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A novel pyrazole–pyridine derivative (PPD) targets specific biological pathways in the larval stages of the northern house mosquito *Culex pipiens* Linnaeus (Diptera: Culicidae)

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Abstract

Background Mosquitoes cause a variety of health problems in humans and pets. So, the control of mosquito larvae is one of the best ways to avoid health problems arising from diseases transmitted by these insects. There are various control mechanisms including mechanical, biological and chemical control. The latter, despite the presence of some obstacles associated with its use, is preferred because of its ability to supply rapid management results.

Result A novel laboratory-synthesized chemical compound containing pyrazole and pyridine moieties (pyrazole–pyridine derivatives, PPD) was used to control and address the biological effects on *Culex pipiens* mosquito second larval instar. A sublethal concentration (LC30) of PPD inhibited larval growth by about 50%. Furthermore, the developmental time of larvae into pupae and the emergence of adults from the pupal stages were increased by about 20% and 17%, respectively. The ultrastructural studies on the midgut cells revealed that treated larvae suffered dramatic degeneration in the gastric caeca and the posterior midgut cells, while the anterior midgut epithelium appeared with an abundance of lysosomal activities. Additionally, treated larvae showed fluctuated activities in the levels of the detoxifying enzymes and increased levels in total antioxidants.

Conclusions These results clearly show that pyrazole and pyridine moieties containing compounds can be used against larval stages of *C. pipiens*.

Keywords Mosquito, Pyrazole, Pyridine, Insecticide, *Culex pipiens*

Background

Mosquitoes affect several health issues related to humans and domestic animals. For instance, several genera of mosquito can transmit pathogenic agents to human including viruses, bacteria and eukaryotic protozoa (Melhorn, 2008; Melhorn et al., 2012). This medical importance attracted entomologists to look for valuable methods to regulate such insects. Mosquito management is achieved by several control methods including mechanical, behavioral, chemical and biotechnology-based methods (Benelli, 2015; Lees et al., 2014). Resistance to insecticides by mosquitoes of different genera emerged more than 45 years ago in all continents of the

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world; this resistance occurs frequently due to a loss of sensitivity of the insect's acetylcholinesterase enzymes to organophosphate and carbamate insecticides (Toutant, 1989). Thus, it is needed to always develop novel insecticides overcoming such limitation.

Pyrazole is a commonly used insecticide as it acts directly on the γ -aminobutyric acid (GABA) chloride channels in insects to disrupt neuronal signaling. Structurally, it has a 5-membered ring of three carbon atoms and two adjacent nitrogen atoms. Naturally, it is present in the seeds of watermelon (Noe et al., 1959). This compound has a broad spectrum of medical applications including anti-tuberculosis, anti-malignant, antimicrobial, anti-inflammatory, antidepressant and antiviral effects (Faisal et al., 2019; Naim et al., 2016). Pyridine compounds are heterocyclic organics having the chemical formula of C_5H_5N . The pyridine ring is present in a broad spectrum of compounds, including agrochemicals, pharmaceuticals, and vitamins (Gehad et al., 2009a, 2009b). Besides their wide existence in nature, pyridines have several applications including antimicrobial, antioxidant, anticancer, and insecticidal activities (Altaf et al., 2015; Bakhite et al., 2014; Zaki et al., 2017). Imidacloprid and thiacloprid (neonicotinoid-based insecticides) are pyridine derivatives representing the most widely used insecticides between 1999 and 2018 because their toxicity to birds and mammals was considered low compared to organophosphates and carbamates (Ihara & Matsuda, 2018). In 2018, some European countries banned them but recently in 2020 the European Food Safety Authority EFSA unblocked the use of these insecticides (EFSA, 2020).

The current work develops new insecticidal molecule based on the following criteria: incorporations of the substructural unit of pyridine moiety into the backbone of pyrazole resulting in pyrazole amides containing a pyridine substructure. In this work, a novel pyrazole–pyridine derivative (PPDs) with the IUPAC name 6-amino-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*b*]pyridine-5-carboxamide was synthesized and used as a potential larvicidal compound against *C. pipiens*. A sublethal concentration of PPD was used to understand the subsequent effects of this compound on some biological and physiological parameters in *C. pipiens* larvae.

Methods

Insect culture

A stable laboratory culture of *C. pipiens* is established at the laboratory of Entomology, Assiut University. Mature males and females are cultured in the laboratory under fixed dark–light cycles with 12:12 h light: dark and approximate culturing temperature of 25 ± 1 °C. Briefly, mosquito eggs are laid on a water surface that is covered

with green algae. Eggs hatch into the larval stage with approximate life span of 6–7 days under the mentioned conditions. Later, they develop into the pupal stage, and finally, adult's emergence is achieved within a couple of days (Megan et al., 2023).

