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### Sublethal Effects of the Milky Latex of Sodom Apple, *Calotropis procera* (Alton) on the Growth, development, and some Physiological Aspects of the Greater Wax Moth, *Galleria mellonella* (L.) (Lepidoptera: Pyralidae)



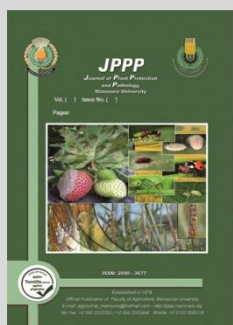
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#### ABSTRACT

The lethal, sublethal and some physiological effects of crude latex of Sodom apple, *Calotropis procera* 9Alton) were assessed using the third instar larvae of the greater wax moth, *Galleria mellonella* (L.) as a model insect species. Latex showed LC<sub>50</sub> of 37 µl latex/g diet after 48 hours of feeding on latex incorporated artificial diet. Larvae were continuously fed, until pupation, with artificial diet containing LC<sub>20</sub> of latex. Feeding inhibition and accumulated larval mortality of 53.0 ± 15% and 73 ± 2.6%, respectively, were observed before pupation for the LC<sub>20</sub> fed larvae. The incorporation of *C. procera* latex into the diet significantly prolonged the larval stage duration and decreased larval and pupal weight than untreated larvae. Furthermore, latex significantly reduced relative consumption rate (RCR), relative growth rate (RGR) and efficiency of conversion of ingested food (ECI). While there were no significant effects of latex on activities of both invertase, and esterases ( $\alpha$  and  $\beta$  Esterases) it showed a strong and significant inhibitory effect on  $\alpha$ -amylase. However, latex induced an obvious and significant increase in alkaline phosphatase activity. It is suggested that the antifeedant effect of *C. procera* latex could be due to the digestion inhibition through the inhibition of  $\alpha$ -amylase activity, while a significant portion of the digested food were used by *C. procera* latex fed larvae for synthesis alkaline phosphatase. These results provide more evidences regarding the mode of action of the lethal and sublethal effects of *C. procera* latex on *G. mellonella*. as a model insect species.

**Keywords:** *Calotropis procera* ,Latex ,*Galleria mellonella* , Growth, Development, Nuteation, Physiology



#### INTRODUCTION

Screening of toxicity of various plants against insect pests is the primary steps for isolation of new biologically active compounds, which could lead to the discovery of new pesticides. During the last decades, studies on natural plant products against agricultural insect pests proved that many of them can be used as insect toxicants, growth inhibitors, reproduction inhibitors or repellents alternatives to synthetic chemical insecticides (Singhi et al., 2004 and Bakavathiappan, et al., 2012) Among the studied plants, "Sodom apple" have been reported to show promising insecticidal properties against several important crop pests (Ramos et al., 2007 and 2010 and Dhileepan 2014). Sodom apple, *Calotropis procera* (Alton) is wild plant species belonging to the plant family Asclepiadacea. It is distributed in the desert, tropical and subtropical regions of the world (Tahir et al., 2013). In Egypt, *C. procera* which popularly known as Oshar grows wildly, in Nile-Faiyum, oases of the West Desert, East Desert, Red sea coastal region, Sinai pensaula, and in the southeast corner of Egypt at the Sudan frontier (Moustafa et al., 2010; El-Bakry et al., 2014 and Singh et al., 2015). It is a shrub or small tree with large leaves, pod-like fruits, and tufted silky-haired seeds (El-Bakry et al., 2014 and Tahir et al., 2013). When injured, the plant exudes a milky sap known as plant latex. Latex of *C. procera* has been shown to have toxic effect against several insect pests

(Moursy, 1997; Morsy et al., 2001; Singhi et al., 2006 and Tahir et al., 2013). However, the physiological effects of *C. procera* latex on insects are still not fully understood.

