Anti-feedant and growth inhibitory effects of seed extracts of custard apple, *Annona squamosa* against Khapra beetle, *Trogoderma granarium*

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The anti-feedant and growth inhibitory effects of seed extracts of custard apple in hexane, ethyl acetate and methanol were tested against neonate and 7 days old larvae of the Khapra beetle, *Trogoderma granarium*, using a feeding bioassay. The LC_{50} values for hexane, ethyl acetate and methanol extracts were 1195.41, 305.36 and 1446.32 ppm for neonate larvae; 5805, 1300 and 5815 ppm for 7 days old larvae, respectively. Drastic reduction in larval weight was observed in all extracts as compared with the control (acetone). The weight of five larvae in the control (acetone) was 6.03 mg after 15 days of release of neonate larvae. In case of *Annona squamosa* seed hexane extract the larval weight near LC_{50} (1250 ppm) was 0.65 mg, whereas in ethyl acetate (250 ppm) and methanol extract (1500 ppm) it was 0.91 and 0.90 mg, respectively. Ethyl acetate extract at 1250 ppm showed maximum anti-feedant activity of 44.50 and 59.50% after 10 and 15 days of release, respectively, whereas 35.68 and 53.45 % for hexane extract and 26.07 and 38.65% for methanol extract was observed at same concentration after 10 and 15 days of release, respectively. *Annona squamosa* seed ethyl acetate extract produced 55.73% anti-feedant activity for 7 days old larvae while hexane and methanol extracts produced 50 and 25.04%, respectively. The present study conclusively showed that *A. squamosa* seed ethyl acetate extract was superior to hexane and methanol extracts and first such study against Khapra beetle.

Key words: *Annona Squamosa*, anti-feedant activity, seed extracts

Introduction

Insects often cause extensive damage to stored grains and grain products, amounting to 5-10% loss in temperate regions and 20-30% in the tropical regions (Nakakita, 1998). In India, post harvest losses caused exclusively by insect pests is 12% (Mohan, 2003). At present pest control measures in storage rely heavily on the use of synthetic insecticides and fumigants. Their indiscriminate use in storage, however, has sometimes led to a number of

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problems including toxic residues in food grains (Fishwick, 1988) and environmental pollution (Wright *et al*., 1993). These problems together with the development of insect resistance have made the problem much more complicated. The use of insecticides of natural origin are therefore an important development in storage pest control as they have short residual action, low mammalian toxicity and reduced environmental pollution.

The effectiveness of many plant products for use against stored grain pests have been reviewed by Jacobson (1958, 1975, 1983, 1989). The effects of plant products so far reported include insecticidal, repellent and anti-feedant activities (Huang *et al*., 1998). The seeds of custard apple, *Annona squamosa* are known to show insecticidal and vermicidal activity. The seeds contain chemicals known as acetogenins, which are toxic to insects. In the Philippines, coconut oil extract of this seed is used to control lice in human hair. Farmers in Vietnam use seed oil to control rice leafhoppers and plant hoppers (Brady *et al*., 1978). The petroleum ether (40-60ºC) extract of *A. squamosa* seed was toxic to *Musca nebulo* adults as a contact poison (Qadri and Rao, 1977). Kawazu *et al*. (1989) reported pronounced activity of ethyl acetate extract of the defatted seeds. Santosh Babu *et al*. (1996) reported that chloroform extract from seeds showed high feeding deterrence against *Longitarsus nigripennis*.

Some reports are also available on efficacy of leaves of custard apple to several microbes and pests. Alkaloids isolated from custard apple showed larvicidal, growth regulating and chemosterilant activities against *Anopheles stephansi* (Saxena *et al*., 1993). Hemlata *et al*. (2001) tested foliar extracts and reported anti-microbial activity to common microbial infestants of pulses and insecticidal activity against pulse beetle, *Callasobruchus chinensis*. So far, most of the work on custard apple seed extracts has been done against field pests and most of the authors used either petroleum ether extract or methanolic extract and the information available is scanty to apply against stored grain pests.

