



ORIGINAL ARTICLE

Feeding deterrence towards *Helicoverpa armigera* by *Tithonia diversifolia* tagitinin C-enriched extract



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Abstract The use of botanical insecticides is a tactic that can be highly useful for the management of *Helicoverpa armigera* populations in the field. However, most botanical insecticides are produced using primarily macerated leaves. The problem with this process is that all components of the leaves are present in the extract, and not just the plant defence chemicals. Therefore, the objective of this research was to evaluate the feeding deterrence of a tagitinin C-rich leaf rinse extract (TagCE) obtained from *Tithonia diversifolia* (which concentrates mainly the plant defence substances) towards *H. armigera* larvae. The RP-HPLC-PDA analytical method allowed the identification and quantification of the major constituent TagCE, showing that this compound is suitable to be considered as its chemical marker. Regarding biological activity, we observed high insect mortality, with up to 80%, and high antifeedant activity for *H. armigera* larvae for all host plant species treated with *T. diversifolia* extract. These results open up the possibility to use *T. diversifolia* extract as a potential botanical insecticide, mainly against plant-defoliating Lepidoptera.

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1. Introduction

On a global level, the species *Helicoverpa armigera* (Hübner, 1805) (Lepidoptera: Noctuidae), with an extensive geographical distribution and polyphagous habits, is among the main pests that attack agricultural crops, causing significant damage to the economy (Jones et al., 2019; Gonçalves et al., 2019). The first record of this species in the Brazilian territory was in 2013 in the state of Goiás (Czepak et al., 2013). In its larval stage, *H. armigera* feeds on more than 100 plant species of native and cultivars of approximately 47 families, including Solanaceae, Poaceae, Malvaceae, Fabaceae and Asteraceae. It gener-

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ally feeds on the leaves and stems of these plants and may also reach inflorescences and fruits of some species (Jallow et al., 2004). In Brazil, the larvae mainly affect cotton, crotalaria, beans, guandu, millet, corn, soybean, sorghum, tomato and wheat crops, including some weeds such as *Conyza bonariensis* (Bueno and Sosa-Gómez, 2014).

The control tactics available for *H. armigera* are chemical control using synthetic chemical insecticides and biological control using *Bacillus thuringiensis* bioinsecticides. However, there are records of *H. armigera* populations resistant to both chemical insecticides (Yang et al., 2013; Bird, 2015; Bird, 2018) as well as to bioinsecticides (Estela et al., 2004; Avilla et al., 2005). Studies have shown that there may be future risks in the use of Bt crops mainly due to increased resistance of *H. armigera* populations (Ahmad et al., 2019; Leite et al., 2018; Dandan et al., 2019).

In this context, new plant-derived natural insecticides, known as “botanical insecticides”, can help to avoid these problems because instead of being lethal to several non-target arthropods, they act specifically as impediments to oviposition and feeding of insects (Cameron et al., 2014). Deterrent activity against *Chlosyne lacinia* (Lepidoptera: Nymphalidae) larvae was observed using leaves of *T. diversifolia* (Ambrosio et al., 2008). Thus, the use of botanical insecticides is a tactic that can be highly useful for the management of *H. armigera* populations in the field (Mabou Tagne et al., 2018). However, most botanical insecticides are produced using primarily macerated leaves (Arivoli and Tennyson, 2012; Amoabeng et al., 2019; Cerda et al., 2019), and the issue with this process is that all the components of the leaves are present in the extract and not just the plant defence chemicals. Therefore, in this study, we evaluated the feeding deterrence of a *T. diversifolia* leaf rinse extract obtained from leaf trichomes (TagCE, which concentrates only the plant defence substances) in *H. armigera* larvae.

2. Material and methods

2.1. General experimental procedures and reagents

The Liquid Chromatography system consisted of a three-pump Shimadzu chromatograph model LC-20AR Prominence equipped with a SIL-10AF auto sampler, a CTO-20A column oven, a CBM-20A communications bus module, a DGU-20A3R in-line degasser, and an SPD-M20A photodiode array detector. The Lab solution® software was used to process the data; the analyses were conducted in a Shim-pack VP-ODS column (250 × 4.6 mm i.d., 5 µm; Shimadzu). Acetonitrile (chromatographic grade) was supplied by Mallinckrodt Baker Inc. (Phillipsburg, NJ, USA), and water was purified with a Milli-Q-plus filter system (Millipore, Bedford, MA, USA). Classic and vacuum liquid chromatography (respectively CC and VLC; glass columns of 450 × 25 mm and 50–100 mm i. d.) were used to purify tagitinin C (Fig. 1A) by using silica

