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Toxicity of Piperine Amide Analogs toward the Tomato Pinworm *Tuta absoluta* (Lepidoptera: Gelechiidae) and Risk Assessment for Two Predators

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Abstract: Nineteen amides (three of them unpublished) were synthesized and tested on *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae), an important pest of Solanaceae plants worldwide. Three of these compounds (**14**, **15** and **16)** presented high acute toxicity toward the pest, with LD_{50}s of 3.68 (CI_{95} = 2.83 − 4.47), 6.46 (CI_{95} = 5.85 − 7.20), and 13.52 µg/mg (CI_{95} = 11.06 − 15.95), respectively. Amide **14** presented the fastest action (LT_{50} = 1.2 minutes, CI_{95} = 1.03 − 1.37), followed by amide **16** (LT_{50} = 18 minutes, CI_{95} = 9.96 − 26.04), and amide **15** (LT_{50} = 3.7 hours, CI_{95} = 0.69 − 6.71). When applied at a sublethal dose, they did not affect weight gain and leaf consumption of the pest. Bioassays were also conducted using the ant *Solenopsis saevissima* (Hymenoptera: Formicidae) and wasp *Polybia ignobilis* (Hymenoptera: Vespidae) to assess the toxicity of the amides against *T. absoluta* predators. Amides **14**, **15,** and **16** were harmless to *S. saevissima* but presented high toxicity toward *P. ignobilis*. Amides **14**, **15** and **16** are potential hit compounds for the development of insecticides for *T. absoluta* control. However, their use should be carried out following the principles of ecological selectivity to mitigate potential adverse effects on non-target organisms.

Keywords: invasive pest; natural-based insecticide; tomato

1. Introduction

The tomato pinworm *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae) is an important pest of Solanaceae [1,2]. Native to South America, this pest was introduced into Europe in the last decade and currently can be found throughout Europe, Africa, and Asia [3–7]. The damage of this pest is greater in tomato crops (*Solanum lycopersicum* L.), where its larvae build galleries in leaves and burrow into fruits and branches [8]. Thus, crop yield and fruit marketability are compromised [9].

Alternatives such as cultural and biological controls have been sought for the management of *T. absoluta*, but the use of insecticide remains the primary control measure used by tomato growers, especially in South America [2]. Continuous application of insecticides contributes to the development of *T. absoluta*-resistant strains, which reduces the efficacy of several products registered to control this pest [10–15]. Another problem related to the use of insecticides are the side-effects of some molecules on non-target organisms, such as predators, pollinators, and detritivore arthropods [16–18].

In this sense, studies related to the development of new insecticidal molecules are important, aiming at the discovery and synthesis of active compounds against pests and less harmful to the

environment [19]. Natural products have been consistently used as models in the synthesis of new insecticides [20]. Pyrethroids and neonicotinoids are examples of synthetic insecticides based on molecules extracted from plants [21]. Piperine is an amide found in fruits of Piperaceae plants with insecticidal activity against several pests [22,23], and has been used as a model for the synthesis of analogous products [24–26].

Given the demand for the development of new insecticidal molecules for *T. absoluta* management, this study evaluated the potential of 19 piperine analogs as hit compounds for the development of insecticides against this pest.

2. Materials and Methods

2.1. Synthesis

(2*E*,4*E*)-Hexa-2,4-dienoic acid (sorbic acid), a diene with six carbon atoms, presents the right functional group for amide formation and is readily available for the preparation of piperine analogs. The precursor acid chloride was prepared by treatment of sorbic acid with oxalyl chloride. Amides with conjugated double bonds were prepared by the reaction of hexa-2,4-dienoyl chloride with the corresponding amines [27]. The amides were characterized as (2*E*,4*E*)-*N*-cyclohexylhexa-2,4-dienamide (**1**), (2*E*,4*E*)-*N*-benzylhexa-2,4-dienamide (**2**), (2*E*,4*E*)-*N*-phenethylhexa-2,4-dienamide (**3**), (2*E*,4*E*)-*N*-phenylhexa-2,4-dienamide (**4**), (2*E*,4*E*)-*N*,*N*-diphenylhexa-2,4-dienamide (**5**), (2*E*,4*E*)-*N*,*N*-di(propan-2-yl)hexa-2,4-dienamide (**6**), (2*E*,4*E*)-*N*, *N*-(diethyl)hexa-2,4-dienamide (**7**), (2*E*,4*E*)-1-(pyrrolidin-1-yl)hexa-2,4-dien-1-one (**8**), (2*E*,4*E*)-*N*-hexylhexa-2,4-dienamide (**9**), and (2*E*,4*E*)-1-(piperidin-1-yl)hexa-2,4-dien-1-one (**10**).

