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Insecticidal Activity of Seed Extracts of *Annona squamosa* L., Against The Cotton Leafworm *Spodoptera Littoralis* (Boisd.)

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Abstract

The custard apple, *Annona squamosa* have been reported to have toxic and biological activities against various insect pests.Twelve natural chemical compounds were identified from *A. squamosa* seeds extract.Acetone and methanol extracts of *A. squamosa* seeds were laboratory prepared to investigate their toxicity and other biological effects against *Spodoptera littoralis* larvae. Toxicity of acetone and methanol seed extracts of *A. squamosa* against 2nd and 4th instar larvae of *S.littoralis* were tested at different w/v concentrations using the leaf dipping bioassay technique. The results showed that, the acetone extract was more effective against the 2nd instar larvae of *S. littoralis*, LC_{50} values were1.9, 1.7 and 1.1%, compared with the methanol extract, LC_{50} values were2.8, 2.4 and1.6% on 8, 11 and 14 exposure days, respectively. Regarding the efficiency of both *A. Squamosa* seeds extracts against the 4th instar larvae, the acetone extract was again more toxic than the methanol one. The LC_{50} values of the acetone extract were 2.9, 2.1, and 0.97%, while the LC_{50} values of the methanol extract were 3.2, 2.1 and 1.7% on 8, 11 and 14 exposure days, respectively. In addition, the data exhibited that, the treatment of the 2nd instars larvae of *S. Littoralis* with acetone seed extract of *A. squamosa* at LC_{25} and LC_{50} prolonged the larval and pupal duration and reduced the pupal weight. Malformations of pupae and adults were recorded.

Keywords: *Spodoptera littoralis*, custard apple seeds, Toxicity, Malformations

1. Introduction

The cotton leafworm, *Spodoptera littoralis* (Boisd), is considered one of the most injurious and destructive polyphagous lepidopterous insect pests that attack crops, vegetables and fruit trees worldwide [1, 2].Commonly, control of this pest has primarily been dependent on chemical insecticides [3]. Considering the adverse effects of chemical pesticides such as; evolution of resistance, injuring non-target organisms, dismantling natural enemies and switching the inoffensive species, seeking for natural ecofriendly pesticides are in demand.

Botanical extracts offer desirable alternatives to synthetic chemicals in agro-systems where protection of the environment and preservation of beneficial organisms are essential [4]. More than 6000 plant species are known to have various activities against pests and part of these plants are used as pesticides by the local farmers in some developing countries around the world [5].These botanical pesticides have been recognized to cause fewer environmental injuries or human health dangers alike synthetic pesticides [6]. *Annona squamosa* L. (Annonaceae), also known as "custard apple, or sugar apple" is a tropical, endemic species of the West Indies, South and Central America, Ecuador, Peru, Brazil, India, Mexico, Bahamas, Bermuda, and Egypt [7,8,9]. *A. squamosa* has been utilized as natural medicine and in various other food applications, e.g., its pulp is utilized as flavouring agent in ice cream, and 50-80%

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of custard apple fruit is edible and can be pulped as juice. It contains appreciable vitamin C in the range of 35-42 mg per 100 g, and dietary fibre, vitamin B1 (thiamine), and potassium contents are also notably high [10]. Custard apple contains numerous phenol-based compounds, e.g., proanthocyanidins, with 18 different phenolic compounds, mainly alkaloids or flavonoids [11]. Extract of *A.squamosa* L., has shown potential pest control across an extensive array of insect pests. Laboratory and field experiments proved that custard apple extract effectively managed different field crop pests and stored grain pests [12, 13, 14, 15, 16, 17]. Ethanolic and hexane extracts of *A. squamosa* have been reported to have larvicidal activity against *S.litura* [18, 19]. Also, the biological effects of *A. squamosa* seed methyl alcohol extract on *S. litura* were the highest records among transgenic cotton varieties. The extract affected the pupation, adult emergence, fecundity as well as fertility of *S. litura*. [20]. Therefore, the present work aimed to study the toxicity and other effects of acetone and methanol seed extracts of *A. squamosa* against *S.littoralis.*

2. Experimental Design

2.1. Insect rearing

The laboratory- susceptible strain of cotton leaf worm, *S. littoralis* was obtained from the Plant protection laboratory, Agriculture Research Center, Alexandria. This strain was reared in the laboratory for several years without pesticides exposure under control conditions (25± 2°C and RH 60% ± 10)[21].

