Research Article

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Dose–response relationships for the antifeedant effects of *Humulus lupulus* extracts against larvae and adults of the Colorado potato beetle

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Abstract

BACKGROUND: Dose–response relationships for antifeedant effects of *H. lupulus* extracts against larvae and adults of the Colorado potato beetle (CPB) were determined in laboratory conditions. The larval and adult beetles were fed on potato leaflets treated with *H. lupulus* extract ranging from 0.4 to 40 mg mL^{-1} in a no-choice situation. Their feeding behavior was recorded, and larval growth and antifeedant indexes were calculated.

RESULTS: *H. lupulus* treatments significantly affected larval growth rate, and at higher concentrations the larval weights were significantly reduced over the course of the assay. Adults of CPB were more sensitive to the extracts than the larvae, and, even at lower doses, adult beetles were arrested for longer periods than larvae.

CONCLUSION: These results indicate that *H. lupulus* extracts may have potential for control of CPB, particularly in organic farms where conventional insecticides are not available.

Keywords: hop; *Leptinotarsa decemlineata*; larvae; adult; antifeedant

1 INTRODUCTION

The Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say), is a serious pest of potatoes and other crops worldwide, distributed throughout most of the United States, Europe and Asia.[1] The adults and larvae feed primarily on the leaves of potato plants and have the potential to defoliate host plants completely. Extensive use of conventional pesticides to control this pest led to the development of resistance to all major insecticide classes, e.g. carbamates, pyrethroids and neonicotinoids.[2–4] Therefore, in addition to new classes of insecticides, chemicals that have different modes of action (e.g. antifeedants and repellents) are urgently needed in the potato industry around the world to maintain stable food production. Azadirachtin, for example, derived from the neem tree (*Azadirachta indica*), is both an antifeedant and a toxicant and is successfully used against various insect pests.

Most plants defend themselves from herbivory through production of secondary compounds such as terpenes, phenolics and nitrogen-containing compounds.[5] Some of these secondary compounds, including pyrethrin and rotenone, produce direct toxicity, while the majority of them cause either physiological disruption as caused by growth regulators or behavioral effects as repellents, attractants or antifeedants.[6–11] While the neurotoxic properties of plant secondary compounds have been exploited primarily in insect pest management programmes, the behavior-modifying aspects of plant-derived compounds provide additional suitability for sustainable pest control because resistance to these compounds requires both physiological and behavioral adaptations.[12]

Recently, Gökçe *et al*.[13] reported that larvae of *L. decemlineata* were deterred from feeding on potato, *Solanum tuberosum*, leaves by surface residues of 2 and 200 g kg^{-1} of extracts from four plant species found commonly in Turkey. Application of 2 g kg^{-1} of extracts did not cause a significant reduction in larval feeding. In the same study, behavioral observations during interactions of *L. decemlineata* larvae with potato leaves treated with varying concentrations of the plant extracts suggested that the reduction in leaf consumption was caused by larvae rejecting the leaves upon contact. This indicates that non-volatile chemicals detected by tarsal or mandibular sensors are responsible for the insect's response. Gökçe *et al*.[13] also found that extracts of *Humulus lupulus* inflorescences were the most active at protecting leaves from feeding and caused complete inhibition of feeding behaviors in larvae at 20 and 200 g kg^{-1} concentration.

The high activity of *H. lupulus* extracts and the relative ease of collection from raw plant material led to this further investigation of how extracts from this plant disrupt feeding by both pestiferous

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life stages of *L. decemlineata*. Adult and larval feeding on potato leaves treated with a range of concentrations was measured. These two life stages of *L. decemlineata* were also observed during interaction with treated leaves to determine how the extracts affected acceptance and rejection behaviors by this pest.

2 MATERIALS AND METHODS

2.1 Insect culture

L. decemlineata beetles were obtained from the Alampi Beneficial Insect Laboratory, New Jersey Department of Agriculture, Trenton, New Jersey. The insects were reared on potato plants, *S. tuberosum* cultivar Superior, at 26 ± 1 °C and 45% RH in a 16 : 8 h light : dark photoperiod regime. The colony has been routinely used as a reference by many universities in monitoring pesticide resistance (Palmer D, private communication, 2006). Adults and third-instar larvae used in bioassays were starved for 24 h prior to each study.