gel 60 (Merck, art. 9385) and silica gel 60H (Merck, art. 7736). Commercial hexanes and ethyl acetate (EtOAc) were purified by distillation in our facilities. The NMR spectra were run on a Bruker DPX 400 spectrometer (400 MHz for ¹H and 100 MHz for ¹³C). The samples were dissolved in CDCl₃, and the spectra were calibrated with the solvent signals at 7.26 (¹H) and 77.0 (¹³C). We used HPLC grade *p*-coumaric acid (Fig. 1B, CA), a natural phenylpropanoid that is not found in *T. diversifolia* leaves, as Internal Standard, acquired from Sigma-Aldrich (≥98% purity, Darmstadt, Germany).

2.2. Plant material

Tithonia diversifolia leaves were collected in Patrocínio (Minas Gerais state, Brazil; S18° 56' 38" W46° 59' 34") by Gabriel da Costa Inacio. Plant identification was carried out by Rosângela de Oliveira Araújo from the University Center of the Cerrado-Patrocínio (UNICERP), Patrocínio, Minas Gerais, Brazil, and the voucher specimen was deposited in the Herbarium Uberlandense (HUFU) under the number HUFU 73.901.

2.3. Obtaining of tagitinin C-rich leaf rinse extract (TagCE) and isolation of tagitinin C

Initially, 8.0 kg of *T. diversifolia* leaves were rinsed with dichloromethane (6 L) for 3 min to obtain 23,290 g of TagCE after filtering and solvent evaporation (Ambrosio et al., 2008). About 9.0 g of the extract were then chromatographed over 600.0 g of silica gel 60H (Merck, art. 7736), using vacuum liquid chromatography (VLC) (Pelletier et al., 1986) with increasing amounts of ethyl acetate in *n*-hexane as eluent (2 L for each fraction). This procedure delivered seven fractions that were analysed through TLC, using silica gel PF254 (Merck art. 9385; 1 mm thickness) and isocratic *n*-hexane: ethyl acetate 1:1 as mobile phase after solvent evaporation. The second and third fractions (0.511 and 2.309 g, respectively) were combined and then submitted to another fractionation using VLC (150.0 g of silica gel 60H; increasing amounts of *n*-hexane: ethyl acetate – 1 L for each fraction). After TLC analysis using the conditions previously described, the tenth fraction showed a single spot and was identified as tagitinin C after ¹H and ¹³C NMR analyses and comparison with literature data (Baruah et al. 1979). The NMR data also allowed us to conclude that the purity of the isolated compound was above 99%.

2.4. Development and validation of the analytical method

Chromatographic analyses of TagCE were performed through RP-HPLC-PDA using a Shim-pack VP-ODS column (250 mm × 4.6 mm i.d., 5 µm; Shimadzu) and an isocratic system consisting of acetonitrile and water with 0.1% acetic acid (45:55 v/v) as mobile phase, which was established through initial experiments using the gradient scouting run evaluation as previously described (Snyder and Dolan, 1996). The temperature of the column was set at 40 °C, the flow rate was 1.0 mL min⁻¹, the injected volume was 20.0 µL and the detection wavelength was set at 254 nm. The developed and validated analytical method complies with the requirements established by the National Agency for Sanitary Vigilance in Brazil (ANVISA, 2003).

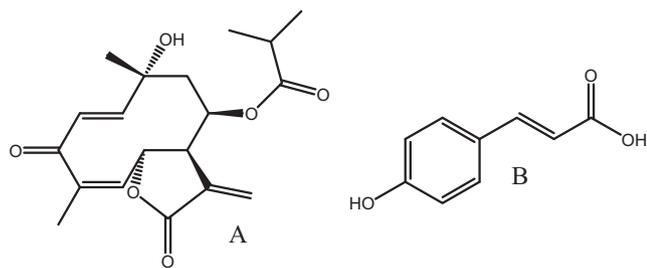


Fig. 1 Molecular structures of tagitinin C (A) and *p*-coumaric acid (B).

2.5. Preparation of sample solutions, construction of linear analytical curves, determination of limits of detection (LOD) and quantification (LOQ)

The analytical solutions were prepared by dissolving 1.0 mg of the sample plus 0.5 mg of *p*-cumarinic acid (CA), used as internal standard in 5 mL of acetonitrile, followed by filtration through a UNIFLO 25/0.2 PTFE syringe filter (Whatman/Schleicher & Schuell, Maidstone, UK).

