

Extraction and isolation of anti-tryptic castor-bean (*Ricinus communis* L.) substances and their effects on *Spodoptera frugiperda* (Smith, 1797) (Lepidoptera: Noctuidae)

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The search for alternative methods aiming to the control of agricultural pests that cause less environmental impact has been a recurring theme in several studies. For this purpose, the factors influencing the extraction and isolation of the trypsin inhibitor found in castor-bean (*Ricinus communis* L.) cake and seeds were studied, and its effect on the development of fall armyworm *Spodoptera frugiperda* (Smith, 1797) was evaluated. Therefore, the type and proportion of the solvent (w/v), extraction period and oil content were evaluated. The castor-bean cake extract with oil content of 20% in water at a ratio 1:40 (w/v) for 30 min showed the greatest trypsin inhibition, with 21.23 mUTI g⁻¹ seed. The isolation of the trypsin inhibitor contained in the extract was performed by flash chromatography, with inhibition peaks of up to 8906.12 mUTI g⁻¹ fraction, as well as inferring that the best eluent was ethyl acetate with a small percentage of ethanol (< 30%). In order to evaluate the effect of the inhibitor on the development of this insect, the aqueous extract of castor bean cake with oil content of 20% was incorporated into the diet that was provided to the caterpillars at a concentration of 4000 ppm. There was reduction in the oviposition period and an increase in the concentration of proteins in the feces, with 20.55 ± 3.73 d and 177.70 ± 16.90 μmol g⁻¹, respectively. The increase in protein excretion indicates that the trypsin inhibitor was able to reduce the absorption of essential amino acid residues by the insect without, however, impairing the development of *S. frugiperda*.

Key words: *Zea mays*, fall armyworm, *Ricinus communis*, trypsin inhibitor, alternative control.

INTRODUCTION

The use of plant extracts aiming the control of fall armyworm (*Spodoptera frugiperda* Smith, 1797) is considered promising, since the plants defend themselves from the attack by insects and other organisms through several mechanisms, among which stand out the synthesis and accumulation of toxic proteins, e.g. trypsin inhibitors. Since the discovery of protease inhibitors as a plant defense mechanism, numerous attempts have been made for its use in plant breeding (Mosolov and Valueva, 2008).

When insects cause injury to a specific part of the plant this can induce production of endopeptidase inhibitors in various parts of the plant, which usually act on the digestive system of insects. This plant internal communication mechanism is activated by the systemin, a phytohormone that acts on genes producers of protease inhibitors (Lopes et al., 2004).

Protease inhibitors are produced by several species of plants and act on the regulation of proteolysis, amino acid stock and defense against pathogens and pests. Inhibitors of serine proteases are of greater abundance and variety in nature, being divided into eight families: Kunitz inhibitor, Bowman-Birk type inhibitors, inhibitors of potato families

I and II, trypsin inhibitors found in pumpkins, trypsin and α-amylase inhibitors from cereal, trypsin inhibitors found in mustard, and the inhibitors from the family of the Serpins, while there is only one family of inhibitors of cysteine proteases, which are phytocystatins (Mosolov and Valueva, 2005).

The most pronounced effect of these inhibitors occurs when forming a stable complex with digestive enzymes, limiting amino acid hydrolysis and making it extremely slow (Jongsma and Beekwilder, 2011). This mechanism interferes on the development, due to the deficiency of essential amino acid residues available. The use of protease inhibitors as a means of control of *S. frugiperda* was evaluated by Paulillo et al. (2000), and it was discovered that the insect adapts to Kunitz and Bowman-Birk inhibitors. Brioschi et al. (2007) reported that the mechanism of resistance of the fall armyworm occurs through the expression of several isoenzymes, some of them immune to these inhibitors.

Trypsin inhibitors described in the literature are typically protein, unlike the inhibitor found in castor-bean leaves, a polar and non-proteic molecule, whose molecular structure remains unknown. The mechanisms of inhibition for some protease inhibitors have been described. However, it is still not known for sure how a non-protein inhibitor influences the enzymatic hydrolysis. Understanding how this mechanism works can contribute to new research that aim to use this substance in the control of pests of economic importance (Benchabane et al., 2010; Rossi et al., 2010).