2.2 Plant material

The detailed experimental protocol for preparing *H. lupulus* extract was described by Gökçe *et al*.[13] Hop cones were collected during the spring and summer seasons of 2008–2009 in Tokat, Turkey. Dried and ground hop cones were treated with methanol for 24 h. Thereafter, the suspension was sieved through cheesecloth, and excess methanol was removed using a rotary evaporator (RV 05 Basic 1B, IKA Group, Wilmington, NC) at 32 ± 2 °C. Residue of hops was diluted with sufficient HPLC-grade acetone (Sigma-Aldrich, Milwaukee, WI) to give a series of 400 mg mL^{-1} stock solutions. The stock suspension was then diluted with acetone to give solutions containing 0.4, 0.8, 4, 8 or 40 mg mL^{-1} of plant suspension. These concentrations were selected because a previous study[13] showed that these concentrations were not phytotoxic to potato plant samples and produced behavioral responses in CPB larvae.

2.3 Behavioral test

Gökçe *et al*.[13] described the experimental protocol for testing *H. lupulus* extract against Colorado potato beetle larvae. To standardize leaf area, potato leaflets were removed from healthy potato plants, *S. tuberosum* L., a 20 mm^2 disc was cut from each using a cork borer and the leaflet discs were immediately dipped into one of the *H. lupulus* treatment solutions or the solvent control for 5 s. The discs were dried in a fume cabinet for 15 min at room temperature, and then each disc was transferred into a 60 mm diameter Petri dish and a third-instar larva or an adult *L. decemlineata* was placed on the disc. The dish was set 70 mm beneath a black and white CCD camera (Shebar, Burton, MI) and illuminated with a cold-source dissecting lamp (Schott, Elmsford, NY); ambient light was minimized. The cameras were connected to a computer (Gateway, Irvine, CA) with video input and TVR 2.0 digital video recording software (Homestretch, Austin, TX). Video recording for 15 min commenced immediately following the placement of each insect on the leaf disc. Recordings lasting 5 min were made every 2 h over a 24 h period to assess insect feeding activity throughout exposure to the extracts. The bioassays were conducted at 26 ± 2 °C in a 16 : 8 h light : dark photoperiod. A completely randomized design was used in this study, as all the bioassays could not be conducted on a single occasion. Ten larvae or adult CPB were assigned randomly among each of the five plant extract concentrations and control treatments and observed for behavioral analysis. The whole experiment was carried out over ten consecutive days.

Video recordings from the first 15 min of the observations were analyzed using the Observer 5.0 behavior analysis software (Noldus Information Technologies, Wageningen, The Netherlands). The duration and frequency of the following behavioral states were recorded: feeding, resting and walking. Leaf rejection, defined as the insect leaving the leaflet and remaining away until the end of the observation, was also recorded. From these data, the amount of gustatory interaction and leaf rejections were calculated.

2.4 Damage rate test

The damage rate of CPB larvae and adults on potato leaflet discs treated with *H. lupulus* extract or control were assessed at 24 h intervals for 144 h. Leaflets were treated with *H. lupulus* concentrations or control, left to dry and supplied to adults or larvae as described above. The insects were incubated at 26 ± 2 °C in a 16 : 8 h light : dark photoperiod. Every 24 h, the leaflets were replaced with new ones. The leaflets that were removed from the Petri dishes were dried at 40 °C in a gravity convection incubator (GCA Precision, Winchester, VA) for 24 h and were then weighed on an electronic balance (Sartorius, Goettingen, Germany) to assess the amount of leaf tissue remaining. Leaves treated with acetone were placed into Petri dishes for 24 h in the bioassay room and were also weighed to provide a positive control. The remaining weights of treated and control leaves and the positive control were used to calculate a damage rate, $DR = [(T - C)/PC]$, where C and T are the remaining weights of control and plant-extract-treated leaf discs, and PC is the weight of the positive control. A rating of 0–4 was given, based on the following criteria:

$0 - DR = 1$
$1 - 0.75 \leq DR < 1$
$2 - 0.50 \leq DR < 0.75$
$3 - 0.25 \leq DR < 0.50$
$4 - 0 \leq DR < 0.25$

Ten larvae and adults were used for each concentration of *H. lupulus* and control treatments.