A dilution series of metabolite tagitinin (400.0, 300.0, 200.0, 100.0, 80.0, 40.0, and 20.0 $\mu\text{g}\cdot\text{mL}^{-1}$) with internal standard (CA; 100.0 $\mu\text{g}\cdot\text{mL}^{-1}$) was prepared in acetonitrile. Then, 20.0 μL aliquots of these solutions were injected into the HPLC equipment in triplicate. Linear analytical curves were obtained by plotting the area ratio of the individual chromatographic standards to the internal standard CA. The correlation coefficient was calculated using Microsoft Excel®.

The LOD and LOQ values were determined on the basis of the standard deviation of the response (σ) and of the slope of the analytical curve (S), using the expressions $\text{LOD} = 3.3\sigma/S$ and $\text{LOQ} = 10\sigma/S$.

2.6. Selectivity

The selectivity of the method was evaluated by comparing DAD spectral data in the ascending, upper and descending regions of the respective peaks of tagitinin C in the linear analytical curve and in the sample (Souza et al., 2012). All the spectra matched, confirming that no other metabolites co-eluted with the target compound.

2.7. Precision and accuracy

Intra-day precision was evaluated by analysing six samples, and inter-day precision was assayed on two consecutive days. The concentration and the relative standard deviation (RSD) of the analyte were determined in TagCE. For the inter-day precision, the concentration values of tagitinin C were compared by applying the *t* test ($p < 0.05$), using GraphPad Prism®.

The accuracy of the method was determined by spiking known contents of tagitinin C into TagCE at low, medium and high levels (respectively 25, 50 and 100% of the compound content determined in the precision experiment) in triplicate. The spiked samples were analysed, and the recoveries were calculated by comparing the measured to the spiked concentrations (Moreira et al., 2013, 2016).

2.8. Robustness

The robustness of the chromatographic method was assessed by following the experimental design proposed by Plackett-Burman for seven factors and eight experiments, as previously described (Vander Heyden et al., 2001; Moreira et al., 2016). Briefly, the selected operational factors related to the chromatographic method were as follows: mobile phase flow rate (Flow), column oven temperature (Temp.), percentage of organic solvent in the mobile phase (%B), detection wavelength (λ) and volume of injected samples (Inj.). Two dummy factors had to be included to reach a saturated design and vari-

ation levels for each of these operational factors (Table 1). Robustness values were expressed as RSD (%) of the responses.

2.9. Feeding deterrence on artificial diet

The artificial diet used in the bioassay was prepared according to Greene et al. (1976). The extract was sprayed when the diet temperature decreased and reached 25 °C, to avoid the potential degradation of compounds present in the extract. Dichloromethane extract was tested at concentrations of 100, 500 and 1000 mg mL^{-1} . In the control treatment, only dimethyl sulfoxide (DMSO) solvent was used. The extract was dissolved in DMSO at a ratio of 1% (w/w) extract on the dry weight of the artificial diet. A 0.1-mL aliquot of the diluted extract was applied to the diet with a micropipette, and the diet remained at room temperature for 30 min for drying. The procedure was repeated for each tested extract concentration, for the control (DMSO) and for the positive control (flubendiamide insecticide used at the manufacturer's recommended dose of 0.35 $\mu\text{L mL}^{-1}$). After diet drying, 10-day-old third instar larvae were added; each repetition consisted of a Petri dish (90 mm diameter) containing an artificial diet and one larva. Evaluations were performed every 24 h until pupae formation and subsequent emergence of adults. The pupae were transferred to 1000-mL plastic cups where they remained until the emergence of the adults. The experiment was conducted in a BOD chamber adjusted to a temperature of 25 ± 2 °C, a relative humidity of $70 \pm 10\%$ and a photoperiod of 14 h light and 10 h dark. The experimental design was completely randomised, with 10 replications for each treatment. The mortality of the larval and pupal insects was evaluated.

