



Morphological analysis of the alimentary canal of *Plutella xylostella* (Linnaeus, 1758) (Lepidoptera: Plutellidae) submitted to microencapsulated formulation of *Annona muricata* L. (Annonaceae)

Leonara Evangelista de Figueiroa¹ Priscylla Costa Dantas¹ Ellen Carine Neves Valente¹
Alice Maria Nascimento Araújo¹ José Pedro da Silva² Roseane Cristina Predes Trindade^{1*}

¹Universidade Federal de Alagoas (UFAL), Campus de Engenharias e Ciências Agrárias, 57100-000, Rio Largo, AL, Brasil. E-mail: rcpt@ceca.ufal.br.

*Corresponding author.

²Instituto Federal de Alagoas, Murici, AL, Brasil.

ABSTRACT: This study obtained information about the external and internal morphology of the alimentary canal of the main pest of brassica, *Plutella xylostella* L. (Lepidoptera: Plutellidae), when it is exposed to a microencapsulated formulation of the seed extract of *Annona muricata* L. (Annonaceae). The toxicity of the microencapsulated formulation of the caterpillars fed for 12, 24, and 48 hours plus the control was evaluated to verify and evaluate external and internal morphological changes caused by the extract that may have killed the caterpillars. After the assays, the alimentary canal of the caterpillars was dissected, fixed, dehydrated, blocked, cut, and colored. Then, histological slides were produced and studied under light microscopy and scanning microscopy (SEM). The microencapsulated formulation of *A. muricata* caused irreversible damage to the alimentary canal of *P. xylostella*, with disorganization, cell stratification, and modifications in the morphology of the cells considered essential for survival. SEM verified that the peritrophic membrane and the muscular layer remained intact during all the assays. The effects were more severe with more hours of exposure. These results demonstrated that after contact by ingestion, the insect dies due to intoxication and cellular disorganization, inhibiting its ability to continue feeding.

Key words: Soursop, diamondback moth, light microscopy, scanning electron microscopy (SEM).

Análise morfológica do canal alimentar de *Plutella xylostella* (Linnaeus, 1758) (Lepidoptera: Plutellidae) submetida à formulação microencapsulada de *Annona muricata* L. (Annonaceae)

RESUMO: O objetivo deste estudo foi obter informações sobre a morfologia externa e interna do canal alimentar da principal praga das brássicas, *Plutella xylostella* L. (Lepidoptera: Plutellidae), quando exposta a uma formulação microencapsulada do extrato da semente de *Annona muricata* L. (Annonaceae). A toxicidade da formulação microencapsulada das lagartas alimentadas por 12, 24 e 48 horas mais o controle foi avaliada para verificar alterações morfológicas externas e internas causadas pelo extrato que possam ter causado a morte das lagartas. Após os ensaios, o canal alimentar das lagartas foi dissecado, fixado, desidratado, bloqueado, cortado e colorido. Em seguida, lâminas histológicas foram produzidas e estudadas em microscopia óptica e microscopia de varredura (MEV). A formulação microencapsulada de *A. muricata*, causou danos irreversíveis ao canal alimentar de *P. xylostella*, com desorganização, estratificação celular e modificações na morfologia das células consideradas essenciais para a sobrevivência. Na análise por MEV, verificou-se que a membrana peritrófica e a camada muscular permaneceram intactas durante todos os ensaios. Os efeitos foram mais graves com mais horas de exposição. Esses resultados demonstraram que após contato por ingestão, o inseto morre por intoxicação e desorganização celular, inibindo sua capacidade de continuar se alimentando.

Palavras-chave: graviola, traça-das-crucíferas, microscópio óptico, microscopia eletrônica de varredura.