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Due to the damage caused by *S. frugiperda* to corn (*Zea mays* L.) and its tolerance to a wide range of insecticides, the search for new control methods, especially those that cause less environmental impact, can be justified, since the application of insecticides usually causes biological imbalances, intoxication of applicators and environmental contamination. In this context, the aims of this study were to evaluate techniques for extraction and isolation of trypsin inhibitors in castor-bean (*Ricinus communis* L.) seeds and cake, and the effect of these inhibitors on the development of *S. frugiperda*.

MATERIALS AND METHODS

The experiments were carried out in the Biochemistry (Chemistry Department) and in the Integrated Pest Management (Entomology Department) laboratories, at Federal University of Lavras (UFLA), Brazil. The castor-bean seeds, cv. Guarani moisture content 3.34%, were provided by the technicians from the Seed Laboratory at UFLA.

Preparation of the castor-bean seeds and cake extracts

Seeds were ground, so that they could be degreased with ethyl ether in a system for fat determination (model TE-044-8/50, Tecnal, Piracicaba, São Paulo, Brazil) for different periods of time, aiming to obtain castor-bean cakes (solid waste) with different oil contents, in order to make it possible to evaluate their interference in the extraction of the trypsin inhibitor.

The first extraction process was carried out with castor-bean cake with 0% oil content, and the extracts were obtained according to the methodology proposed by Carvalho (2009). The castor-bean cake static percolation was performed in a solvent for 7 d at a ratio of 1:20 (w/v) using ethyl acetate, and after this period the extract was filtered and named Ac0%. The remainder castor-bean cake of this first extraction was subjected to the same process, but using ethanol, and the obtained extract was named ET0. This process was also carried out with methanol, thereby obtaining the extract Me0%. The extracts obtained had their solvent removed in a roto-evaporator (Model 208, Fisatom, São Paulo, Brazil) at 20 °C below the boiling point of the solvents used and the solid residue was dissolved in water for later analysis of the trypsin inhibition of *S. frugiperda*.

The second procedure involved agitation of ground seeds in presence of ethanol and water. The ethanolic extract of seeds (Et_{seed}) was obtained by orbital shaking for 60 min at a ratio of 1:80 (w/v); this mixture was centrifuged and the supernatant was filtered, and then concentrated on a roto-evaporator. The residue obtained was dissolved in ethyl ether at a ratio of 1:80 (w/v). In a dropping funnel, a liquid/liquid partition was performed and the ether was washed with distilled water, using double the amount of water used in the dissolution; the

etheral fraction was discarded. The aqueous fraction was concentrated on the roto-evaporator and the solid residue was dissolved in 10 mL ethanol for use in *in vitro* trypsin inhibition tests. The seed aqueous extract (Aq_{seed}) was obtained by 60 min agitation of ground seeds with water at a ratio of 1:40 (w/v); the mixture was then centrifuged and the supernatant was filtered to be used in the *in vitro* inhibition tests.

The aqueous extracts of cake with oil contents of 20, 26, 30, 34, 41, 45, and 48% (extracts Aq20%, Aq26%, Aq30%, Aq34%, Aq41%, Aq45%, and Aq48%, respectively) were obtained the same way as the Aq_{seed} was, and their inhibitory potential was evaluated in *in vitro* *S. frugiperda* trypsin inhibition assays, aiming to determine the oil effect on the anti-trypsin activity.

The extract Aq20% suffered optimization during agitation period and in the proportion of solvent; four periods of time were tested (30, 60, 90, and 120 min) and four ratios (1:10, 1:20, 1:30, 1:40, 1:50, and 1:75 w/v) in order to obtain a greater amount of inhibitor for use in biological assays and chromatographic fractionation. For this reason, the extract was oven dried at 45 °C and *in vitro* trypsin inhibitory activity of solid residue was evaluated after dissolution of 2.5 mg mL⁻¹ distilled water. The choice of Aq20% extract was based on the reliability of the absorbance readings during enzyme kinetic assay of trypsin inhibition experiments, in addition to making it possible to calculate mass/volume concentration in preparation of the diet provided to the caterpillar, and in the chromatographic fractionation.

