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Effect of the Aqueous Extracts of the Seeds of *Talisia esculenta* and *Sapindus saponaria* on Fall Armyworm

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ABSTRACT

The effect of aqueous extracts of Talisia esculenta *(T.E.) and* Sapindus sapo*naria (S.S.), were evaluated on the development and mortality of 8-14th day-life* Spodoptera frugiperda, *an important pest of maize. Corn leaves were dipped in the aqueous extracts (1% w/v) and offered as food to the caterpillars. The treated corn leaves with the extracts caused larval mortality (26.71%/T.E.; 63.3%/S.S.) and also showed effect on the larval weight (237.50 mg/T.E.; 86.65 mg/S.S.) when compared with the control* (*11.3% and 293.45 mg*)*, mortality and larval weight, respectively. The electrophoresis with gelatin* (*0.1%*) *showed two very clear white areas (trypsin activity) in the caterpillars midgut of all the treatments. Only the caterpillar treated with* S. saponaria *presented significant differences, showing trypsin activity* (*10.59%*). Sapindus saponaria *appeared better than* Talisia esculenta *and showed good potential to be used as control agent for* S. frugiperda.

Key words: *Talisia esculenta*, *Sapindus saponaria*, Sapindaceae, *Spodoptera frugiperda*, natural insecticide

INTRODUCTION

Plants are rich sources of natural substances that can be utilized in the development of environmentally safe methods for insect control (Sadek, 2003; Caramori et al., 2004). Simple crude extracts from plants have been used as insecticides in many countries for centruries (Crosby, 1971; Leatemia and Isman, 2004). Crude plant extracts often consist of complex mixtures of active compounds. Use of complex mixtures as pest control agents could be advantageous as natural mixtures may act synergistically (Berenbaum, 1985; Leatemia and Isman, 2004). They may show greater overall bioactivity compared to the individual constituents (Berenbaum et al., 1991; Chen et al., 1995), and insect resistance is much less likely to develop with mixtures (Feng and Isman, 1995). These reasons support the use of crude, chemically unrefined plant extracts, and the former will be simpler and cheaper to prepare if the plant materials are locally available (Leatemia and Isman, 2004). Much research has focused on screening of the Meliaceae for limonoids (triperpene derivatives). This has been driven by the well-documented bioactivity of azadirachtin, a

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limonoid from the neem tree (*Azadirachta indica*) (Wheeler et al., 2001; Hirose et al., 2001). The deleterious effects of certain purified phytochemicals or crude extracts on insects are manifested in several ways, including toxicity, growth retardation, feeding inhibition, oviposition deterrence, suppression of calling behaviour and reduction of fecundity and fertility (Mordue and Blackwell, 1993; Breuer and Schmidt, 1995; Hiremath et al., 1997; Zhao et al., 1998; Wheeler & Isman, 2001; Muthudrishnan and Pushpalatha, 2001). An advantage of the neem extract is of being little toxic for endothermic animals and its activity does not persist for a long time (Schmutterer, 1990; Koul et al., 1990). Such a wide variety of effects provide potential alternatives to the use of synthetic chemical insecticides (Sadek, 2003).

Talisia esculenta (St. Hil.) Radlk., locally known as pitomba, belongs to the Sapindaceae family and occurs in northern and northeastern Brazil. The fruit of *T. esculenta* is consumed by humans and birds. The latter act as seed dispersers. *Sapindus saponaria* L. (Sapindaceae), commonly known as Saboneteira, is a tree used by the population to prepare homemade soap. The fruits are not eatable, and are considered toxic to bovine, bats and birds. Their seeds present insecticide action. The fall armyworm, *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), is an economically important pest of maize and other graminaceous crops throughout a great part of tropical and subtropical America (Sparks, 1979; Andrews, 1980; Chapman et al., 2000). On maize, the favorite host, the larvae feed almost exclusively within the wrapped leaves of the developing whorl (Labatte, 1993). The aim of this work was to evaluate the effects of aqueous seeds-extracts two plants of Sapindaceae family, *T. esculenta* and *S. saponaria*, in the development and midgut trypsin activity of *S. frugiperda*.