Synthesis

of 6-amino-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*b*]pyridine-5-carboxamide

The synthetic route of the targeted compound (6-amino-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*b*]pyridine-5-carboxamide) is established according to the following: A solution of 5-amino-3-methyl-1-phenyl pyrazole-4 carbaldehyde (0.01 mol) in ethanol (50 mL) was mixed with (0.01 mol) cyanoacetamide. The reaction mixture was treated with a few drops of piperidine and then refluxed for 3 h. The solid product formed was collected by filtration and recrystallized from ethanol. The chemical structure of the compound can be found in Fig. 1.

Experimental designs

Second-instar larvae were used to address the impact of PPD on the growth rate, developmental periods, midgut ultrastructural changes, and the biochemical manipulations in selected detoxification enzymes and the total antioxidant content of *C. pipiens* larvae. The sublethal concentration LC₃₀ calculated from a previous study (unpublished data) was used in these assays. Samples were collected 48 h following treatments and used for subsequent analysis.

Transmission electron microscopy (TEM)

Ultrastructural studies on the midgut of *C. pipiens* larvae were performed on both control and treated larvae (treated with a sublethal concentration (LC₃₀) of PPD). The selected tissue was fixed in 2.5% glutaraldehyde, washed in 0.1M phosphate buffer, pH = 7.3. Subsequently,

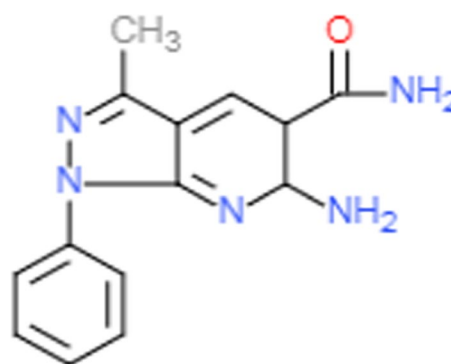


Fig. 1 Chemical structure of the synthetic compound 6-amino-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*b*]pyridine-5-carboxamide (PPD)

tissues were dehydrated in a graded series of ethanol. Ultrathin sections were photographed with a JEM, 100CX11 USA, TEM at the electron microscope unit of Assiut University (Bancroft et al., 1982).

Biochemical analyses

Superoxide dismutase (SOD), alkaline phosphatase (ALP), catalase (CAT), glutathione transferase (GST), and the total antioxidant capacity (TAC) were measured in control and LC30-treated larvae using commercial kits (Biodiagnostics, Cairo, Egypt) according to manufacturer instructions.

Statistical analyses

All treatments were presented as mean \pm standard deviation. The sublethal concentration (LC30) was calculated by Probit analysis (Finney, 1971). Larval and pupal periods were analyzed by starting with 105 larvae for both control and treatment. All biochemical assays were performed based on three independent replicates and analyzed statistically by Student's *t* test using Graph Pad Prism 5 statistical software (GraphPad Software, San Diego, CA, USA).

Results

Growth inhibition and developmental dysfunctions in *C. pipiens* larvae-treated PPD

The calculated LC30 value recorded by Probit analysis was 3.13 mg/L. For all assays, 0.313 mg/100 mL was prepared in ddH₂O containing 10-s instar larvae. A sublethal concentration of the newly synthesized PPD compound was used against the second instar of *C. pipiens* larvae to either address the changes in growth rate or developmental alterations. The treated larvae suffered from observable retardation in the growth rate compared to control larvae (Fig. 2a, b). Larvae treated

with LC30 of the pesticide showed extended larval and pupal periods (Fig. 2c). The recorded larval period in treated larvae increased by about 2 days to control ($t=5.512$ $df=106$; $P<0.0001$) (Fig. 3a), while the pupal period was extending by about 0.2 days compared to control ($t=2.033$ $df=106$; $P=0.0446$) (Fig. 3b).