Enzyme kinetic assay of trypsin inhibition

Sixth instar of *S. frugiperda* caterpillars from laboratory rearing, were immobilized at -20 °C for 10 min and their digestive tubes were removed with the aid of forceps and scissors, and then macerated in Potter homogenizer at a ratio of one digestive tract to 4 mL of distilled water at 4 °C. The crude extract was then filtered on a 100 µm nylon mesh and centrifuged at 10 000 g at 4 °C for 30 min. The supernatant, considered the enzyme extract, was then stored in a freezer at -20 °C until its use (Rossi et al., 2010).

The substrate used in the trypsin inhibition assays was the N- α -benzoyl-DL-arginine-4-nitroanilide hydrochloride (BAPNA) at 1.25 mmol L⁻¹ and in a 0.1 M glycine-sodium hydroxide (Gly-NaOH) buffer (pH 9.7). To prepare the solution, 54.5 mg BAPNA were solubilized in 1.25 mL of dimethyl sulfoxide (DMSO) and then added up to 100 mL with Gly-NaOH buffer.

The assay was kinetically performed, with four periods of reaction time: 30, 60, 90, and 120 min. For each inhibition assay, 200 µL extract containing inhibitor, 200 µL suitably diluted enzyme (enzyme extract) and 800 µL substrate solution were added to the reaction mixture, thus initiating the reaction. The control test was performed without inhibitor to occur at full speed. The reaction was

stopped with 200 μL 30% acetic acid and absorbance was read at 410 nm, according to the methodology proposed by Erlanger et al. (1961).

It is noted that 1 UTI (unit of trypsin inhibited) corresponds to 1 μmol of p-nitroanilide, which ceases to be produced due to the presence of the inhibitor; whereas the results in this study were expressed in mUTI g^{-1} seed in the extraction procedures and in mUTI g^{-1} fraction and 200 μL in the chromatographic purification procedures.

Fractionation of Aq20% extract by adsorption flash chromatography

A glass column (3 cm diameter \times 50 cm) was filled with silica gel 60 Merck mixed with chloroform; 113.80 g silica were suspended in chloroform for the mixture became fluid. This fluid was poured into the column forming the stationary phase (Rossi et al., 2010).

As the detection method used was based on a kinetic assay of trypsin inhibition, which does not allow real-time detection, three chromatographies were required to determine the best concentration and better eluents. The Aq20% extract was diluted in distilled water and subjected to fractionation in concentrations of 0.06, 0.01, and 0.5 g mL^{-1} distilled water, and 1 mL of these dilutions was injected.

The fractions were collected in 100 mL containers, concentrated on a roto-evaporator and transferred to 15 mL vials. The remaining solvent was evaporated in an oven at 45 $^{\circ}\text{C}$. The residue was dissolved in 2 mL distilled water in an orbital shaker for 1 h. Detection was carried out by an inhibition enzymatic assay in all the fractions obtained from the three chromatographies performed.

The first two chromatographies showed the peak of trypsin inhibition in the ethyl acetate/ethanol interphase, even with different sample concentrations and eluent volumes, which indicates that the best elution range was a mixture of ethyl acetate/ethanol. In the third chromatography, a run was performed with the sample in the concentration of 0.5 g mL^{-1} and it was eluted with the following eluotropic series: ethyl acetate; ethyl acetate solution/ethanol in a 70:30 ratio; ethyl acetate solution/ethanol in a 30:70 ratio, and pure ethanol.

