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Larvicidal effect of an N-isobutyl-(2E,4E,8Z,10E/Z)-dodecatetraenamides-rich extract of *Salmea scandens* on fall armyworm

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Abstract

Aim of the study: To determine if secondary metabolites present in an ethanolic extract of *Salmea scandens* could be considered as a viable alternative for the control of fall armyworm (*Spodoptera frugiperda* larvae), as this is the most important maize pest in terms of economic losses to agriculture worldwide.

Area of study: S. scandens shrubs were collected in San Rafael Toltepec, Oaxaca, Mexico. The laboratory assays were conducted at CIIDIR Oaxaca, and preliminary field assay was carried out in Zaachila Oaxaca.

Material and methods: N-isobutyl-(2E,4E,8Z,10E/Z)-dodecatetraenamides-rich extract of *S. scandens*, corroborated by nuclear magnetic resonance (^{1}H NMR and ^{13}C NMR) and Fourier transform infrared (FTIR), was obtained from *S. scandens* by 10-day maceration in ethanol. The effect of the extract on the mortality of *S. frugiperda* larvae was investigated in the laboratory (in vitro) by topical application, and in the field (in situ), testing both topical and spraying applications.

*Main results:*The ^{1}H NMR, ^{13}C NMR and FTIR spectra evidenced the obtention of the alkylamides-rich ethanol extract of *S. scandens*. Mortality of *S. frugiperda* in vitro increased with dose and monitoring time, reaching up to 80%. Under field conditions 63% of mortality was recorded at a dose of 0.30 mg μL^{-1} per larva by topical application, and 49% by spray application at a dose of 0.15 mg μL^{-1} per larva.

Research highlights: The ethanolic extract of *S. scandens* can be considered a viable alternative for controlling fall armyworm *S. frugiperda*.

Additional key words: bio-controllers; maceration; N-isobutyl-(2E,4E,8Z,10E/Z)-dodecatetraenamides; pest control; plant extract.

Abbreviations used: FAW (fall armyworm larvae); FTIR-ATR (Attenuated Total Reflectance-Fourier-Transform Infrared Spectroscopy); ^{1}H NMR (Proton Nuclear Magnetic Resonance); $CDCl_3$ (deuterated chloroform); TLC (Thin Layer Chromatography); Rf (retention factor); EtOAc (ethyl acetate).

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Introduction

Fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) is a polyphagous pest native to tropical and subtropical regions of the America and is the most important maize pest because of economic losses to agriculture worldwide (CABI, 2020). This lepidopteran pest feeds primarily on maize leaves, seriously reducing the production up to 100% when the plants are infested (Fotso-Kuate et al., 2019) . The most damaging effect occurs during the larval stages of the insect when it consumes a large amount of foliage.

To control this pest, various synthetic insecticides have been used, for example, chlorinated hydrocarbons, organophosphates, carbamates and pyrethroids. However, there are several concerns associated with the extensive use of these compounds such as developed resistance, damage to the environment, negative health impacts and killing of non-target organisms (Jayaraj et al., 2016).

The search for environmentally-friendly alternatives to improve insect pest control has focused on the use of plant extracts; however, their effectiveness and toxicity are usually low compared with the toxicity of synthetic insecticides. An alternative method to controlling insect pests is the use of secondary metabolites obtained from certain plants; these metabolites cause no apparent collateral effects like those caused by synthetic insecticides (De Souza Tavares et al., 2009; Kortbeek et al., 2019). For example, secondary-metabolite-based insecticides are less toxic to non-target species and more degradable than chemical insecticides (Walia et al., 2017).

Secondary metabolites such as pyrethrum, terpenoids and alkylamides are present in plant families such as Asteraceae, Rutaceae, Piperaceae, and Solanaceae (Greger, 1984; Boonen et al., 2012). Alkylamides are metabolites widely distributed in plants with a broad range of biological activities (Wynendaele et al., 2018). The isobutyl amides are the mayor active secondary metabolites from *Salmea scandens,* and these are strongly associated of larvicidal activity on *Anopheles albimanis* and *Aedes aegypti* (Villa-Ruano et al., 2015). Furthermore, different investigations have shown the insecticidal potency of alkylamides as an alternative control against a variety of pests such as *Helicoverpa zea*, *Plutella xylostella* and *Tuta absoluta* (Ramsewak et al., 1999; Clifford et al., 2002; Sharma et al., 2012).

In summary, a review of the literature shows evidence that most of the research on the use of alkylamides to control *S. frugiperda* have been carried out mostly in the laboratory. Furthermore, although the stem bark of *S. scandens* is rich in N-isobutyl-(2E,4E,8Z,10E/Z)-dodecatetraenamides, no studies on the efficacy and effectiveness of *S. scandens* extracts on *S. frugiperda* mortality have been reported. Thus, this study aims to evaluate the larvicidal effects of an alkylamides-rich extract of *S. scandens* stem bark on the mortality of *S. frugiperda* in laboratory and a preliminary field condition assay to test the hypothesis that this plant extract causes a significant reduction in the larvae population.

