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Insecticidal activity and changes in midgut histology of the generalist herbivore, *Spodoptera litura* F. (Lepidoptera: Noctuidae) in response to seed extract of *Annona squamosa* Linn.

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Abstract
The tobacco caterpillar, *Spodoptera litura* F., is one of the most devastating, cosmopolitan polyphagous pests affecting major crops that significantly impact agricultural productivity. The present study aimed to evaluate the toxicological effect of seed extract of the medicinal plant, *Annona squamosa* L. and the histological effect on the midgut of *Spodoptera litura* F., under laboratory conditions. The crude methanolic extract of *A. squamosa* seed was tested against the third instar larvae of *S. litura* at five different concentrations *viz.,* 0.5%, 1.0%, 1.5%, 2.0% and 2.5 % by leaf dip bioassay method. The seed extract exhibited larval mortality of 96.67%, 83.37 %,70%, 53.33% and 40 % at the concentrations 2.5 %, 2.0%, 1.5%, 1.0% and 0.5%, respectively.The results indicated that the response of larval mortality to the seed extract was dose-dependent. The dose of 0.5% showed the lowest mortality (40.00%), while the dose of 2.5 % showed maximum larval mortality (96.67%). Hence they were subjected to histological analysis. The anatomical sectioning of *S. litura* larval midgut treated with 0.5% concentration showed disruption in the peritrophic membrane and striated border of epithelial cells. The midgut of larvae treated with 2.5 % concentration showed an irregular epithelium and high vacuolization in the cytoplasmic cells. The results indicated that methanol extract of *A. squamosa* seed extract has the ability to cause changes in the midgut region, thereby affecting the digestion and nutrient absorption of *S. litura* larvae, which will lead to a debilitating effect on the development of larvae. Based on the present study *A. squamosa* seed extract will be a potential biopesticide for managing *S. litura*.

Keywords: *Annona squamosa* seeds, Histology, Methanolic extract, Midgut, *Spodoptera litura*

INTRODUCTION

The tobacco caterpillar, *Spodoptera litura* F. (Noctuidae: Lepidoptera), is a polyphagous and devastating insect pest which is reported on 112 cultivated plants belonging to 40 families in tropical and subtropical areas causing monetary loss of 25.8-100 % in many economically important crops includes cotton, pulses and vegetable crops (Sahu *et al.*, 2020). Farmers have been using synthetic pesticides to manage *S. litura,* but this pest has developed resistance against most of the commonly used pesticides like indoxacarb, abamectin, fipronil (Ahmad *et al.,* 2008) and organophosphates, carbamates, pyrethroids (Armes *et al.*,

1997). There is a need for a holistic approach towards managing *S. litura,* including using botanicals to overcome these issues. Plants are an abundant source of compounds with insecticidal principles that can be employed in the development of environmentally safe alternatives for insect pest management (Souto *et al*., 2021).

Conventional pesticides negatively impact the environment compared to botanical insecticides, which are made primarily from compounds derived from plants. The custard apple, *Annona squamosa* Linn. (Magnoliales: Annonaceae), is cultivated in almost all tropical and subtropical countries, including India. It is a potential medicinal plant with a wide range of therapeutic and insecticidal applications. Owing to its antimicrobial, cytotoxic, antioxidant, anti-tumor, insecticidal and anthelmintic properties, it is traditionally used in herbal remedies and consumed as fruit (Mondal *et al.,* 2018). The Annonaceous plants possess acetogenins, a polyketide group which has the insecticidal properties and can be used in the management of lepidopteran (Hidalgo *et al*., 2018), coleopteran (Anita *et al.,* 2012), hemipteran (Lin *et al.,* 2009) and dipteran (Kamaraj *et al.,* 2011) pests.

The midgut region of insects is of endodermal origin and is responsible for digestion and nutrient absorption (Caccia *et al*., 2019). The midgut occupies a major share of space in the hemocoel and is an important part of the alimentary canal. Apart from that, it is also crucially involved in multiple physiological regulations, including metabolism, immune response, homeostasis of electrolytes, osmotic pressure, and circulation (Takeda, 2012). Consequently, disrupting any of these functions could provide a target and tactic for future insect pest management practices.