MATERIAL AND METHODS

The study was developed from February to July 2004 under laboratory conditions in the Escola Superior de Agronomia Luiz de Queiroz-ESALQ/USP/Piracicaba and Universidade Federal de Mato Grosso do Sul-UFMS/Três Lagoas/ Brazil. The *S. frugiperda* caterpillars were from a laboratory colony provided by Dr J. D. Vendramim (Departamento de Entomologia, Fitopatologia e Zoologia Agrícola, Escola Superior de Agricultura Luiz de Queiroz, Universidade de São Paulo, Piracicaba, SP, Brazil). The photoperiod used for the colony was L14:D10, and the larvae were maintained in an incubator at 25±1^{o}C and a relative humidity of 60±10% during the feeding trials. The maize plants, cv AG5020, susceptible to fail army worm, were used in the experiments but neither synthetic nor botanical insecticide was applied. *T. esculenta* and *S. saponaria* seeds were collected in the States of Ceará and São Paulo (Brazil), respectively. After drying, the seeds were triturated in an electric mill to obtain a flour of fine granulation. Flours were stored at -20ºC for subsequent use.

The aqueous extracts were obtained through the dissolution (distilled water) of the flours (Viana and Prates (2004). The solutions were stored in glass containers, covered, maintained at room temperature in dark conditions for 24h to extract the compounds soluble in water. The aqueous extracts were filtered through fine fabric of *voile* before use, and stored up to three days at 4^{0}C (Bouamama et al, 2006). The maize leaves, collected from plants with age between 35 and 45 days after the plantation (V7-V9) (Busato et al, 2002), were cut in pieces of 5 x 3 cm and immersed in the respective extracts at room temperature. Maize leaves immersed only in distilled water were used as control. The effect of the treatment was observed daily, as well as the change of the maize leaves which, after having evaporated the water excess, were offered to the caterpillars.

The experiment was composed of three treatments (control, *T. esculenta*, and *S. saponaria*) using glass tubes (8.5 x 2.5 cm) covered with cotton to observe the biological development of the caterpillars of *S. frugiperda* conditioned together with the maize leaves. Each treatment presented 150 caterpillars properly individualized (n=150) to avoid cannibalism.

The caterpillars were weighed at 8 and 14 days of development. In the pupal stage, they were weighed again after 24 h. Insects were kept in tubes until adults emergence. Viability and duration of the larval and pupal stages were also evaluated. The experiment was developed under controlled conditions of temperature (25±2ºC), humidity (80±10ºC) and light (14 h).

Twenty 4th - instar caterpillars, were removed at random of each treatment and cold-immobilized. Their midguts were surgically removed in cold 150 mM NaCl using tweezers and stored frozen (-20ºC). Later the midguts were homogenized in 150 mM NaCl, centrifuged at 20,000 x g for 30 min at 4 ºC and the supernatants pooled and kept on ice for in vitro enzymatic assays.

Feces of the caterpillars were collected during the experiment and frozen (-20ºC). When necessary, they were macerated, homogenized in 200 mM Tris-HCl buffer (Tris - Hydroxymethyl aminomethane), pH 8.5, centrifuged at 20,000 x g for 30 min at 4 ºC and supernatants were used for in vitro enzymatic assays.

Protein concentrations were determined as described by Bradford (1976), using bovine serum albumin (BSA) as a standard. Absorbance at 595 nm was also used to determine the protein content in both aqueous seeds-extracts and midgut/feces supernatants of caterpillars.