Material and methods

Plant material

Salmea scandens shrubs were collected in San Rafael Toltepec Oaxaca, Mexico (15°88' N and 96°45' W, 341 masl) during July 2019. The bark was manually removed from the stem and dried under shade until a moisture content of 12% was reached, three days were necessary (AOAC, 2000). Afterwards, a total of 300 g of dry stem bark was ground in a mill (FRITSCH® model Pulviressette 19) and sieved through a 2-mm mesh. The samples were stored in vacuumed-sealed polyethylene bags at room temperature and kept in complete darkness until used.

Preparation of plant extracts

Maceration in ethanol was used as the extraction method to obtain 3- and 10-day extracts. Because of the limited information regarding the maceration extraction time of alkylamides from *S. scandens* stem bark, a 3-day maceration time was implemented as a starting point. This time has been reported as the optimal for alkylamides extraction time for *Echinacea purpurea* using an ethanolic extract (Spelman et al., 2009). Additionally, this time was used because *E. purpurea* and *S. scandens* belong to the same family of Asteraceae and contain essentially the same type of alkylamides (Bauer & Remiger, 1989; Villa-Ruano et al., 2015). Regarding the 10-day maceration time, it was selected based on a previous study where the extraction process of alkylamides from *S. scandens* reached equilibrium (maximum concentration of alkylamides) at this time (Pacheco-Esteva, 2018). Powdered samples were placed in a flask with a 1:5 weight ratio of dried stem bark (g) and anhydrous ethanol Fermont® and left at room temperature (13°C during the night and 28°C as the highest temperature in the shadow during the day) for 3 and 10 days. The extracts were filtered and concentrated using a rotary vacuum evaporator (Buchi ®, R-100) under reduced pressure at 40°C. A total of 3.78 and 4.23 g of concentrate were obtained for 3 days and 10 days, respectively. Finally, the concentrate was stored at 4°C in a desiccator until the day of the test. The extraction process was made in triplicate and the percentage of extraction was determined according to Eq. (1) proposed by Prasad et al. (2009):

$$\%\ yield\ of\ extraction = \frac{g\ concentrate}{g\ dried\ plant\ material} \times 100 \qquad (1)$$

The relative concentrations of alkylamides of the 3- and 10-day extracts were determined by thin layer

chromatography (TLC). First, the extracts were eluted in hexane/AcOEt in a 2:1 proportion; then, the areas of the spots corresponding to N-isobutyl-(2E,4E,8Z,10E/Z)-dodecatetraenamides (retention factor R_f = 0.5) for each extract were measured (corroborated as described below). Finally, the relative concentration of alkylamides was estimated by the ratio between the two areas. The areas were determined five times.

Since isobutyl amides are strongly associated to larvicidal activity (Villa-Ruano et al., 2015), the presence of alkylamides in *S. scandens* were verified prior to the bioactivity tests. Considering a R_f of 0.5 in hexane/AcOEt (2:1) reported for N-isobutyl-(2E,4E,8Z,10E/Z)-dodecatetraenamides (Bauer & Remiger, 1989), a semi-purified fraction was obtained from an ethanolic extract from the stem bark of *S. scandens* by using a preparative layer plate, silica gel coated with flourescent indicator F254, size 20 x 20 cm, 1.5 mm layer thickness (Merck®). The semi-purified fraction was dissolved in deuterated chloroform ($CDCl_3$) to be characterized by Proton and Carbon Nuclear Magnetic Resonance (^{1}H NMR, ^{13}C NMR) in one and two dimensions using a JEOL ECA 500 MHz spectrometer. The semi-purified fraction was also characterized by Attenuated Total Reflectance-Fourier-Transform Infrared spectroscopy using a Nicolet 6700® FTIR-ATR spectrometer from 4000 to 650 cm^{-1}, with 32 scans accumulated for the spectrum.

Insects

The FAW larvae used for the tests were collected from local landrace maize plants 'Criollo bolita', from San Antonio de la Cal and Zaachila (17°03'10" N and 96°69'80" W, 1500 masl), in Oaxaca, Mexico. According to the farmer, no insecticide was applied to the local landrace maize plants before the larvae were collected. We identified the larvae based on specific morphological characteristics such as the inverted Y on the head and the presence of four raised dots on body segments (FAO, 2018; CABI, 2020). For the in vitro testing, 500 larvae were carefully collected from the field and kept individually in plastic vessels with maize leaves and perforated lids. The larvae were taken to the laboratory where the following selection process was implemented. First, the larvae were acclimated in the laboratory for three days at room temperature (25 ± 2°C) and fed with fresh maize leaves (FAO, 2018). The lengths of 30 larvae were measured, which ranged between 21.10 and 34.40 mm; and the average weight of that larvae was of 0.252 ± 0.042 g. Therefore, the larvae used for this study corresponded to the 5th and 6th instars, which cause large crop damage and consume a considerable amount of corn foliage (Guzmán Prada et al., 2016). Finally, to select uniform larvae, it was decided to use only those close to the average weight; in consequence, the smallest and the largest were discarded. For the in situ testing, the presence of the larvae in the plant was corroborated and the larvae were visually selected for the tests in accordance with their morphological characteristics; as in the in vitro test, plants with small larvae were also discarded from the experiment.