Effects of addition of Aq20% extract on the diet at different developmental stages of *S. frugiperda*

Second instar of *S. frugiperda* caterpillars from laboratory rearing, were transferred to glass tubes (3 cm diameter \times 10 cm) in the proportion of one caterpillar per container, containing the diet prepared according to the methodology proposed by Kasten Jr. et al. (1978). The bioassay consisted of a control treatment containing distilled water and a treatment containing aqueous extract of castor-bean cake at 4 g L^{-1} . This value was used as a result to be among the concentrations of 1.6 g L^{-1} and 9.6 g L^{-1} of the methanol extract of seeds used by Ramos-López et al. (2010). The concentration of 1.6 g L^{-1} methanol extract

was able to alter the development of the fall armyworm and at 9.6 g L^{-1} all caterpillars died.

In the control treatment, 270 mL of diet were added to 30 mL distilled water and, in the treatment with Aq20% extract, 30 mL extract were added, whose final concentration in the diet was 4 g L^{-1} .

In order to evaluate if the inhibitor altered the amino acid uptake by the insect, protein concentration in the feces of *S. frugiperda* caterpillar was determined, based on the methodology developed by Bradford (1976). After the end of the larval period, 45 mg feces were collected, 200 μL distilled water and 200 μL 1 M perchloric acid were added to the feces, and this mixture was homogenized and placed in an ice bath for 10 min. Samples were centrifuged and supernatant was discarded, the precipitate was resuspended in 200 μL 0.1 M NaOH and its protein concentration was determined spectrophotometrically at 594 nm, taking as a basis a standard curve of BSA (bovine serum albumin), with protein masses ranging from 2 to 20 μg .

The design used in the experiment was completely randomized, with two treatments and 72 replicates, each one consisting of one *S. frugiperda* second instar caterpillar. The duration of larval and pupal period, pupal weight, protein concentration in feces, female and male longevity, pre-oviposition period, oviposition period, and number of eggs produced per couple were evaluated.

Statistical analysis

All data obtained were subjected to ANOVA. Data from solvent for extraction of trypsin inhibitor found in castor-beans, duration of larval and pupal period, pupal weight, protein concentration in feces, female and male longevity, pre-oviposition period, oviposition period, and number of eggs per couple had their averages compared by the Scott-Knott test at 5% significance level. Those data associated with proportion of water, extraction time, and oil content in the extraction of trypsin inhibitor found in castor-beans were analyzed through regression. All statistical analyzes were performed using the software R (R Core Team, 2012).

RESULTS AND DISCUSSION

Extraction and evaluation of the extractant, time, proportion and oil content on the trypsin inhibitor of castor-bean seeds

Extracts Aq20% and Et_{seed} caused trypsin inhibition of *S. frugiperda* and the highest average (65.76%) was observed in extract Et_{seed} treatment (Table 1). When it comes to enzyme assays, the range of acceptable percentage for calculating UTI should vary between 80 and 40%. Values above these range may be masked, that is, the inhibition is greater than that observed, and smaller values may be due to dilution of the inhibitor in the extraction and, therefore, only extracts Aq20% and Et_{seed} allowed calculating mUTI.

The extracts Ac0%, Et0%, and Me0% did not show trypsin inhibition, and it was not possible to evaluate

Table 1. Comparison of trypsin inhibition (%) and milliunits of trypsin inhibited (mUTI) caused by different extracts, obtained from castor-bean seeds and cake.

Extracts	Inhibition of trypsin	
	%	mUTI g ⁻¹ seeds
Ac0%: ethyl acetate extract from castor-bean cake 0% oil	10.53 ± 2.40a	-
Et0%: ethanol extract from castor-bean cake 0% oil	6.31 ± 0.90a	-
Me0%: methanol extract from castor-bean cake 0% oil	8.77 ± 0.88a	-
Aq20%: aqueous extract from castor-bean cake 20% oil	40.44 ± 3.94b	21.23 ± 2.07a
Aq _{seed} : aqueous extract from ground castor-bean seeds	-	-
Et _{seed} : ethanol extract from ground castor-bean seeds	65.76 ± 6.97c	6.90 ± 0.73b
	F = 61.63; p < 0.001	F = 27.63; p < 0.01

Means followed by the same letter in each column are not statistically different according to Scott-Knott test at 5% significance level.