INTRODUCTION

The search for active botanical compounds against pests has intensified due to their possible use in plant protection as an alternative method for the conventionally used synthetic insecticides as well as their low impact on ecosystems (MEDHINI et al., 2012; MARQUES et al., 2016; ALVES et al., 2018). The Annonaceae family is one of the most promising (ISMAN & SEFFRIN, 2014) because it produces phenolic compounds, alkaloids, and terpenes (CHOWAŃSKI et al., 2016; MARQUES et al., 2016), but mostly because of the acetogenins,

which are the bioactive constituents reported in specific genera of annonaceae (MIAO et al., 2016). The microencapsulated formulation of the ethanol extract of *Annona muricata* L. (Annonaceae) was demonstrated to be an excellent and promising alternative (MACIEL et al., 2019) in the search for this more gradual, effective, and selective control for both *Plutella xylostella* L. (Lepidoptera: Plutellidae) (GOMES et al., 2016).

Substances derived from the secondary metabolism of plants are very promising for their influence on various biochemical components, such as proteins, carbohydrates, and lipids within the

body of insects. These substances can alter their internal metabolism and reduce activity or cause mortality of the insect (MEDHINI et al., 2012), due to the reduction in the levels of proteins, lipids and total carbohydrates present in the insect's body. This reduction may be due to the increased manipulation of proteins to detoxify the active ingredients present in plant extracts, which can affect the nutritional status, causing problems in the insect's development (ROYA & SENDI, 2010; MEDHINI et al., 2012) which makes annonaceae species very interesting for the development of bioinsecticides.

The mode of action of the active constituents of plant insecticides has not been well explored, especially in relation to ingestion. Studies are needed to elucidate what occurs in the digestive system of insects upon contact with these extracts. Thus, this study described the morphology of the alimentary canal of *P. xylostella* submitted to treatment with a microencapsulated formulation of the ethanol extract of *A. muricata* in lethal and sub-lethal concentrations for 12, 24, and 48 hours after ingestion, to assess impairment at the cellular level in the digestive system.

MATERIALS AND METHODS

Rearing of Plutella xylostella

The colony of *P. xylostella* was maintained at the Laboratory of Entomology: Alternative Pest Control (LECAP), Center of Agrarian Sciences at the Federal University of Alagoas, Brazil under conditions of 25 ± 2 °C, of $67 \pm 2\%$ relative humidity, and 12 h photophase according to the methodology of BARROS et al. (2012), using cabbage leaves, *Brassica oleraceae* var. *acephala* cultivar Georgia grown in beds filled with a mixture of black soil, manure, and filter cake at the same proportions.

Preparing of extracts and Microencapsulation of the ethanol extract of Annona muricata

The soursop seed ethanol extract was prepared and processed at the Laboratory of Natural Resources Research of the Institute of Chemistry and Biotechnology, Universidade Federal de Alagoas/UFAL, according to the methodology adopted by TRINDADE et al. (2018).

The microencapsulated formulation of the ethanol extract of *A. muricata* was carried out at the Technology Laboratory to Control Medicines at UFAL, through spray drying using the Buchi® Mini Spray Dryer B-290 (Switzerland), with a 1-mm atomizer nozzle, at an inlet temperature of 200 °C

and a temperature of 100 °C, feed rate of 10 ml/min., according to the methodology of MACIEL et al. (2019) under patent deposit BR 10 2018 008313 9.

Determination of LC_{50} and LC_{99} of the microencapsulated formulation of Annona muricata ethanol extract to Plutella xylostella

For lethality studies, the lower (5% mortality) and upper (95% mortality) limits were determined to be submitted to the Bliss formula (1934): $q = (an \div a1) 1/n + 1$, where q = geometric progression ratio (pg); n = number of concentrations to be extrapolated; an and $a1$ = upper and lower limits of pg, respectively. The concentrations tested for the microencapsulated formulation of the ethanolic extract were: 0.82; 0.5; 0.113; 0.0461 and 0.016 mL/L, which were solubilized in distilled water with the addition of the emulsifier Tween 80 (0.05%). The control treatment was with distilled water plus Tween 80 (0.05%). A separate test was carried out with the formulation containing only the polymers, without the *A. muricata* extract.

Cabbage leaf discs measuring 8 cm in diameter were sprayed with the different treatments, using a Potter tower (POTTER, 1952), at a pressure of 5 psi/in² using a spray volume of 2.3 mL, which corresponds to a deposit of 1.9 ± 0.37 mg/cm², in accordance with recommendations by IOBC/WPRS (REIS et al., 1998).