To compare the insecticidal activity of amides with conjugated double bonds, nine saturated amides were synthesized by the reaction of hexanoic anhydride with the corresponding amine. The products were characterized as *N*-phenylhexanamide (**11**), *N*-benzylhexanamide (**12**), *N*-cyclohexylhexanamide (**13**), 1-(pyrrolidin-1-yl)hexan-1-one (**14**), *N*-pentylhexanamide (**15**), *N*-phenethylhexanamide (**16**), *N*-(4-nitrophenyl)hexanamide (**17**), *N*-(4-chlorophenyl)hexanamide (**18**), and *N*-(3-chlorophenyl)hexanamide (**19**). To our knowledge, this is the first study to report the synthesis of amides **3**, **9,** and **19**. Chemical structures of the evaluated amides and the details of the experimental procedures and physical and spectroscopic data (IR, ^{1}H NMR, ^{13}C NMR, and MS) used for the complete structural characterization of the new compounds are provided in the supplementary material.

2.2. Insecticidal Bioassays

2.2.1. Amide's Susceptibility Bioassay

Bioassays were performed with second instar larvae of *T. absoluta*. The population used has been maintained in the laboratory for more than ten years [28]. Larvae were fed tomato leaves (*Solanum lycopersicon* L. var. Santa Clara) from plants cultivated under greenhouse conditions without any insecticide application. The treatments were the 19 amides, piperine (positive control), and acetone (negative control). Piperine was used as the positive control due to its reported insecticidal action against lepidopteran pests, in addition to its structural similarity with the synthesized compounds [22,24]. The compounds were diluted in acetone at a dose of 30 µg/mg of body weight and topically applied at the dorsal thorax (0.5 µL per insect) using a Hamilton microsyringe. This dose was adopted based on previous studies that recommend doses from 10 to 50 µg/mg of body weight to select substances with insecticidal activity [29,30]. The experimental unit was a plastic pot (6 cm diameter × 5 cm height) containing ten *T. absoluta* larvae. The design was completely randomized with three replicates. Mortality was evaluated after a 48-h exposure to the treatments. Insects were considered dead when they did not move when touched by a fine brush. Mortality data were checked for normality (Shapiro-Wilk test) and for homoscedasticity of residuals (Bartlett test), and subjected to ANOVA, followed by the Scott–Knott cluster analysis at $P = 0.05$ [31,32]. Amides causing mortality rates ≥80% to

T. absoluta larvae were selected for the subsequent bioassays since this is the minimum value required by Brazilian legislation to select potential insecticidal molecules [33].

2.2.2. Dose-Response Bioassay

The same procedures of the previous bioassay were used. The treatments were the most efficacious amides (selected in the previous bioassay) applied at different doses, piperine, and acetone control. Five to six doses causing mortalities between 1% and 99% were used for each treatment. Mortality data were subjected to probit analysis (PROC PROBIT, SAS 9.2, SAS Institute Inc, Cary, NC, USA) to estimate the dose-mortality curves. Lethal doses (LD_{25}, LD_{50}, and LD_{80}) and their respective confidence intervals (CI_{95}) for the most efficacious amides were estimated by regression analysis of probit mortality to *T. absoluta* larvae versus log10 of dose. The goodness-of-fit was assessed using Pearson's chi-square test. *P*-values >0.05 of Pearson's chi-square test indicated a significant fit between the observed and expected regression models. Estimates of the lethal doses were significantly different when their CI_{95} did not overlap.