whether Aq_{seed} extract caused inhibition or not, because the reaction mixture became turbid, due to the presence of oil, which made the reading in spectrophotometer impossible. Most trypsin inhibitors described in literature are protein and therefore their extraction is performed through saline or buffer solutions due to their effectiveness to solubilize proteins. Purification of these protein inhibitors is similarly affected by techniques as centrifugation, ammonium sulfate precipitation, acetone precipitation at -20 °C, and affinity chromatography, which are the most common for purification of proteins (Benjakul et al., 2000; Tsybina et al., 2001; Carlini and Grossi-de-Sá, 2002; Brioschi et al., 2007; Zhou et al., 2008). However, trypsin inhibitory activity present in extracts of castor-bean leaves is concentrated in polar solvents and does not change at high temperatures or protein denaturing agents (Rossi et al., 2010).

When identifying compounds, extraction by solvents with increasing polarity is common, aiming grouping substances with similar characteristics, in order to facilitate their further characterization. Therefore, in the present work, this extraction technique was initially used in castor-bean cake with 0% oil. Carvalho (2009) obtained the trypsin inhibitor of castor-bean leaves using this methodology; however, in the present work, this technique was not effective for the extraction of the inhibitor in castor-bean cake with 0% oil (Table 1), probably due to the removal process of the oil present in castor-bean seeds. The presence of trypsin inhibitor in the castor-bean seed and cake was confirmed in extracts Aq20% and Et_{seed}, whose oil was removed by partition with ethyl ether.

For the proportions evaluated, only the ratios 1:10, 1:20, 1:30, and 1:40 (w/v) caused significant inhibition, and a linear increase for the trypsin inhibition in *S. frugiperda* was observed (Figure 1). Ratio 1:50 and 1:75 do not exhibit acceptable percentage to calculate inhibition. When subjected to different extraction conditions, trypsin inhibitory activity of castor-bean cake with 20% oil showed a positive correlation with the increase in water proportion in the extraction of up to 1:40 (w/v). The increase in the trypsin inhibition can be explained by the greater availability of water during the extraction since, in higher proportions, the dilution

effect was predominant and it did not cause significant trypsin inhibition. Moreover, referring to the extraction time of the inhibitor, there was no increase or decrease in the trypsin inhibition caused by the aqueous extract of the castor-bean cake with 20% oil, as shown in Figure 2.

Oil content in castor-bean cake caused an increase in the inhibition, which was proportional to its own increase (Figure 3). The oil content in castor-bean cake influenced the extraction of the trypsin inhibitor and, the higher the content, the greater the trypsin inhibitory activity. All extracts used in this experiment were aqueous; therefore the oil was not responsible for inhibition due to its insolubility in water. Thus, the linear relationship between oil content/trypsin inhibition can be explained by

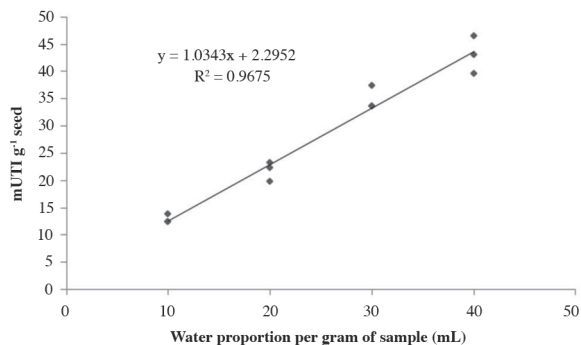


Figure 1. Curve of the effect of the water proportion on the extraction of the inhibitor present in the castor-bean cake on the inhibitory activity of trypsin in *Spodoptera frugiperda*. F = 175.87; p < 0.001.