There are a variety of botanical insecticides encompassing toxicological properties which are exploited towards the management of the *S. litura* insect populations (Devanand and Rani, 2008; Chauhan and Mishra,2016) however, the seed extract of *A. squamosa* has been reported to be toxic against *Plutella xylostella* (Plutellidae: Lepidoptera) and *Trichoplusiani* (Noctuidae: Lepidoptera) infesting cabbage crop and less susceptible to its natural enemies, *Chrysoperla carnea* (Stephens) (Neuroptera: Lepidoptera) larvae and *Orius insidiosus* (Say) (Hemiptera : Anthocoridae) adults (Leatemia and Isman (2004). In this context, the toxicological and histological changes in *S. litura* exposed to a methanolic extract of *A. squamosa* seeds were evaluated to contribute to developing newer strategies for managing this insect pest.

MATERIALS AND METHODS

Preparation of the extract

Ripened fruits of *A. squamosa* were collected and the seeds from the pulp were separated and washed with tap water and shade dried for four weeks. The shade-dried seeds were ground and sieved with 40-60 mesh sieve to obtain uniform-sized particles. It was then weighed and stored in air-tight containers. About 860g of seed powder was collected from one kg of seeds. From the seed powder, extraction was done using methanol by following the maceration method (Azwanida, 2015), in which 10 g of sample (seed powder) was soaked in 100 ml of solvent in a conical flask and allowed to stand for three days with frequent agitation in a magnetic stirrer at 800 rpm. After extraction, the extracts were filtered and concentrated under a vacuum in a Rotary vacuum evaporator at 30°C to obtain the viscous concentrate (Patil, 2009).

Larval rearing

Larvae were mass cultured with the semi-synthetic diet (Urs and Subramanya, 1974) at the Department of Agricultural Entomology, Tamil Nadu Agricultural University, Coimbatore - Tamil Nadu, in the year 2022. The rearing tray and other materials used for rearing purposes were disinfected with 0.1% formaldehyde solution. The diet composition was as follows: Fraction A comprising of Chickpea flour-105g, Methyl para hydroxyl benzoate-2g, Sorbic acid-1g, Streptomycin sulphate - 0.25g, Fraction B with Agar-agar -12.75g and Fraction C containing Yeast-40g, Ascorbic acid – 3.25g, Multivitamin-2 capsules, Vitamin E-1g, Formaldehyde 10%-2ml, Distilled water-780ml. Fraction ‘A’ of the diet was mixed thoroughly in a blender with 390 ml of water for two minutes. Another 390 ml water was added with fraction ‘B’ and boiled. Then the fractions ‘A’ and ‘B’ were mixed in the blender for one minute. Finally, fraction ‘C’ was added to the admixture of ‘A’ and ‘B’ and mixed for one minute. At the end 1ml of 10% formaldehyde solution was added and thoroughly mixed. Then the diet was poured into the pre-sterilized container. The larvae that emerged from the egg mass were released using a camel hair brush (number 00) into the container having a semi-synthetic diet. Initially, the larvae were reared in bulk and from the third instar onwards, they were transferred to the sterilized plastic containers. Every day the diet was changed with a clean tray containing a fresh diet. The third-instar larvae of *S. litura* without malformations were utilized in the bioassays and histological analysis.

Bioassay

The third instar larvae of *S. litura* were used for the leaf dip bioassay method. Fresh castor leaves from castor plants maintained at the TNAU Orchard without any prior exposure to chemicals and free from insect infestation were used for the bioassay. The leaf discs of 4cm diameter were cut from the fresh castor leaves, washed with tap water, and allowed to shade dry free

of moisture. The leaf discs were dipped in the test solution 0.5%, 1.0%, 1.5%, 2.0%, and 2.5 % concentrations of methanolic extracts of *A. squamosa* seeds with control (methanol solvent). Ten pre-starved (4 hr) third instar larvae were introduced into the circular polystyrene container(90x40mm) containing the leaf discs and were covered with a cap having 40mm diameter of ventilation hole made with mesh. The bioassay container was lined with moist filter paper to maintain the turgidity of the leaf disc. The experiment was conducted in a completely randomized design and each treatment was replicated four times. The larval mortality was recorded at 24, 48 and 72 hours after treatment and moribund larvae were counted as dead after prodding with a soft camel hair brush. The midgut of the S. *litura* larvae treated (0.5 % and 2.5% concentration) and untreated were subjected to histological analysis.