Preparation of the stock solution

The stock solution was prepared by dissolving 3.00 g of the ethanolic extract concentrate from *S. scandens* and 0.08 mL of Tween 20 as an emulsifier (corresponding to 0.8% w/w) in 10 mL of distilled water. The stock solution was used to prepare the different doses by diluting it with distilled water. A solution of distilled water with 0.8% Tween 20 was used as a control for all the tests. The details of the doses used for each test are described in the corresponding section.

Larvicidal activity of extracts

The efficacy of the 3-day maceration extract was evaluated by topical application in an in vitro screening test. The efficacy of the 10-day maceration extract was evaluated also by topical application; however, an additional lower dose value was included based on the results from the previous screening test with 3-day maceration extract. Finally, the effectiveness of the 10-day maceration extract was evaluated in the in situ tests by both topical and spraying methods.

Laboratory experiments

To evaluate the efficacy of the *S. scandens* extracts, tests were carried out following the method of topical application proposed by The American Entomological Society (Brazzel, 1970) with slight modifications. The modifications consisted in applying 50 µL of extract at specific concentration to the larvae instead of 20 µL because 5th and 6th instars larvae were evaluated in the present study instead of second instar larvae. The selected larvae were taken with three fingers covered by latex gloves, and the emulsified extract was applied on the prothorax with a micropipette (Fig. S1 [suppl]). After the application, the larvae were kept in an individual plastic vessel with a perforated lid at room temperature and with fresh maize leaves as food. The monitoring of mortality (Eq. 2) was carried out after 12, 24 and 48 h post application and larvae that did not react to a poking stimulus were considered dead:

$$\%\, mortality = \frac{X - Y}{X} \times 100 \qquad (2)$$

where X=larvae living in the control after treatment, Y=larvae living after the extract application.

For the screening test, the 3-day extract was used at doses per larva of 0.10, 0.20 and 0.30 mg μL^{-1} (see Appendix [suppl] for calculations). Eight larvae per dose and four replicates were considered for this test besides a control group.

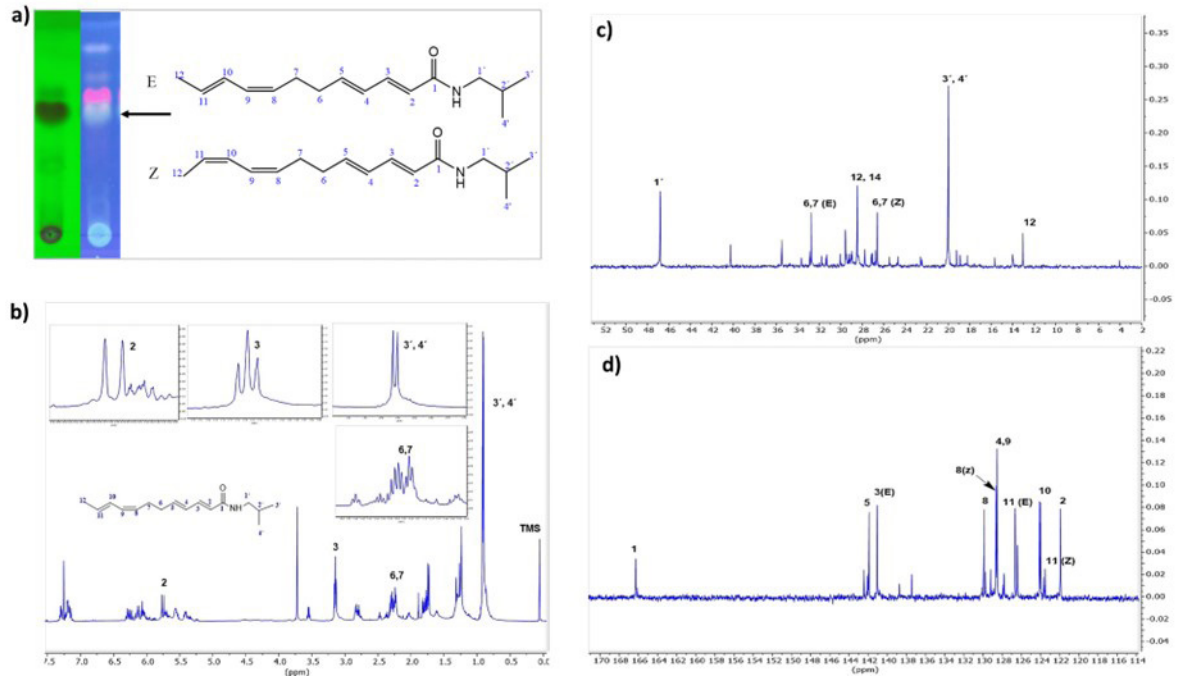

Figure 1. Characterization of *Salmea scandens* extract: a) thin layer chromatography of *Salmea scandens* extract eluted in hexane/EtOAc (2:1) view with light at 254 nm (green) and 365 nm (blue) (a); ^{1}H NMR spectrum (500 MHz) (b); ^{13}C NMR spectrum of semi-purified N-isobutyl-(2E,4E,8Z,10E/Z)-dodecatetraenamides in $CDCl_3$ (c and d).

For the 10-day extract, doses per larva were complemented to 0.05, 0.10, 0.15, 0.20 and 0.30 mg µL^{-1} to evaluate the effect at lower doses. For this test, 10 larvae per dose, four replicates and a control were considered.