2.10. Feeding deterrence on plants

The effect of feeding deterrence, besides being tested on the artificial diet, was also confirmed in host plants. The plant species used in the experiments (cotton, beans, corn, soybeans, sorghum and tomato) were selected due to the frequent occurrence of *H. armigera* in these crops in Brazil (Czepak et al., 2013; Specht et al., 2013; Pratisoli et al., 2015). The experiment was conducted in a BOD chamber adjusted to a temperature of 25 ± 2 °C, a relative humidity of $70 \pm 10\%$ and a photoperiod of 14 h light and 10 h dark. The arena was made up of a 10-cm diameter acrylic Petri dish. Each experimental unit consisted of an arena containing six discs of 1-cm diameter leaf, interspersed with discs treated and untreated with the extract. In each arena, there were discs of only one host plant. Prior to the experiment, the 10-day-old third instar larvae were kept 24 h without food. One larva was inserted into the centre of each arena, and after 3 h, the leaf discs were removed, and the leaf area was measured to record the consumption in a Lee Tools device, with an accuracy of 0.01 cm^2 . After measuring the leaf area, the discs were oven-dried at 60 °C for 48 h and weighed on a precision scale.

The procedure was repeated for each tested extract concentration (100, 500 and 1000 mg mL^{-1}) and each host plant (cotton, beans, corn, soybeans, sorghum and tomato). The control used was DMSO, and for the positive control, we used fluben-

Table 1 Selected operational factors and variation levels related to the chromatographic method.

Factors	Unit	Limit (\pm)	Level (-1)	Level (+1)	Nominal
Flow	mL·min ⁻¹	0.1	0.9	1.1	1.0
Temp.	° C	5	35.0	45.0	40.0
%B	%	1	40	50	45.0
λ	nm	5	259	249	254
Inj.	μ L	1	19.0	21.0	20.0

diamide insecticide at the manufacturer's recommended dose of 0.35 μ L mL⁻¹. The experimental design was completely randomised, with 10 replications for each treatment.

The feeding deterrence index was calculated using the following equation (Simmonds et al., 1989):

$$I = (Ac - At)/(Ac + At);$$

Ac = control disc consumed area (mm²)

At = treated disc consumed area (mm²).

Feeding deterrence values above 0.50 and 0.90 are considered significant and highly significant, respectively (Simmonds et al., 1989).

2.11. Data analysis

Data on the feeding deterrence on artificial diets and on plants for *H. armigera* were submitted to the Kolmogorov and Bartlett tests to verify the normality of their residuals and the

homogeneity of their variances, respectively. The data met the assumptions and were then submitted to analysis of variance (ANOVA). Treatment means were compared using Tukey's test ($P < 0.05$). All analyses were conducted using the SAS software (SAS Institute, 2015).

3. Results

3.1. Chemical analysis

After obtaining TagCE through the leaf rinse process, its tagitin C content was determined using the developed and validated reverse-phase HPLC analytical method, whose validation parameters are presented herein. In this sense, the linear calibration curve obtained by plotting the area ratio of tagitin C to that of the internal standard (CA) against the standard concentrations, and a correlation coefficient of 0.9998 was achieved. The linear regression equation ($y = 0.0070x + 0.0029$) and the values of LOD (2.8670 μ g·mL⁻¹)