2.2.3. Time-Response Bioassay

In order to estimate the speed of action of the most efficacious amides, 100 second instar larvae of *T. absoluta* were treated with the LD_{90} of the treatments (amides and piperine). The death of the larvae was monitored for 48 h by noting the time at which each insect died. The mortality was assessed manually every 10 min during the first hour of the experiment, every 1 h up to 24 h and subsequently, every 4 h up to 48 h. Insects were considered dead when they did not move when touched by a fine brush. Experimental data were subjected to survival analysis using Kaplan–Meier estimators (PROC LIFETEST, SAS 9.2) to obtain survival curves and estimates of median lethal times (LT_{50}s). Overall similarity among the survival curves and LT_{50}s values was tested using the Log-Rank test, and pairwise comparisons among the curves were tested using the Holm–Sidak's test at $P = 0.05$.

2.2.4. Sublethal Effects on Weight Gain and Leaf Consumption

Sublethal effects of the most efficacious amides to *T. absoluta* were investigated using second instar larvae treated with the LD_{25} for the pest. The larvae were weighed individually and treated by topical application. The control consisted of the application of acetone. The larvae (25 per treatment) were individualized in plastic pots (6 cm diameter × 5 cm height) and fed tomato leaflets. After 96 h, the leaflets were photographed using an EOS Digital Rebel XS camera (Canon Inc., Taiwan, China) and the larvae were removed from the mines and weighed. Leaf area consumed was calculated using the software ImageJ (version 1.50i; National Institutes of Health, Bethesda, MD, USA). Data of weight increment of the larvae (mg) and leaf area consumed (cm^2) were subjected to ANOVA (PROC GLM, SAS 9.2) at $P = 0.05$.

2.2.5. Risk Assessment for *T. absoluta* Predators

Adults of *Solenopsis saevissima* Smith (Hymenoptera: Formicidae) and *Polybia ignobilis* Haliday (Hymenoptera: Vespidae) were exposed to the most efficacious amides (LD_{80} for *T. absoluta*) in order to assess the toxicity of the amides toward *T. absoluta* predators. They were collected from nests located at the Universidade Federal de Viçosa campus and kept in the laboratory to acclimatize for at least two hours before the bioassays. The experimental units comprised either a round plastic container (6 cm diameter × 5 cm height) or a Petri dish (9 cm diameter × 2 cm height) containing ten insects for *S. saevissima* and *P. ignobilis*, respectively. The experiment was performed with six replicates. The wasps were immobilized by placing them in a freezer at −18 °C for 4 minutes. After the application, the insects were kept in an acclimatized room at 25 ± 0.5 °C and 75 ± 5% relative humidity. *Polybia ignobilis* was subjected to a photoperiod of 12 h while *S. saevissima* was kept in the dark during the bioassay. Mortality data were subjected to ANOVA, followed by the Tukey's test at $P = 0.05$ (PROC TTEST, SAS 9.2).

3. Results

3.1. Amide Susceptibility Bioassay

There was a significant effect of the treatments in the mortality of *T. absoluta* larvae (ANOVA: $F_{20, 42} = 33.48$, $P < 0.001$). Amides **14** and **15** and the positive control (piperine) caused the highest mortality rates (>95%). Amide **16** caused 78% mortality to *T. absoluta*, a value not statistically different from 80% (One sample *t*-test: $t = -0.275$, $P = 0.81$), the minimum mortality rate required by Brazilian legislation to select potential insecticidal molecules. The other tested amides caused low mortalities (<45%) (Figure 1).