Field experiments

To validate the effectiveness of the alkylamides-rich extract of *S. scandens* obtained by 10-day maceration on the larvae in situ, two experiments were performed, one by topical application and the another by spraying. The experiments were carried out in a maize plantation in Zaachila, Oaxaca, Mexico (16°55´51.0" N; 96°43´21.8" W; 1550 masl), in late August 2019 when the temperature and solar radiation were high (mean temperature of 30°C, no clouds present). Since the testing was carried out in an uncontrolled environment, the mortality (Eq. 3) as the reduction of the larvae population after the extract treatment was determined according to Henderson & Tilton (1955):

$$\%\, mortality \vee reduction = \left[1 - \frac{TA \times CB}{TB \times CA}\right] \times 100 \qquad (3)$$

where CA=larvae population of control after treatment, TA=larvae population after the extract application, CB=larvae population of control before treatment, and TB=larvae population before the extract application.

a) Field test by topical application

A completely randomized experimental design was used to evaluate the reduction of the FAW larvae population caused by *S. scandens* extract at doses per larva of 0.10, 0.20 and 0.30 mg µL^{-1}. Dried ethanolic extract (50 µL) emulsified in distilled water at the corresponding dilution was applied directly over the larvae localized in the plant. The experimental unit consisted of 10 larvae (one larva present in each plant) and 4 replicates made per dose. A control group receiving distilled water with 0.8% Tween 20 was included. The reduction of the larvae population produced by the *S. scandens* extract was evaluated 24 h post-application using Eq. (3).

b) Field test by spraying

In the maize plantation, an area of 20 × 20 m was delimited to apply two doses of emulsified *S. scandens* extract by spraying 0.50 ± 0.06 mL into each maize whorl using a hand sprayer; the equivalent doses per larva were 0.05 and 0.10 mg µL^{-1}. The application was carried out using a completely randomized experimental design with 40 plants per experimental unit and where the presence of one larva of FAW had been previously corroborated. In addition, distilled water with 0.8% Tween 20 was used as a control over 40 plants with larvae. The reduction of the larvae population produced by the *S. scandens* extract was evaluated 24 h post-application using Eq. (3).

Table 1. Chemical shifts of alkylamides in the semi-purified fraction from *Salmea scandens* stem bark extract.

^{1}H NMR (ppm)			^{13}C NMR (ppm)		
H	**E (Trans)**	**Z (Cis)**	**C**	**E (Trans)**	**Z (Cis)**
2	5.76 d (15)	5.76 d (15)	1	166.5	166.5
3	7.18 dd (15, 10)	7.20 dd (15, 10)	2	122.1	122.1
4	6.15 dd (15, 10)	6.15 dd (15, 10)	3	141.3	141.3
5	6.08 dt (15, 7)	6.08 dd (15, 7)	4	128.5	128.8
6	2.30 m	2.24 m	5	142.2	142.2
7	2.30 m	2.24 m	6	32.9	26.8
8	5.35 dt (10, 7)	5.41 dt (10, 7)	7	33.1	26.8
9	6.00 dd (11, 11)	6.30 dd (11, 11)	8	130.1	130.1
10	6.31 dd (15, 10)	6.31 dd (15, 10)	9	128.8	128.8
11	5.56 m	5.76 m	10	124.2	124.3
12	1.79 dd (7, 1)	1.74 dd (7, 2)	11	126.8	123.7
1’	3.15 t (7)	3.15 t (7)	12	13.3	28.7
2’	1.79 m	1.79 m	1’	47.0	47.0
3’	0.91 d (7)	0.91 d (7)	2’	28.7	28.7
4’	0.91 d (7)	0.91 d (7)	3’	20.2	20.2
5’			4’	20.2	20.2

J: Coupling constant (in Hz) is given in parenthesis. d: doublet. t: triplet. dd: doublet of doublets. dt: doublet of triplet. m: multiple.

Statistical analysis

Prior to the analysis of the data, the mortality data of *S. frugiperda* were corrected for control mortality by using Abbott’s correction formula (Abbott, 1925). The data of larvae survival probabilities were subjected to the survival analysis using the non-parametric Kaplan-Meier model as a function of time. The log-rank (Mantel-Cox) test was employed to evaluate the significance of the pair-wise comparisons of the mortality and survival curves. All tests were performed to a significance level of α =0.05. The IBM Statistical Package for Social Sciences (SPSS) version 25® was used for all the analyses.

Results

Chemical identification of N-isobutyl-(2E,4E,8Z,10E/Z)-dodecatetraenamides, percentage of extraction and relative concentrations of alkylamides

The ethanolic extract was firstly analyzed by TLC (Fig. 1a), and the most prominent spot corresponded to alkylamides, as the spectroscopic characterization showed. As described in the methodology, the compounds assigned to alkylamides were fractionated to characterized them. The signals observed in the ^{1}H NMR spectrum from the semi-purified fraction of N-isobutyl-(2E,4E,8Z,10E/Z)-dodecatetraenamides (Fig. 1b) agree with all expected protons, and these were assigned based on the data reported by Bauer et al. (1988) and corroborating the coupling constant. Additionally, the corresponding signals of ^{13}C were assigned by 2D HSQC and HMBC (see Figs. S2 and S3 [suppl], respectively) spectra and the signals observed in the ^{13}C NMR spectrum are shown in the Fig. 1c and 1d. The details of the ^{1}H and ^{13}C NMR signals are shown in Table 1.