The trypsin-like enzyme activity, in the midguts and feces, was determined using N-benzoyl-dl-arginine-p-nitroanilide (BAPNA) as substrate. Samples of midgut and feces supernatants (50 µg of protein) were incubated in 200 mM Tris-HCl buffer, pH 8.5, in a final volume of 0.2 ml for 10 min before the addition of 1 ml of mM substrate. The reaction was allowed to proceed at 37°C for 20 min and then stopped by adding 0.2 ml of 30% (v/v) acetic acid. The resulting absorbance was read at 410 nm (Macedo et al, 2000). To evaluate the anti-trypsin activity of both the aqueous seeds-extracts (*T. esculenta* and *S. soponaria*), samples of 50 µl were used (10 µg and 17 µg of proteins respectively) in assays of serial dilutions, containing 200 mM Tris-HCl buffer, pH 8.5 and added 50 µl of trypsin bovine enzyme (0.3 mg/ml). After 10 min, solution of BAPNA was added as substrate and the reaction was interrupted with acetic acid (30%) after 20 min (absorbance read at 410 nm).

Incubations were carried out for at least three periods and were calculated for the initial rates of hydrolysis. The assays were determined under conditions in which enzyme activity was proportional to protein concentration and the time of incubation. One enzyme unit was defined as the amount of protein that catalyzed the cleavage of 1 µmol of substrate/min.

SDS polyacrylamide gel (12.5%) electrophoresis (SDS-PAGE) was run according to Laemmli method (1970). Caterpillars feces samples were boiled for 2 min before applying. The proteins used as molecular mass standards were phosphorylase B (94 kDa), BSA (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa) and α-lactalbumin (14.4 kDa). The proteins were detected by staining with 0.1% Coomassie brilliant blue R-250.

SDS-PAGE (10%) containing gelatin (0.1%) was carried out to verify total proteolytic activity (Michaud et al. 1993; Novillo et al. 1997). To prevent the irreversible inactivation of the proteinases, the samples were not previously boiled as done in SDS-PAGE described above. The same samples was previously incubated with trypsin inhibitor - Nα-p-tosyl-L-lysine chloromethyl ketone (TLCK) for 30 min. Following electrophoresis at 5ºC, the gels were washed with 2.5% (v/v) Triton X-100 solution for 2 h with shaking to remove the SDS, after which the gels were incubated with 100 ml of activating solution (Tris-HCl 100 mM, pH 8.5) for 2 h. The gels were subsequently stained with Coomasie brilliant blue R-250.

All data were examined using one-way analysis of variance (ANOVA) (General Linear Models on GLM procedure). Tukey's test was used to identify the means which differed whenever the ANOVA test indicated significance. A P value <0.05 was considered to be significant.

RESULTS

The mean weight of the caterpillars after 8 days of development showed that only the *S. saponaria* treatment caused significant differences, with a mean weight 72.41% smaller (Table 1). However, after 14 days of development, the caterpillars presented a significant reduction in the means weights for both, *T. esculenta* (19.06%) and *S. saponaria* (70.47%) treatments, when compared to the control. The results showed that the larval viability was significantly reduced in both the aqueous seeds-extract treatments. But, there were no significant differences in the mean duration of the larval period of *S. frugiperda* for both the treatments. The pupal weight was not significantly different (Table 2). During the pupal period, effects that caused deformities or mortality for the insect did not occur. There were no significant

differences for viability and duration of the pupal period either.

Table 1 - Weight, duration of larval period and viability of *S. frugiperda* caterpillars fed maize leaves treated with *T. esculenta* and *S. saponaria* aqueous seeds-extracts (1% w/v). Weight (mg) at the eighth[1] and fourteenth[2] days of development.

		Larval stage			
Treatment	n	Weight (mg)[1]	Weight (mg)[2]	Period (days)	Viability (%)
Control	139	13.56±1.2 a	293.45±15.42 a	18.3±0.85 a	88.7±3.62 a
T. esculenta	129	11.18±1.05 a	237.50±10.7 b	19.17±0.91 a	73.29±6.58 b
S. saponaria	136	3.74±0.48 b	86.65±8.39 c	21.68±1.06 a	36.85±2.23 c

[a]Means (± EP) followed by the same letter, in the column, are not significantly different (P≤0.05), according to Tukey's test.