Ten newly hatched caterpillars were transferred to each disc, with 5 replications per treatment. The assessment of larval mortality began on the third day of the experiment. Lethal concentrations (LC) were estimated by Probit using the SAS statistical program (SAS Institute, 2003).

Toxicity tests of the alimentary canal of Plutella xylostella

For a detailed study of the morphological structure of the alimentary canal, initially 30 fourth-instar caterpillars were dissected, and the intestines were packed in Eppendorf tubes with Zamboni fixing solution (STEFANINI et al., 1967). Subsequently, the samples were investigated for description of morphology.

In the toxicity bioassays, the same procedures used to estimate the LCs were performed, where 10 fourth-instar caterpillars fed on the cabbage leaves were treated with the LC_{50} and LC_{99} of the microencapsulated formulation of *A. muricata*. Dissections were performed after 12, 24, and 48 hours. For the control treatment, the caterpillars were fed only on cabbage leaves treated with distilled

water plus Tween 80 (0.05%). Samples from the alimentary canal were standardized for SEM and light microscopy studies.

After each determined test period, all caterpillars were dissected by the dorsal region and the alimentary canal removed and stored in tubes containing fixing solution for subsequent microscopic analyses.

Scanning Microscopy

Sample preparation was performed at the Devices and Nanostructures Laboratory at the Universidade Federal de Pernambuco (UFPE).

After dissection, the samples of the insects' alimentary canal were immersed in a fixative solution (glutaraldehyde 7%, pH 7.2) and submitted to the following protocol: washing in distilled water (three times for 5 min); post-fixation in osmium tetroxide 1% in distilled water (30 min); dehydration in increasing series of ethyl alcohol; and drying in a critical point device CPD 020 (Balzer Union). The samples obtained were mounted on aluminum stubs, with double-sided carbon tape, placed on an aluminum foil film, covered with carbon and observed in SEM Zeiss LEO EVO 40XVP to analyze the structures of the alimentary canal.

Production of histological slides and light microscopy

After fixation, the samples were dehydrated in a series of increasing alcoholic concentration at 30, 50, 70, and 90% (15 min each) and then in two 10-min baths each in 99% ethyl alcohol. Then the samples were embedded in Histoessin (Leica) and sectioned 5 μ m thick with stainless steel razors in a Leica RM 2155 microtome, stained with hematoxylin (10 min) and eosin (30 sec), and then analyzed and photographed with a light microscope (Zeiss AxioCam ERc5s) at the CECA-UFAL microscopy laboratory.

RESULTS AND DISCUSSION

The estimated lethal concentrations for microencapsulated ethanolic extract of soursop for LC_{50} (IC 95%) were 0.15mL/L (0.12-0.20mL/L) and for CL_{99} (IC 95%) were 2.15mL/L (1.50-5.12mL/L) with slope ($\pm$ EP) of 1.93 ($\pm$ 0.18) and ($\chi^2=6.24$; $df=3$).

The alimentary canal of *P. xylostella* larvae is an elongated tube with little morphological differentiation from mouth to anus. The anterior region is narrower, with dilation in the middle region, for greater stretching capacity during feeding (Figures 1A and 1B).