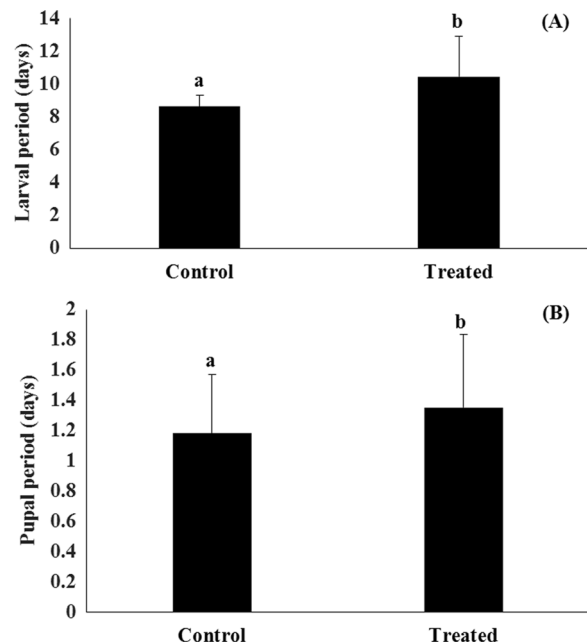


Fig. 3 Developmental dysfunctions in the second-instar larvae and pupae of *C. pipiens* treated with LC₃₀ of a pyrazole–pyridine derivative (PPD). Comparison between the longevity (in days) of both control (Control) or LC₃₀-treated (Treated) larvae (A) and pupae (B). All data are presented as mean \pm SD based on three independent replicates. Letters above error bars represent statistical difference at type error $P \leq 0.05$ (Student's *t* test)

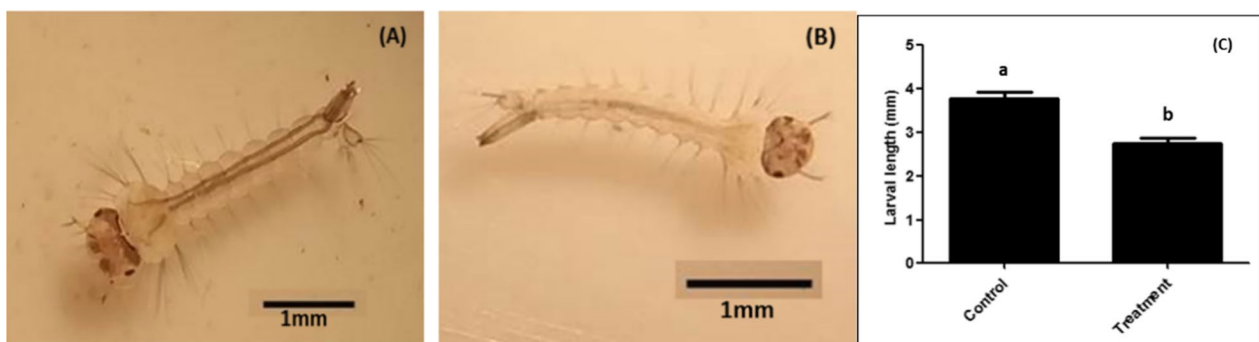


Fig. 2 A pyrazole–pyridine derivative (PPD) inhibits the growth of *C. pipiens* larvae. Photomicrographs of *C. pipiens* showing control (A) or LC₃₀-treated (B) larvae to compare the differences in the growth rate. A diagram comparing the differences in growth rate between untreated larvae (Control) and larvae treated with PPD (Treatment) (C). Letters above error bars represent statistical differences at type error $P \leq 0.05$ (Student's *t* test)

Midgut ultrastructural abnormalities in *C. pipiens* larvae treated with PPD

The midgut pieces isolated from larvae treated with LC30 of PPD compound or those isolated from control larvae were fixed and prepared for TEM analysis. The gastric caeca, which are projections from the midgut, are characterized by epithelial cells with intact cytoplasm, nucleus with scattered chromatin and extended microvilli on their surface (Fig. 4a, b). Gastric caeca of treated larvae showed abundant areas of empty cytoplasm and short disorganized microvilli (Fig. 4c, d).

The anterior part of the midgut of control larvae was characterized by intact cytoplasm and normal

appearances of the nucleus (Fig. 5a, b). On the contrary, treated larvae showed upregulation in the lysosomal activity and clear condensation in the nuclear chromatin (Fig. 5c, d).

The posterior part of the control midgut has a similar ultrastructure to that of the gastric caeca where the epithelial cells have intact cytoplasm, long microvilli and nuclei having scattered chromatin (Fig. 6a, b). Similarly, the response of this part of the alimentary canal to PPD compound was similar to the gastric caeca where the microvilli were very short or missed, the cytoplasm was characterized by empty spaces, and in some cells the chromatin was condensed (Fig. 6c, d).