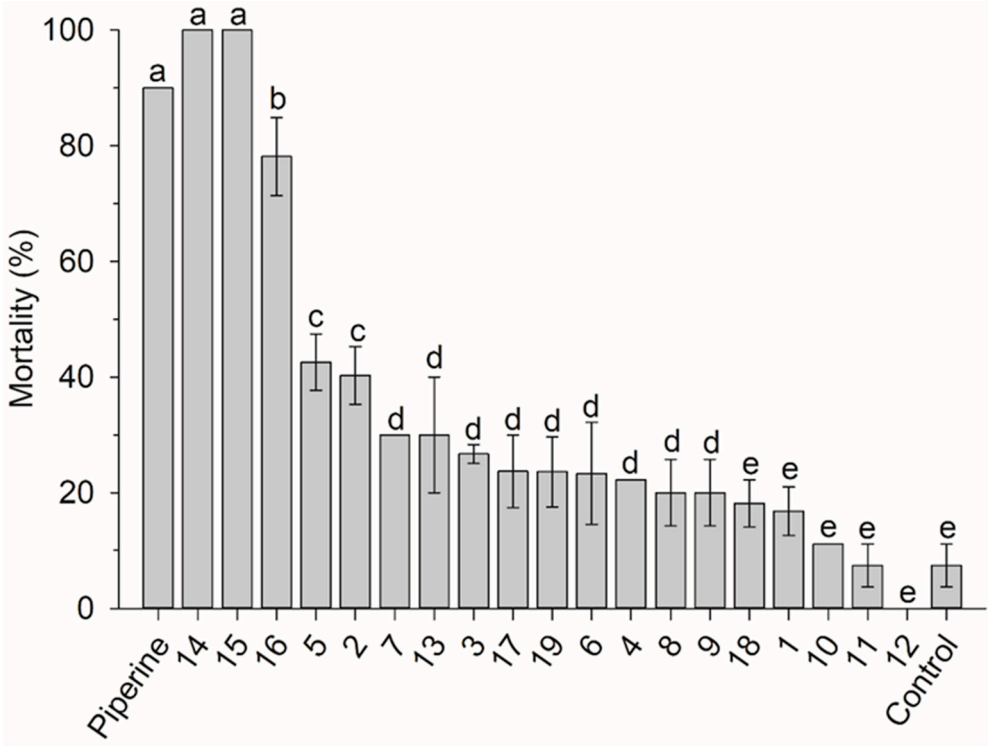

Figure 1. Mortality (%) of *Tuta absoluta* larvae 48 h after topical application of 19 amides and piperine at the dose of 30 µg/mg of body weight. Different letters above bars indicate significant differences in mean mortalities according to ANOVA and Scott–Knott test ($P < 0.05$). Control = acetone.

3.2. Dose-Response Bioassay

Pearson's Chi-square test statistic (all values of $P > 0.05$) indicated that the data fitted the regression models. The lethal doses of the amides against *T. absoluta* are shown in Table 1. Amide **14** (LD_{50} = 3.68 µg/mg, CI_{95} = 2.83 − 4.47) was as toxic as piperine (LD_{50} = 3.80 µg/mg, CI_{95} = 2.92 − 4.81). The LD_{50} of amides **15** and **16** were 6.46 (CI_{95} = 5.85 − 7.20) and 13.52 µg/mg (CI_{95} = 11.06 − 15.95), respectively.

Table 1. Dose-mortality curves of the most efficacious amides and piperine (positive control) to second instar larvae of *Tuta absoluta* 48 hours after topical application.

Compound	N [a]	Slope [b]	LD_{50} (µg/mg) [b]	*Chi-square* [d]	*P-Value* [d]
Amide **14**	240	3.14 (2.29–3.99)	3.68 (2.83–4.47)	0.22	0.90
Amide **15**	300	5.29 (3.82–6.76)	6.46 (5.85–7.20)	1.82	0.61
Amide **16**	420	2.47 (1.96–2.98)	13.52 (11.06–15.95)	3.34	0.65
Piperine [c]	300	1.79 (1.38–2.20)	3.80 (2.92–4.81)	0.11	0.99

[a] Number of insects; [b] The numbers in parenthesis are the confidence intervals at $P = 0.05$ (CI95); [c] Positive control; [d] Pearson's chi-square: *P-values* > 0.05 indicate that the observed regression model is not significantly different from the expected model.

3.3. Time-Response Bioassay

Survival analysis of *T. absoluta* larvae exposed to the control and the amides indicated a significant difference between the treatments (log-rank test: $\chi^2 = 303.46$, *d.f.* = 3, $P < 0.001$). Survival curves for all treatments differed by the Holm-Sidak method ($P < 0.05$). Amide **14** presented the fastest action (LT_{50} = 1.2 minutes, CI_{95} = 1.03 − 1.37), followed by amide **16** (LT_{50} = 18 minutes, CI_{95} = 9.96 − 26.04) and amide **15** (LT_{50} = 3.7 hours, CI_{95} = 0.69 − 6.71) (Figure 2).