The Khapra beetle, *Trogoderma granarium* is considered one of the world's most destructive pests of stored products, originated from regions now including India and Bangladesh, but has since spread to other areas including northern and eastern Africa, southern Europe, the Mediterranean region and to the entire Asia. In India it was spread to all parts when Punjab wheat was distributed during an emergency (Pruthi and Singh, 1950). The problem of preventing the beetle's spread is further compounded by its ability to survive for several years in the larval stage with little or no food, and its habit of hiding in cracks and crevices. No reports are available on efficacy of custard apple seed extracts against Khapra beetle. Based on these facts, the present study was

undertaken to evaluate the anti-feedant and growth inhibitory effects of custard apple (*Annona squamosa*) seed extracts against Khapra beetle.

Materials and methods

Extraction

Fully ripened fruits of custard apple, *Annona squamosa* were collected in and around regions of Hyderabad, India. Seeds were removed manually from these fruits, washed in water and shade dried and was crushed in to fine powder by using iron mortar and pestle. The solvents hexane, ethyl acetate and methanol were used for successive extraction in soxhlet based on polarity. Extraction was done for 48 hours for each solvent until the solvent extracted have no colour. After extraction distillation was carried out with rotary evaporator to remove the solvent and the extract was separated out and used in the feeding bioassay.

Insect culture

The culture of Khapra beetle was maintained in wheat at the temperature of 35 ± 1ºC and 70 ± 5% relative humidity in a BOD incubator. The neonate and 7-days old larvae were used for the experiment. New adults were obtained from laboratory-bred culture maintained on wheat grain (sterilized and conditioned). These fresh adults were kept in wheat flour sieved through 100 mesh. After every 24 hours the flour was sieved through 80 mesh so that the flour passed through the mesh and eggs remained on the top. These eggs kept in Petri dishes were allowed to hatch. Freshly hatched larvae were used for study. To obtain 7-day-old larvae, the freshly hatched larvae were transferred into artificial diet (Pant, 1956) and allowed to feed for seven days.

Feeding bioassay

All the experiments were carried out using an artificial diet. All ingredients were mixed thoroughly using a mortar and pestle. One gram of diet was placed in a plastic Petri dish (5 × 1.5 cm) for neonate and 7 days old larvae, respectively. The required concentrations of various extracts were prepared by serial dilution by using acetone as the solvent. For neonate larvae 250, 500, 750, 1000, 1250 and 1500 ppm of hexane, 50, 250, 500, 750, 1000 and 1250 ppm of ethyl acetate and 500, 750, 1000, 1250, 1500 and 2000 ppm of methanol extract (with the solvents removed) were tested. For 7-day-old

larvae 1000, 2500, 5000, 7500, 10000, 15000 and 20000 ppm of hexane, 250, 500, 1000, 2500, 5000, 7500 and 10000 ppm of ethyl acetate and 1000, 2500, 5000, 7500, 10000, 15000 and 20000 ppm of methanol extract were tested. Fifteen larvae were inoculated into each replication and three such replications were maintained. Acetone control and other control without acetone were maintained for all the three extracts. All experiments were conducted in the dark at the rearing conditions described above. Observations were taken on the 10^{th} and 15^{th} day after inoculation for neonate larvae, but on the 8^{th} day after inoculation in case of 7-day-old larvae. Larval mortality, larval weight, weight of diet consumed was recorded. Per cent anti-feedant activity was computed by using the formula given by Luco *et al*. (1994). Larval weight was recorded for 5 larvae. The weight of diet consumed after 10 and 15 days of larval inoculation was recorded. Corrected percentage mortality, the logarithm of concentration and LC_{50} were estimated by a log-probit plot.

Results

Efficacy to neonate larvae

Among the three *Annona squamosa* seed extracts tested, ethyl acetate showed pronounced activity by causing larval mortality at a lowest concentration of 50 ppm and maximum mortality at 1250 ppm (Table 1). The other two *A. squamosa* seed extracts required higher concentration of 250 and 500 ppm to initiate larval mortality. The mortality was dose dependant in all the extracts. At 1000 ppm concentration, *A. squamosa* seed ethyl acetate extract caused 64.47 and 80% larval mortality on the 10^{th} and 15^{th} day respectively, whereas, in hexane and methanol extract it was 17.8 and 28.87; 22.2 and 24.47% respectively.