Table 2 - Weight, duration of pupal period and viability of *S. frugiperda* pupae originated from caterpillars fed maize leaves treated with *T. esculenta* and *S. saponaria* aqueous seeds-extracts (1% w/v). Weight (mg) at the 24 h of development.

		Pupal stage		
Treatment	n	Weight (mg)	Period (days)	Viability (%)
Control	98	198.32±5.25 a	10.6±0.18 a	97±1.73 a
T. esculenta	62	203.05±5.72 a	11±0.23 a	91.94±2.88 a
S. saponaria	23	217.42±10.2 a	10±0.34 a	91.3±3.37 a

[a]Means (± EP) followed by the same letter, in the column, are not significantly different (P≤0.05), according to Tukey's test.

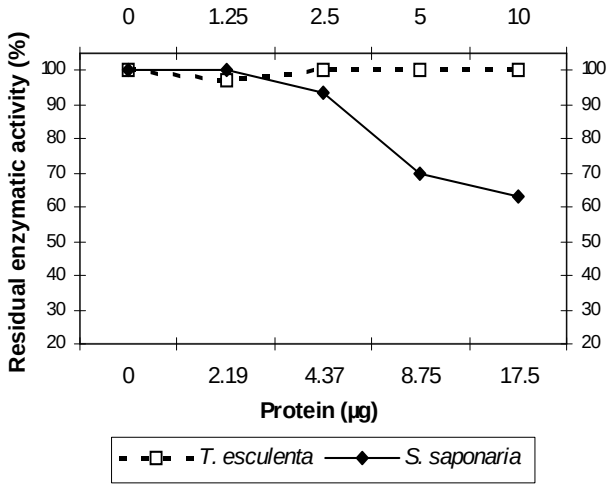

Figure 1 - Anti-trypsin activity (bovine tripsin), samples of serial dilutions, of the *T. esculenta* (10 µg protein) and *S. saponaria* (17.5 µg protein) aqueous seeds-extracts (1% w/v)

The anti-trypsin action of the aqueous extracts of the seed were evaluated in relation to enzymatic activity of the bovine trypsin (Fig. 1). The results showed that *T. esculenta* did not inhibit the activity of the trypsin enzyme. On the other hand, *S. saponaria* inhibited the trypsin activity around 30% in the samples with concentration above 9.0 µg of proteins.

Through *in vitro* assays, the trypsin activity of the caterpillars midguts supernatants was evaluated (Fig. 2). Compared to the control, an increase of 6.8% in the trypsin activity of the caterpillars in the *T. esculenta* treatment was not significant. However, a significant reduction of 10.59% occurred in the caterpillars of *S. saponaria* treatment (Fig. 2).

The *in vitro* assays with fecal material of the caterpillars showed that *T. esculenta* treatment presented non-significant values for trypsin activity; but the caterpillars *S. saponaria* treatment, presented a significant reduction (34.37%) in same enzyme activity (Fig. 3).

In SDS-PAGE, 50 µg of proteins of the samples of feces of each treatment were applied. Figure 4 showed that the digestion products presented the same hydrolyzis patterns.

