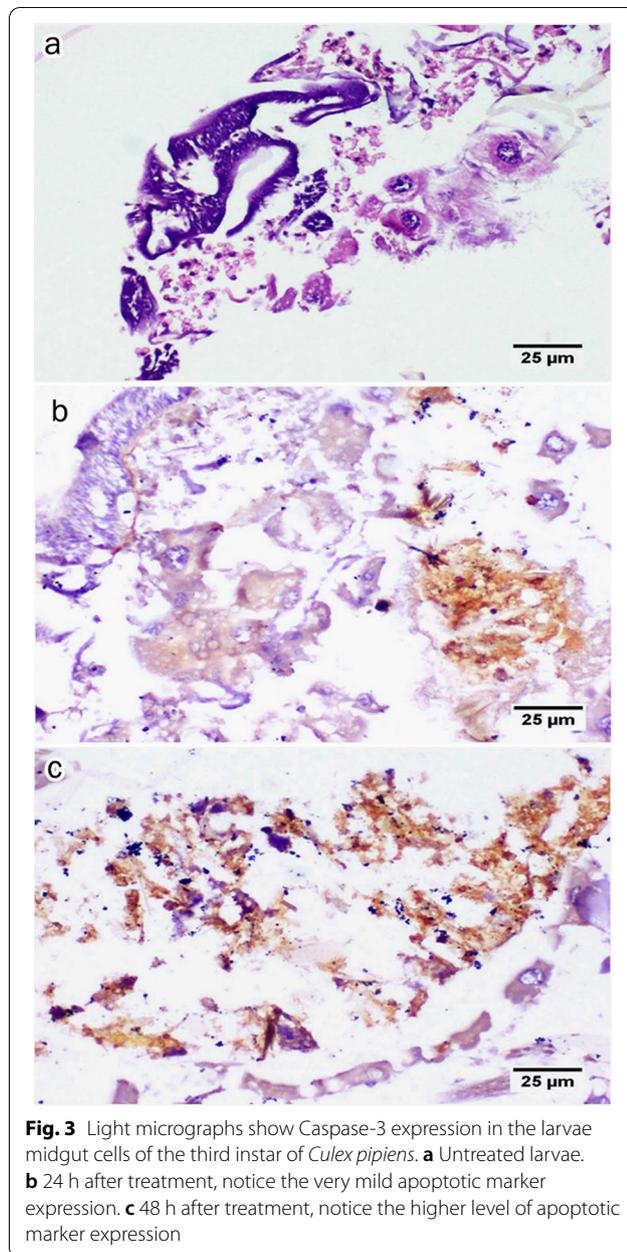
Discussion

The expansion of insecticide resistance and the increasing awareness of environmental problems associated with synthetic insecticides have prompted research into alternatives to environmentally damaging insecticides. Among the biological agents employed to control mosquito larvae, products that use entomopathogenic fungi are under development (Butt et al. 2013; Greenfield et al. 2015). These fungi generate a wide cocktail of extracellular hydrolytic enzymes, including proteases, lipases, phospholipase C, catalase, and chitinases (Santi et al. 2010). In the present study, the in vitro production of extracellular tyrosinase was done using fungi species isolated from soil. Particularly, *As. tamaritii* NRC3 showed high tyrosinase activity and was therefore selected to conduct the bioassays. The effect of this enzyme on the larval development of *Cx. pipiens* was assessed for the first time, in order to develop a new bio-larvicidal agent that can be used against *Cx. pipiens*. Proteolytic enzymes are the only ones that are recognized to launch simple, intact proteins in vitro. Tyrosinase can interact with naked tyrosyl side chains in polypeptides (Fairhead and Thöny-Meyer 2010). In this study, tyrosinase showed high pupation inhibition when *Cx. pipiens* third-instar larvae are treated, and the answer is concentration dependent. One would anticipate that, with insects, as with other animals, mortality would occur as a result of protein and tissue damage induced by proteolytic enzymatic activity. A previous in vitro study suggested high inhibitory activity of *Streptomyces* tyrosinase on egg development of the animal parasitic nematode *T. vitulorum* and severe cuticular alterations in the treated adult worms (Shalaby et al. 2020).