Histological analysis

Preparation of *S. litura* midgut

The treated and untreated larvae were subjected to dissection to remove the larvae midgut region. In order to remove the blood and impurities from the midgut region NaCl 0.9% solution was used. After washing, the midgut was immersed directly into Davidson's alcohol formalin acetic acid fixative solution. For the preparation of one litre of fixative solution, 95% ethyl alcohol (330ml), 37-39% formaldehyde (220ml), glacial acetic acid (115ml) and tap water (335ml) were added. The midgut was dissected at 3-5mm thickness, and tissue processing was done. Dehydration of tissues was done with a series of ethyl alcohol prepared at 70%, 80%,90% and 100% for one hour for each concentration before clearing with xylene twice for one hour each, followed by impregnation of paraffin wax twice for each solution for 1-2 hours approximately. After processing, the tissues were placed in a tissue holder with melted wax. Orientation of tissues in melted paraffin which, when solidified, provided a firm medium for keeping all parts of the tissue intact when sections were cut. Cassettes were placed onto cold plate and when the wax solidified, the samples were removed from the molds and stored at room temperature until further microtome sectioning. LEICA 2125RTS manual rotatory microtome was used for taking sections with section thickness set to the appropriate level of 4-5µ (Bancroft and Gamble, 2008).

Staining process

For the staining of sections, the method given by Sheehan and Hrapchak (1980) was used. The microtome sections were stained with xylene twice for 3 mins each. The samples were then soaked with ethyl alcohol solution 100%, 90%, 80% and 60% each for 15 dips then with distilled water for 1-2min followed by Hematoxylin (H&E) stain for 1-2 min and was kept in running tap water 4-5min followed by phloxine/eosin for 1 min. The next step was soaking in 95%, 100% and 100% ethyl alcohol each for 2 mins, followed by dipping in xylene twice for 2 minutes each. After H&E staining, the organ preparation was done. A drop of D.P.X (mountant) was added to the cover slip and slides were inverted onto the cover slip. Care was taken to avoid air bubbles and to avoid touching the slide or cover slip, as it could damage the section. The observations were made with the help of LEICA DM 750 stereomicroscope with image capture using Las X software version 3.0. at 10x10 magnification.

Statistical analysis

Mortality data collected were subjected to arc-sine transformation and the transformed values were used for performing one-way ANOVA. For comparison of means, Tukey's Honestly Significance Difference (HSD) ($p<0.05$) test was performed. All the statistical analyses were performed with the IBM SPSS v. 20 statistical program.

RESULTS AND DISCUSSION

The toxicity of methanol extract obtained from *A. squamosa* tested against third-instar larvae of *S. litura* are presented in Table 1. The numbers of dead larvae were counted at 24, 48 and 72 hours after exposure to different doses of *A. squamosa* methanol extract (0.5, 1.0, 1.5, 2.0 and 2.5%). The corrected mortality was calculated using Abbott's formula (Abbott, 1925). In the current experiment, the least mortality rate of the third instar larva of *S. litura* was 40 per cent at the concentration of 0.5% seed extract whereas a maximum of 96.67 per cent mortality was recorded at the concentration of 2.5%. The larval mortality of *S. litura* treated with methanolic extract of *A. squamosa* seed started 24 hours after exposure. On the first day, the mortality of *S. litura* larva reached 46.67 % at 2.5 % concentration. At a concentration of 2.5 per cent, 60 % larval mortality was recorded 48 hours after exposure. Likewise, the leaf dip bioassay method was used to investigate the toxicity of acetone and methanol seed extracts of *A. squamosa* against 2nd and 4th instar larvae of *S. litura* at various doses (Mahmoud and Hassan,2022). The results demonstrated that the acetone extract was more efficient with LC_{50} values of 1.9, 1.7 and 1.1% on 8^{th}, 11^{th} and 14^{th} days of exposure, respectively, when compared to the methanol extract, where the LC_{50} values were 2.8, 2.4 and 1.6% on 8^{th}, 11^{th} and 14^{th} days of exposure respectively. Similarly, the effectiveness of crude aqueous extracts of eight different plant species was tested against *S. litura* larvae in controlled laboratory conditions at the concentrations of 25, 50, 75, and 100% alone and in conjunction with *Bacillus thuringiensis* sub sp. *kurstaki*. The most efficient leaf extracts

were those from *A. squamosa* and *Acacia arabica,* which at 25% concentration within 3 days of treatment caused 83.33% and 76.66% larval mortality, respectively (Rajguru and Sharma, 2012). Vetal and Pardeshi (2019) reported that *A. squamosa* seed extracts in hexane and ethanol had insecticidal effects on the third instar larva of *S. litura* with the LD_{50} values 13.9878 mg/ml and 22.48 mg/ml for the hexane extract and ethanol extract, respectively.

