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Contact Toxicity of Six Plant Extracts to Different Larval Stages of Colorado Potato Beetle (*Leptinotarsa decemlineata* SAY (Col: Chrysomelidae))

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ABSTRACT

Discovery of new eco-friendly methods for insect pest management is very important in integrated pest management program. Contact toxicity of six plant extracts i.e. *Acanthus dioscoridis* L. (Acanthaceae), *Achillea millefolium* L. (Asteraceae), *Bifora radians* Bieb. (Apiaceae), *Heracleum platytaenium* Boiss (Apiaceae), *Humulus lupulus* L. (Cannabaceae) and *Phlomoides tuberosa* (L.) Moench (Lamiaceae), were tested on the 1st to 4th instar larvae of Colorado potato beetle (*Leptinotarsa decemlineata* Say. (Coleoptera: Chrysomelidae)). The *H. platytaenium* and *H. lupulus* extracts were the most effective among the tested extracts, so dose-response bioassay was carried out only with *H. lupulus* and *H. platytaenium* against larval stages of Colorado potato beetle. The *H. platytaenium* extract was the most effective extract with calculated LD_{50} values 0.126, 0.204, 0.206 and 0.458 µL insect^{-1}, LD_{90} values were calculated as 0.345, 0.342, 0.402, 0.566 µL insect^{-1} for 1st, 2nd, 3rd and 4th instars larvae respectively. These results indicate that *H. platytaenium* and *H. lupulus* extracts have great potentials as insecticides in the management of larvae of *L. decemlineata*.

Keywords: Colorado potato beetle; Plant extracts; *Heracleum platytaenium*; *Humulus lupulus*; Contact toxicity

Altı Bitki Ekstraktının Patates Böceğinin (*Leptinotarsa decemlineata* SAY (Col: Chrysomelidae)) Farklı Dönemlerdeki Larvaları Üzerine Kontakt Etkileri

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ÖZET

Zararlı böcekler ile mücadelede yeni çevre dostu metodların keşfi entegre zararlı yönetiminde çok önemlidir. Farklı familyalara ait altı bitki ekstraktının (*Acanthus dioscoridis* L. (Acanthaceae), *Achillea millefolium* L. (Asteraceae), *Bifora radians* Bieb. (Apiaceae), *Heracleum platytaenium* Boiss (Apiaceae), *Humulus lupulus* L. (Cannabaceae) and *Phlomoides tuberosa* (L.) Moench (Lamiaceae)) kontakt toksisiteleri patates böceğinin (*Leptinotarsa decemlineata* (Coleoptera:Chysomelidae)) 1-4 dönem larvalarına karşı laboratuvar koşullarında test edilmiştir. *H. platytaenium* ve *H. lupulus* ekstraktları test edilen ekstraktlar arasında tüm larval dönemler için en yüksek toksik etkiye sahip olmuştur. Çalışmanın ikinci kısmında, *H. lupulus* ve *H. platytaenium* ekstraktların ile Patates böceğinin farklı larva dönemlerinde doz-etki ile denemeleri yürütülmüştür. *H. platytaenium* ekstraktı en yüksek toksik etkiye sahip olmuş ve bu bitki ekstraktı için LD_{50} değerleri birinci, ikinci, üçüncü ve dördüncü dönem larvalar için sırasıyla 0.126, 0.204, 0.206 ve 0.458 µL böcek^{-1} olarak, LD_{90} değerleri ise 0.345, 0.342, 0.402, 0.566 µL böcek^{-1} olarak hesaplanmıştır. Bu sonuçlar *H. platytaenium*'un patates böceği ile mücadele de potansiyele sahip olduğunu göstermektedir.

Anahtar Kelimeler: Patates böceği; Bitki ekstraktı; *Heracleum platytaenium*; *Humulus lupulus*; Kontakt toksisite

1. Introduction

Colorado potato beetle (*Leptinotarsa decemlineata* SAY) (CPB) is a polyphagous insect-pest causing damage to various Solanaceae plants including potato, tomato and eggplant (Hsiao 1978; Hare 1990). In absence of control tactics, yield loss can rise to even 100% (Christie et al 1991). This cosmopolitan insect is spread over an area of 12 million km^2 in the world including North America, Asia and Europe (Alyokhin 2009). It feeds on different sections of the host plants and is also vectors of certain viral plant diseases e.g. potato spindle tuber viroid (PTSVD) (Borror & DeLong 1966; Kısmalı 1973; Jolivet et al 1988; Booth et al 1990).