The greater wax moth, *Galleria mellonella* is found almost everywhere on earth where it considers as a serious pest of beehives and stored bee wax (Fathy et al., 2017). It is also used as a host for rearing entomopathogenic nematode and as an excellent model insect for evaluating toxicity of diverse chemicals and pathogens in many different physiologic and toxicology studies on insects (Zorlui et al., 2018)

Accordingly, objectives of this study were: (i) To evaluate the lethal and sublethal effects of whole latex of *C. procera*, on third-instar larvae of the greater wax moth, *G. mellonella* as an experimental model insect species; (ii) To investigate the effects of *C. procera* latex on nutritional physiology and specific enzymatic systems in *G. mellonella* larvae.

#### MATERIALS AND METHODS

##### Insect

The greater wax moth, *G. mellonella* larvae were obtained from infested hives and reared in the laboratory at Pest Physiology Research Dept., Plant Protection Research Institute, Agricultural Research Center, Giza, Egypt under controlled conditions (25 ± 2 °C, 60 ± 10% RH) according to rearing method of Birah et al., (2008). Larvae were fed on an artificial diet composed

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of wheat flour (350 g), corn flour (200 g), milk powder (130 g), glycerine (150 ml), honey (100 ml), and backing yeast powder (70 g) (Metwally *et al.*, 2012).

**Plant material:**

Whole small branches of healthy and non-cultivated plants growing in the vicinity of 6-October - Egypt, were collected during July-August 2018, and carried to the laboratory for latex collection. The plant materials were identified by experts at Plant Protection Research Institute, Dokki, Giza, Egypt.

**Latex collecting:**

The latex was extracted in the laboratory at the same day of branch collection by cutting the petiole of the fresh leaves and left to flow off in Eppendorf tubes. The latex was gently agitated during collection to overcome the tendency of the coagulation-like effect and was immediately used in the experiments (Ramos *et al.*, 2011).

**Toxicity Bioassay**

The method for determining the toxicity of *C. procera* latex on the third-instar larvae of *G. mellonella* was carried out by mixing an artificial diet with latex. First, a preparatory test was required to find the effective concentration ranges. Then, latex was added to the artificial diet at concentration of 12.5, 25.0, 50.0, 75.0, and 100.0 µl latex/g diet and mixed thoroughly. Newly active third-instar larvae were selected and starved for 4 hours before the experiments. Ten grams of latex-containing diet was put into covered 9-cm petri dish with 10 larvae. The control was given an artificial diet containing distilled water and the experiment was performed in five replications. Mortality was assessed after 48 hrs. Larvae were considered dead if they become immobile and have shown no detectable response to the external stimuli. Mortality data were subjected to probit analysis according to Finney's method Finney (1971).

**Effect of Sodom apple latex on growth, survival and feeding indices of *G. mellonella* larvae.**

Latex concentration equal to the calculated LC<sub>20</sub> was used to evaluate the effect of sublethal concentration of Sodom apple latex on growth, survival and the feeding indices of *G. mellonella* larvae according to Xu *et al.*, (2016). Briefly, ten grams of the latex containing diet was put into 9-cm covered petri dish with 10 active pre-weighted third-instar larvae. The control was given an artificial diet containing distilled water instead of latex and the experiment was performed in five replications. Larvae were continuously fed with treated diet until pupation. Petri dishes were checked daily for larval mortality and every 5 days, alive larvae were removed from diet with soft forceps, weighted individually and the remaining portions of the diet were weighed. This experiment was continued until adult emergence from pupae in control group, then accumulated mortality, duration of larval stage, pupal weight and the consumed diet weights were calculated.

The feeding inhibition percentage and feeding indices were estimated after 15 days of feeding using the following formulae (Waldbauer, 1968 and Jinguji *et al.*, 2018):

$$\text{Feeding inhibition (FI)} = (E_{\text{con}} - E_{\text{latex}} / E_{\text{con}}) \times 100$$

**Relative consumption rate**

$$\text{(RCR)} = E / TA;$$

**Relative growth rate**

$$\text{(RGR)} = P / TA;$$

**Efficiency of conversion of ingested food**

$$\text{(ECI)} = P / E \times 100,$$

where:

T = duration of experimental period.