The results of the present experiment showed that larval mortality increased with an increase in concentration up to 72 hours after exposure to methanolic extract of *A. squamosa* seed (Table 1). These results are comparable to Prijono *et al.* (1997), who reported cent per cent mortality of Cabbage head caterpillar, *Crocidolomiabinotalis,* with a concentration of 0.8 % of acetone extracts of *A. squamosa* seeds after two days of treatment. Similarly, Leatemia and Isman (2004) also reported that the crude ethanolic seed extracts of *A. squamosa* performed significantly better than those of *A. muricata* in terms of their ability to limit *S. litura* larval growth in a dose-dependent manner. They were significantly 20-fold more active than of *A. muricata.* In another experiment by Deshmukhe *et al*. (2010), the fourth instar larvae of *S. litura* were treated with ethyl alcohol extract of *A. squamosa* seeds at various concentrations from 0.5-25%.The result showed that 80 per cent cumulative mortality of larvae wasobserved when treated with 25% concentration of seed extract. Jose *et al.* (2021) synthesized the silver nanoparticles with aqueous seed extract of *A. squamosa* exhibited larvicidal effect against third instar mosquito larvae with an LC_{50} value of 22.44 g/mL. The methanol extract of *A. squamosa* seeds was more toxic to cabbage caterpillar, *Crocidolomia pavonana* larvae, with LC_{50} and LC_{95} values of 0.04 % and 0.16 %, respectively (Nenotek *et al*.,2022).

Histological changes have been reported to occur in larval midgut following the consumption of toxic compounds by the insects, severely affecting the insect physiology (Sreeletha and Geetha, 2012; Farder *et al.*, 2022). In the present experiment, to study and compare the effect of different concentrations of *A. squamosa* seed extract on insect midgut, among the five concentrations of *A. squamosa* seed extracts used to test the mortality of *S. litura* larva, the lowest concentration of 0.5% and the highest concentration of 2.5% were selected to evaluate the histological changes in the midgut region of third instar larva of *S. litura*. The histological experiment was conducted through the leaf dipping method; the larvae fed the treated leaves, and thereby the active ingredient of the *A. squamosa* seed extracts entered inside the midgut of the insect larvae. A healthy lepidopteran larval midgut consisted of four types of cells which were involved in digestion and nutrient absorption, namely, the columnar cells responsible for the absorption and secretion of enzymes; the goblet cells involved in the ion homeostasis process; the endocrine cells involved in endocrine function and the regenerative cells involved in the replacement of new epithelial cells in which damaged and lost cells are replaced during digestion (Franzetti *et al.,* 2016). The microvilli help in increasing surface area and nutrient intake for maximising the absorption process (Jackson and McLaugh-

Table 1. Insecticidal activity of methanolic extract of *A. squamosa* seed against tobacco caterpillar, *S. litura* larva

Treatments	Conc. (%)	Cumulative mortality (%)		
		24 HAT	48 HAT	72 HAT
T1	0.5	16.67±0.96 (24.09)^{c}	23.33±0.50 (28.88)^{c}	40.00±0.82 (39.23)^{d}
T2	1.0	20.00±0.58 (26.57)^{bc}	33.33±0.58 (35.26)^{bc}	53.33±0.82 (46.91)^{c}
T3	1.5	26.67±0.50 (33.21)^{bc}	46.67±0.58 (43.09)^{ab}	70.00±0.50 (56.79)^{b}
T4	2.0	30.00±0.82 (31.09)^{b}	53.33±0.82 (46.91)^{a}	83.33±0.50 (65.91)^{ab}
T5	2.5	46.67±0.58 (43.09)^{a}	60.00±1.29 (50.77)^{a}	96.67±0.50 (79.48)^{a}
T6 – Untreated check	-	0.00 (4.05)^{d}	0.00 (4.05)^{d}	0.00 (4.05)^{e}
SEd		1.26	0.82	0.60
F-value		15.03	54.05	160.87
p(significance)		0.000	0.000	0.000

Mean values of four replications are represented as mean± standard deviation; Figures in the parentheses are arc sine transformed values (x+0.5); Means followed by the same letter are not significantly different from each other by Tukey's test ($p \leq 0.05$); SEd: Standard Error of the difference; ** Highly Significant; HAT- Hours after treatment

lin, 2009).

In the untreated larvae, midgut structures were clear, not lost or damaged and the midgut region of *S. litura* larvae showed an intact peritrophic membrane. The epithelial cells had a well-developed striated brush border arranged tightly adjacent to each other to form columnar epithelial cells with an elongated nucleus and decondensed chromatin followed by regenerative cells are attached at the surface of the basement membrane and well developed longitudinal muscular layers and goblet cells (Fig. 1). This condition confirms that the healthy functioning of midgut cells enables the metabolic activity of the insect larvae (Suryani *et al.,* 2020).