2.5 Larval growth test

Larval growth of CPB feeding was measured on treated leaflets with plant extracts in a no-choice context. Five concentrations of *H. lupulus* extract, 0.4, 0.8, 4, 8 or 40 mg mL^{-1}, were prepared in acetone, and potato leaflets were dipped into one of these concentrations or the control (described above). Leaflets were left to dry and then transferred to Petri dishes. Prior to introducing the larvae to leaflets, all larvae were weighed using the electronic balance. A freshly moulted third-instar larva was placed into each petri dish. The larvae were incubated at 26 ± 2 °C and in a 16 : 8 h light : dark photoperiod for 7 days. A fresh potato disc was supplied to larvae every 24 h for 7 days. After the incubation period, larval weight was measured again for each insect and a growth inhibition index (GHI) was calculated for each concentration using a modified equation from Abdelgaleil and El-Eswad:[14] $GHI = [(C - T)/T]$, where C is the larval weight gain in the control and T is the larval weight gain in the treatment. Ten larvae were exposed to each concentration or control treatment.

2.6 Analysis

The frequency and duration of each CPB behavior observed on each larva were determined during the 15 min feeding observations, and these were compared among treatments using analysis of variance (ANOVA), followed by Fisher's protected

least significant difference (PLSD) test. The proportion of time during each observation for which the insects exhibited each recorded behavior was compared across treatments for each of the behaviors using analysis of variance (ANOVA) on arcsine-transformed values, followed by comparison among means using Fisher's PLSD test. The damage rates were analyzed by means of the Kruskal–Wallis test, which corrects for ties, to determine whether the extract concentration had a significant effect on observed scores. The growth inhibition test data were analyzed by a Kruskal–Wallace test ($P < 0.05$).

3 RESULTS

The frequency with which movement-related behaviors were observed in the assays varied with extract concentration (Fig. 1), and adult and larval *L. decemlineata* vary in the nature of their behavioral responses to extracts. Adult beetles exposed to potato leaves treated with the *H. lupulus* extracts exhibited an increasing frequency of walking as the extract concentration increased ($F = 2.41$; df = 5, 54; $P = 0.047$). For adult beetles, resting time on the dish increased with increasing extract concentration ($F = 4.33$; df = 5, 54; $P = 0.0022$), while resting time on leaflets decreased following transfer from extract treated leaves ($F = 4.11$; df = 5, 54; $P = 0.003$). Walking frequency of larvae on the leaf or surfaces in the arena dish yielded similar patterns to those exhibited by adult beetles (Fig. 1), with significant increases in the frequency of walking on the dish as the extract concentration increased ($F = 5.49$; df = 5, 54; $P = 0.0004$). In contrast to the adults, larval resting in the arena was rarely observed and, as with adults, was more commonly encountered on treated leaf surfaces (Fig. 1). Larval leaf resting frequency showed a significant non-linear relationship, with higher resting frequency as extract concentration increased.

Leaf biting and feeding behaviors varied between *L. decemlineata* larvae and adults, and among concentrations (Fig. 1). Extract concentration significantly reduced biting behavior in adults ($F = 3.87$; df = 5, 54; $P < 0.0045$), but not in the larvae ($F = 0.52$, df = 5, 54; $P = 0.76$). Leaf feeding declined to zero with increasing extract concentration between 0.4 and 4 mg mL^{-1} in adult beetles, with no feeding above that concentration. Larvae did not exhibit a gradual decline. Rather, feeding was observed at a similar rate up to the 4 mg mL^{-1} concentration, and all feeding ceased in larvae at concentrations higher than 0.8 mg mL^{-1}.

The behavioral basis of the reduced leaf consumption with increasing concentration of extract described above was also seen in the distribution of time spent in different feeding-related behaviors. Adult and larval *L. decemlineata* spent 63% and 85% of their time, respectively, feeding on leaves without the extract, and these values were reduced dramatically to cessation on leaves receiving the 4 mg mL^{-1} or higher extract (Fig. 2). As feeding decreased, adult beetles spent a significantly greater amount of their time resting or walking on the dish. Time spent in resting behaviors also increased with increasing concentrations. Like the adults, larvae spent an increasing amount of time resting on the leaf as the extract concentration increased (Fig. 2) progressively until, at 4 mg mL^{-1} concentration, over 95% of larval time was spent resting on the leaf.