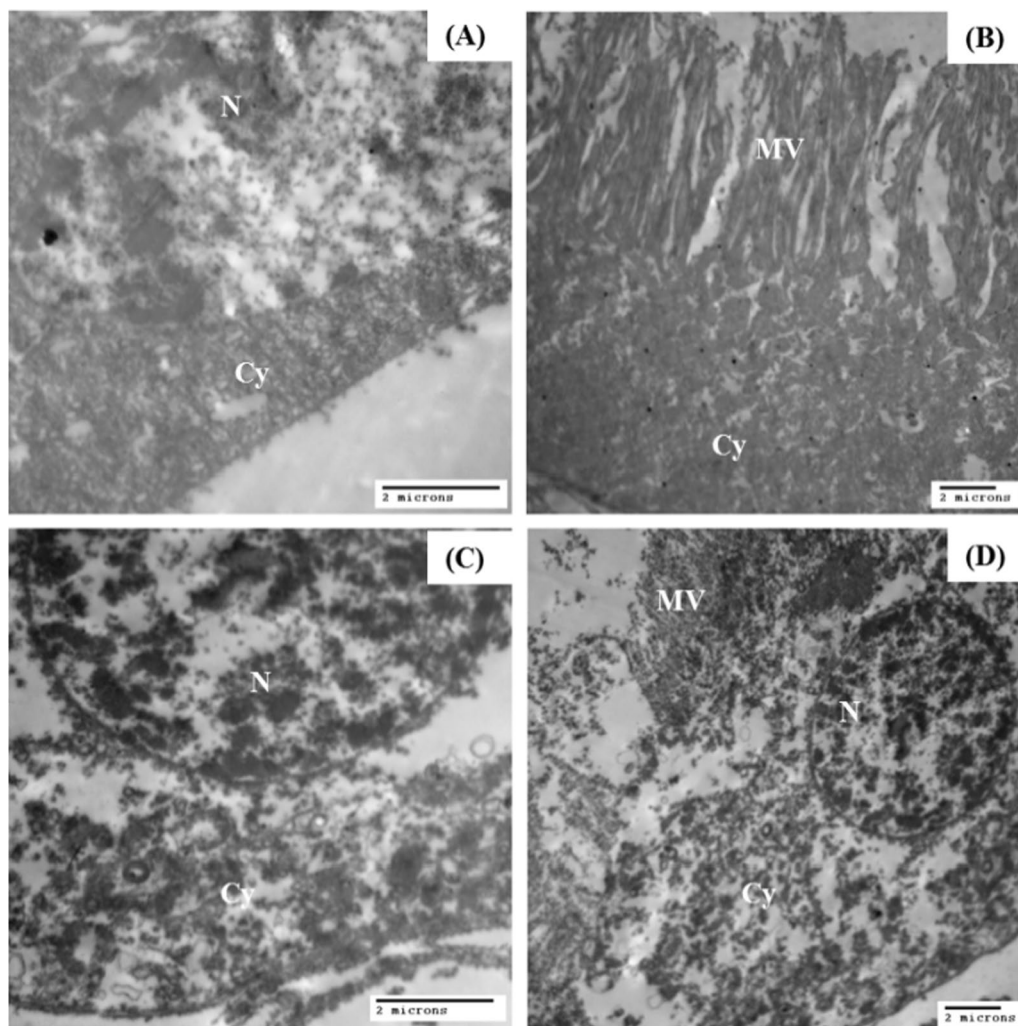


Fig. 4 Transmission electron micrographs of the epithelium of the gastric caecum from the alimentary canal of *C. pipiens* larvae. Normal alimentary canal of *C. pipiens* larvae showing the normal appearance of the nucleus, cytoplasm and microvilli (A, B). Treatment with the pyrazole–pyridine derivative (PPD) altered the appearance of the cytoplasmic components and induced serious damage in the microvilli in *C. pipiens* larvae (C, D). N (nucleus), MV (microvilli), and Cy (cytoplasm)