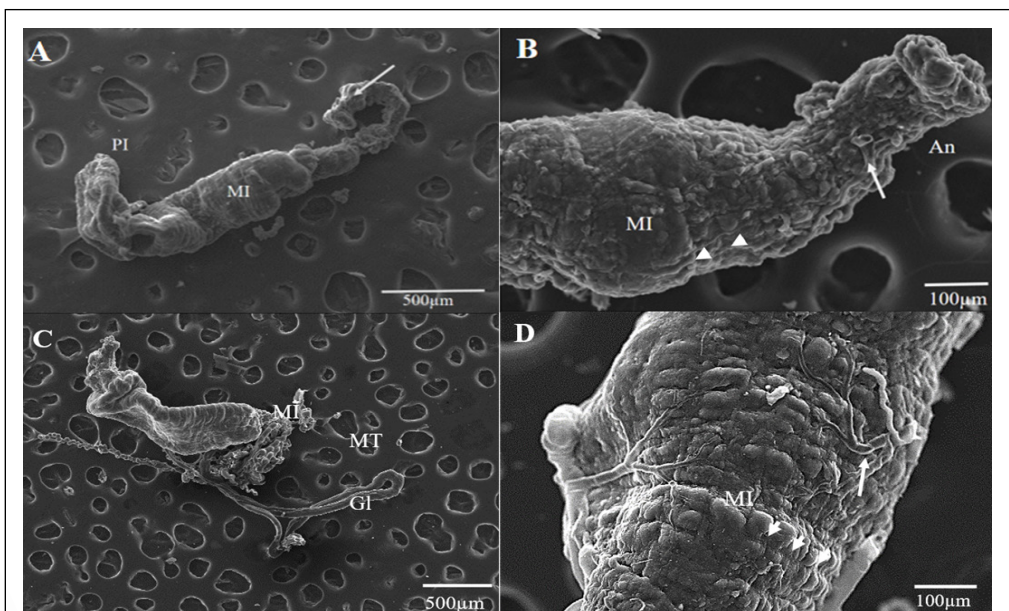


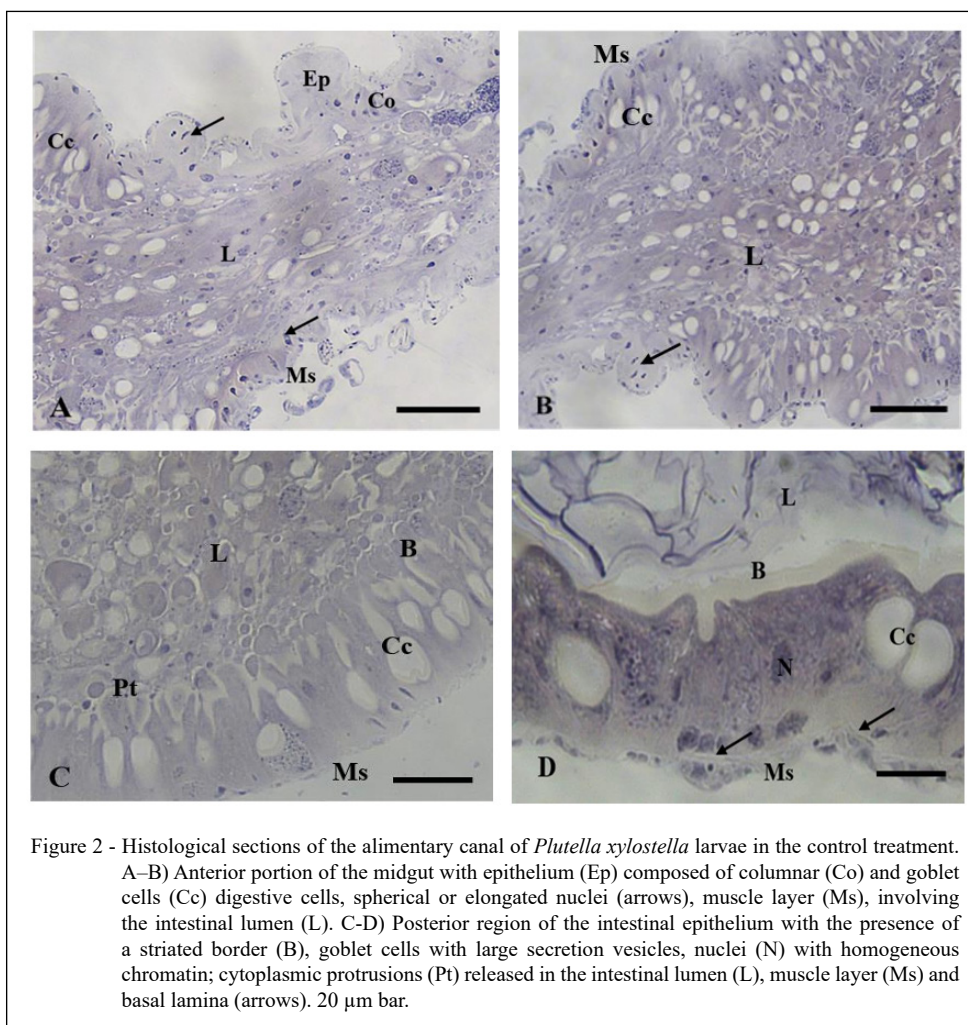
Figure 1 - Scanning electron microscopy for the morphological description of the alimentary canal of *Plutella xylostella* A) Divided into anterior intestine (arrow), medium intestine (MI), posterior intestine (PI). B) Anterior intestine (An), medium intestine (MI), tracheal tube (long arrow), longitudinal muscles (arrowhead). C) Food canal with Malpighi tubules (MT) between the median and posterior portion of the intestine (MI), presence of the accessory gland (GI). D) Alimentary canal with detail of the tracheal trunks branches (arrows) involving the tissue of the medium intestine (MI), circular muscular layer (arrowheads).