Within 24 h of exposure to leaves treated with extracts, adult *L. decemlineata* exhibited a significant decrease in their potato leaf consumption rankings when treated with different concentrations of plant extract (Kruskal–Wallis $H = 52.4$, $P < 0.0001$), with decreasing leaf consumption as extract concentration increased

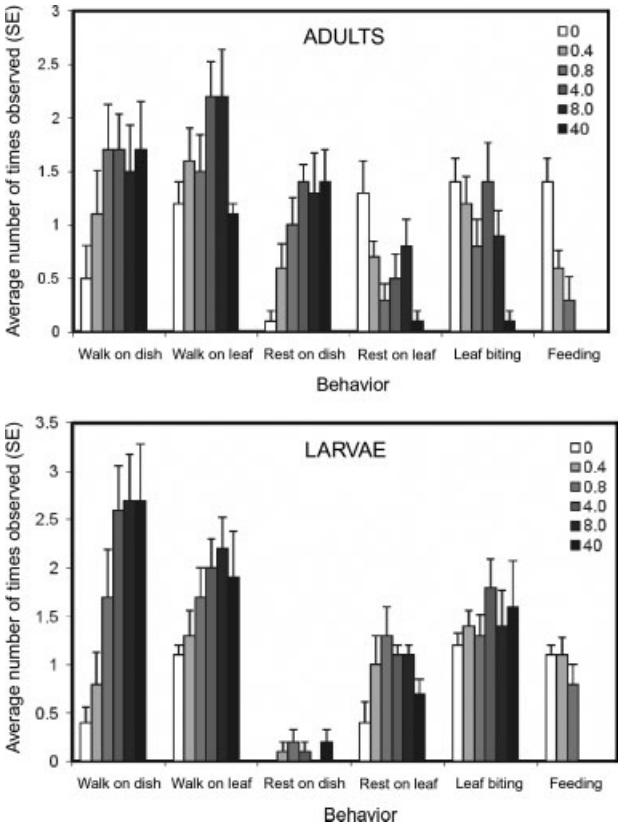

Figure 1. Average frequency (± SE) of behaviors exhibited by adult and larval *Leptinotarsa decemlineata* in an arena containing a *Solanum tuberosum* leaf treated with varying concentrations of *Humulus lupulus* extract ($n = 10$).

(Table 1). A similar pattern of feeding was found for *L. decemlineata* larvae (Table 1). These findings were exhibited consistently across the duration of the assays, while extract concentrations significantly reduced adult and larval leaf feeding ($H > 38.3$, $P < 0.0001$). Comparison of the relative sensitivity of adult and larval *L. decemlineata* to the extracts indicated that the adults were more sensitive, yielding greater leaf protection at lower concentrations (Table 1).

Larval growth results confirmed previous results that CPB feeding on treated potato leaflets decreased as the dose of *H. lupulus* increased in the treatments (Fig. 3). Increasing concentration of *H. lupulus* in treatments significantly affected larval growth rate ($H = 32.1$, $P < 0.00$), and at 40 mg mL^{-1} concentration the larvae lost weight until they weighed less than their initial weight. Interestingly, larvae that were fed on leaf discs treated with *H. lupulus* at the 0.4 mg mL^{-1} concentration gained more weight than larvae in the control. This is in accordance with the results observed in the behavioral and damage rate test. Desensitization of larvae to *H. lupulus* extract was not observed during the experiment, which eventually led to some mortality in the treatment (unpublished data), likely owing to starvation of larvae.

4 DISCUSSION

Host plant recognition by *L. decemlineata* is mediated through chemosensory inputs received from host plants before contact with plants.[15–18] Once in contact with the plant, feeding behavior is