The LC_{50} values for hexane, ethyl acetate and methanol extracts of *Annona squamosa* seeds were 1195.41, 305.36 and 1446.32 for neonate larvae after fifteen days of inoculation (Table 3). Ethyl acetate extract of *A. squamosa* seeds was 3.91 and 4.74 times more toxic than hexane and methanol extract, respectively. Drastic reduction in larval weight was observed in all *A. squamosa* seed extracts as compared with the control. Among *A. squamosa* seed extracts, ethyl acetate caused maximum reduction in larval weight (Table 1). The weight of five larvae in the acetone control was 4.23 mg and 6.03 mg after 10 and 15 days of release of neonate larvae, respectively. In case of *A. squamosa* seed hexane extract the larval weight near LC_{50} (1250 ppm) was 0.65 mg, whereas in ethyl acetate (250 ppm) and methanol extract (1500 ppm) it was 0.91 and 0.9 mg, respectively. In *A. squamosa* seed ethyl acetate extract

Table 1. Anti-feedant activity of seed extracts of custard apple against neonate larvae of Khapra beetle.

Conc. (ppm)	Larval Mortality (%)		5 Larvae Weight (mg±SD)		Diet fed (mg)	
	10th day	15th day	10th day	15th day	10th day	15th day
Hexane Extract						
250	0	11.13	1.58±0.14	2.17±0.06	66.1	80.5
500	4.47	11.13	0.83±0.06	1.43±0.08	75.1	84.6
750	11.13	17.8	0.74±0.12	1.13±0.09	64.9	70.9
1000	17.8	28.87	0.60±0.05	0.75±0.25	63.6	71.8
1250	48.87	53.33	0.45±0.15	0.65±0.1	64.9	70.1
1500	75.33	82.2	0.32±0.04	0.42±0.15	58.3	61.5
Ethyl Acetate Extract						
50	4.47	20. 00	1.42±0.14	1.65±0.10	73	86.2
250	24.47	33.33	0.72±0.28	0.91±0.09	65.6	73
500	26.67	55.53	0.49±0.19	0.62±0.08	72.4	81
750	51.13	57.8	0.41±0.08	0.51±0.11	62.9	69.5
1000	64.47	80.00	0.48±0.14	0.53±0.12	59.8	66
1250	84.47	91.13	0.29±0.01	0.32±0.08	56	61
Methanol Extract						
500	6.67	8.87	1.73±0.14	2.93±0.15	78	96.6
750	13.34	17.8	1.44±0.16	2.73±1.21	74.1	93.1
1000	22.2	24.47	0.96±0.15	1.90±0.95	75.2	98.2
1250	20	26.67	0.65±0.03	1.40±0.1	74.6	92.4
1500	48.87	57.73	0.46±0.04	0.90±0.12	58	85
2000	66.67	77.73	0.42±0.09	0.50±0.15	53.4	62.5
AC	0	0	4.23±0.4	6.03±0.49	100.9	150.6
C	0	0	5.10±0.3	6.45±0.45	94.9	161.6

Each value is average of three replications, mg – milligram, AC – Acetone Control, C – Control, SD – Standard Deviation from mean.

at 1000 ppm, the weight of 5 larvae was 0.48 mg on 10th day and 0.53 mg on 15th day as compared to 4.23 and 6.03 mg in acetone control. This indicates that hardly any growth of surviving larvae took place in 5 days. The total diet consumed by larvae was again dose dependant, and the larvae failed to feed upon when compared with acetone control at higher concentrations in all extracts tested. After 10 and 15 days of release, diet consumed in acetone

Table 2. Anti-feedant activity of seed extracts of custard apple against 7-day-old larvae of Khapra beetle.