The posterior intestine is smaller, and at the end, it has a slight dilation where the Malpighian tubules are (Figure 1C). The arrangement of the musculature throughout the canal follows a uniform pattern, as described in the literature for Lepidoptera (CHAPMAN, 1998). The epithelial layer has a heterogeneous surface with cellular protuberances throughout the intestine and is lined by two well-developed muscle layers, the innermost supporting the circular and thin epithelium (Figure 1D), and the outermost is longitudinal (Figure 1B). Tracheal branches occur along the length of the intestine (Figures 1B and 1D).

The larvae treated with the microencapsulated formulation of *A. muricata* extract exhibited no morphological changes in the anterior, middle, or posterior intestine regions. The external epithelial structure was maintained

with its original arrangement, with no rupture or disorganization of muscle tissue. This study of the structures of the alimentary canal of *P. xylostella* examined by SEM was the first description, as it was different from the histological study carried out by RIBEIRO et al. (2013).

In the histological analysis of the control treatment, the characteristics of the alimentary canal were similar in both the anterior and posterior regions (Figures 2A and 2D). The morphological organization exhibits simple epithelium, composed of layers of columnar and goblet-type digestive cells (Figures 2A and 2B). Goblet cells are located throughout the intestinal epithelium, interspersed by columnar cells, and are characterized by the presence of an extracellular cavity that partially connects to the lumen, as described by CAVALCANTE & CRUZ-LANDIM (1999) and PINHEIRO et al.



(2003). In this cavity, secretion vesicles accumulate in the supranuclear cytoplasm, with its nucleus compressed in the cell base. The microscopy images show the presence of the striated border (Figures 2C and 2D) and the peritrophic membrane surrounding the lumen.

The peritrophic membrane plays an important role for insects. In addition to compartmentalizing digestion, it serves as a physical barrier against mechanical damage and pathogenic microorganisms (KONNO & MITSUHASHI, 2019). These characteristics are similar to those described by RIBEIRO et al. (2013) in *P. xylostella* larvae and CORREIA et al. (2009) in *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae) larvae. According to SOUSA et al. (2009), these characteristics are commonly found in larval stages of Lepidoptera.

The epithelium rests on the basal lamina (Figure 2D) where it is supported by two muscular layers, one circular and one longitudinal (Figures 2A–2D). Digestive cells have a spherical or elongated nucleus with uncondensed chromatin and cytoplasm with some granules (Figures 2A and 2B). Digestive cells have a striated border on their apical surface and small vacuoles are found in the cytoplasm of digestive cells (Figures 2C and 2D).

Throughout the epithelial extension, cytoplasmic protrusions with different sizes and shapes occur (Figure 2C) that are eliminated by the cells and released in the lumen region. PINHEIRO et al. (2003) stated that protrusions are the result of the process of apocrine secretion of digestive enzymes into the lumen. Cytoplasmic protrusions are mainly related to degeneration processes, serving to eliminate cellular components (DE PRIESTER, 1971). The alimentary canal morphology of *P. xylostella* submitted to sublethal concentration of the microencapsulated formulation of *A. muricata* extract demonstrated changes in the intestinal epithelium beginning at the 12-h toxicity assessment, where disorganization and cell stratification were identified (Figures 3A and 3B). The changes intensified after 24 hours (Figures 3C and 3D), where cell organization of the epithelium occurred with changes in the structure of the striated border. Changes in the epithelium were also verified in a study of the alimentary canal of *Tuta absoluta* (Meyrick, 1917) (Lepidoptera: Gelechiidae) with a methanol extract of *Annona mucosa* Jacq. (Annonaceae) (BASTOS et al., 2018).