2.2. Crude extract preparation

Whole custard applefruits were purchased from the local market and their species were identified. Seeds of *A.squamosa* were dried in the shade at room temperature, grounded in a domestic electrical mill. The obtained powder (250 g) was immersed in acetone, the solvent was removed at reduce pressure in a rotary evaporator at 50°C. The extracted material was left to dry and then, was successively extracted with methanol. The crude extracts were stored at 4 °C for further use.

2.3.GC-MS Analysis of *A. squamosa* seeds extract

The extract of acetone (which displayed high mortality rate) in preliminary bioassays was analyzed by using A Thermo Scientific gas chromatograph GC Trace 1300 coupled with an EI Mass spectrometer ISQ Single Quadruple mass spectroscopy with direct capillary column TG-5MS (30 m in length × 250 µm in diameter × 0.25 µm in thickness of film). Analysis conditions were 20 min at 100 °C, column temperature 235 °C for 3min and 240 °C for injector temperature, helium was used as carrier gas, , and the split ratio was 5:4. The sample (2 µl) was evaporated in a split-less injector at 300°C with a run time 29 min. The compounds were identified by comparison of mass spectra of each peak with those of the library.

2.4. Bioassay technique

Leaf dipping bioassay technique [21]was implemented on the second and fourth instar larvae of *S. littoralis*. Castor oil leaves were dipped for 30 seconds in aqueous dilutions (1,2, 3 and 4%, w/v) of acetone and/or methanol extracts separately and left to dry for an hour at room temperature. Each ten larvae were placed in cups covered with muslin lids and were fed upon treated leaves, freshly provided till the pupation. The used concentrations were replicated three times. The mortality was observed on 8, 11and 14 days of treatment. The larvae in the control groups were feed on leaves treated with tap water. The 2ndinstar larvae of *S. littoralis* was treated with sub-lethal concentrations of acetone seed extract of *A. squamosa*, LC_{50} and LC_{25} and compare with untreated groups (four replicates / each group and 25 larvae/ replicate). The mean larval-pupal duration and the pupal weight were recorded.

2.5. Statistical analysis

The mean lethal concentrations (LC_{50} s), confidence limits and slops were estimated based on [22] using Ldp Line ® software. The means of larval, pupal duration and the pupal weight were subjected to ANOVA analysis (Costat Statistical Software, 1990, https://www.cohort.com).

3. Results

3.1.GC-MS Analysis of *A. squamosa* seeds extract

The GC-MS analysis revealed that twelve major compounds were present in *A. squamosa* seeds extract. The retention times, names of compounds, peak area percentages and molecular formula were noted (Table 1). Analysis of *A. squamosa* seeds extract indicted twelve chief compounds with significant matches to myo-Inositol (65.63%), in addition, the presence of N-Methoxy-N-methylacetamide (11.56%), 1,5-Heptadiene (3.48%), d-Mannose (2.52%), Oxirane, (1,1dimethylbutyl)

(2.42%), 3-Trifluoroacetoxypentadecane (2.03%), Oleic Acid (1.91%), Cyclopentaneundecanoic acid, methyl ester (1.86%), n-Hexadecanoic acid (1.70%), Dianhydromannitol (1.57%), 9-Octadecenoic acid (Z), methyl ester (1.51%) and Carboisopropoxyisopropoxy sulfide (1.24%) .

Table 1
Chemical composition of *A. squamosa* seeds extract analyzed with GC-MS

S. No	RT (min)	Compound name	Peak area (%)	Molecular formula
1	5.12	Oxirane, (1,1dimethylbutyl)	2.42	$C_{18}H_{16}O$
2	5.19	Carboisopropoxyisopropoxy sulfide	1.24	$C_7H_{14}O_3S$
3	5.28	1,5-Heptadiene	3.48	C_7H_{12}
4	5.55	3-Trifluoroacetoxy pentadecane	2.03	$C_{17}H_{31}F_3O_2$
5	8.17	N-Methoxy-N-methylacetamide	11.56	$C_4H_9NO_2$
6	9.07	Dianhydromannitol	1.57	$C_6H_{10}O_4$
7	15.34	D-Mannose	2.52	$C_6H_{12}O_6$
8	16.83	Cyclopentaneundecanoic acid, methyl ester	1.86	$C_{17}H_{32}O_2$
9	17.25	n-Hexadecanoic acid	1.70	$C_{16}H_{32}O_2$
10	18.18	9-Octadecenoic acid (Z), methyl ester	1.51	$C_{19}H_{36}O_2$
11	18.52	Oleic Acid	1.91	$C_{18}H_{34}O_2$
12	19.75	Myo-Inositol	65.63	$C_6H_{12}O_7$