Histological changes were found in the midgut of *S. litura* after the treatment of *A. squamosa* seed extracts at 0.5 per cent concentration, as observed 72 hours after exposure. A substantial effect was observed when larvae were treated with 0.5 % concentration. The peritrophic membrane and striated border of epithelial cells were disrupted (Fig. 2). In the larvae treated with a higher concentration of 2.5 per cent the maximum damage was observed. The midgut showed an irregular epithelium and high vacuolization in the digestive cells, and goblet cells (Fig. 3). Symptoms like the detachment of epithelial cells from the basement membrane, and collapse of the lumen due to rupture of peritrophic membrane (Fig. 4) were observed. The effect was observed to be dose-dependent. The present results were similar to those observed by Fiaz *et al*. (2018) and Suryani*et al*. (2020) in the digestive cells of the midgut of *Anticarsia gemmatalis* and *S. litura* larvae, respectively, exposed to plant extracts. The midgut of *S. litura* treated with LC_{90} showed irregular epithelium with high vacuolation in the cytoplasm and peritrophic membrane was affected in comparison with larvae treated with LC_{50} dosage. Same pattern of dose dependant toxicity response occurs in the *A. gemmatalis* larvae treated with squamocin (Fiaz *et al*.,2018) and *S. exigua* fed on goniothalamin (Senthil Nathan *et al*., 2008). Khalil *et al.* (2021) also reported that, when exposed to Bt Cry 1C

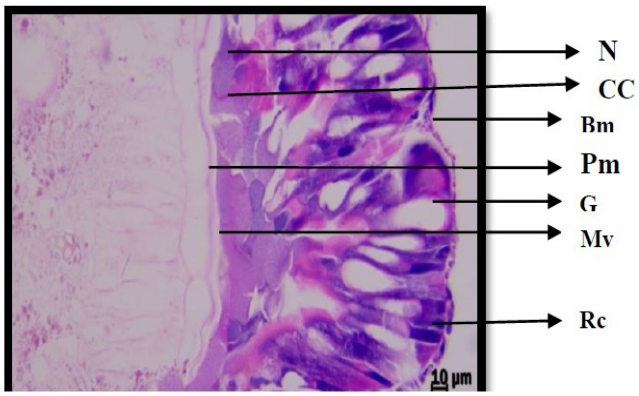

Fig. 1. *Untreated S. litura larval midgut. Magnification: 10x10. Staining: Harris Hematoxylin-Eosin (HE). Description: G-Goblet cells, Rc- Regenerative cells, Bm-Basement membrane, Mv-Microvilli, N-Nuclei, Pm-Peritropic membrane, CC- Columnar cells*

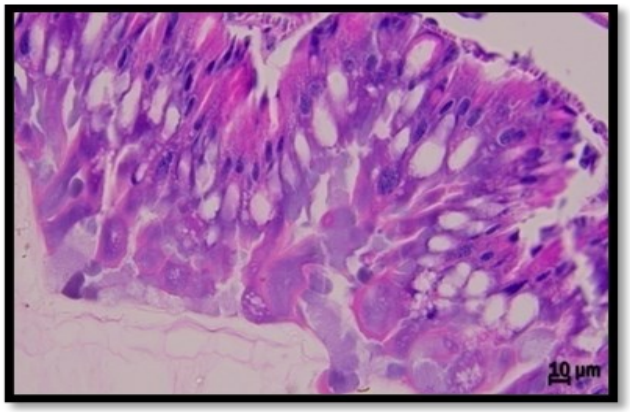

Fig. 2. *Larva fed with 0.5 % concentration of methanolic extract of A. squamosa seed showing Disruption of Peritrophic membrane and striated border of epithelial cells.Magnification: 10x10. Staining: Harris Hematoxylin-Eosin (HE).*