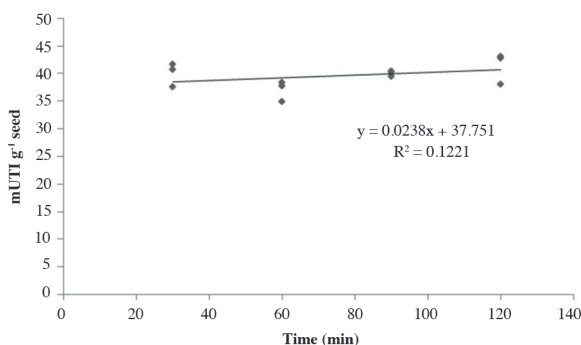


Figure 2. Curve of the effect of the extraction period of the castor-bean cake inhibitor on the inhibitory activity of trypsin in *Spodoptera frugiperda*. F = 1.5425; p = 0.2426.

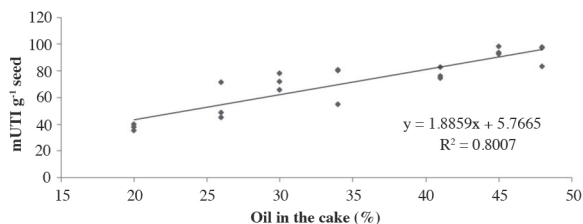


Figure 3. Curve of trypsin inhibition *in vitro* caused by castor-bean cake extracts with different oil contents. $F = 76.33$; $p < 0.001$.

the degreasing time needed to obtain castor-bean cakes because, the longer the oil extraction (therefore, lower oil content), the lower the inhibition. This fact shows that this molecule able to inhibit trypsin in *S. frugiperda* may be organic because, even with their affinity for polar solvents in the extraction, it also has some affinity with non-polar solvents required for degreasing, besides explaining the lack of inhibition in the extracts Ac0%, Et0%, and Me0%, due to the use of castor-bean cake without oil in the extraction of trypsin inhibitor.

Even with all this evidence, there is the possibility that this molecule might be a lipoprotein because of its affinity for polar and non-polar solvents; however, the fact that the inhibitory activity has remained unchanged when Rossi et al. (2010) treated the castor-bean leaf extract with β -mercaptoethanol indicates that this trypsin inhibitor is not a protein.

Fractionation of Aq20% extract by adsorption flash chromatography

Before being subjected to chromatography, Aq20% extract showed 795.20 mUTI g^{-1} extract. The eluents used were: ethyl acetate, ethanol, and methanol in increasing order of polarity. The extract was solubilized in distilled water at a concentration of 0.06 $g mL^{-1}$ for the first chromatography. Through the enzymatic assay performed with the fractions, it was possible to observe that the inhibitor began being eluted from fraction 17, corresponding to the interphase ethyl acetate/ethanol. The inhibitory activity for this peak was 66.80 mUTI g^{-1} fraction (Figure 4).

The formation of a tail in the chromatographic curve was observed, indicating the possibility of a highly concentrated sample, and therefore a new chromatography was performed at a concentration 0.01 $g mL^{-1}$ extract (Figure 5).

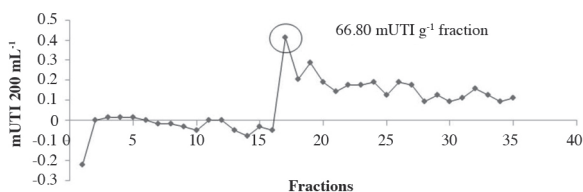


Figure 4. Chromatogram 1. Detection of the inhibitor via enzymatic assay of trypsin inhibition in *Spodoptera frugiperda*. Sample: 0.06 $g mL^{-1}$ of distilled water. Fractions: 1-16 = Ethyl acetate; 17 = Interphase; 18-25 = Ethanol; 26 = Interphase; and 27-35 = Methanol.

The chromatography presented in Figure 6 shows variations in the baseline, and it was possible to observe a peak in fraction 10, with a trypsin inhibitory activity 221.81 mUTI g^{-1} fraction, corresponding to the interphase ethyl acetate/ethanol. The extract was applied to the column at a concentration of 0.5 $g mL^{-1}$ water, and the inhibitor was eluted by the interphase formed between the ethyl acetate and the ethyl acetate/ethanol solution in the ratio of 70:30 (v/v), corresponding to the fractions 5-11, with a peak of trypsin inhibitory activity of 8906.12 mUTI g^{-1} fraction (Figure 6).