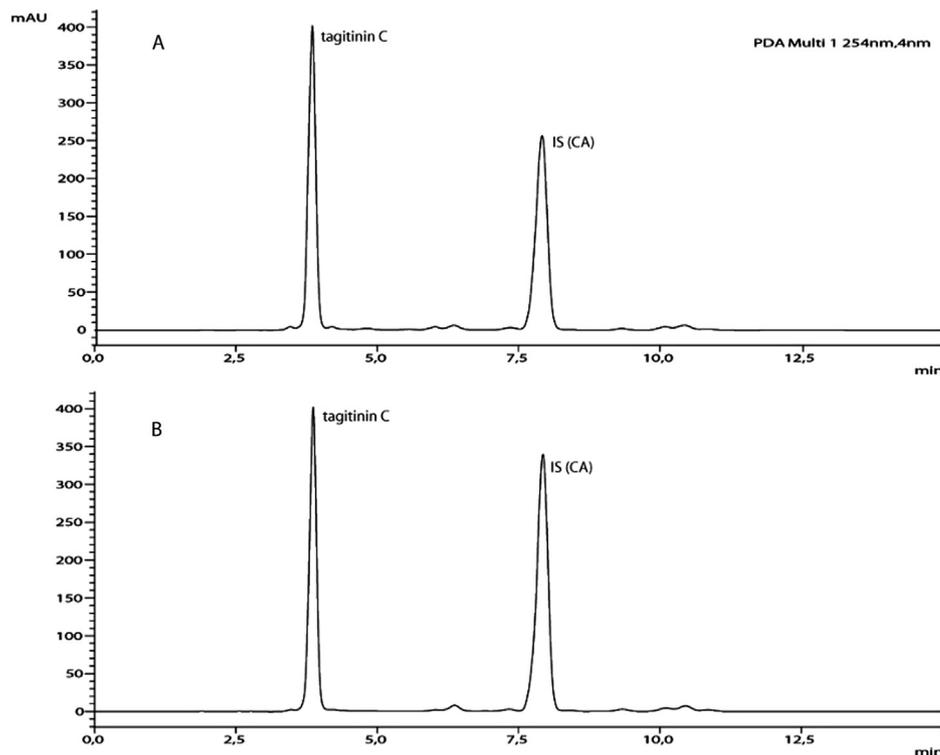


Fig. 2 HPLC-DAD chromatograms showing (A) TagCE and *p*-coumaric acid (CA; internal standard) and (B) the analytical standard (tagitin C) and *p*-coumaric acid (CA; internal standard) peaks, obtained in the established analytical conditions (isocratic mobile phase system of 45% acetonitrile in Milli Q water containing 0.1% acetic acid, mobile phase flow rate of 1 mL·min⁻¹, column temperature of 40 °C, injected sample volume of 20 μ L and detection at 254 nm).

Table 2 Larval and pupal mortality of *Helicoverpa armigera* in feeding deterrence bioassay using artificial diets with various concentrations of *Tithonia diversifolia* extract.

Concentration (mg mL ⁻¹)	Mortality (%)	
	Larval	Pupal
Control	0.0 ± 0.00 b ¹	0.0 ± 0.00 b
100	30.0 ± 0.42 a	50.0 ± 0.50 a
500	50.0 ± 0.50 a	70.0 ± 0.42 a
1000	50.0 ± 0.50 a	80.0 ± 0.32 a

¹ Means ± standard error followed by the same letter in the column do not differ by Tukey's test (P > 0.05).

and LOQ (8.6879 µg·mL⁻¹) indicate that the developed method is highly sensitive and adequate to determine the tagitinin C content in TagCE. Fig. 2 displays a comparison between typical HPLC-DAD chromatograms obtained for the sample (TagCE plus CA) and for the analytical standard (tagitinin C plus CA).

Intra-day precision was determined by analysis of tagitinin C in six samples of TagCE, and inter-day precision was assayed on two consecutive days. The obtained concentrations were respectively 98.724 µg·mL⁻¹ and 101.763 µg·mL⁻¹, which corresponded to about 11.36% of tagitinin C content in TagCE, thus confirming the previously reported high contents of this compound in *T. diversifolia* and leading us to propose this compound as the chemical marker of TagCE (Ambrosio et al., 2008; Baruah et al. 1979; Orsomando et al., 2016; Sut et al., 2018). The RSD values for tagitinin C concentrations

were below 5%, and the means obtained for different days did not differ statistically (p < 0.05). To determine the accuracy of the method and after the addition of known contents of tagitinin C (25, 50 and 100%), the RSD values for all concentrations obtained, in triplicate, were also below 5%, indicating that the results were adequate for the analytical purpose of the developed method. Moreover, the robustness of the method was assessed as previously described by Moreira et al. (2013) and satisfactorily met the requirements of the main Brazilian regulatory agency (Anvisa, 2003).

3.2. Feeding deterrence

In the feeding deterrence bioassay, spraying the extract on the artificial diet caused larval mortality of 50.0% and pupal mortality of 70.0 and 80.0% at concentrations of 500 and 1000 mg mL⁻¹, respectively (Table 2). Positive control using flubendiamide insecticide showed 100.0% mortality of individuals tested within 24 h.

The *T. diversifolia* extract showed feeding deterrence activity for *H. armigera* larvae in the six host plants studied. The concentrations of 500 and 1000 mg/mL presented higher levels of feeding deterrence, ranging from 0.5 to 1.0, and higher leaf dry mass due to the lower leaf consumption at these concentrations (Table 3).

4. Discussion

This is the first study that evaluated the feeding deterrence towards *H. armigera* by *T. diversifolia* extract. Dichloro-

Table 3 Feeding deterrence index and leaf dry mass of plant species treated with different *Tithonia diversifolia* extract concentrations against *Helicoverpa armigera*.