3.2.Toxicity of *Annona squamosa* extracts against *Spodoptera littoralis*

The toxicity of acetone and methanol extracts obtained from *A.squamosa* against the 2nd and 4th instar larvae of *S. littoralis* are presented in Tables 2 and 3. Data in (Table 2) showed that no mortality against the second instar larvae of *S. littoralis* up to 5 days of exposure to the methanol extract while the acetone extract provided toxicity since the LC_{50} was 3.2 %. Furthermore, the acetone extract was more effective against the 2nd instar larvae of *S. littoralis* compared with the methanol extract. The LC_{50} values were 1.9, 1.7 and 1.1%, for the acetone extract and 2.8, 2.4 and 1.6% for the methanol extract consecutively on8, 11 and 14 exposure days. However, the toxicity difference between both extracts was significant and the toxicity was increased in a time-dependent manner. Regarding to the efficiency of bothextracts against the fourth instar larvae (Table 3), the acetone extract was again significantly more toxic compared with the methanol extract, the estimated LC_{50} values were 2.9, 2.1 and 0.97% with acetone extract and 3.2, 2.1 and 1.7% with methanol extract on 8, 11, 14 exposure days, respectively. Likewise, the biological activity of both extracts was increased as the time of exposure was elongated.

Table 2
Toxicity of *A.squamosa* seed extracts of acetone and methanol against 2nd instar larvae of *S. littoralis* at different days post-treatment

Solvent	Days after treatmen	LC_{50} (% w/v)	95% Confidence limits (%)		Slope±SE	χ^2
			Upper	lower		
Acetone	5	3.2	4.4	2.7	1.6±0.3	3.6
	8	1.9	2.5	1.3	1.1±0.28	4.8
	11	1.7	2.1	1.3	1.3±0.29	4.9
	14	1.1	1.4	0.73	1.6±0.29	1.1
Methanol	5	-	-	-	-	-
	8	2.8	3.9	2.2	1.2±0.29	0.69
	11	2.4	3.3	1.7	1.1±0.280	0.17
	14	1.6	1.9	1.2	1.5±0.29	1.8

Table 3

Toxicity of *A.squamosa* seed extracts of acetone and methanol against 4th instar larvae of *S. littoralis* at different days post-treatment

Solvent	Days after treatmen	LC_{50} (% w/v)	95% Confidence limits (%)		Slope±SE	χ^2
			Upper	lower		
Acetone	8	2.9	3.7	2.5	1.7±0.29	4.9
	11	2.1	2.6	1.6	1.3±0.29	3.1
	14	0.97	1.3	0.39	1.1±0.29	0.12
Methanol	8	3.2	4.7	2.6	1.4±0.29	0.73
	11	2.1	2.6	1.6	1.3±0.28	3.1
	14	1.7	2.0	1.2	1.33±0.29	2.8

Table 4

Effect of sub-lethal concentrations of acetone seed extract of *A. squamosa* on the larval, pupal duration and pupal weight of *S. littoralis*

Treatments	Mean larval duration	Mean pupal duration	Mean pupal weight (g)
LC_{25}	23.5^{b}±0.5	18.9^{ab}±0.5	0.255^{b}±0.0
LC_{50}	27.2^{a}±0.5	19.5^{a}±0.5	0.216^{c}±0.0
Control	21.4^{c}±0.0	18.3^{b}±0.0	0.281^{a}±0.0
LSD (5%)	1.17	1.12	0.02

Means with the same letter are not significantly different ($p<0.05$)

3.3. Effect of sub lethal concentrations of acetone seed extract of *A. squamosa* on some biological aspects of *S. littoralis*:

Data in (Table 4) presented that, the acetone extract of *A. squamosa* seed treatment of the 2nd larval instar of *S. littoralis* extended the larval duration up to 23.5 and 27.2 days, at concentrations of LC_{25} and $LC_{50,}$ respectively compared with the untreated larvae 21.4 days. Yet, there was no significant effect of extract at LC_{25} value on the mean pupal duration, while, a significant increase in pupal duration was recorded with LC_{50} treatment (19.5 days) while the pupae of control lasted only 18.3 days. Furthermore, the mean of pupal weight was significantly reduced (0.255 and 0.216 g) due to treatment with LC_{25} and LC_{50} comparable with control (0.281g).