Modifications were observed in the striated border with changes in the goblet cell morphology

(Figures 3C and 3D) located throughout the midgut epithelium and interspersed by columnar cells. The main function of the goblet cells is to transport potassium from the hemolymph to the lumen, maintaining ionic homeostasis and cooperating with columnar cells in the absorption of metabolites (BRADLEY, 2009).

Cellular changes in the epithelium and goblet and columnar cell morphology were also observed in *Dione juno juno* Cramer larvae, 1779 (Lepidoptera: Nymphalidae) treated with extract of neem *Azadirachta indica* L. (Meliaceae) (MORDUE (LUNTZ) & NISBET, 2000). Studies claim that azadirachtin, the active ingredient in neem, is absorbed by digestive cells, which inhibits cell division. These effects also corroborate with our study using microencapsulated formulation of *A. muricata*, since it is known that acetogenins, the main active ingredient of *Annona* species, effects the mitochondria with cellular alterations (ISMAN & SEFFRIN, 2014) and contributes to this behavior in the alimentary canal.

Cytoplasmic protrusions were observed in caterpillars before treatment with the microencapsulated formulation, but the protrusions increased after treatment with the extract (Figure 2C). Similar results have been identified in *Spodoptera litura* (Fabricius, 1775) (Lepidoptera: Noctuidae) caterpillars after ingestion of *Bacillus thuringiensis* delta toxin (PANDEY et al., 2009).

The dense granules in the cytoplasm and altered goblet cells were reported, but the peritrophic membrane remained intact surrounding the alimentary bolus in the lumen (Figures 3E and 3F).

JARIAL (2005) observed in *Cenocorixa bifida* (Hungerford, 1926) (Hemiptera: Corixidae) exocytosis of secretion granules, as well as cytoplasmic protrusions in the process of enzymatic secretion and cell degeneration. Cytoplasmic protrusions are mainly related to degeneration processes, serving to eliminate cellular components (DE PRIESTER, 1971). The evaluation of lethal treatment (LC₉₉) demonstrated evident cellular disorganization (Figures 4A and 4B).

At 48 hours after ingestion of the microencapsulated formulation of *A. muricata* extract, the peritrophic membrane remained intact. This integrity of the peritrophic membrane was also verified in the study of the alimentary canal of *S. frugiperda* fed with the neem formulation (neemseto®) (CORREIA et al., 2009).