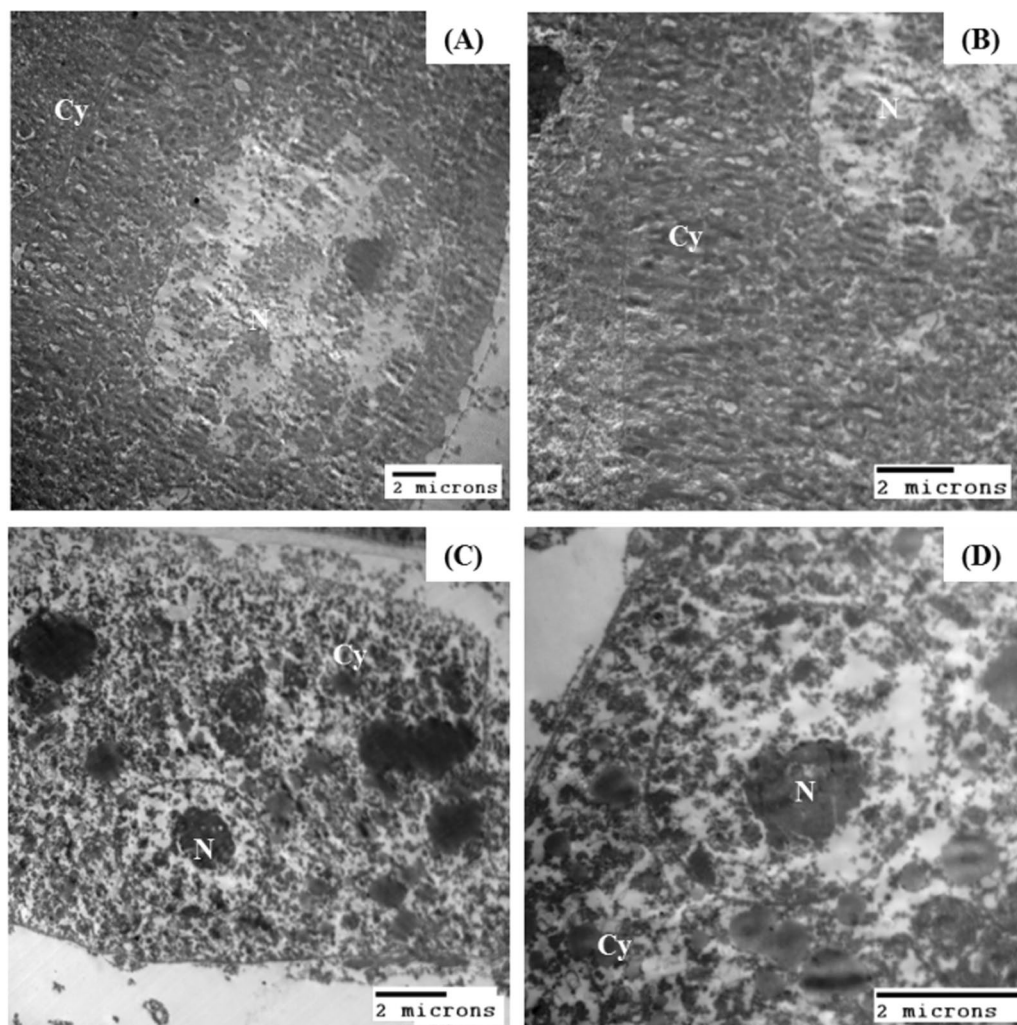


Fig. 5 Transmission electron micrographs of the epithelium of the anterior midgut from the alimentary canal of *C. pipiens* larvae. Normal alimentary canal of *C. pipiens* larvae showing the normal appearance of the nucleus with scattered chromatin, cytoplasm and cell organelles (A, B). Treatment with the pyrazole–pyridine derivative altered the nuclear structure leading to chromatin condensation and induced lysosomal activity in *C. pipiens* larvae (C, D). N (nucleus), and Cy (cytoplasm)

Biochemical dysfunctions in the detoxification system of *C. pipiens* larvae treated with PPD

In response to insecticide treatment, larval mosquitoes activate a detoxification response consisting of some detoxifying enzymes and nonenzymatic antioxidants. In the current study, we measured the changes in several detoxifying enzymes including catalase (CAT), glutathione-S-transferase (GST), superoxide dismutase (SOD) and alkaline phosphatase (ALP) together with the total antioxidant capacity (TAC) in response to LC30 of PPD. On the other side, treated larvae showed significant increase in SOD activity by about 5 folds compared to control (Fig. 7a) ($t=3.698$ $df=4$; $P=0.0209$). On the contrary, ALP activity was decreased by about 6 folds

in treated larvae compared to control (Fig. 7b) ($t=3.007$ $df=4$; $P=0.0397$). The total antioxidant capacity (TAC) was doubled in treated larvae compared to control (Fig. 7c) ($t=3.137$ $df=4$; $P=0.0349$).

Discussion

This study addressed the insecticidal activity and the biological effects of a novel synthetic compound containing a pyrazole and pyridine backbone against larval stages of *C. pipiens* mosquito. Both moieties present in the compound showed insecticidal activity against different insect taxa and were used previously in pesticide design (Mondal et al., 2017; Wei et al., 2019; Xu et al., 2018; Yanyan et al., 2018). However, the subsequent