A = mean weight of larvae during T,

E = mean weight of food eaten by larva during T

E<sub>con</sub> = mean weight of food eaten by control larva during T

E<sub>latex</sub> = mean weight of food eaten by latex fed larva during T

P = mean weight gain of larva at the end of T.

**Effect of Sodom apple latex on some enzymatic systems:**

The survived *G. mellonella* larvae after 48 hrs of feeding on artificial diet incorporated with LC<sub>20</sub> of latex were used for various biochemical tests. A known weight of larvae (whole body) was homogenized in appropriate amount of distilled water using mechanical homogenizer, centrifuged at 10,000g for 5 min at 4 °C and the supernatant was used for biochemical tests. For calculation the absolute activities of the selected enzymes, total soluble protein per gram of larval weight were determined according to the method of (Bradford, 1976). The activity of carbohydrate digestive enzymes α-amylase and invertase were measured by the procedure of Ishaaya and Swirski (1976) using 1% soluble starch and 2% sucroses as substrates for α-amylase and invertase, respectively. Alkaline phosphatase activity was measured by the method of Bessey *et al.*, (1946) using p-nitrophenyl phosphate as substrate. The activity of α- and β- esterases was determined according to the method of Van Asperen, (1962) using α-naphthylacetate and β-naphthylacetate as substrates.

**Statistical analysis**

Probit analysis was done to calculate the median lethal concentration (LC<sub>50</sub>) and LC<sub>20</sub> values of *C. procera* latex using LdP-Line® software (Bakr 2007). Each experiment was repeated at five times each with ten larvae (n = 50). The data were statistically analysed by means of analysis of variance (ANOVA) (Tukey's test) at P<0.05 using CoStat® software (Costat, 2007). Most of the results were expressed in percentage, although actual numbers were used for statistical tests. Results were recorded as mean ± standard deviation (SD).

## RESULTS AND DISCUSSION

### Results

**Toxicity of *C. procera* crude latex to third-instar larvae of *G. mellonella*:**

Because the dose-dependent effect of *C. procera* crude latex incorporated in the artificial diet of third-instar larvae of *G. mellonella* was not apparent in 24-h, LC<sub>50</sub> of latex was calculated after 48 hrs of feeding. Results tabulated in Table (1) shows that the LC<sub>50</sub> of *C. procera* crude latex on the third-instar larvae after 48 h of treatment was 37.0 µl/g of artificial diet, while the LC<sub>20</sub>, value was 22.5, µl/g of artificial diet.

**Sublethal effects of *C. procera* crude latex on the development and growth of *G. mellonella***

Data illustrated in Table 2 shows that the sublethal concentration of *C. procera* crude latex (22.5 µl/g) incorporated in artificial diet of 3<sup>rd</sup> instar larvae of *G. mellonella* was significantly prolonged the duration of larval stage compared with the control (20 vs 15 days,

respectively). The sublethal concentrations of latex significantly decreased both the mean wet weight of larva and the amount of food consumed by larva all along the larval development period (Fig 1 and 2).

**Table 1. Toxicity of Sodom apple crude latex on third-instar larvae of *G. mellonella* after 48 h of treatment**

LC	Concentration $\mu\text{l latex/g diet}$	Lower limit $\mu\text{l latex/g diet}$	Upper Limit $\mu\text{l latex/g diet}$
20	22.5	17.5	26.0
50	37.0	31.0	44.0