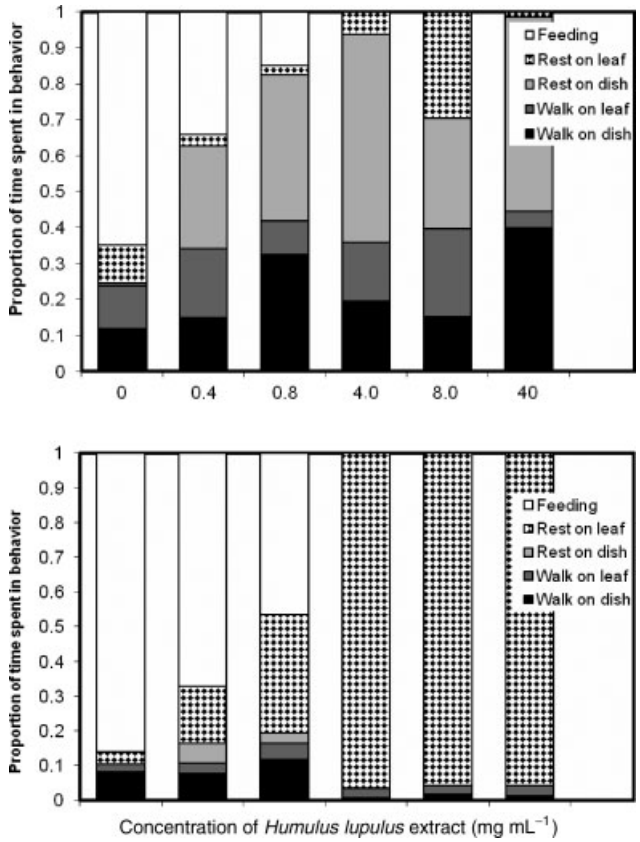

Figure 2. Average proportion of time spent in different behaviors by *L. decemlineata* adults and larvae when exposed to *Solanum tuberosum* leaves treated with varying concentrations of *Humulus lupulus* extract ($n = 10$).

affected by physical (leaf trichomes, cuticular waxes) and chemical (leaf volatiles and secondary metabolites) host factors.[19] Potato plant contents, especially L-alanine, L-serine, GABA, sucrose and phospholipids, act as a phagostimulants and are reported to have important roles in continuation or sustained feeding by *L. decemlineata*.[20,21] Compounds masking or causing concentration changes in the plant sap can deter, reduce or stop feeding.[21–23] Many organic or inorganic chemicals aimed at reducing or arresting host feeding have been tested where host secondary plant compounds have been cited as putative agents of this behavior.[13,24–28] The present studies confirmed and also extended information that *H. lupulus* extracts can disrupt feeding by *L. decemlineata* on the potato host plant. Hop plant extracts contain many secondary compounds, including flavonoids, alpha acids, beta acids and essential oils.[29–31] Some of these compounds have been reported as having both repellent and antifeedant activity to arthropods.[11,32,33]

Both larvae and adult feeding patterns were affected by hop extracts in this study, with adults more sensitive than larvae. In adult beetles, feeding also ceased at lower concentrations than those observed for larvae. These results support previous studies showing differential response between larvae and adult *L. decemlineata*.[25,34] These observations may be related to the adult's host-finding behavior, as adults are more mobile than the larvae. Given that adult *L. decemlineata* are mobile and can fly, they have access to greater resources in the environment, which may, in turn, allow them to be more selective than the larvae.

The feeding behavior of both larvae and adult beetles was significantly affected by the *H. lupulus* extract. Their response was also dose dependent. These results are in agreement with previous studies showing similar dose-dependent feeding behavior changes in CPB when they were force fed on alkaloid- or limonoid-treated leaflets.[35,36] The concentration threshold for evoking these behaviors appeared to be around 4 mg mL^{-1} for adults and 8 mg mL^{-1} for larvae, which are lower than previously reported.[25,28]

Forcing the CPB larvae to feed on the extract-treated potato leaflets resulted in reduction in larval growth in a dose-dependent manner. This suggests that responses of larvae to the extract are behavioral, such as a repellency or antifeedant action, rather than toxic effects, as no mortality was observed at lower concentrations where larvae consumed all or most of the potato leaflets. Although there is no report of direct comparison of hops extract on larval

Table 1. Average (± SE) damage scores[a] of potato leaves treated with different concentrations of *Humulus lupulus* extract and exposed to individual adult or larval *Leptinotarsa decemlineata* for 5 days. For each life stage, values in a column followed by the same letter are not significantly different ($P > 0.05$) ($n = 10$)