Plant species	Common name	Concentration (mg mL ⁻¹)	Feeding deterrence index ²	Leaf dry mass (mg)
<i>Glycine max</i>	Soybean	Control DMSO	0.0	0.040 b ¹
		100	0.0	0.089 b
		500	1.0	0.115 a
		1000	1.0	0.119 a
<i>Gossypium herbaceum</i>	Cotton	Control DMSO	0.0	0.187 b
		100	0.0	0.193 b
		500	0.5	0.210 a
		1000	1.0	0.215 a
<i>Phaseolus vulgaris</i>	Bean	Control DMSO	0.0	0.091 b
		100	1.0	0.147 a
		500	1.0	0.155 a
		1000	1.0	0.152 a
<i>Solanum lycopersicum</i>	Tomato	Control DMSO	0.0	0.093 b
		100	1.0	0.143 a
		500	1.0	0.173 a
		1000	1.0	0.190 a
<i>Sorghum bicolor</i>	Sorghum	Control DMSO	0.0	0.141 b
		100	0.5	0.164 a
		500	0.5	0.170 a
		1000	1.0	0.193 a
<i>Zea mays</i>	Corn	Control DMSO	0.0	0.174 b
		100	0.0	0.154 b
		500	0.6	0.222 a
		1000	1.0	0.210 a

¹ Means followed by the same letters in the column do not differ by Tukey's test (P > 0.05).

² Feeding deterrence index = (Ac - At)/(Ac + At) ranging from -1 to 1. Ac = mean control disk consumed area (mm²), At = mean treated disk consumed area (mm²).

methane rinse extracts of the leaves and inflorescences were chemically investigated, and 16 compounds were isolated and identified: 14 sesquiterpene lactones, one flavonoid and one diterpenoid (Ambrosio et al., 2008). We showed that tagitinin C is a main metabolite of the *T. diversifolia* extract obtained from foliar glandular trichomes. To determine if this compound is responsible for the observed antifeedant activity, HPLC qualitative analysis of the leaf trichomes extract and quantitative analysis of tagitinin C, the main metabolite, were performed.

The extract used in this study was obtained by leaf washing. We dipped the fresh, intact leaf into the organic solvent so that only the glandular trichomes were ruptured. Since these structures concentrate the defence substances (in this case of *T. diversifolia*, the sesquiterpene lactones), we actually lent the sesquiterpene lactones to the studied crop plants (cotton, beans, corn, soybeans, sorghum and tomato), which have no inherent defence at all.

The reported RP-HPLC-PDA analytical method allowed the identification and quantification of the major constituent of *T. diversifolia* in the rinse leaf extracts, showing that this compound is suitable to be considered as the chemical marker of TagCE. Also, the developed analytical method described herein is simple, reliable and should be used routinely in analyses of *T. diversifolia* rinse leaf extracts as well as in other studies, since it met the requirements of the Brazilian Regulatory Agency.

The results show high mortality, with up to 80% of larvae, and antifeedant activity for all plant species tested using *T. diversifolia* extract for *H. armigera* larvae. This suggests that *H. armigera* larvae avoid feeding when *T. diversifolia* extract is present in the host plants. Previous studies have shown that *T. diversifolia* dichloromethane leaf rinse extract presented an antifeedant activity for other pest insects such as *Chlosyne lacinia* (Lepidoptera: Noctuidae) (Ambrosio et al., 2008). In addition, *T. diversifolia* leaf extract also showed high bioactivity on mortality of *Callosobruchus maculatus* (Coleoptera: Bruchidae) (Adedire and Akinneye, 2004).

Our results not only help in understanding that tagitinin C is a main compound present in the glandular trichomes from *T. diversifolia* leaves, but also clarify the mode of action of *T. diversifolia* extract. A previous study characterising the mode of action of sesquiterpene lactones on *Trypanosoma cruzi* showed that psilostachyin accomplished its antiparasitic effect by interacting with hemin, while psilostachyin C interfered with sterol synthesis, and both sesquiterpene lactones induced parasite death by apoptosis (Sülsen et al., 2016).

This opens up the possibility to use *T. diversifolia* extract as a potential botanical insecticide, mainly for plant-defoliating Lepidoptera. In Brazil, there are only five botanical pesticides available to use in crop protection. Most insecticides of botanical origin are extracts containing a group of active ingredients of diverse chemical synthetics and could be used as a good strategy in insect resistance management. Plant origin insecticides also have a great advantage by being compatible with other low-risk options which are acceptable for insect management, such as entomopathogenic fungi, predators and parasitoids, which greatly increase the probabilities of being integrated in IPM programs. Further studies should be performed to understand the relationships between the isolated compounds from *T. diversifolia* extract and the antifeedant activity for Lepidoptera pests.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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