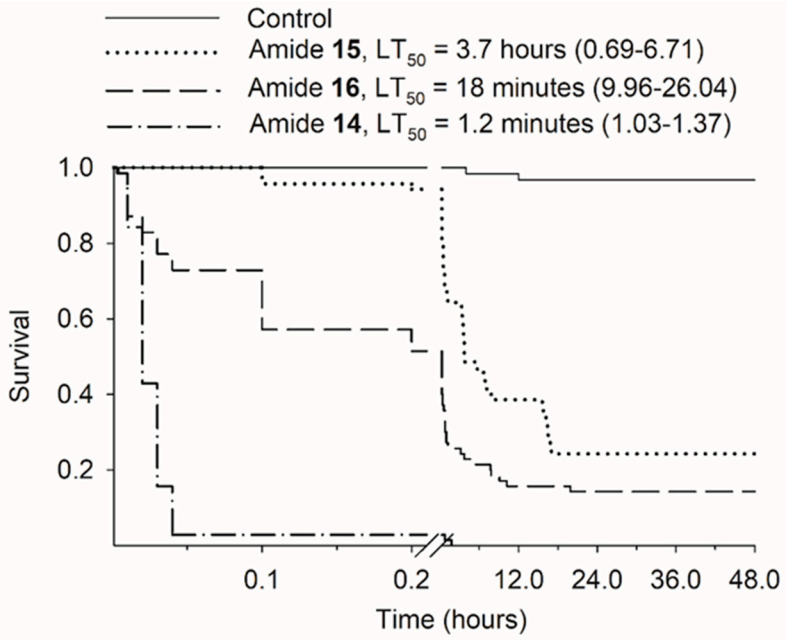

Figure 2. Survival curves of *Tuta absoluta* larvae topically treated with the LD_{90} of the amides **14**, **15**, and **16.** Control = acetone.

3.4. Sublethal Effects on Weight Gain and Leaf Consumption

Weight gain ($F_{3, 76}$ = 1.23, P = 0.31) and leaf consumption ($F_{3, 99}$ = 0.54, P = 0.66) of *Tuta absoluta* larvae were not affected by the LD_{25} of amides **14**, **15**, and **16** (Figure 3).

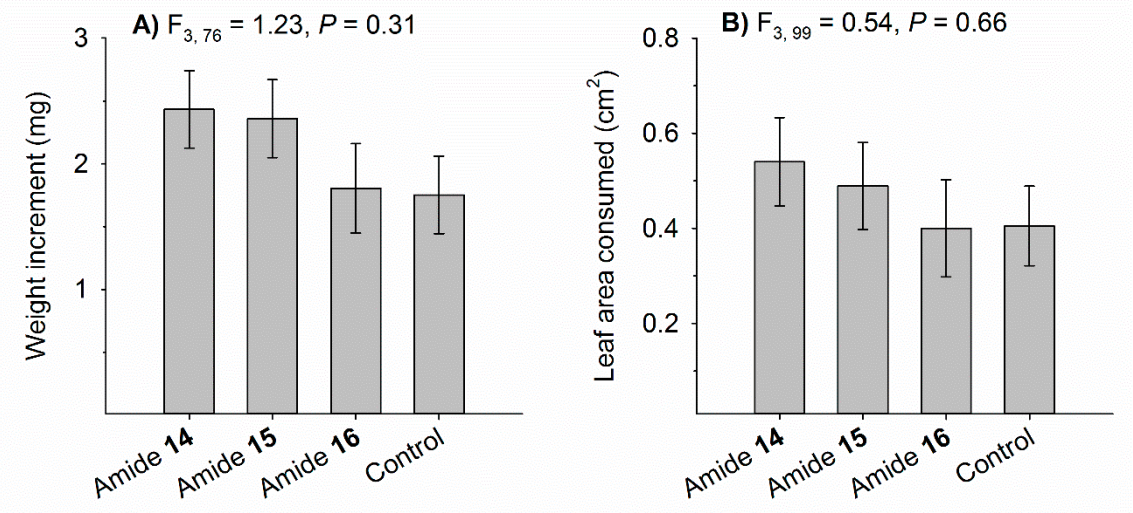

Figure 3. Weight increment (**A**) and leaf area consumed (**B**) by *Tuta absoluta* larvae topically treated with the LD_{25} of amides **14**, **15,** and **16**. Control = acetone.