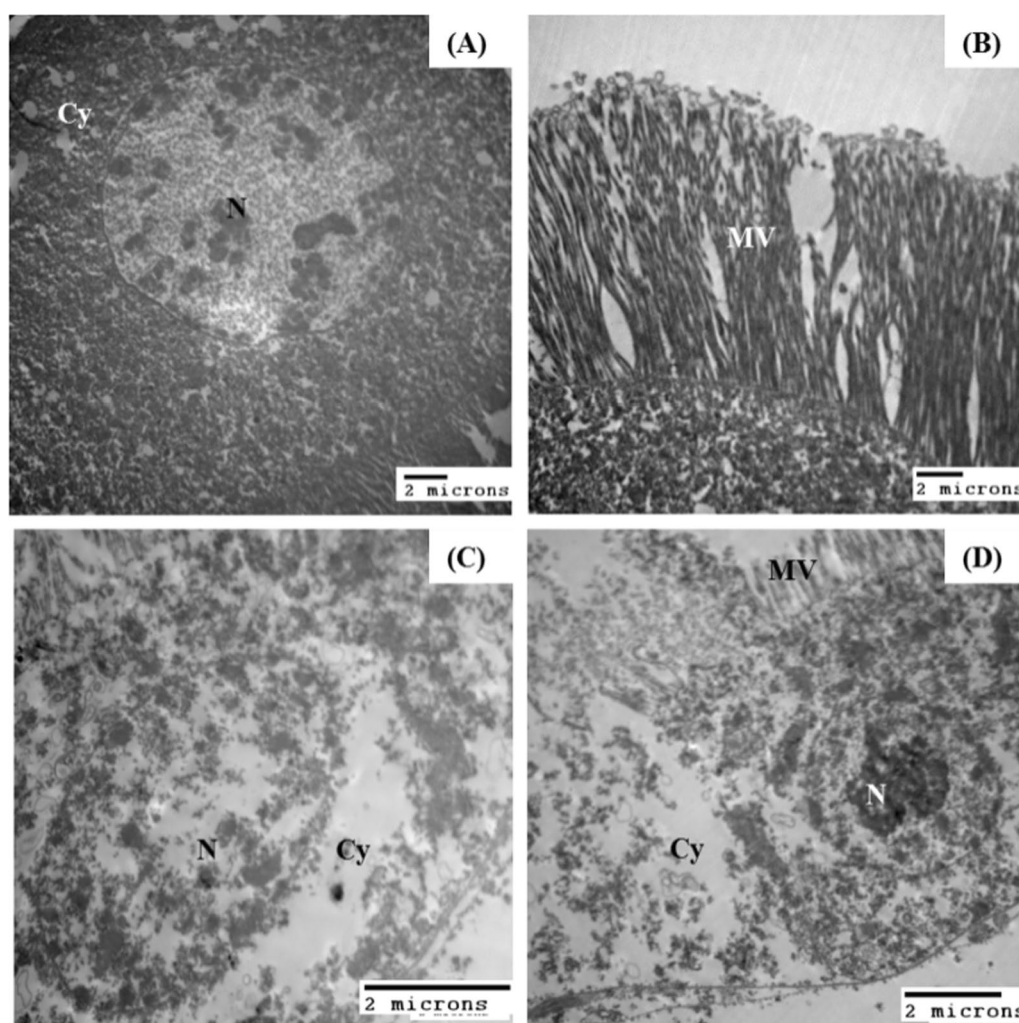


Fig. 6 Transmission electron micrographs of the epithelium of the posterior midgut from the alimentary canal of *C. pipiens* larvae. Normal alimentary canal of *C. pipiens* larvae showing the normal appearance of the nucleus with scattered chromatin, the normal shape of the microvilli, cytoplasm and cell organelles (A, B). Treatment with the pyrazole–pyridine derivative showed pathological signs in the cytoplasm and destruction of the microvilli in *C. pipiens* larvae (C, D). N (nucleus), MV (microvilli) and Cy (cytoplasm)

biological and developmental effects were poorly studied for compounds containing both moieties. Therefore, such effects needed further attentions. Additionally, this study assumed that a combination between pyrazole and pyridine moieties may lead to synergistic physiological effects based on the fact that each moiety has its mode of action against insects.

Treatment of the second-instar *C. pipiens* with PPD inhibited the larval growth rates compared to the untreated larvae. Growth inhibition is likely happened due to the inability of larvae to ingest enough food to digest and absorb the ingested diet. Flonicamid, which is a pyridine-containing compound, is classified as a feeding blocker against insects (Maienfisch, 2019). Pymetrozine, which is a neuroactive pyridine azomethine,

inhibited feeding behavior in Homoptera (Nauen et al., 2013). Treatment of the PPD compound also extended the larval and pupal developmental time than control larvae. The development of larvae into pupae in mosquitoes is arrested by the expression of juvenile hormones (Schooley, 2021). A pyridine derivative, pyriproxyfen, is considered as a juvenile hormone analog mimics the action of juvenile hormone and guarantee the extension of the larval period (Fiaz et al., 2019). A broad survey in the research literature did not give a direct link between the pyrazole ring and dysfunctions in growth and development, suggesting that it might interfere with *C. pipiens* larvae via other pathways.