The FTIR-ATR spectrum obtained from the semi-purified N-isobutyl-(2E,4E,8Z,10E/Z)-dodecatetraenamides from *S. scandens* (Fig. S4 [suppl]) showed the characteristic bands associated with alkylamides in accordance with Bauer et al. (1988). The characteristic bands identified were N-H stretching band at 3286 cm^{-1}, and C=C and C=O stretching bands at 1656 and 1544 cm^{-1}, respectively. The C-H stretching double bond was observed at 3020 cm^{-1} and the C-H stretching and bending double bond at 1628 cm^{-1} and 997 cm^{-1}, respectively.

The average percentages of the concentrates extracted at 3-day and 10-day macerations were 2.52 ± 0.13 and 2.82 ± 0.16, respectively. These results indicate that the yield significantly increased when the maceration time increased from 3 to 10 days (F=10.58, df=1; p=0.012). In addition, the relative concentrations of alkylamides increased from 2.49 to 3.52 when the maceration time increased from 3 to 10 days, respectively. A significant difference between

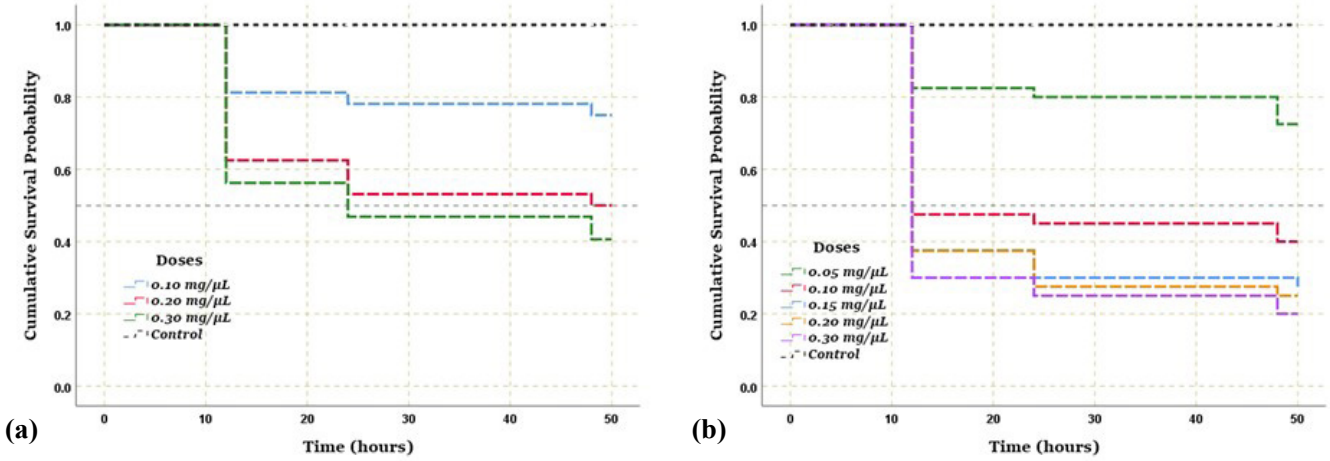

Figure 2. Survival probability of fall armyworm larvae exposed to: three doses of the ethanolic 3-day maceration extract from *Salmea scandens* (a), five doses of the ethanolic 10-day maceration extract from *Salmea scandens* (b).

relative concentrations of the two maceration times was also found (F=470.41; df=1, p<0.05).

In vitro toxicity

a) The 3-day maceration extract (screening test)

The mortalities caused by the 3-day maceration extract from the in vitro screening test were recorded after 12, 24 and 48 h. The results analyzed as cumulative survival probability of *S. frugiperda* are shown in Fig. 2a. They indicate that the survival probability of *S. frugiperda* larvae decreased with the increase in the dose of *S. scandens* extracts and the monitoring time. The decrease in survival probability with the increase in dose followed the same pattern for the different monitoring times, which allows to preliminarily conclude that no apparent dose and monitoring time interactive effect occurs. It is worth noting that no larvae mortality was recorded for the control. The survival probability indicated significant differences between doses ($p<0.05$) at 48 h post application. The highest larval survival was recorded by 0.10 mg μL^{-1} with 71.87 ± 2.09%, followed by 0.20 mg μL^{-1} with 50 ± 2.81%, and the lowest by 0.30 mg μL^{-1} with 40.62 ± 3.73%.

The Log Rank (Mantel-Cox) test results indicate that the survival distributions were significantly different (X^2 = 27.23; df = 3; p = 5×10^{-6}). Pair-wise comparisons detected that the survival of the control was significantly different than the applied doses ($p<0.05$); this means that 0.10 mg μL^{-1} per larva caused a significant mortality against *S. frugiperda* larvae (p = 1×10^{-6}).