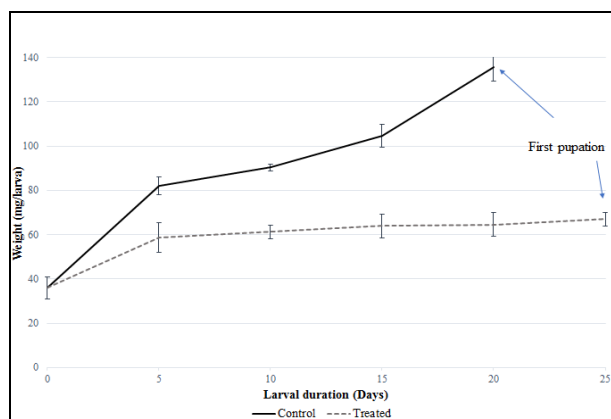
Slope =  $3.9 \pm 0.14$

**Table 2. The sublethal effects of Sodom apple crude latex on the development and growth of *G. mellonella***

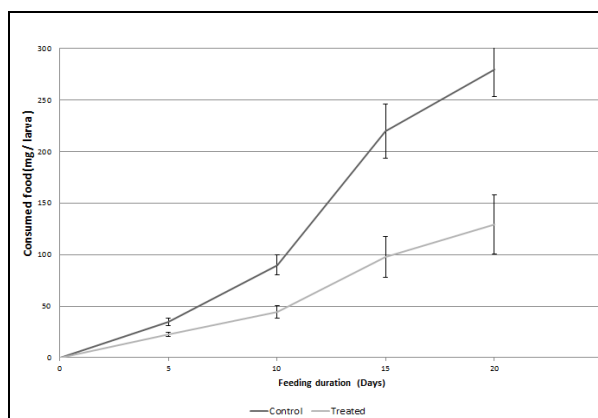
Parameters	Control	Treated
	Means $\pm$ SD	
Larval duration (days)	15 $\pm$ 2a	20 $\pm$ 1.5b
Last instar larval weight (mg)	136 $\pm$ 6.0a	65 $\pm$ 2.3b
Accumulated larval mortality (%)	3 $\pm$ 1a	73 $\pm$ 2.6b
Pupation (%)	97 $\pm$ 6a	27 $\pm$ 1.7b
Pupal weight (mg)	145 $\pm$ 6.4a	70 $\pm$ 2.2b
Adult emergence (%)	97 $\pm$ 6a	0b

Values are means  $\pm$  standard deviation (SD) of five replicates, each set up with ten larvae (n = 50).

Means followed by different letters within the same row are significantly different (Tukey's test,  $p < 0.05$ )



**Figure 1. The progress in weight of *G. mellonella* larvae fed on sublethal concentration of *C. procera* whole latex (Mean  $\pm$  SD)**



**Figure 2. Mean weight of food consumed by *G. mellonella* larvae fed on sublethal concentration of *C. procera* whole latex during larval stage (Mean  $\pm$  SD).**

The amount of diet consumed per larva during larval period decreased by more than half compared to the control (Fig. 1). Moreover, high accumulated mortality percentage was recorded among latex fed larvae (73%) compared with control larvae (3%), so that only 27% of treated larvae reached pupal stage compared with 97.0 percentage pupation among control larvae. The weights of pupae were significantly much lower in treated groups than that of control groups (70 vs 145 mg/pupa). All pupae from control groups developed to adults while no adults were emerged from pupae of latex fed larvae.

**Sublethal effects of *C. procera* crude latex on nutritional physiology and enzymatic activities.**

Results in Table 3 shows that RCR, RGR, and ECI values were significantly reduced in the larvae that fed on diets containing LC<sub>20</sub> of latex compared with control larvae. RCR was slightly reduced in the larvae that fed diets containing fed on latex compared with control larvae (0.107  $\pm$  0.016 vs 0.139  $\pm$  0.01, respectively). However, RGR was highly reduced after ingestion of the latex (0.06  $\pm$  0.02 vs 0.26  $\pm$  0.008 for latex fed and control larvae, respectively). Five-fold reduction in the efficiency of conversion of ingested food (ECI) was observed due to latex ingestion (4.94  $\pm$  2.45 vs 19.6  $\pm$  2.8 for latex fed and control larvae, respectively). The overall feeding inhibition (FI) percentage due to latex feeding was 53.0  $\pm$  15% of control.