Life stage	Extract conc. (mg mL^{-1})	Time after exposure (h)				
		24	48	72	96	120
Adult	0	4.00 ± 0.00a	3.80 ± 0.13a	3.50 ± 0.22a	3.30 ± 0.39a	4.00 ± 0.00a
	0.4	3.50 ± 0.17b	3.70 ± 0.15a	3.60 ± 0.22a	3.40 ± 0.27a	3.90 ± 0.10a
	0.8	1.30 ± 0.15c	0.60 ± 0.22b	1.40 ± 0.58b	0.80 ± 0.53b	1.20 ± 0.61b
	4.0	0.30 ± 0.15d	0.00 ± 0.00c	0.00 ± 0.00d	0.00 ± 0.00b	0.00 ± 0.00c
	8.0	0.30 ± 0.15d	0.00 ± 0.00c	0.20 ± 0.13c	0.00 ± 0.00b	0.00 ± 0.00c
	40	0.00 ± 0.00d	0.00 ± 0.00c	0.00 ± 0.00d	0.00 ± 0.00b	0.00 ± 0.00c
Larva	0	3.80 ± 0.13a	3.80 ± 0.13a	3.90 ± 0.10a	3.90 ± 0.10a	3.90 ± 0.10a
	0.4	3.40 ± 0.40ab	3.60 ± 0.40a	3.60 ± 0.40a	3.60 ± 0.40a	3.60 ± 0.40a
	0.8	3.10 ± 0.43b	2.80 ± 0.49ab	3.20 ± 0.53a	2.70 ± 0.52ab	2.80 ± 0.51b
	4.0	2.20 ± 0.36c	1.90 ± 0.35bc	1.80 ± 0.33b	2.10 ± 0.38b	2.40 ± 0.40b
	8.0	1.00 ± 0.29d	1.11 ± 0.31c	1.33 ± 0.41c	1.00 ± 0.29c	0.89 ± 0.26c
	40	0.00 ± 0.00e	0.00 ± 0.00d	0.10 ± 0.10d	0.10 ± 0.10c	0.00 ± 0.00c

[a] Scoring system: 0 = DR = 1; 1 = 0.75 ≤ DR < 1; 2 = 0.50 ≤ DR < 75; 3 = 0.25 ≤ DR < 0.50; 4 = 0 ≤ DR < 0.25.

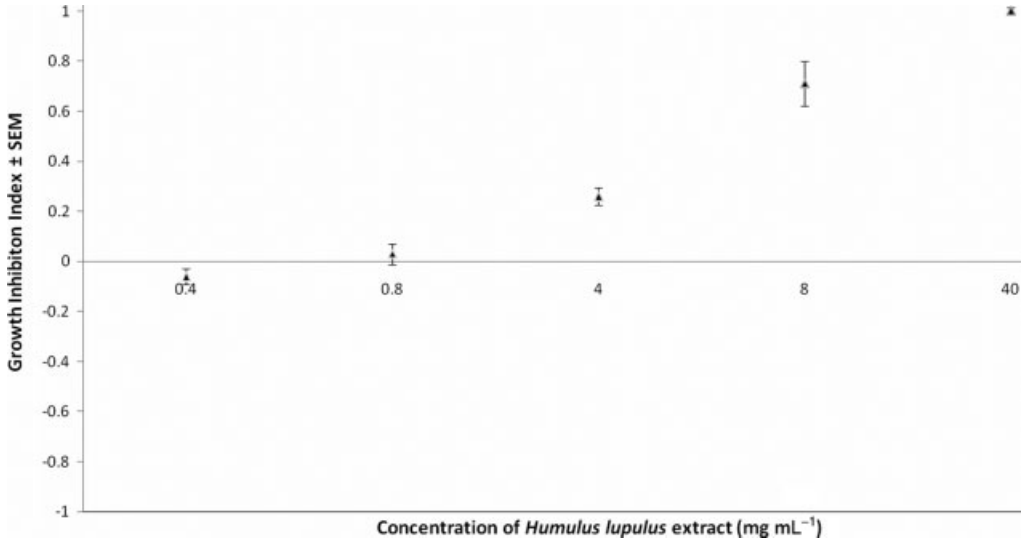

Figure 3. Growth inhibition indexes of *Leptinotarsa decemlineata* larvae reared on potato leaf discs treated with different concentrations of *Humulus lupulus* extract ($n = 10$).

growth of CPB, similar larval growth reduction of CPB has been reported with other plant extracts, and the present results are in accordance with those earlier findings.[34]

In a recent study, *H. lupulus* extracts were observed to elicit contact toxicity to both adults and larvae.[37] Therefore, applications of *H. lupulus* extracts or *H. lupulus* active constituents as a control strategy for *L. decemlineata* may be more durable, as these extracts exhibit both sensory and toxic effects on multiple life stages. Further studies are needed to determine how the combined antifeedant and toxic effects of *H. lupulus* affect potato crop injury levels from *L. decemlineata* under field conditions including exposure to ultraviolet light and/or rain.

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