3.5. *Risk Assessment for* T. absoluta *Predators*

Amides **14**, **15**, and **16** caused low mortality rates (<8%) to *S. saevissima*. Conversely, they were highly toxic (mortality >93%) toward *P. ignobilis* (Figure 4).

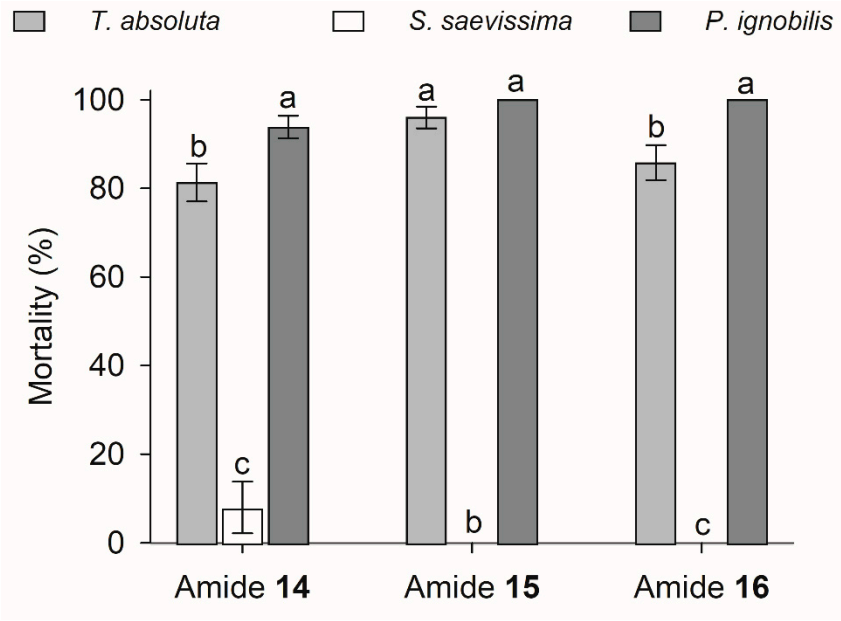

Figure 4. Mortality (%) caused by LD_{80} of amides **14**, **15**, and **16** to *Tuta absoluta* larvae and the predators *Solenopsis saevissima* and *Polybia ignobilis*, 48 hours after topical exposure. Means followed by the same letter in each column group (amide) do not differ by Tukey's test ($P < 0.05$).

4. Discussion

The most efficacious compounds (**14**, **15**, and **16**) are illustrated in Figure 5. They are structurally similar to amides **8**, **9,** and **3**, respectively, differing mainly in relation to the absence of double bonds in carbons 2 and 4 of the main chain (Supporting material, Schemes S1 and S2). This fact indicates that saturation may be related to the higher insecticidal activity of the molecules against this pest. In addition, the most efficacious amides and four more of the tested amides have the same main chain, evidencing that the constitution of the side chain is also important for the biological activity of these molecules.

The similar LD_{50} of the amide **14** and piperine indicates that the former is a promising substance for use in the control of *T. absoluta* since piperine is a botanical insecticide recommended for the control of insect pests [34,35]. The two most toxic amides (**14** and **15**) had the highest curve slopes. Steep slopes in dose mortality-curves indicated, for the same increase in mortality, less proportional increment in dose, increasing the chance of application error [36]. However, this does not exclude the potential of these compounds for use as insecticides, since formulations can increase their efficiency and stability [37]. Amides have been shown to be efficient in controlling insecticide-resistant pest populations [34,38], as well as acting synergistically with other pesticides [39,40]. Thus, insecticide mixtures containing the amides and other active ingredients can be developed for *T. absoluta* control, contributing to the management of insecticide resistance.