**Table 3. Nutritional indices of *G. mellonella* after 15 days of feeding third instar larvae on artificial diet containing LC<sub>20</sub> (22.5  $\mu\text{l/g}$  diet) of Sodom apple crude latex.**

Indices (Mean $\pm$ SD)	Control	Treated
Relative consumption rate (RCR) (gm food consumed/gram body weight/day)	0.139 $\pm$ 0.01a	0.107 $\pm$ 0.016b
Relative growth rate (RGR) (gm biomass acquired/g body weight/day)	0.26 $\pm$ 0.008a	0.06 $\pm$ 0.02b
Efficiency of conversion of ingested food (ECI) (g weight gain/g of food eaten %)	19.6 $\pm$ 2.8a	4.94 $\pm$ 2.45b
Feeding inhibition (FI) (%)	0a	53.0 $\pm$ 15%b

Values are means  $\pm$  standard deviation (SD) of five replicates, each set up with ten larvae (n = 50).

Means followed by different letters within the same row are significantly different (Tukey's test,  $p < 0.05$ )

The effect of LC<sub>20</sub> (22.5  $\mu\text{l/g}$  diet) of *C. procera* latex incorporated in artificial diet of *G. mellonella* 3<sup>rd</sup> instar larvae on activities of specific larval carbohydrate digestive enzymes and detoxification enzymes after 48 h of contentious feeding was assayed and tabulated in Tables 4.

**Table 4. Activities of certain carbohydrate digestive enzymes and detoxification enzymes in *G. mellonella* third instar larvae after 48hrs of continuous feeding on LC<sub>20</sub> of *C. procera* crude latex.**

Enzyme	Control	Treated
$\alpha$ -amylase ( $\mu\text{g glucose/min/g protein}$ )	95.2 $\pm$ 4.2a	28.13 $\pm$ 0.7b
Alkaline phosphatase ( $\mu\text{g phenol/min/g protein}$ )	908.2 $\pm$ 24a	1488.4 $\pm$ 43b
$\alpha$ -Esterase ( $\mu\text{g } \alpha\text{-naphthol/min/g protein}$ )	41.4 $\pm$ 1.92a	39.2 $\pm$ 1.6a
$\beta$ -esterase ( $\mu\text{g } \beta\text{-naphthol/min/g protein}$ )	19.4 $\pm$ 1.60a	21.8 $\pm$ 1.8a
Invertase ( $\mu\text{g glucose/min/g protein}$ )	28.6 $\pm$ 2.3a	28.9 $\pm$ 1.8a

Values are means  $\pm$  standard deviation (SD) of five replicates, each set up with ten larvae (n = 50).

Means followed by different letters within the same row are significantly different (Tukey's test,  $p < 0.05$ )

Activity of  $\alpha$ -amylase enzyme decreased significantly in latex fed larvae compared with the control ( $28.13 \pm 0.7$  vs  $95.2 \pm 4.2$   $\mu$ g glucose/min /g protein, respectively). The reduction of  $\alpha$ -amylase activity reached more than 3-folds. Also, the activity of alkaline phosphatase in latex fed larvae increased significantly compared with the controls ( $1488.4 \pm 43$  vs  $908.2 \pm 24$  mg phenol/min/g protein, respectively). Activities of other detoxifying and carbohydrate hydrolysing enzymes such as  $\alpha$ -Esterase,  $\beta$ -Esterase and invertase did not differ significantly between control and latex fed larvae (Table 4).