The mortality caused by the dose 0.10 mg μL^{-1} per larva was significantly different than that caused by 0.20 mg μL^{-1} (X^2 = 4.24; df = 1; p = 0.039) and by 0.30 mg μL^{-1} (X^2 = 7.69; df = 1; p = 0.006). Finally, the mortality caused by the 0.20 mg μL^{-1} per larva was similar to the mortality by 0.30 mg μL^{-1} per larva (X^2 = 0.52; df = 1; p = 0.471). The highest mortality was 59.38% using the dose of 30 mg μL^{-1} of ethanolic extract from *S. scandens* at 48 h.

b) The 10-day maceration extract

The mortality caused by the 10-day maceration extract from the in vitro test was recorded after 12, 24 and 48 h and analyzed in terms of the cumulative survival probability of *S. frugiperda* (Fig. 2b). The survival analysis results indicated a significant decrease in *S. frugiperda* larvae with increasing doses of *S. scandens*, as well as with the monitoring time. It was also observed that a similar cumulative survival probability can be reached with a low dose at a longer monitoring time, i.e. the survival at dose of 0.20 mg μL^{-1} at 48 h was higher than that at 0.30 mg μL^{-1} at 24 h. The highest larval survival was recorded at 0.05 mg μL^{-1} with 72.50 ± 1.26%, followed by 0.10 mg μL^{-1} with 40.0 ± 0.82%, and the lowest larval survival was caused at 0.30 mg μL^{-1} with 20.0 ± 1.17%. Less than the 50% of larvae populations survived with a dose of 0.10 mg μL^{-1} until the end of the experiment.

The Mantel-Cox test results indicated significantly different survival distributions between doses (X^2 = 29.42; df = 4; p = 6×10^{-6}). Pair-wise comparison results indicated that the survival of the control was significantly different from the rest of the doses ($p < 0.05$); this means that the application of the *S. scandens* extract, even at a low dose, caused a significant mortality of the *S. frugiperda* larvae.

The mortality caused by the 0.05 mg μL^{-1} dose per larva was significantly lower than for the other studied doses: 0.10 mg μL^{-1} (X^2 = 9.12; df = 1; p = 0.003), 0.15 mg μL^{-1} (X^2 = 17.58; df = 1; p = 2.8×10^{-5}), 0.20 mg μL^{-1} (X^2 = 19.89; df = 1; p = 8×10^{-6}) and 0.30 mg μL^{-1} (X^2 = 24.60; df = 1; p = 7×10^{-6}). Furthermore, the mortality caused by 0.10 mg

Table 2. Results of the different application methods and monitoring times of ethanolic extracts from *Salmea scandens* against fall armyworm larvae (FAW).

Test	Extract	Application method	Time (h)	Highest mortality (%)
In vitro	3-day maceration	Topical	12	44
			24	53
			48	59
	10-day maceration	Topical	12	70
			24	75
			48	80
In situ	10-day maceration	Topical	24	63
		Spraying	24	49

μL^{-1} per larva was similar to that caused by the 0.15 (X^2 = 1.38; *df* = 1; p = 0.240) and by 0.20 mg μL^{-1} (X^2 = 1.99; *df* = 1; p = 0.157) per larva doses, but lower than that at 0.30 mg μL^{-1} (X^2 = 3.83; *df* = 1; p = 0.041) per larva. Mortality caused by the 0.15 mg μL^{-1} per larva dose was also similar to those caused by the 0.20 (X^2 = 0.49; *df* = 1; p = 0.826) and 0.30 mg μL^{-1} (X^2 = 0.62; *df* = 1; p = 0.432) per larva doses. Finally, the mortality caused by 0.20 mg μL^{-1} per larva was similar to the 0.30 mg μL^{-1} per larva doses (X^2 = 0.31; *df* = 1; p = 0.579).

In situ toxicity by topical and spraying application

The results of mortality, analyzed as survival probability, from the in situ test by topical and spraying applications of the 10-day maceration extract recorded after 24 h are summarized in Fig. 3. Fig. 3a shows the cumulative survival probability by topical application in larvae of *S. frugiperda*; the dose factor was significant; this means that the increase in dose caused a significant decrease in survival of larvae. According to survival probability, the highest larvae survival was recorded by 0.10 mg μL^{-1} with 57.5 ± 1.71%, followed by 0.20 mg μL^{-1} with 45.0 ± 1.17%, with the lowest larval survival caused by 0.30 mg μL^{-1} with 37.5 ± 1.26%. Less than the 50% larvae populations survived with a dose of 0.20 mg μL^{-1} at 24 hours. No larvae mortality was recorded for the control.

The Mantel-Cox test results indicate significantly different survival distributions (X^2 = 37.95; *df* = 3; p = 2.9×10^{-8}). Pair-wise comparisons between the control and different doses were significantly different: 0.10 mg μL^{-1} (X^2 = 20.06; *df* = 1; p = 8×10^{-6}), 0.20 mg μL^{-1} (X^2 = 29.97; *df* = 1; p = 4.4×10^{-8}) and 0.30 mg μL^{-1} (X^2 = 35.91; *df* = 1; p = 2.1×10^{-9}). The comparison also indicates that the mortality caused by 0.10 mg μL^{-1} per larva was similar to those caused by the 0.20 (X^2 = 1.25; *df* = 1; p = 0.264) and 0.30 mg μL^{-1} (X^2 = 3.20; *df* = 1; p = 0.07) per larva.