Generally, the midgut epithelium is the primary target for pesticide toxicity. It is formed of cuboidal or low

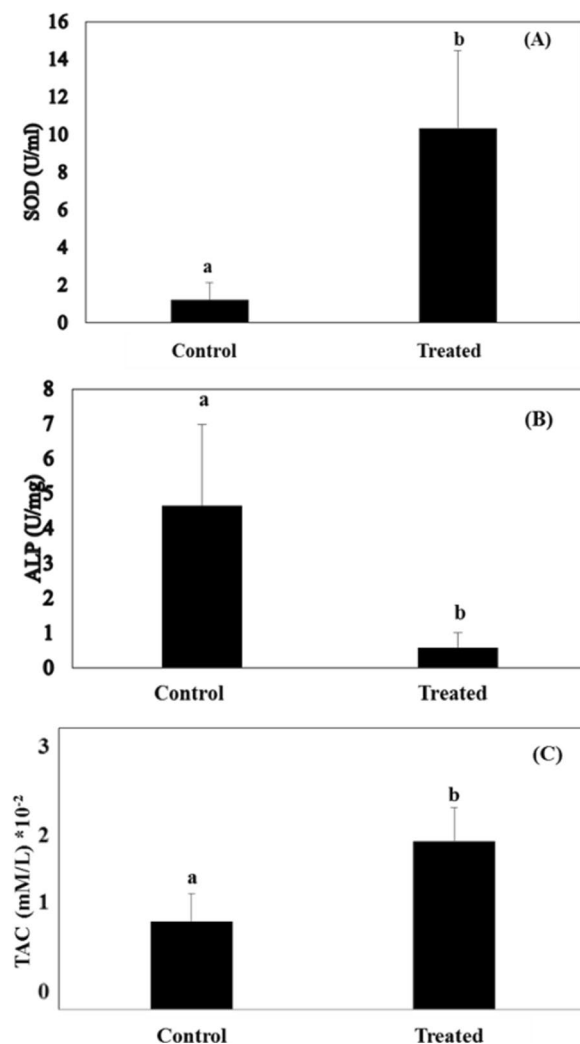


Fig. 7 Biochemical alteration in the detoxification system of *C. pipiens* larvae either cultured on ddH₂O supplied with artificial diet (Control) or treated with artificial diet in aqueous suspension of a pyrazole–pyridine derivative (PPD) (LC₅₀). **A** Superoxide dismutase (SOD). **B** Alkaline phosphatase (ALP). **C** Total antioxidant capacity (TAC). All data are presented as mean ± SD based on three independent replicates. Letters above error bars represent statistical difference at type error $P \leq 0.05$ (Student's *t* test)

columnar cells resting on a basement membrane. The cells have well-developed microvilli and central rounded nuclei (Al-Doaiss et al., 2021; Yu Cheng et al., 2011). However, the midgut itself has three main regions: the gastric caeca, the anterior midgut and the posterior midgut. The ultrastructural studies were performed to supply information regarding how mosquito larvae detoxify chemicals. Treatment of *C. pipiens* larvae with a sublethal concentration of PPD caused several histopathological signs in the midgut ultrastructure. The gastric caeca, which are projections of the midgut, appeared to have

numerous vacuole-like empty spaces in the cytoplasm and disorganization in the brush borders in treated larvae. Such signs in insecticide-treated insects are clear indicators that these cells are undergoing death (Alves et al., 2010). The histopathological patterns observed in the posterior midgut cells treated with the PPD compound were similar to that of the gastric caeca. The microvilli on the cell border were greatly shorter than the control and the cytoplasm was characterized by empty spaces and the nuclear chromatin was condensed.

The anterior midgut of treated *C. pipiens* larvae showed several pathological signs. The nuclei of anterior midgut cells in treated larvae showed condensed chromatin, while numerous vacuoles appeared in the cytoplasm together with abundant lysosomal activity. The impact of insecticidal compounds on the anterior midgut ultrastructure was studied in depth. Unfortunately, pyrazole and pyridine compounds did not have enough attention. The sublethal concentration of imidacloprid on the predatory insect *Podisus nigrispinus* Dallas caused serious damage in the anterior midgut including cytotoxic features, irregular border epithelium, vacuolation in the cytoplasm and apocrine secretions 6 h following exposure to this compound. The digestive cells reach to apoptosis after 12 h of treatment (Martinez et al., 2019). Fipronil (a pyrazole derivative) and boric acid activated cell death in the midgut digestive cells of the honey bee *Apis mellifera* workers (da Silva Cruz et al., 2010).