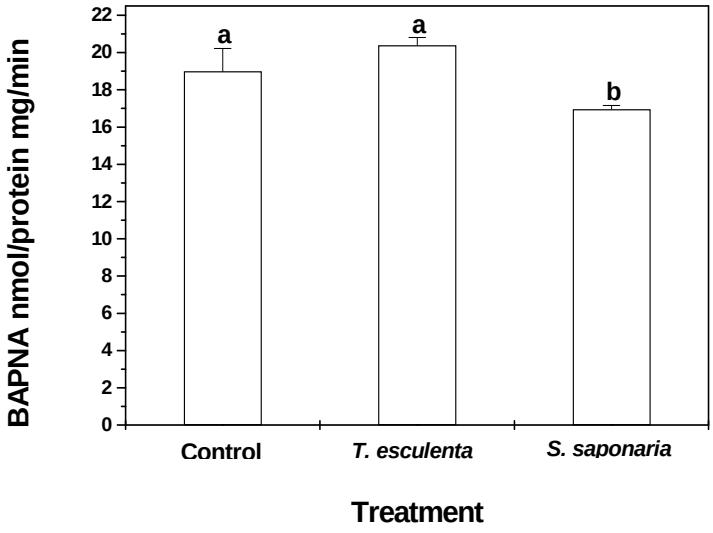

Figure 2 - Trypsin activity (n =150) of the *S. frugiperda* 4th instar caterpillars midgut (50 µg protein) fed maize leaves treated with *T. esculenta* and *S. saponaria* aqueous seeds-extracts (1% w/v). Bars show means±SE and the same letters indicate that there are no significant statistical differences (P≤0.05, Tukey's test)

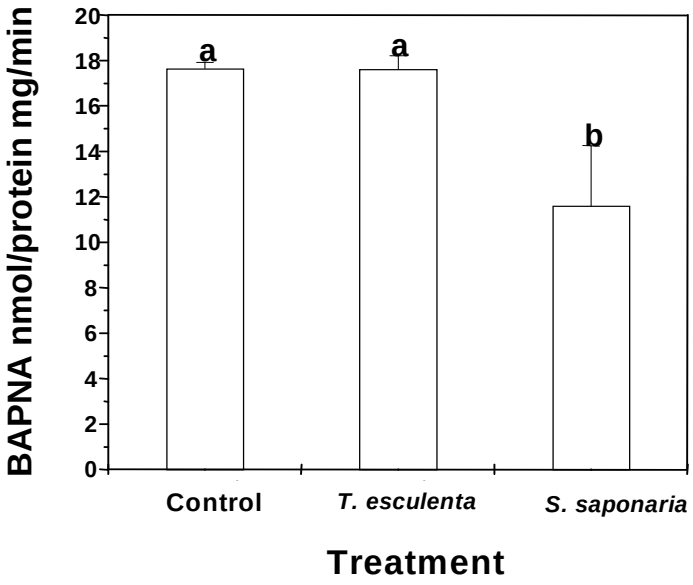

Figure 3 - Trypsin activity (n = 150) of the *S. frugiperda* caterpillars feces (50 μg protein) fed maize leaves treated with *T. esculenta* and *S. saponaria* aqueous seeds-extracts (1% w/v). Bars show means±SE and the same letters indicate that there are no significant statistical differences (P≤0.05, Tukey's test).

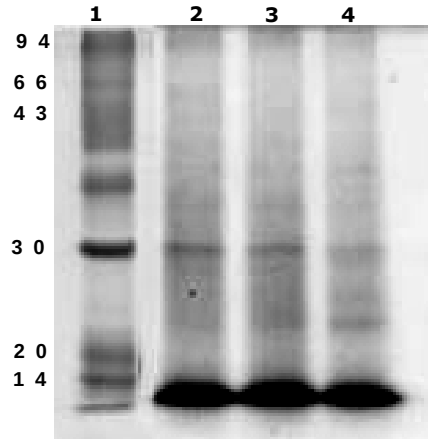

Figure 4 - SDS-PAGE (12.5%) of the *S. frugiperda* caterpillars feces. 1: molecular markers (kDa); 2: control caterpillars feces; 3 and 4: caterpillars feces fed with maize leaves treated with *T. esculenta* and *S. saponaria* aqueous seeds-extracts (1% w/v), respectively.