#### Discussion

Analysis of experimental data showed clearly that *C. procera* latex has concentration and time dependent toxic effect on larvae of *G. mellonella*. In the previous studies *C. procera* latex also has been reported to have toxic effects against several of other insect species (Moursy, 1997; Morsy et al., 2001; Singhi, 2004 and Tahir et al., 2013). Moreover, results of this study clearly indicate that *C. procera* latex at sublethal concentration cause delayed mortality of *G. mellonella* larvae due to feeding inhibition. These latent lethal effects are very serious compared to the acute toxicity effect. Furthermore, it is appearing that with low food intake, larvae take longer to reach the critical weight for ecdysis, which implies an increase of the larval stage duration. Such delays in larval development due to feeding inhibition may result in impaired larval development and unsuccessful emergence of adults. The high value of FI percentage, calculated in this study proves that *C. procera* latex possess feeding inhibition properties as, generally, the higher the value of FI, the lower the rate of feeding (Szczepek et al., 2016 and Jinguji et al., 2018). The feeding inhibition of *C. procera* leave extract against another two lepidopteran larvae; *Spodoptera littoralis* and *Spodoptera lithura* has been reported by Abdel-Rahman and Al-Mozini (2007) and Bakavathiappan, et al. (2012), respectively. Such extract, also, has been reported to possess potent lethal and growth reducing activity to mosquito larvae (Singhi et al., 2004).

The feeding inhibition properties of *C. procera* latex were further assessed using feeding deterrence parameters such as the relative consumption rate (RCR), the relative growth rate (RGR), and the efficiency of conversion of ingested food (ECI). RCR indicates the amount of food ingested by larva per gram of body weight per day, RGR, indicates the gain of biomass by the insect in relation to body weight per day, while ECI, indicates the percentage of ingested food that is converted into biomass (Giongo et al., 2015). It is likely that this low feeding rate is due to the inhibitory effect of the latex on  $\alpha$ -amylase activity, and at least in part, this contributes to the decrease in growth rate. Alpha-amylase is a major digestive enzyme, that catalyses the hydrolysis of starch into sugars (Nasr and Zibae, 2017). As the artificial diet of *G. mellonella* larvae is composed mainly of starch, it obvious that  $\alpha$ -amylase plays an essential role in digestion of ingested food. Incorporation of LC<sub>20</sub> of *C. procera* latex in the artificial diet caused a serious reduction in  $\alpha$ -amylase activity. As a result, digestion of food in the elementary canal of *G. mellonella* larva takes longer time, which lowering the rate of feeding. This conclusion could be supported by Batista-Pereira et al., (2002) and Abdel-Rahman and Al-Mozini (2007) who mentioned that the

mode of action of the *C. procera* as antifeedant may be due to the digestion inhibition through inactivation of digestive enzymes. Also, the results of this study confirmed the suggestion of Upadhyay (2013) that the deleterious effects of *C. procera* latex on insect feeding may be due to presence of  $\alpha$ -amylase inhibitors.

Feeding inhibition from sublethal exposure to *C. procera* latex appeared to be responsible for decreases in the growth, body size, RCR and RGR of larvae. Furthermore, the significant reduction in both RCR and RGR due to consumption of *C. procera* latex incorporated in artificial diet may be due to not only a low food intake and digestibility but also due to a toxic effect caused by the latex. The calculated of ECI among latex fed larvae confirmed this conclusion. The low conversion of food into biomass as reflected by the low ECI value justifies the delay in larval development observed with latex fed larvae, and indicates that it causes toxic effect, which can be considered secondary phagodeterrence (reduced consumption caused by toxicity) according to Giongo et al., (2015). Since the biomass conversion (ECI) was low, it could be concluded that significant portion of the digested food was utilized for purposes other than growth, such as synthesis of detoxification enzymes as suggested by Giongo et al., (2015). To test this hypothesis, the activities of three of the main detoxification enzymes in insects were assayed in this study; namely:  $\alpha$  and  $\beta$  esterases, and alkaline phosphatase. Esterases ( $\alpha$ -EST and  $\beta$ -EST) are an important detoxifying enzyme which hydrolyse the esteric bond in toxic chemicals, they have been reported to possess detoxification ability against synthetic and botanical insecticides (Zibae, 2011). Alkaline phosphatase is mainly found in the intestinal epithelium of insects and its role is to hydrolyse phosphomonoesters under alkaline conditions and provide phosphate ions for different metabolic processes (Nasr and Zibae, 2017). Although the results of this study indicate that feeding *G. mellonella* larvae on artificial diet contains *C. procera* latex for 48 hrs did not affect significantly both of 3rd instar larvae  $\alpha$  and  $\beta$  esterase activities, it induced an obvious and significantly increase in alkaline phosphatase activity. Increasing alkaline phosphatase activity level indicates that the enzyme is involved in detoxification of certain toxic compounds that could be present in *C. procera* latex and confirmed the suggestion that portion of the digested food were used by *C. procera* latex fed larvae for synthesis of specific detoxification enzymes. However, further work, to isolate and identify the insecticidal compound(s) in *C. procera* latex, especially that responsible for the serious reduction in *G. mellonella*  $\alpha$ -amylase activity is in progress.