3.4. Morphogenetic effects

Treatment of the 2nd larval instars of *S. Littoralis* with acetone seeds extract of *A. squamosa* produced a noticeable increase in the pupalmal formation at concentrations of LC_{25} and LC_{50} values, (Fig.1b-d) and (Fig.1e-g) compared with the typical pupa (Fig. 1a). Regarding the adults malformation, the 2nd instars larvae treatment with acetone seeds extract of *A. squamosa* at concentrations of LC_{25} and LC_{50} values presented wing malformations in the adult stage, the deformed adults emerged with crumpled and undeveloped wings (Fig. 2b and c) compared to normal adults (Fig. 2a).

Fig.1.Pupae of *S. littoralis*, (a) Normal pupa (control); (b-d) pupal malformations at LC_{25} value, (e-g) pupal malformations at LC_{50} value of acetone seeds extract of *A. squamosa*

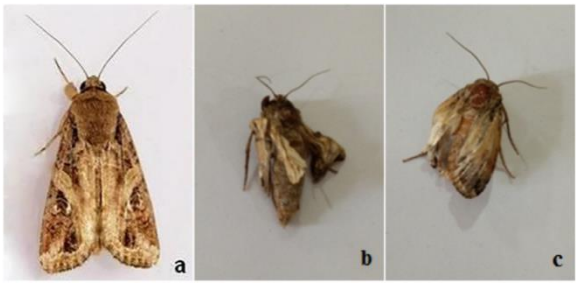

Fig.2. Adults of *S. littoralis*, (a) Normal adult (control); (b and c) Emerged adults from LC_{50} or LC_{25} treatment, had crumpled and undeveloped wings

4. Discussion

According to the US Environmental Protection Agency (EPA), biorational insecticides exhibit minimum risk to the environment due to their quick break down, the minimum residue, the safety for the applicant, and the comparatively low concentrations required for controlling the target organisms [23]. *A. squamosa* seeds extract indicted twelve chief compounds, some of which may have insecticidal properties. Myo-Inositol, For example, showed the highest percentage at 65.63% peak area .Myo-Inositol and phytate are toxic to formosan subterranean termites, when consuming cellulose. Myo-Inositol significantly caused mortality when applied to food source filter paper discs at 640.8 and 1,281.7µg/mm^3in three independent bioassay [24]. D-mannose had a peak area percentage of 2.52%, DB1 was classified into the monocot mannose-binding (GNA-related) lectin family and had a high sequence homology to GNA and showed insecticidal activity of *Helicoverpa armigera* when incorporated in an artificial diet at 0.01%, resulting in 33% survival rate of larva and pupae [25]. 9-Octadecadienoic acid, methyl ester at 1.51% of peak area was isolated from *Citrullus colocynthis* fruits and *Mentha microphylla*by [26] who recorded the toxicity this compound against whitefly (*Bemisia tabaci*) and aphid (*Aphis craccivora*). Also, [27] proved the toxicity of this compound as insecticide against cabbage hooper, *Trichopulsia ni* larvae. The compound, n-Hexadecanoic acid had a peak area percentage of 1.70 %. [28] reported n-Hexadecanoic acid to be implicated in biological activities such as Antioxidant, Hypocholesterolemic Nematicide, Pesticide, Lubricant, Antiandrogenic, Flavor, Hemolytic. Seed extract of *A. squamosa* contains oleic acid at 1.91% peak area. Oleic acid, eicosyl ester was found to be the most effective larvicide against *Aedesaegypti* (LC_{50}/24 h -8.51 ppm) and *Culex quinquefasciatus*(LC_{50}/24 h-12.5 ppm) [29].The present research revealed that, both acetone and methanol seed extracts of *A. squamosa* had insecticidal activity against the 2^{nd} and 4^{th} instar larvae of *S.littoralis*. Earlier findings stated that, the ethanolic extract from *A. mucosa* seeds showed significant acute toxicity (LC_{50} = 842.9mgkg^{-1}) against *S. frugiperda* after 7 days of exposure [30]. Also, methanolic extract of *A.squamosa* was reported to cause larvae mortality percentages of *S. litura* ranged between 50.6 and 68.0% [31]. Our results closely resemble these finding [32]; they reported that, the hexane and ethanol seed extracts of *A. squamosa* showed insecticidal activity against the third instar larvae of *S.litura*. (LD_{10} and LD_{50} of the hexane extract were LD_{10}=5.91mg/ml, LD_{50}=13.9878 mg/ml and the same values of the ethanol extract were (LD_{10}=11.72 mg/ml, LD_{50}= 22.48 mg/ml), respectively. Similarly, leaf application of cold ethyl alcohol extract of *A.squamosa* seeds against fourth instar larvae of *S. litura* exhibited LC_{50}= 25.75 % [33]. Studing the efficacy of crude aqueous extracts of eight plant species against *S. litura* larvae and found that, the leaf extracts of *Acacia Arabica* and *A. squamosa* used 76.66% and 83.33% larval mortality, respectively at 25% concentration within 3 days of treatment [34].