In the SDS-PAGE, where the proteins were under non-denaturing conditions, total enzymatic activity can be visualized through white areas against a dark blue background (Fig. 5). In the first group of samples (wells 1, 2 and 3), the total enzymatic activity of the midgut was observed, where a similarity for *T. esculenta* and a clear reduction in the proteolytic activity for *S. saponaria* in relation

to the control caterpillars occurred. Compared to the samples of the second group (wells 4,5 and 6), incubated with TLCK, an inhibition was observed in the proteolytic activity, showing the suppression of two very clear areas, indicating to be trypsin activity.

All the samples were also under non-denaturing conditions and visualization as described for electrophoresis above (Fig. 6). In the wells (1), (2) and (3), it was possible to observe the total enzymatic activity in the fecal material of the insects, with similarity for *T. esculenta* and smaller activity for *S. saponaria* caterpillars treatments, when compared to control caterpillars. Although the fecal material did not allow the visualization of defined white areas, it was still possible to see that in the samples, previously incubated with TLCK, applied in the wells (4), (5) and (6), a decrease in the total proteolytic intensity occurred, confirming the trypsin activity present in the insects feces .

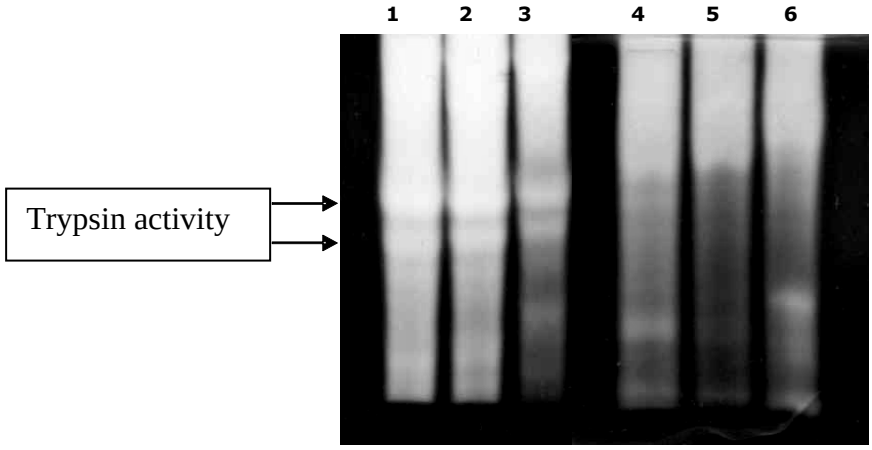

Figure 5 - Total enzymatic activity in SDS-PAGE (10%) containing gelatin 0.1% of the *S. frugiperda* caterpillars midgut (4th instar). 1: control caterpillars midgut; 2 and 3: caterpillars midgut of the *T. esculenta* and *S. saponaria* treatments, respectively; 4: control caterpillars midgut incubated with TLCK; 5 and 6: caterpillars midgut of the *T. esculenta* and *S. saponaria* incubated with TLCK, respectively.

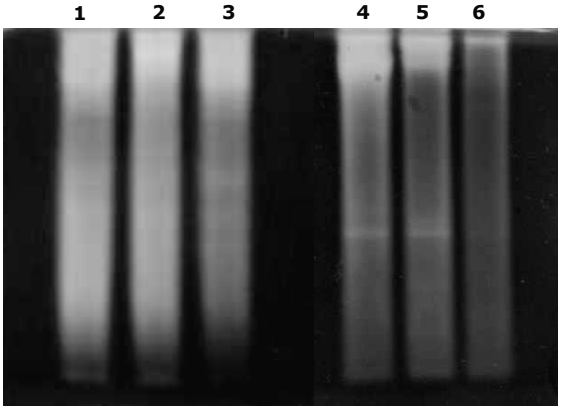

Figure 6 - Total enzymatic activity in SDS-PAGE (10%) containing gelatin 0.1% of the *S. frugiperda* caterpillars feces. 1: control caterpillars feces; 2 and 3: caterpillars feces of the *T. esculenta* and *S. saponaria* treatments, respectively; 4: Control caterpillars feces incubated with TLCK; 5 and 6: Caterpillars feces of the *T. esculenta* and *S. saponaria* treatments incubated with TLCK, respectively