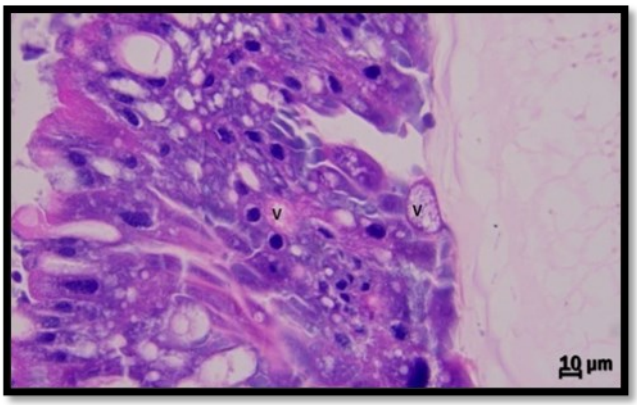

Fig. 3. *Larva fed with 2.5 % concentration of methanolic extract of A. squamosa seed showing vacuolization (V) in the digestive cells, goblet cells.Magnification: 10x10. Staining: Harris Hematoxylin-Eosin (HE)*

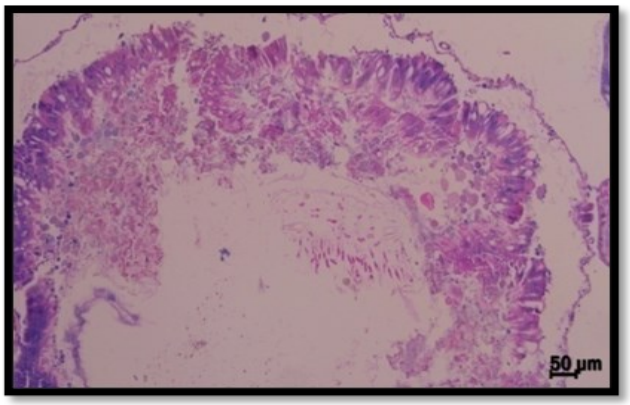

Fig. 4. *Larva fed with 2.5 % concentration of methanolic extract of A. squamosa seed showing detachment of epithelial cells from basement membrane and disrupted lumen due to the rupture of Peritrophic membrane(Pm) and cytoplasm. Magnification: 10x10. Staining: Harris Hematoxylin-Eosin (HE)*

toxin, late third instar larvae of the tolerant population of *S. littoralis* exhibited vacuolization of the epithelium and disintegration of both the peritrophic membrane and the striated border in comparison to the susceptible strain. The results are also in congruence with Pinto *et al.* (2011), who investigated the insecticidal activity and the histopathological effects of bacterial suspension and purified Cry1Ba protein from *B. thuringiensis* strain 4412 in the midgut epithelial cells of *S. frugiperda* larvae. The result revealed that the tested strain of *B. thuringiensis thuringiensis*4412 was highly toxic with cent per cent mortality. After 3 hours of treatment, histological analysis showed a progressive loss of epithelial cells.Similarly,the larvae of *S. litura* treated with asatone and isoasatone isolated from *Asarum ichangense* resulted in some morphological and cellular damage to epithelial columnar cells and appeared disordered in the midgut (Ling *et al.,* 2019). Sreeletha and Geetha (2012) studied the effects of *A. squamosa* leaf powder extracts on the reproductive physiology of male *Oryctes rhinoceros* Linn. (Coleoptera:Scarabaeidae) beetles reared on 5% leaf powder cow dung medium. Significant decline in the number and size of spermatids, along with the fat body with symptoms like the breakdown of cell membrane and pycnotic nucleus, were reported. Histological examination of the midgut of *H. armigera* larvae fed a diet containing 0.005%–0.05% stem extract of *Thevetia neriifolia* revealed significant vacuolization, distortion, and shrinkage of the gut tissues and peritrophic membrane, which resulted in the destruction of epithelial, goblet, and regenerative cells. The damage got worse as the concentration increased (Mishra *et al.,* 2015).The present study indicated that among the several flora of the tropical ecosystems reported to have insecticidal properties, the seeds of *A. squamosa* are potential sources of biopesticides for managing economically important pest like *S. litura* in an ecofriendly manner.

Conclusion

The present study concluded that the methanolic extract of *A. squamosa* seeds increased mortality and induced the histological changes in the midgut region of the pest, *S. litura*. The midgut showed an irregular epithelium and high vacuolization in the cytoplasmic cells. The effects were dose-dependent. A substantial effect was observed when larvae were treated with lower concentration (0.5%).The maximum damage was observed in the larvae treated with a higher concentration (2.5 %. The potential of *A. squamosa* as bio-pesticide has been studied in relation to histological changes in the midgut of *S. litura* larvae and mortality under laboratory conditions suggested as an alternative to chemical insecticides for the management of *S. litura*. Further field trials should be done to evaluate the effectiveness of this bio -pesticide in light of this perspective.

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Conflict of interests

The authors declare that they have no conflict of interest.

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