The current results revealed that the mean pupal weight was significantly reduced, as a response to the LC_{50} of the acetone extract treatments of the 2nd instar larvae of *S. littoralis*, whereas the larval and pupal duration increased. Other researchers reported similar results, pupae length, weight and pupal period were affected by methanol extracts of twelve leaves plants including *A. squamosa*, as the weight and length reduced and the pupation period prolonged when the larvae of *S. litura* were treated with 2% of the extraction [31]. Also, treatment of *H. armigera* and *S. litura*, by ononitol monohydrate isolated from sicklepod, a dicot legume, *Cassia tora* L. increased pupal duration and decreased pupal size [35]. Similar research concluded that, the pupal duration of *S. littoralis* increased due to treatment with leaf extracts of *Adhatoda vasica* Nees [36].

We observed pupae and adults malformations in the acetone extract treatment groups. Accordingly, [37] found pupal mortality and wing malformations of the adult stages of *S. frugiperda* treated with *V. nebularum* natural products. Morphological deformities like deformed wings and mouthparts were also observed [38] in *Manduca sexta*. [39] observed the abnormalities like attachment of exuviae to the abdomen and deformed wings in *S. litura* treated with azadirachtin. This work expressed that, both acetone and methanol extracts of *A.squamosa* showed toxicity and malformation against *S. littotals* stages which may be attributed to the plant extracted components. The toxicity of *A. squamosa* could be due to the presence of phytochemicals reported in other research including alkaloids, flavonoids and terpenoids [40,41]. The pesticide activities of *A. squamosa* were found to be relay on about thirty active compounds of acetogenins. Also, sesqui-terpenes, and monoterpenes were reported as main components [42, 43]. Acetogenins have been reported to be occurring naturally in *Annona* spp. [44, 45]. The toxicity of *Annona* spp. has been reported to refer to acetogenins, although several chemical classes were detected in their extracts [46, 30]. In addition, the pupal weight reduction could be revealed to the effects of *A. squamosa* tested extracts on food uptake rate. [47, 48, 49] reported a positive relation between pupal biomass and food intake inhibited by extracts contents. As well feeding inhibition of *Annona* spp. had been reported [18, 50].

Moreover, adverse quantitative and qualitative effects of *A. squamosa* seed extract on the total protein contents of *S. litura* [51]. Furthermore, Acetogenins acts as potent inhibitors of complex I (NADH: ubiquinone oxidoreductase) of the mitochondrial electron transport system and NAD Hoxidase of the insect's plasma membrane, which induces apoptosis due to ATP redaction [52, 53].

The present study showed that the crude seed extracts of *A. squamosa* have insecticidal activity against *S. littoralis*. Botanical antifeedant and insecticidal agents can play a significant role as an effective element of the Integrated Pest Management tactics. However, to convert these botanical extracts to practical use, further researches are mandatory for investigation environmental and mammalian toxicity as well as the formulation and application rates.

5. Conclusion

The results of this study indicate that seed extracts of *A.squamosa* have insecticidal activity against larval stages of *S.littoralis*.

6. Conflicts of interest

The authors declare that there is no conflict of interest.

7. References

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