The responses of the cells in the midgut regions to PPD showed high lysosomal activity in the anterior regions of the midgut (anterior midgut). This response was not found in the gastric caeca and the posterior midgut. Structurally, several insects possess a resemblance between gastric caeca and distal posterior midgut cells (Clark et al., 1999; Ferreira et al., 1981). On the contrary, the anterior midgut cells, unlike the other parts of the midgut, have abundant mitochondria and small microvilli on the apical part of the cell (Ferreira et al., 1981). Functionally, there is no evidence to answer which part of the midgut is responsible for detoxification of toxic chemicals in mosquito larvae. However, abundance of lysosomal activity in the anterior midgut might be indicator that the anterior part of midgut is actively tolerating PPD toxicity. Lysosomal activity is a key step in the detoxification of xenobiotics. For instance, heavy metals can reach various insect organs where they are managed by lysosomes to detoxify (Sun et al., 2007).

Treatment with PPD altered the detoxification system of *C. pipiens* larvae. Treated larvae showed enhanced SOD and total antioxidant activity, while the ALP activity was significantly reduced. Additionally, some other enzymatic antioxidants were unaffected by the treatment (supplementary data). The midgut epithelial cells are

specialized for the production of enzymes that metabolize and detoxify insecticides and xenobiotics. There are two key reasons for the limited toxicity of insecticides against pests and other harmful insects. The first is their metabolic degradation in the midgut and the reduced penetration of insecticides through midgut walls (Cheng Zhu et al., 2011). Several enzymes are involved in the detoxification process to reach metabolic degradation of the insecticides (Ibrahim & Ali, 2018; Ibrahim et al., 2023; Wang et al., 2020). When challenged by insecticides, insects rely on antioxidants and detoxifying enzymes to return their normal state. Selection of the appropriate defense mechanism against the used insecticide is still unknown. It is necessary to keep into consideration that inhibition of some detoxifying enzymes, such as ALP, indicates that these enzymes either play no role in the detoxification of the tested compounds or the used insecticide impairs the activation pathway of these enzymes (Abd El-Aziz & El-Sayed, 2009).

The return to chemical control methods in mosquito control is built upon two main reasons. The first reason is that insects develop multiple resistance mechanisms against different insecticide categories. Resistance mechanisms in insects can be divided into two categories. The first is (i) metabolic resistance (manipulations in the expression levels of certain proteins or activities of detoxification enzymes), while the second category of resistance is (ii) target-site resistance (reduced susceptibility of the sodium channel, acetylcholinesterase, and GABA receptor) (Dang et al., 2017; Hemingway et al., 2004; Liu, 2015). The second reason is the appearance of serious disadvantages in modern methods of control. Current control methods for mosquitoes have wide range of approaches. Nanotechnology-based methods are promising tools in management but they are restricted by their possible toxic effects to human and domestic animals (Yang et al., 2021). Sterile insect technique by releasing sterile males is limited by the need to release large number of sterile males. Insect repellents showed serious health issues in both humans and other animals (Potera, 2008). Transgenic mosquitoes suffer from lower developmental and reproductive fitness compared to wild mosquitoes (Ramayasoma et al., 2021). In the near future, it appears that synthetic chemical insecticides will continue in the forefront of selection for pest management.

Conclusions

Control of *C. pipiens* larvae using a novel, synthetic chemical (PPD) was achieved successfully in the current study. Further, based on the fact that two different moieties were incorporated in the compounds under investigation (pyrazole and pyridine moieties), it was needed to evaluate the developmental and physiological

dysfunctions associated with its use. As speculated, the developmental time was arrested in treated larvae, the growth rate was impacted and several physiological impairments were also detected.

Abbreviations

PPD	Pyrazole–pyridine derivative
LC30	Sublethal concentration
<i>C. pipiens</i>	<i>Culex pipiens</i>
TEM	Transmission electron microscopy
SOD	Superoxide dismutase
ALP	Alkaline phosphatase
CAT	Catalase
GST	Glutathione transferase
TAC	The total antioxidant capacity
GABA	γ-Aminobutyric acid

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Author contributions

DSM, NAA, AAO, and AMI did conception and designed the work, supplied materials, conceptualization, methodology, formal analysis, investigation, writing—original draft, resources, data curation. AAO contributed to synthesis of 6-amino-3-methyl-1-phenyl-1H-pyrazolo[3,4-b]pyridine-5-carboxamide. All authors reviewed the manuscript. The authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article. Please contact authors for data.

Declarations

Ethics approval and consent to participate

All experimental protocols were performed according to regulations set by the National Institutes of Health guidelines, Egypt.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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