Carvalho (2009) performed a chromatographic isolation in the ethanol extract of castor-bean leaves, and the recovered fraction with the greatest inhibitory activity was eluted by the ethanolic mobile phase, with 2100 mUTI g^{-1} extract. In the present work, the activity of Aq20% extract was 795.2 mUTI g^{-1} extract before being purified, and the highest inhibitory activity was obtained at the peak corresponding to the fractions 5-11 in chromatography 3 with 8906.1200 mUTI g^{-1} fraction (Figure 6). This recovered inhibitory activity probably occurred due to the optimization of the mobile phase with a lower concentration of ethanol that may have eluted the trypsin inhibitor more effectively. In all the runs, it was possible to observe the presence of inhibition peaks, but it was not possible to obtain a defined peak of trypsin inhibition. It was observed that the inhibitor was eluted by a small percentage of ethanol in ethyl acetate.

Effects of Aq20% extract addition on the diet at different developmental stages of *S. frugiperda*

It was found that caterpillars fed with the diet containing trypsin inhibitor excreted larger amounts of protein, with an average of $215.61 \pm 24.82 \mu mol g^{-1}$ feces ($F = 7.9729$;

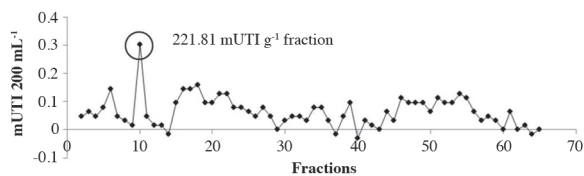


Figure 5. Chromatogram 2. Detection of the inhibitor via enzymatic assay of trypsin inhibition. Sample: 0.01 $g mL^{-1}$ of distilled water. Fractions: 1-8 = Ethyl acetate; 8 = Interphase; 10-37 = Ethanol; 38 = Interphase; 39-62 = Methanol.

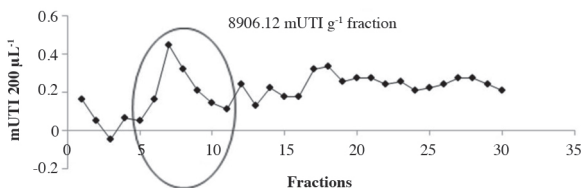


Figure 6. Chromatogram 3. Detection of the inhibitor via enzymatic assay of trypsin inhibition. Sample: 0.5 $g mL^{-1}$ of distilled water. Fractions: 1-6 = Ethyl acetate; 7 = Interphase; 8-11 = Ethyl acetate/Ethanol 70:30; 12 = Interphase; 13-21 = Ethyl acetate/Ethanol 30:70; 22 = Interphase, and 23-30 = Ethanol.

$p = 0.0224$). For the other biological characteristics negative influence of trypsin inhibitor was not observed (Table 2). The adult insects from caterpillars fed with diets containing the inhibitor had their oviposition period reduced. However, the duration of larval period, duration of pupal period, pupal weight, number of eggs per couple, pre-oviposition period, longevity, and fecundity were not affected.

It is known that insects have defense mechanisms against the secondary metabolites produced by plants. When it comes to digestion inhibitors, two mechanisms are the most common: the first involves the accentuated expression of protease inhibited, seeking to increase its activity against the inhibitor whereas, in the second one, several isoenzymes are synthesized, aiming that some of them are not inhibited. Both mechanisms deviate the essential amino acids for growth to the synthesis of digestive enzymes, thus causing a shortage in the development. Therefore, it is necessary an *in vivo* assay, in addition to an *in vitro* detection of trypsin inhibition, since the plant-insect interactions are far more complex nature, and cannot be evaluated only under laboratory conditions (Paulillo et al., 2000; Brioschi et al., 2007).

The results obtained by Ramos-López et al. (2010) demonstrated negative effects on larval duration, larval and pupal survival, and pupal weight of insects treated with methanol extract of castor-bean seeds. There was an increase in larval duration, as well as pupal and larval weight and survival reduction at 1.6 g L^{-1} ; and at 9.6 g L^{-1} none insects survived.