A variety of insecticides are registered for the management of CPB. Extensive use of insecticides against this pest has led to serious problems like resistance, phytotoxicity and environmental contamination problems (Ioannidis et al 1991; Stewart et al 1997; Mota-Sanchez et al 2000). CPB has developed resistance to 54 insecticides belonging to different chemical classes with various modes of actions (Whalon et al 2013). These problems have led to exploration of different control methods like bio-pesticides including plant-based compounds against this pest. Although promising outcomes were reported with plant extracts especially acute toxicity and also behavioral effects (Hough-Goldstein 1990; Scott et al 2003; 2004; Gökçe et al 2005; 2006; 2012), however limited numbers of commercialized natural products are available for use (Hassan & Gökçe 2014).

In previous studies, *H. lupulus* and *B. radinas* were tested against CPB using total methanol extracts (Gökçe et al 2006; 2007). However, in the current study, these plants species were treated with solvent using maceration technique. This technique allows to obtain all available secondary plant metabolites using a larger amount of solvent (Hassan & Gökçe 2014) comparing with the previous studies. The other plant species (*Acanthus dioscoridis*, *Achillea millefolium*, *Heracleum platytaenium* and *Phlomoides tuberosa*) used in this study have not been not tested against CPB yet. The objectives of the current study were to evaluate the contact toxicities of six different plant extracts on various larval stages of CPB and to calculate LD_{50} and LD_{90} values for the most promising extracts.

2. Material and Methods

2.1. Materials

Plant species, extracted parts and places of collection are presented in the Table 1. As described in Gökçe et al (2005), the plants were collected in the summer or spring months of 2009. After the

separation of leafs, stems and cones from other parts, they were placed over blotting paper and kept under room temperature (25 ˚C) in dark conditions for two weeks. Subsequent to drying process, the plant materials were grounded into small pieces using a mill (M 20 IKA Universal Mill, IKA Group, Wilmington, NC, USA) and then they were put into 5 liter glass jars and protected in a dark room at 15±5 ˚C until they were used.

2.2. Preparation of plant extracts

Plant extracts were obtained through the maceration method as described in Alkan & Gökçe (2012). Two hundred grams of each plant species were put into a 5 liter glass jar and hexane, ethyl acetate, and methanol were separately added into the jar in an order according to their polarity range. Plant materials were firstly treated with hexane for 48 hours; and then the plant suspension was filtered through Whatman™ No 4 filter paper to obtain hexane fraction. After this process, ethyl acetate was added to the jars, and the plant materials were left again in this solvent for 48 hours at room conditions. Ethyl acetate fraction was filtered through the filter paper followed by separation from plant materials. Lastly, methanol was added to the plant materials and incubated as described above and then the filtration of the suspension was also repeated for methanol fraction. Excess solvents in the suspensions were evaporated using a rotary evaporator (RV 05 Basic 1-B, IKA® werke GmbH & Co. KG, Germany) and plant residues of *A. dioscoridis*, *H. platytaenium* and *P. tuberosa* were obtained. The *H. lupulus*, *B. radians* and *A. millefolium* extracts were prepared using the same technique but only methanol was used as a solvent. All plant extracts were diluted with 70% acetone solution to give the concentration of 15% plant extract/acetone (w v^{-1}). Plant extracts prepared were transferred to glass tubes and then stored at 4 ˚C in the refrigerator.

2.3. Rearing of potato beetles

Larvae of CPB were reared at Gaziosmanpasa University, Faculty of Agriculture, Plant Protection Department. CPB colony was continuously reared on potato plants (*Solanum tuberosum* L. cultivar Granola) which were planted at Gaziosmanpasa University Research Station in Tasliciftlik, Tokat, Turkey. The field was designated for the organic potato production and there was no pesticide application for 3 years prior to the initiation of this project and no pesticide was applied during the study. Granola cultivar was planted in a 0.2 ha potato field. When the potato plants reached to 3 to 5 leaves stage adults of test pest were released into the field and all required stages for the studies were collected from the field.