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### التأثيرات تحت المميّنة لعصارة نبات العشار (*Calotropis procera* (Alton) اللبنيّة على التطور والنمو وبعض الجوانب الفسيولوجية الخاصة بيرقات عثة الشمع الكبرى، *Galleria mellonella* (L.) (Lepidoptera: Pyralidae). أحمد عبد المنعم محمد الحفني

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تم تقييم التأثيرات المميّنة وتحت المميّنة والفسيولوجية لعصارة نبات العشار (*Calotropis procera*) اللبنيّة الخام باستخدام يرقات العمر الثالث ليرقات عثة الشمع الكبرى (*Galleria mellonella*) كحشرة تجارب، وقد أظهرت العصارة تأثيراً مميّناً على اليرقات بجرعة نصف مميّنة تم تقديرها بـ 37 ميكرو لتر من العصارة لكل جرام من الغذاء بعد 48 ساعة من التغذية على نظام غذائي صناعي مضاف اليه العصارة. وقد تم تغذية اليرقات بصفة مستمرة، حتى التعذير، على نظام غذائي اصطناعي يحتوي على جرعة العصارة التي حسب انها تسبب 20% موت (LC<sub>20</sub>) بين اليرقات. وقد لوحظ ان العصارة تسببت في حدوث تثبيط معنوي للتغذية في اليرقات بنسبة 53% ووصلت نسبة الموت المتراكمة بين اليرقات الى 73% خلال الفترة من العمر اليرقي الثالث وحتى التعذير. وقد أدت إضافة العصارة الى النظام الغذائي إلى إطالة مدة الطور اليرقي وانخفاض متوسط وزن اليرقة والشرنقة بشكل معنوي مقارنة باليرقات والعذارى غير المعاملة. علاوة على ذلك، خفضت العصارة بشكل كبير معدل استهلاك اليرقات النسبي للغذاء (RCR)، ومعدل النمو النسبي لليرقات (RGR) وكفاءة تحويل ما تناولته اليرقة من غذاء الى وزن جسم (ECI)، وفي حين أن العصارة لم تظهر أي تأثير معنوي على أنشطة كل من انزيمي الانفرتيز والإستريز العام (ألفا وبيتا الإستريز)، الا انها اظهرت تأثيراً مثبطاً قوياً ومعنوياً على إنزيم ألفا أميليز. وأيضاً، تسببت في زيادة واضحة ومعنوية في نشاط انزيم الفوسفاتيز القلوي. وعلى هذا يُقترح أن يكون التأثير المانع للتغذية لعصارة نبات العشار اللبنيّة هو نتيجة اعاقه هضم اليرقة للغذاء من خلال تثبيطه نشاط انزيم الاميليز الضروري لهضم المواد النشوية المعقدة، بينما اضطرت اليرقة الى استخدام جزء كبير من الطعام المهضوم لانتاج بعض إنزيمات إزالة ومعادلة السموم مثل انزيم الفوسفاتيز القلوي. في المجمع توفر هذه النتائج المزيد من الأدلة على طريقة عمل التأثيرات تحت المميّنة لعصارة نبات العشار اللبنيّة على يرقات عثة الشمع الكبرى.