Fig. 3b shows the cumulative survival probability by spraying application. Similar to the effects by topical application on the larval survival of FAW, the survival analysis results indicated significant differences between doses. The highest larval survival was recorded by 0.05 mg

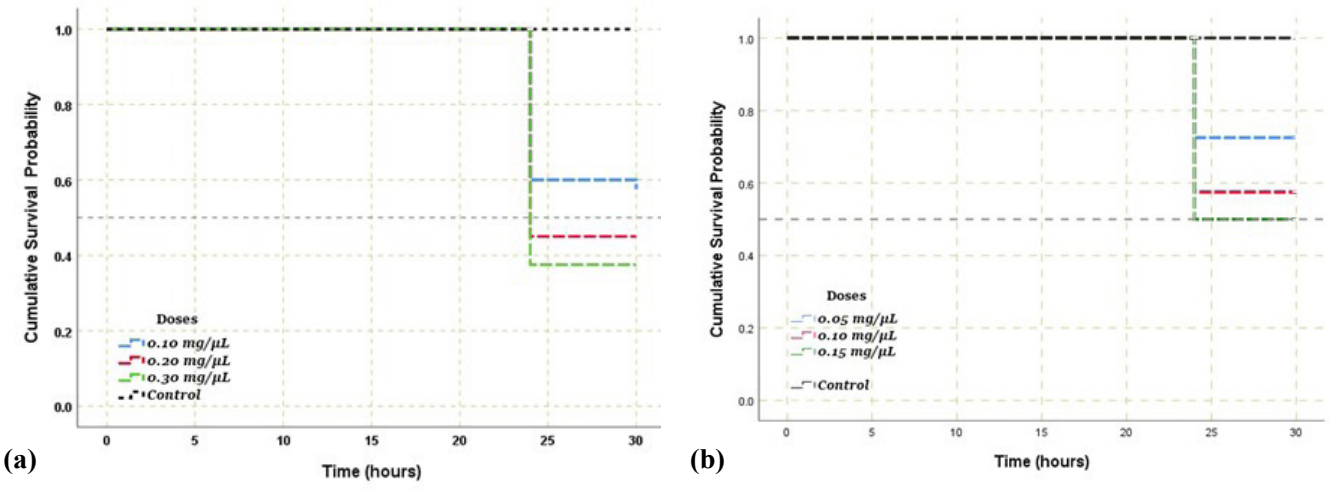

Figure 3. Survival probability of fall armyworm larvae exposed to three doses after 24 h by direct application (a) and spraying application (b).

μL^{-1} with 72.55 ± 2.12%, followed by 0.10 mg μL^{-1} with 57. 60 ± 1.04% per larva and at the dose of 0.15 mg μL^{-1} a 49% reduction of *S. frugiperda* was reached. More than 50% of the larvae populations survived with a dose of 0.10 mg μL^{-1} at 24 hours.

The Mantel-Cox test results indicated significantly different survival distributions (X^2 = 31.32; *df* = 3; p = 2.75×10^{-7}). The control was significantly different than the rest of the doses; 0.05 mg μL^{-1} (X^2 = 12.59; *df* = 1; p = 3.9×10^{-4}), 0.10 mg μL^{-1} (X^2 = 16.76; *df* = 1; p = 4.2×10^{-5}), 0.15 mg μL^{-1} (X^2 = 26.33; *df* = 1; p = 2.9×10^{-7}), which means that even the lowest dose caused a significant mortality of *S. frugiperda* larvae. The mortality caused by the dose 0.05 mg μL^{-1} was similar to that induced by 0.10 mg μL^{-1} (X^2 = 0.52; *df* = 1; p = 0.472) whereas it was significantly different to that induced by 0.15 mg μL^{-1} (X^2 = 4.21; *df* = 1; p = 0.03). Also, the mortality induced by the 0.10 mg μL^{-1} dose was similar to that induced by the 0.15 mg μL^{-1} dose (X^2 = 1.82; *df* = 1; p = 0.178).

Discussion

In contrast to reports of other authors who mention that 3-day maceration is an adequate time to extract alkylamides (Spelman et al., 2009), the relative concentration of alkylamides in the ethanolic extract continued to increase as time increased, reaching equilibrium at 10 days. Although the 10-day extraction only increased the yield extract by 12% with respect to the 3-day maceration, the alkylamides concentration was increased to 41%, thus obtaining in this manner an alkylamides-rich ethanol extract. The biggest TLC spot in Fig. 1a, associated to N-isobutyl-(2E,4E,8Z,10E/Z)-dodecatetraenamides, was corroborated by the spectroscopic characterization of the semi-purified alkylamides fraction, which confirmed the richness of N-isobutyl-(2E,4E,8Z,10E/Z)-dodecatetraenamides in the ethanolic extract of *S. scandens*. All the expected signals in ^{1}H NMR and ^{13}C NMR spectra were assigned.

The screening test (3-day maceration) showed that the highest mortalities of FAW were around the 50% target value. Mortalities achieved with the use of the 10-day extract in the in vitro test were higher than 50% (Table 2); therefore, this method with similar doses was considered as appropriate for the in situ research.