DISCUSSION

The use of aqueous extracts of plants is a promising alternative for the control of phytophagous insects. These extracts facilitate the handling and its application, besides they can be an option of lower cost in relation to the studies chemical control. Many have been done to reduce the increasing use of the conventional insecticides (Amaugo and Emosairue, 2003; Maceda et al. 2003). The responsible use of the synthetic insecticides in addition to alternative strategies of control agricultural pests control, are tools that incorporate the integrated pest management (IPM) (Miresmailli, et. al. 2006), seeking benefits for the economy with possible environmental impact reduction, once human interference in the environment is inevitable. The caterpillars of the *T. esculenta* treatment only presented significant reduction in the mean weight in the second measurement (14 days of development). This indicate that the *T. esculenta* aqueous seeds extract acted more slowly in the physiology of the insect, becoming significant in the final instar of the larval period. The pupae from these caterpillars did not present significant differences in the mean weight either. In conclusion, the caterpillars of this treatment could surpass the action of any potentially toxic substance that was present in the *T. esculenta* aqueous extract . For the *S. saponaria* treatment, the caterpillars presented a significant reduction in the mean weight (first measurement), which was kept until the second weight measurement. These results showed could unit that even in low percentages, some caterpillars adapt to the toxic effects of the extract, but with physiological coats that damage the insects development, depending on its adaptation capacity. It is also important to emphasize that their pupae presented mean weight similar to the control pupae.

The significant reduction in the mean of the caterpillars treatments (*T. esculenta* and *S. saponaria*), can be related to a reduction in the digestive activity and in the use of the food ingested due to the deviation of part of the insect energy in the attempt of degrading or removing the toxic substances (Tanzubil and McCaffery, 1990) contained in these aqueous extracts.

The larval mortality obtained by both the treatments, *T. esculenta* (26.71%) and *S. saponaria* (63.15%), agreed with other studies with the same purpose. Bogorni and Vendramim (2005) tested leaves and twigs aqueous extract (1% w/v) of six species of *Trichilia* (Meliaceae) against *S. frugiperda* and it was observed that leaves of *T. pallens* and twigs of *T. palida* were the most promising in the control of the insect. Crude foliar extracts of species of *Aglaia* (Meliaceae), significantly reduced the larval growth of the polyphagous lepidopteran *Peridroma saucia* (Noctuidae) (Satasook et al., 1994). Aqueous leaf extracts of *Gnidia glauca* Gilg. and *Toddalia asiatica* Lam., produced larval mortality of more than 50% in the concentrations of 0.8 and 1.0% and reduction in the rate of food consumption and larval growth of the insect *Helicoverpa armigera* (Sundararajan and Kumuthakalavalli, 2001). Comparing rate mortality of both aqueous extracts, it was verified that *S. saponaria* presented higher percentage than *T. esculenta*. This might be because their seeds are very oily, originating an equally oily extract and this oil could act as an adjuvant, improving the fixation and distribution of the *S. saponaria* aqueous extract in the maize leaves offered as food for the caterpillars, promoting an increase in the insecticide action. Viana and Prates (2003) observed the same results in a field study, where they tested aqueous extracts (10% w/v) of *A. indica* leaves, with and without the addition of soy oil, against *S. frugiperda* caterpillars and the mortality was 45.6% for the aqueous extract *A. indica* and 71.7% for the aqueous extract *A. indica* + soy oil.

In the anti-trypsin assays the results showed that the aqueous extract of *T. esculenta* did not inhibit the activity of the bovine trypsin enzyme. On the other hand, the aqueous extract of *S. saponaria* inhibited the activity of the same enzyme, indicating that it contained protein (Haq et al., 2004) or secondary metabolites (Bernays, 1989; Juntheikki and Julkunen-Tiitto, 2000), responsible for the inhibition.