Ramos-López et al. (2010) compared mean lethal concentration (LC_{50}) of hexane, methanol, ethyl acetate extracts of castor-beans leaves and seeds and two compounds present in castor-beans, ricinine and castor oil, both purified. The most polar extracts caused the greatest effect on the development of the fall armyworm and the authors concluded that the reduction in the development of this insect was caused by the ricinine (alkaloid) and the castor oil. The toxic effect was attributed mainly to ricinine, compound mostly found in seeds with polar characteristics and $LC_{50} 0.38 \text{ g L}^{-1}$ (Ferraz et al., 1999).

In the present work, the seed was degreased prior to the extraction of the inhibitor with water, and thus

ricinine may have also been extracted; it implies that the same extract at 4 g L^{-1} in the diet was not able to cause significant changes in the development of treated insects. The sublethal effect of Aq20% extract, added to the increase in the concentration of soluble protein in feces, could be an indicative that the inhibitor is ricinine if the other biological characteristics of development evaluated had been modified (Ferraz et al., 1999; Ramos-López et al., 2010); however, it is likely that ricinine is not present in the Aq20% extract obtained in this study, because it did not cause negative effect on the development of *S. frugiperda*.

Yu et al. (2003) studied populations of *S. frugiperda* resistant 562 fold to carbaryl and 354 fold to methyl-parathion. When enzymes responsible for the detoxification of these populations were compared with those of caterpillars reared in laboratory, an increase in the activity of these enzymes was examined, which explains the greater resistance of these insects to the evaluated insecticides.

The lack of changes in the development of fall armyworm caused by the reduction in the absorption of amino acids is probably due to some strategy of defense and/or detoxification of this pest as the one reported by Yu et al. (2003) to carbaryl and methyl-parathion. Another probable reason is the use of Aq20% extract, where the inhibitor concentration is low and may not have achieved the concentration required to cause some effect on the development of fall armyworm. Carvalho (2009) found that when the caterpillar of *S. frugiperda* were fed with a diet containing the chromatographically purified fraction of the ethanol extract from the castor-bean leaves, there was an increase in the larval period and a decrease in the pupal weight.

CONCLUSIONS

The castor-bean seeds contain compounds with anti-trypsin activity in *S. frugiperda*. The castor-bean cake degreased with 20% oil is the most suitable source for obtaining trypsin inhibitor by extraction with water in the ratio of 1:40 (w/v) for 30 min.

The diet supplemented with Aq20% extract causes

Table 2. Biological characteristics of *Spodoptera frugiperda* fed with a diet containing aqueous extract of castor-bean cake.

Characteristic	Treatment		F	p
	Control	4 g L ⁻¹		
Duration of larval period, d	21.44 ± 6.35a	21.25 ± 4.08a	0.05	0.83
Protein in feces, μmol g ⁻¹	215.61 ± 24.82a	177.70 ± 16.90b	7.97	0.02
Duration of pupal period, d	12.73 ± 2.43a	12.66 ± 2.38a	0.03	0.87
Pupal weight, g	0.2782 ± 0.03a	0.2813 ± 0.03a	0.29	0.59
Pre-oviposition, d	4.64 ± 2.29a	5.00 ± 2.55a	0.11	0.74
Female longevity, d	20.27 ± 4.22a	18.33 ± 5.66a	0.88	0.36
Male longevity, d	18.27 ± 4.29a	15.78 ± 6.10a	1.15	0.30
Oviposition, d	20.55 ± 3.73a	15.67 ± 4.82b	6.53	0.02
Eggs/couple	1890.00 ± 665.12a	1691.11 ± 721.27a	0.41	0.53

Means followed by the same letter in each line are not statistically different according to the Scott-Knott test at 5% significance level.

an increase in the protein excretion by caterpillars of *S. frugiperda* and reduces its oviposition period. The ricinine is not the compound responsible for causing trypsin inhibition in fall armyworm.

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