In terms of mosquito control, *Aspergillus* has been studied least (Seye et al. 2009). In a laboratory study, 100% mortality in the first larval stage which decreased



to 60% mortality in the fourth larval stage of *Anopheles gambiae* larvae had been occurred by *Aspergillus parasiticus* (Nnakumusana 1985). Six species of *Aspergillus* (*As. versicolor*, *As. flavus*, *As. niger*, *As. fumigatus*, *As. ochraceus*, and *As. terreus*) were isolated from *An. stephensi* larvae when exposed to water samples collected from buffalo wallows, water tanks, and the Gomti River in India (Sur et al. 1998). Both *Cx. quinquefasciatus* and *Aedes aegypti* larvae showed 100% mortality in the



laboratory, following exposure to *As. clavatus* isolated from a locust (Seye et al. 2009).

The results of the current study were extended to assess the histopathological effects of tyrosinase on larvae of *Cx. pipiens*. This was in order to investigate whether the inhibitory larval development was due to changes in the midgut structure caused by the tyrosinase. Before being exposed to tyrosinase, the midgut epithelial cells displayed a well-developed brush border, normal nuclei, and an ideal basement membrane. After exposure to tyrosinase the disruption and separation of the larval midgut

epithelium was observed. It has been argued that swelling and disruption of the midgut cause the death of the insect (Poopathi et al. 2000). Accordingly, in the current study, the inhibitory larval development induced by tyrosinase might be attributed to failure of the exposed and disrupted midgut epithelium to capture nutrients. Indeed, the midgut epithelium of most insects is lined with a proteinaceous structure—peritrophic matrix (PM)—providing protection to tissue from various chemical, physical, and microbial challenges while at the same time allowing the transport of nutrients, minerals, and water. Chemical compounds that disrupt the PM can lead to a retardation of insect larval growth and even mortality due to a loss of its important functions. Consequently, the insect PM that lined the midgut epithelium was a desirable target for insecticidal proteins (Wang and Granados 2000, 2001).

In the current study, the histopathological effects of tyrosinase observed on the midgut of *Cx. pipiens* larvae appeared to be in line with the results obtained by Hamouda et al. (1996), who reported that the *Cx. pipiens* midgut exposed to *Artemisia judaica* exhibited vacuolization of the epithelial layer and swollen cells. Masses of cellular material appeared in the lumen, and the epithelium lost its usual appearance. Hamouda et al. (1996) also found that larvae exposed to *Anagallis arvensis* exhibited the break-up of the cell wall and the disruption of the peritrophic membrane. Besides, Assar and El-Sobky (2003) observed that the water extract of *Eichhornia crassipes* showed a dramatic effect on larval midgut as degeneration of some epithelial cells and the brush border had been occurred apically; after 48 h and 72 h most epithelial cells had been vacuolated and fully degenerated.

In the current study, the apoptotic effect of the tyrosinase enzyme was studied by monitoring the larval caspase activity. It was suggested that the high pupation inhibition rate caused by tyrosinase might be explained by the high expression of the apoptotic marker in tyrosinase-exposed larvae. In that sense, some of the proteases—particularly those that targeted the PM—acted as “stomach poisons” upon ingestion by an insect. Those proteases could be applied as an insecticide by themselves, without the requirement for an insect pathogen to deliver them to their target sites (Harrison and Bonning 2010). The activation of apoptotic pathways in the treated larvae (involving caspase enzymes) by active agents released by the conidia of fungi could be considered as a possible mechanism that might eventually lead to the death of the larvae. Extracellular proteases were identified as the active agents and contributed significantly to the larval mortality of *Ae. aegypti*, which appeared to be mediated through stress-induced apoptosis (Butt et al. 2013).

Conclusions

This study could offer a bioinsecticide—tyrosinase—with a new mode of action that has the potential to induce high inhibitory activity on the larval development of *Cx. pipiens*. Consequently, it might be combined with other biocontrol agents or low doses of natural insecticides to reduce toxic effects on the aquatic environment and increase efficacy against mosquitoes.

Abbreviations

As. Flavus: *Aspergillus flavus*; *As. Niger*: *Aspergillus niger*; *As. Oryzae*: *Aspergillus oryzae*; *As. tamarii*NRC3: *Aspergillus tamarii* NRC3; *An gambiae*: *Anopheles gambiae*; *An. Stephensi*: *Anopheles Stephensi*; *As. aegypti*: *Aedes aegypti*; *Cx. Pipiens*: *Culex pipiens*; IA: Inhibitory activity; PBS: Phosphate buffer saline; PM: Peritrophic membrane; *T. vitulorum*: *Toxocara vitulorum*.

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Authors' contributions

HAS and HA planned the work. MS conceived the experiments of tyrosinase production. AHE and HA conducted the experiments of bioassay. MTH performed the histological section. HAS and AHE wrote and prepared the manuscript. All the authors read and approved the final version of the manuscript.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are included in this published manuscript.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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