The non-significant increase in the trypsin enzyme activity observed in the caterpillars midgut of the *T. esculenta* treatment (6.8%) might have been an insect answer to compensate the harmful effect of the aqueous extract, once this represented a reduction of 19.6% in the mean weight of these

caterpillars. In the treatment with *S. saponaria* aqueous extract, the caterpillars presented a significant reduction (10.59%) of the midgut trypsin activity, which might have generated a physiological cost for the insect, since those caterpillars presented a reduction around 70% in the medium weight.

Through the assays using the fecal material of the insects, it was verified that there was no significant difference in the trypsin activity between the caterpillars of the treatments control and *T. esculenta*, but for the treatment *S. saponaria* the caterpillars presented a significant reduction of 34.37%. These results indicated that the intestinal epithelium of the insect was not broken or damaged by the action of the toxic substances present in the extracts that could provoke an overflowing of enzymes onto the feces.

SDS-PAGE showed that the products of digestion of the caterpillars presented the same hydrolyzis pattern in all the treatments, even for the caterpillars of the treatment *S. saponaria* that presented the largest harmful effects. So, these caterpillars, even at the cost of a lower trypsin activity and lower mean weight, could maintain a pattern similar to the control caterpillars.

The pattern of total proteolytic activity of the caterpillars midgut of all of the treatments was obtained through SDS-PAGE with gelatin 0.1% and confirmed the results of the *in vitro* assays of the trypsin activity in the midgut.

The total proteolytic activity in the fecal material of the insects, obtained with SDS-PAGE gelatin 0.1%, showed that although the material type did not allow a definition of the areas of enzymatic activity, it showed a similarity for the caterpillars of the treatment *T. esculenta* and a reduction for the *S. saponaria* treatment when compared to the profile of the control caterpillars. When the samples were incubated with the inhibitor TLCK, it was confirmed that the product of digestion of the caterpillars of all the treatments had contact with the trypsin enzyme, observed by the decrease of the proteolytic activity. The results obtained with the *in vitro* trypsin assays suggested that the insect did not have intestinal epithelium broken or damaged to interrupt the process of enzymes cycle that usually occurred in the insect midgut (Terra and Ferreira, 1994). So, it did not provoke an increase of enzymatic activity in the feces that it would indicate an overflowing.

The results showed that the caterpillars of the treatment with *S. saponaria* presented the largest damage effects (decrease in the mean weight, increase in the mortality and smaller trypsin activity). The recovery by these caterpillars in the final instars, originating pupae with same mean weight of the control caterpillars, was surely achieved at the cost of a metabolic damage.

Understanding the mechanism of action of the aqueous extracts on the insect, important to would be understand. It is also important to search insecticide molecules to generate new synthetic products and to obtain natural insecticides for direct use, to produce commercial formulation (Abudulai et al., 2003) or that can be expressed in plants acting as molecular defenses against herbivores insects (Peumans and Van Damme, 1998).

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RESUMO

Este estudo procurou avaliar o potencial inseticida dos extratos aquosos de sementes de *Talisia esculenta* (St. Hil.) Radlk (Pitombal) e *Sapindus saponaria* L. (Saboneteira), ambas da família Sapindaceae, sobre *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), importante praga da lavoura de milho. As folhas de milho foram imersas nos tratamentos com extratos aquosos (1% p/v) e oferecidas como alimento para as lagartas. Os resultados do período larval tais como mortalidade e peso médio foram respectivamente: para *S. saponaria* 63,15%, 86,65 mg; para *T. esculenta* 26,71%, 237,50 mg e para o controle 11,3%, 293,45 mg. A eletroforese com gelatina 0,1% mostrou duas regiões brancas muito nítidas (atividade tríptica) no intestino médio das lagartas de todos os tratamentos. Somente as lagartas do tratamento *S. saponaria*, apresentaram diferenças significativas, com uma atividade tríptica 10,59% menor.

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