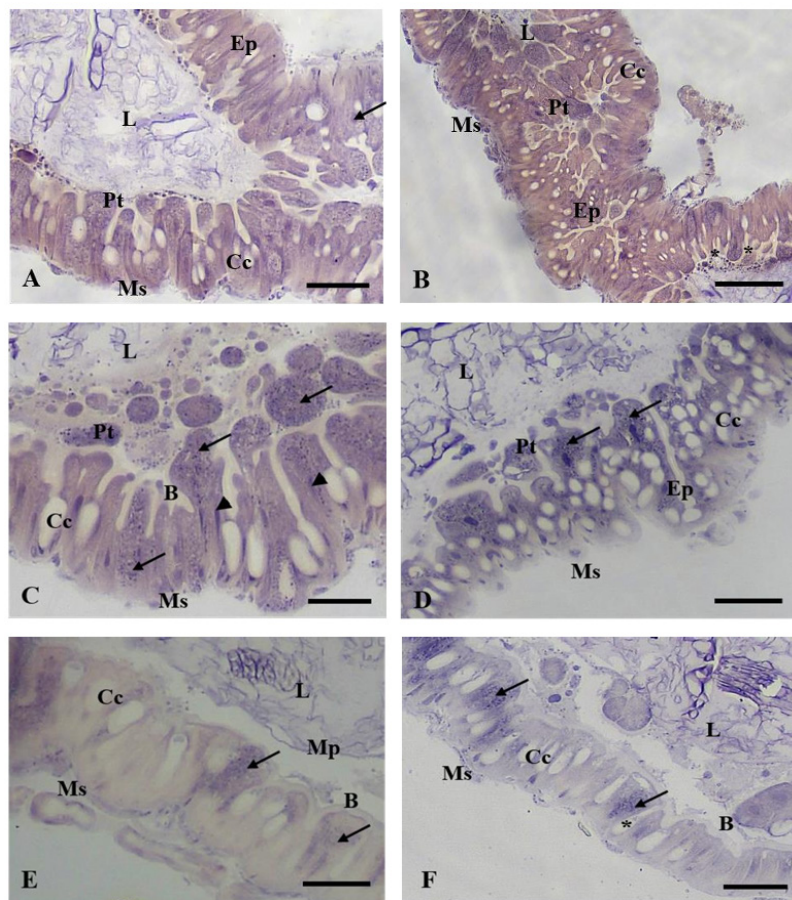


Figure 3 - Light micrographs of the alimentary canal of *Plutella xylostella* feed microencapsulated extract of *Annona muricata* in the concentration LC_{50} . A – B) Intestinal epithelium after 12 h of exposure to the extract, presence of goblet cells (Cc), cytoplasmic protrusions (Pt), muscle layer (Ms), disorganization and prominent stratification of the epithelium (Ep), nucleus (arrow), modification of striated edge structure (asterisks), lumen (L). C – D) Intestinal epithelium after 24 h of exposure to the extract, demonstrating cellular disorganization, with apocrine secretions (Ep) and secretory vesicles (Pt) expelled from the epithelium visible in the lumen (L); presence of dense granules in the cytoplasm (long arrows), goblet cells (Cc) with altered morphology, muscle layer (Ms), irregular nucleus (arrowhead). E – F) Intestinal epithelium after 48 hours of exposure to the extract, with the presence of an intact peritrophic membrane (Mp) surrounding the alimentary bolus present in the lumen (L), muscle layer (Ms), goblet cells (Cc) with secretion (asterisk) (F), striated border (B), dense granules in the cytoplasm (long arrow). A, C, F, 20 μ m bar; B, D, E, 40 μ m bar.

Intense apocrine secretion was observed, and cells exhibited irregularly sized nuclei and goblet cells in the stratification process (Figures 4E and 4F). According to FIAZ et al. (2018), changes in the midgut cells of *Anticarsia gemmatilis* (Hübner, 1818) (Lepidoptera: Noctuidae) induced by squamocin, an acetogenin, caused damage

to microvilli and intense vacuolization of the cytoplasm with autophagy in cells of the midgut, causing cellular disorganization, corroborating with the results found in this research. The same pattern of concentration-dependent toxicity occurred in *Aedes (Stegomyia) aegypti* (Linnaeus, 1762) larvae exposed to squamocin (COSTA et al., 2017).