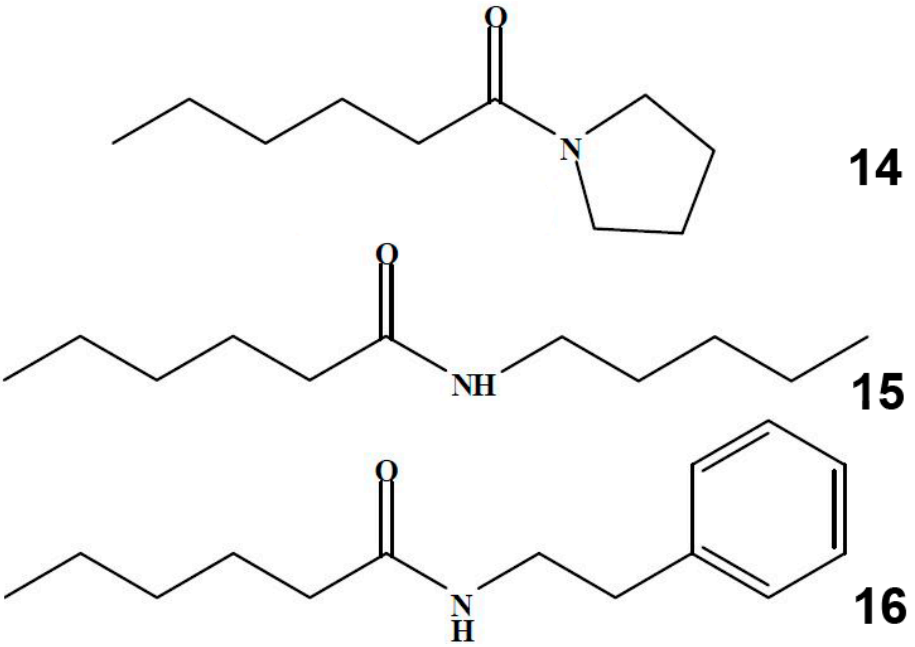

Figure 5. Structures of the most active compounds.

The fast action against *T. absoluta* is important, especially during the reproductive phase of tomato plants, reducing the damage caused by this insect to fruit [28]. In addition, the fast insecticidal activity of amides **14**, **15**, and **16** is indicative of the neurotoxic action of these substances. This hypothesis is reinforced by the fact that neurotoxic action, in addition to inhibition of detoxifying enzymes, was pointed out as the mode of action of other amides [34,41]. Further studies aiming to elucidate the mode of action of these substances should be performed.

Insect exposure to sublethal doses is common in the field due to the degradation of insecticides by environmental conditions [42,43]. Sublethal doses of insecticides can reduce biological parameters of insects [44,45]. On the other hand, undesirable effects can also occur, such as increased consumption, oviposition, and survival, which favors the emergence of resistant insects [44,46]. Here, amides **14**, **15,** and **16** in a sublethal dose did not affect the weight gain and leaf consumption of *T. absoluta* larvae, indicating that *T. absoluta* populations are less likely to be favored when exposed to the lower doses.

The International Organization for Biological Control (IOBC) classifies insecticides in four groups, depending on their toxicity to natural enemies: harmless (<30% mortality), slightly harmful (30%–79% mortality), moderately harmful (80%–99% mortality), and harmful (> 99% mortality) [47]. Amides **14**, **15**, and **16** can be classified as harmless to *S. saevissima*, since they caused low mortality rates (<8%). Conversely, they were harmful (mortality >93%) toward *P. ignobilis* according to the IOBC classification. This contrast may be due to the difference in sensitivity at sites of action and/or detoxifying ability, which may vary between organisms [48]. The tolerance of ants of the genus *Solenopsis* against insecticides has been associated with the activity of the detoxifying enzymes esterases, cytochrome P450 monooxygenases and glutathione S-transferases [49–52]. Natural biological control exerted by predatory species is extremely important in IPM programs, reducing the need for insecticide applications and, consequently, side effects of these molecules [53]. Therefore, the use of the selected amides should be carried out following the principles of ecological selectivity [54], with applications in the late afternoon, when the activity of predatory wasps is lower [55].

5. Conclusions

Amides **14**, **15,** and **16** presented high acute toxicity toward *T. absoluta* and did not favor weight gain and leaf consumption of the pest under a sublethal dose. They are potential hit compounds for the development of insecticides to manage *T. absoluta*. However, despite being selective for the predatory

ant *S. saevissima*, their use should be carried out under the principles of ecological selectivity to reduce the adverse effects on other non-target organisms.

Supplementary Materials: The following are available online at http://www.mdpi.com/2311-7524/5/4/70/s1.

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