Conc. (ppm)	Larval Mortality (%)	5 Larvae Weight before inoculation (mg±SD)	5 Larvae weight after 15 days (mg±SD)	Diet fed (mg)
		Hexane Extract		
1000	20	0.91	1.95±0.2	78.8
2500	33.33	1.03	1.78±0.08	68.8
5000	42.2	0.9	1.00±0.1	66.1
7500	57.8	0.95	0.90±0.12	63.6
10000	66.67	1.15	0.80±0.15	58.5
15000	84.47	1.08	0.70±1.21	54.1
20000	91.13	0.89	0.70±0.05	52
		Ethyl Acetate Extract		
Conc. (ppm)	**Larval Mortality (%)**	**5 Larvae Weight before inoculation (mg±SD)**	**5 Larvae weight after 15 days (mg±SD)**	**Diet fed (mg)**
250	11.13	0.83	1.82±0.18	72.9
500	24.47	0.94	1.23±0.15	70.2
1000	46.67	0.88	0.91±0.06	67.4
2500	68.87	1.08	0.73±0.1	62.9
5000	77.8	0.96	0.60±0.05	61
7500	86.67	1.07	0.60±0.2	54.8
10000	95.53	0.88	0.55±0.1	51.8
		Methanol Extract		
Conc. (ppm)	**Larval Mortality (%)**	**5 Larvae Weight before inoculation (mg±SD)**	**5 Larvae weight after 15 days (mg±SD)**	**Diet fed (mg)**
1000	13.33	1.08	4.37±0.39	100.9
2500	22.2	1.11	3.84±0.09	96.4
5000	40	1.01	3.32±0.21	88.9
7500	55.53	0.93	2.58±0.2	89.3
10000	64.47	0.88	2.17±0.06	87.7
15000	77.8	1.03	1.50±0.18	85.2
20000	84.47	0.94	1.14±0.06	81.3
AC	0	1.05	6.50±0.61	117
C	0	0.96	8.00±0.56	124.2

Each value is average of three replications, mg – milli gram, AC – Acetone Control, C – Control, SD – Standard Deviation from mean.

control (3.31 mg/larvae) was about three times higher when compared with hexane (1.06 mg/larvae) at 1500 ppm.

Ethyl acetate extract at 1250 ppm showed maximum anti-feedant activity of 44.50 and 59.50% after 10 and 15 days of release, respectively, whereas

Table 3. LC_{50} values of different extracts of custard apple against Khapra beetle.

Extract	Stage of insect	Observations (days)	Heterogeneity (χ^2)	Regression Equation Y=	LC_{50} (ppm)	Fiducial Limits
Hexane	Neonate	15	2.459	2.561+2.459X	1195.41	853.03-1675.21
Ethyl acetate	Neonate	15	4.029	1.520+1.400X	305.360	187.83-496.44
Methanol	Neonate	15	2.665	5.852+3.434X	1446.32	1154.47-1811.94
Hexane	7-day-old larvae	8	3.061	5.437+1.853X	5805.73	4155.62-8111.05
Ethyl acetate	7-day-old larvae	8	0.761	6.422+1.634X	1347.78	900.24-2017.80
Methanol	7-day-old larvae	8	0.735	5.399+1.698X	5815.20	4063.69-8321.63

35.68 and 53.45% for hexane extract and 26.07 and 38.65% for methanol extract was observed at same concentration after 10 and 15 days of release, respectively (Figs 1, 2).

Efficacy to 7 days old larvae

Annona squamosa seed ethyl acetate extract could able to produce mortality at a concentration of 250 ppm and caused more than 95% mortality at a concentration of 10000 ppm. The other two extracts required higher concentrations to produce the same effect (Table 2). The LC_{50} values for hexane, ethyl acetate and methanol extracts were 5805, 1300 and 5815 ppm, respectively (Table 3). The hexane and methanol extracts produced more or less equal mortality of larvae based on LC_{50} values. Ethyl acetate extract was 4.46 and 4.47 times more toxic than hexane and methanol extract, respectively.

Larval weight before and after 8 days of inoculation revealed that all the extracts exhibited anti-feedant action and not allowed the larvae to grow. In case of ethyl acetate extract at 10000 ppm concentration, the weight of 5 larvae before release was 0.88 mg and instead of gaining, weight reduced to 0.55 mg

Fig 1: Neonate larvae were allowed to feed on various extracts of custard apple for a period of 10 days