Higher mortalities of *S. frugiperda* were recorded for the in vitro test for the 10-day maceration extract as the dose and the monitoring time increased, similar to previous reports by other authors (Risco et al., 2012; Ponsankar et al., 2016; Silva et al., 2017; Sisay et al., 2019). Moreover, the effect of the increase in mortality with an increment in dose for the different monitoring times was similar to Logita (2015) The same behavior has been reported when using essential oil to control *S. frugiperda* larvae in *Java grass, Ocimum gratissimum,* and *Foeniculum vulgare* (Labinas & Crocomo, 2002).

Table 2 also showed that the highest mortality for the in situ test was 19% lower than the mortality for the in vitro test obtained using the same application method and the same monitoring time. This finding can be explained by the fact that a higher dose is required under field conditions to achieve the same mortality as the laboratory conditions. This occurs because climatic factors affect the concentration of the extract (Ncube et al., 2012) and larvae have greater freedom of movement facilitating the dispersion of the extract. Moreover, the botanical insecticides are sensitive to UV light, oxygen, and microorganisms (Wilson et al., 2020). The application method generally has a significant impact on the effect of the insecticidal agent.

In addition, Table 2 showed that the highest mortality was recorded at the highest dose and the longest monitoring time with 80% control, while 90% has been reported as the threshold value indicating a high toxicity for a plant extract (Chowański et al., 2016). Consequently, it is expected that the toxicity of the extract under evaluation would neither reduce untargeted plant fauna nor cause harm to humans (Teke & Kuete, 2014; Abbas et al., 2018).

Regarding the efficacy and effectiveness of the *S. scandens* extract obtained by the 10-day maceration, it was found that the mortality rate of *S. frugiperda* decreased with the increase in dose when compared to commercial larvicides such as a cypermethrin (Bullangpoti et al., 2012). The extract from *S. scandens* is toxic to insects because the unsaturation configuration of its molecules plays a key role on the toxicity of alkylamides (Boonen et al., 2012; Rios, 2012; Greger, 2016). Despite the significant toxicity of the *S. scandens* extracts caused in *S. frugiperda*, there has been no report of the toxicity to non-target organisms such as birds and mammals. The majority of alkylamides have presented low toxicity (Barrett, 2003) that may be associated to their fast degradation rate in an environment rich in oxygen (Liu & Murphy, 2007). Therefore, *S. scandens* as natural insecticides could require frequent applications and further research to incorporate photostabilizers for increase their efficacy in the field.

The action mechanism of the N-isobutyl-(2E,4E,8Z,10E/Z)-dodecatetraenamides found in *S. scandens* on 5th and 6th instar larval of *S. frugiperda* is not well-known. However, one possible action mechanism of this extract could be the peripheral sodium channel blockade as has been described for sanshool, an alkylamide from Szechuan pepper *(Zanthoxylum piperitum*, Rutaceae) (Dallazen et al., 2018). Additionally, studies have been described that the alkylamides isolated from *Piper* species interfere with the sodium channels of the central and peripheral nervous system, causing nerve discharges, paralysis, and death in insects (Ottea et al., 1989, 1990).

According to the current literature, synthetic insecticides induce mortalities on *S. frugiperda* ranging between 13.9 and 96.7% in the laboratory and from 6.7 to 40% in greenhouses (Sisay et al., 2019). Additionally, Belay et al. (2012) studied the effect of different insecticides for man-

agement of FAW larvae using a direct spray over third-instar larvae. More than 80% mortality was observed with chlorantraniliprole, flubendamide, spinosad, indoxacarb, and fenvalerate treatments 96 h after application. This high efficiency might be attributed to regular pest scouting and applying insecticides in rotation or combination, helping to delay the development of resistance, but in parallel potential effects on non-target organisms such as natural enemies and a higher environmental persistence are achieved.

On the other hand, botanical insecticides induce mortalities on *S. frugiperda* in a range of 5.0 to 90.0% in the laboratory (Phambala et al., 2020). Regarding biocontrol agents of FAW, insect pathogenic viruses are one of the most important. There are different viruses known to infect FAW larvae, such as ascoviruses, baculoviruses, densoviruses, rhabdoviruses, and partition-like viruses, being baculoviruses the most promising (Kumar et al., 2022). However, they are often expensive to produce, the kill rate is slow and residual insecticidal activity is short compared to pest control chemicals. Although Bhele & Popham (2012), reported an average of up to 80.0 % larval mortality, these values decreased to 4.4 after a simulated 3 h exposure to sunlight because viruses are very sensitive to sunlight.

Considering that most of the research carried out on the use of both synthetic and botanical insecticides on *S. frugiperda* considers second and third instar larvae, our 10-day extract obtained from *S. scandens*, which caused mortalities up to 80% under laboratory conditions and up to 63% in the field on 5th and 6th instar larvae, is very promising as an alternative solution against the *S. frugiperda* attack compared to synthetic, botanical and biological ones. It is expected that the extract will not generate resistance or persistence in the environment and with respect to stability at field conditions, alternatives such as photo stabilizers can be used.

In conclusion, the N-isobutyl-(2E,4E,8Z,10E/Z)-dodecatetraenamides rich-extract obtained from the *Salmea scandens* stem bark by the maceration method in ethanol cause significant mortality in *Spodoptera frugiperda* larvae, and the mortality increased proportionally with the dose and the monitoring time. The results confirmed the efficacy and effectiveness of the *S. scandens* extract on the mortalities of fifth and sixth instar larvae under laboratory and field conditions.

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