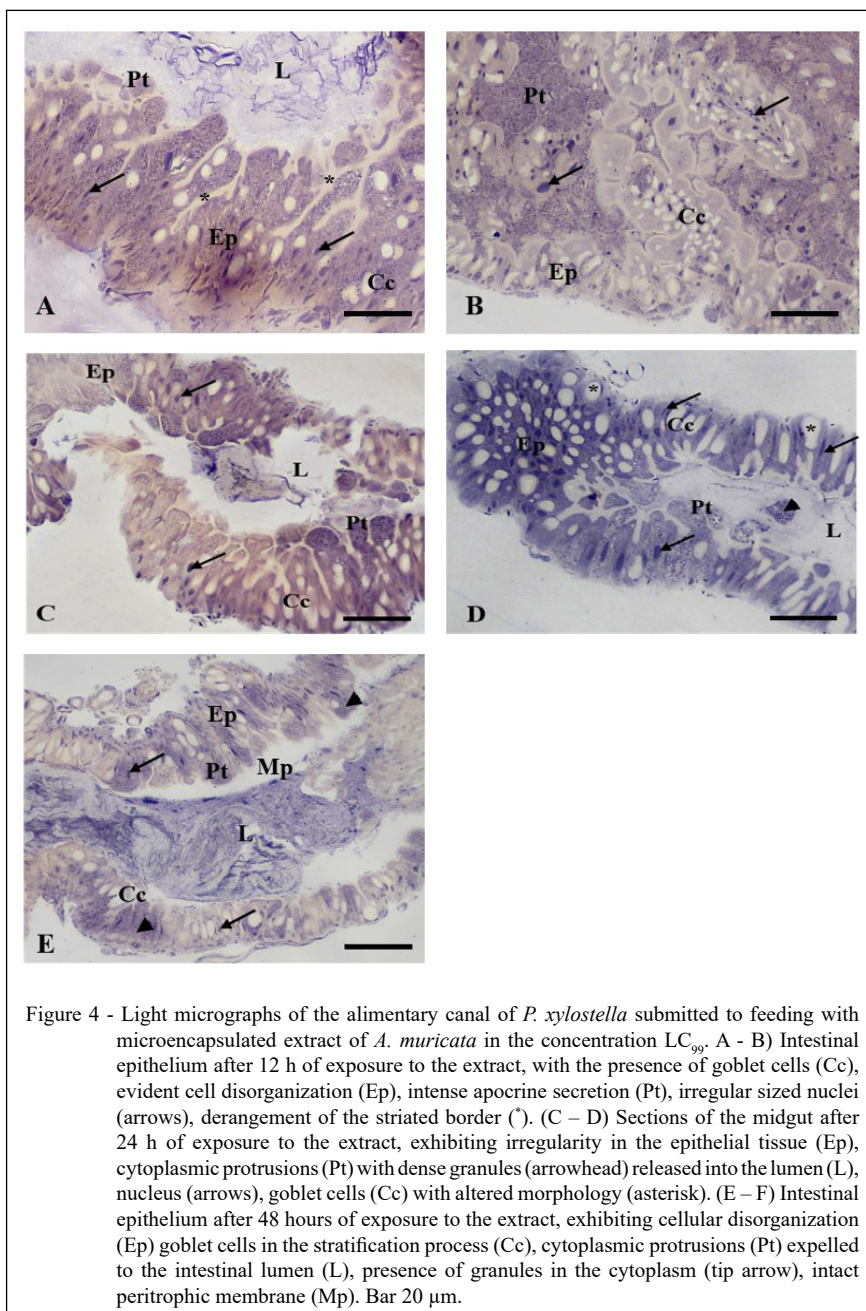


Figure 4 - Light micrographs of the alimentary canal of *P. xylostella* submitted to feeding with microencapsulated extract of *A. muricata* in the concentration LC_{99} . A - B) Intestinal epithelium after 12 h of exposure to the extract, with the presence of goblet cells (Cc), evident cell disorganization (Ep), intense apocrine secretion (Pt), irregular sized nuclei (arrows), derangement of the striated border (*). (C - D) Sections of the midgut after 24 h of exposure to the extract, exhibiting irregularity in the epithelial tissue (Ep), cytoplasmic protrusions (Pt) with dense granules (arrowhead) released into the lumen (L), nucleus (arrows), goblet cells (Cc) with altered morphology (asterisk). (E - F) Intestinal epithelium after 48 hours of exposure to the extract, exhibiting cellular disorganization (Ep) goblet cells in the stratification process (Cc), cytoplasmic protrusions (Pt) expelled to the intestinal lumen (L), presence of granules in the cytoplasm (tip arrow), intact peritrophic membrane (Mp). Bar 20 μ m.

CONCLUSION

There was no change in the external anatomy of the *P. xylostella* alimentary canal assessed by SEM, at any concentration tested. However, the microencapsulated formulation of *A. muricata* caused disorganization in the epithelium of the alimentary canal, modifying the shape and integrity of the cells of *P. xylostella* caterpillars. The increasing the dose and exposure time enhanced the cellular changes in

the alimentary canal of *P. xylostella* caterpillars fed with kale leaves treated with the microencapsulated formulation of *A. muricata*, demonstrated to be a promising alternative for use in controlling this pest.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved of the final version.

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