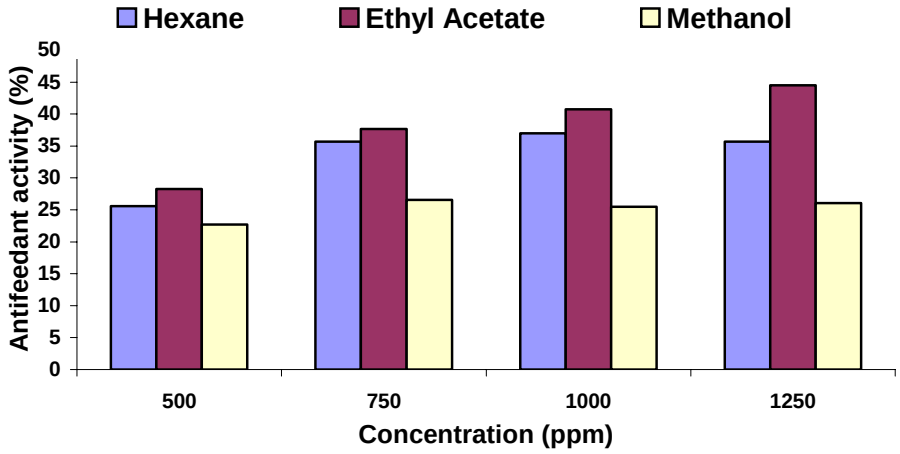
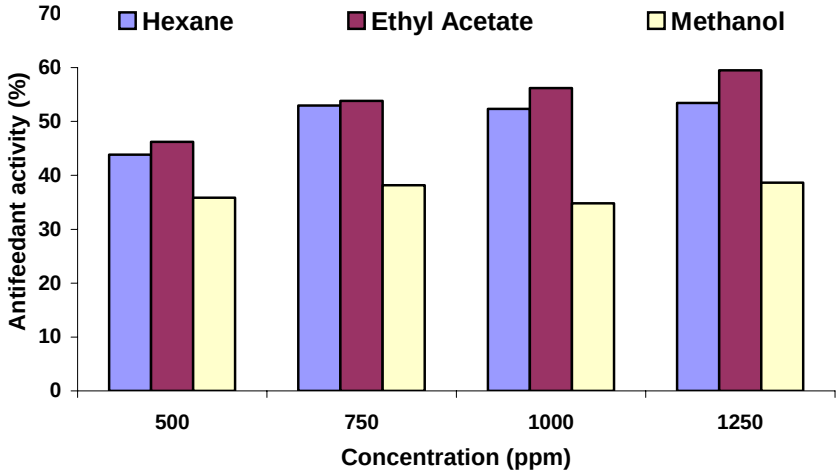
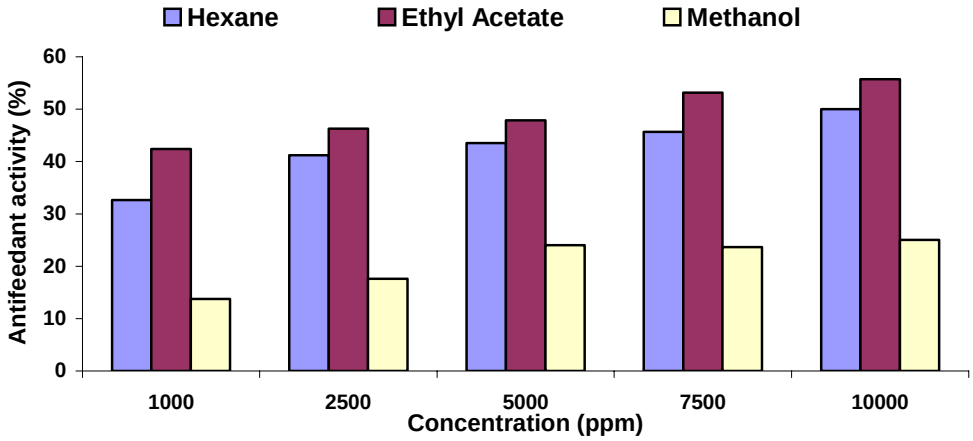


Fig 2. Neonate larvae were allowed to feed on various extracts of custard apple for a period of fifteen days



after 8 days of feeding. This indicates complete retardation in growth and development. The similar trend was observed for *A. squamosa* seed hexane extract at higher concentrations of 10000, 15000 and 20000 ppm, whereas in case of methanol extract the larvae gained very little weight even at higher concentrations of 10000, 15000 and 20000 ppm. The larval weights near LC_{50}

Fig 3: 7 day old larvae were allowed to feed on various extracts of custard apple for a period of 8 days



for *A. squamosa* seed hexane (5000 ppm), ethyl acetate (1000 ppm) and methanol (5000 ppm) extracts were 1.00, 0.91 and 3.32 mg which was 6.5, 7.14, 1.96 times less, respectively when compared to acetone control. The diet consumed by larvae near LC_{50} was 66.1, 67.4, 88.9 and 117 mg for hexane, ethyl acetate, methanol extracts and acetone control, respectively. As such, the ethyl acetate extract produced 55.73% anti-feedant activity while hexane and methanol extracts produced 50 and 25.04%, respectively (Fig. 3). *Annona squamosa* seed ethyl acetate extract showed good efficacy against both neonate and 7-day-old larvae, whereas, hexane and methanol extract produced more or less equal activity in terms of direct mortality. Ethyl acetate and hexane extracts showed good anti-feedant activity against both neonate and 7-day-old larvae. Methanol extract showed good anti-feedant activity (58.50%) against neonate larvae after 15 days of release at 2000 ppm but failed to show same against 7-day-old larvae even at 20000 ppm.

Discussion

The order of activity of three extracts of *Annona squamosa* seeds were ethyl acetate> hexane > methanol for neonate and 7-day-old larvae based on mortality and percent anti-feedant action. The LC_{50} of hexane extract for neonate larvae after 15 days was 1195.41 ppm. Qadri *et al*. (1977) tested cold ether extract of custard apple seed by dry film method against adults of *Callasobruchus chinensis* and *Rhizopertha dominica* and reported the LC_{50}

values of 141.30 and 524.80 µg/dish. Deshmukh *et al*. (1982) reported that hexane extract of seeds of *Annona* proved promising against *Culex quinquefasciatus* and *Musca domestica*.

Fig 4. Comparison between 15 days old larvae in acetone control and hexane extract at 1000 ppm. **Fig. 5.** Comparison between 15 days old larvae in acetone control and 750 ppm of ethyl acetate extract.

In the present study ethyl acetate extract showed maximum activity by recording the LC_{50} values at 305.36 ppm after 15 days for neonate larvae. Kawazu *et al*. (1989) was first to report that ethyl acetate extract had insecticidal activity against *Drosophila*. Later Vyas *et al*. (1999) reported that methanol extract from defatted seeds caused highest percent larval mortality against *Spodoptera litura, Helicoverpa armigera* and *Earias vitella.* The present study conclusively proved that ethyl acetate extract was superior to hexane and methanol extracts and first such study against Khapra beetle.

The weight of larvae was greatly reduced in almost all the concentrations when compared with acetone control. Five larvae weight in control was 4.23 and 6.03 mg after 10 and 15 days of release of neonate larvae, respectively. In case of hexane extract the larval weight near LC_{50} (1250 ppm) was 0.65 mg and 9.28 times lesser than the weight of larvae in acetone control, whereas in *Annona squamosa* seed ethyl acetate (250 ppm) and methanol extract (1500 ppm) it was 6.62 and 6.70 times less. The larvae were very small in size in higher concentrations both in ethyl acetate and hexane extracts. The larvae also turned into black colour, mummified and body covered with dense hairs (Figs 4, 5). Vyas *et al*. (1999) reported that the methanolic extract in addition of

larval mortality also caused reduction in larval growth, prolonged larval duration and lowered larval and pupal weights. Larval weight before inoculation and after 8 days of inoculation for 7-day-old larvae revealed that all the extracts showed anti-feedant action particularly ethyl acetate and not allowing the larvae to feed upon. In case of *A. squamosa* seed ethyl acetate extract at 10000 ppm concentration the weight of 5 larvae before release was 0.88 mg then after the larvae lost its weight instead of gaining and only weighed 0.55 mg i.e. 37.5% reduction in weight was observed after 8 days. The similar trend was observed in case of hexane extract at higher concentrations of 15000 and 20000 ppm.

Seven-day-old larvae were 4.86, 4.41 and 4.02 times less susceptible compared to neonate larvae based on LC_{50}. The extracts of custard apple besides causing direct mortality in larvae, also showed considerable account of anti-feedant activity particularly ethyl acetate (59.50%) and hexane extract (59.16) against neonate larvae. Santosh Babu *et al*. (1996) reported that chloroform extracts of seed showed effective anti-feedant activity against *Longitarsus nigripennis* and the inhibitory activity index was strong at 5000 ppm. *Annona squamosa* seed ethyl acetate and hexane extracts showed good anti-feedant activity against both neonate and 7-day-old larvae. Whereas, methanol extract showed more anti-feedant activity against neonate larvae.

The present study has indicated that *Annona squamosa* seed ethyl acetate and hexane extracts exhibited direct mortality of both neonate larvae and third instar larvae besides producing strong anti-feedant action on surviving larvae resulting in reduction in growth and development. The mummification and blackening of larvae may be attributed to some physiological changes, which took place in larvae.

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