2.4. Single dose contact toxicity screening tests

Single dose contact toxicity of plant extracts were separately tested on 1^{st}, 2^{nd}, 3^{rd}, and 4^{th} instars larvae of CPB. Identification of larval stage was carried out using Boiteau & Le Blancthe (1992)' key. An extract suspension (15% w v^{-1}) was applied at a 2 µL $insect^{-1}$ ratio to the dorsal of larva using a micro-syringe 25 µL microsyringe connected to a microapplicator (Hamilton® Company, Reno, NV). Ten larvae were treated in each replication. After the treatment, 10 larvae were transferred into a 90 mm in diameter glass petri dish in which potato leaflets

Table 1- Plant species and their parts used in the study

Çizelge 1- Çalışmada kullanılan bitkiler ve kısımları

Botanical name	*Family*	*Part used*	*Place collected*
Humulus lupulus	Cannabaceae	Cone	Tokat
Heracleum platytaenium	Apiaceae	Leaf, stem	Trabzon
Achillea millefolium	Asteraceae	Leaf, stem, flower	Tokat
Acanthus dioscoridis	Acanthaceae	Leaf, stem, flower	Erzincan
Phlomoides tuberosa	Lamiaceae	Leaf, stem, flower	Erzincan
Bifora radians	Apiaceae	Leaf, stem	Tokat

were provided. In the control group, the larvae were treated with 70% acetone at 2 µL insect^{-1} dose. An insecticide with spinosad active ingredient was used as a positive control, which was applied at 2 µL insect^{-1} dose as described above. Spinosad (Laser™, Dow Agro Sciences®) was prepared with water at recommended dose for larvae (0.1 mL L^{-1}). After the application, the larvae were incubated at 25±2 ˚C, 60±5% relative humidity (RH) and a 16:8 (Light: Dark) photo period. Mortality of larvae was recorded after 24 hours after treatment (HAT). Bioassays were set up in the randomized complete block design. Experiment was repeated on three different days (blocks) and in each replication all treatment contained three subset groups.

2.5. Dose-response bio-assay

Based on the single-dose screening test results, dose-response bioassays were carried out with *H. platytaenium* and *H. lupulus* extracts that showed high contact toxicity to CPB larvae. These plant extracts were tested against various stages on potato beetle larvae (1st, 2nd, 3rd and 4th instars larvae) in 6 different doses. The doses ranging from 10 to 200 g L^{-1} (10, 25, 50, 75, 100 and 150 g L^{-1} for the 1st , 2nd and 3rd instar larvae, 50, 75, 100, 150, 175 and 200 g L^{-1} for the 4th instars larvae) for *H. lupulus* and from 5 to 250 g L^{-1} (5, 10, 25, 50, 100 and 150 g L^{-1} for the 1st instar lavae, 25, 50, 75, 100, 125 and 150 g L^{-1} for the 2nd and 3rd instar larvae, 125, 150, 175, 200, 225 and 250 g L^{-1} for the 4th instars larvae) for *H. platytaenium* were prepared with 70% acetone and applied to the larvae at 2 µL insect^{-1} dose as stated above. In the control group, the larvae were treated with 70% acetone at 2 µL insect^{-1} dose. Randomized complete block experimental design was used in this study and each block comprised all tested doses and control. Whole treatments were repeated three times. Each trial consisted of 7 treatments i.e. six doses and control group that contained three subset groups.

2.6. Statistical analysis

Single-dose contact toxicity screening test results were firstly converted into percent mortality and then were subjected to arcsine transformation. Variance analysis was carried out with transformed data, and additionally, the differences among treatments were analyzed by means of Tukey multiple comparison test ($P<0.05$). All statistical analyses were conducted with MINITAB® Release 16 package program. Dose-response bioassay results were analyzed using Polo-PC probit package program (LeOra 2002), and confidence intervals were determined with LD_{50} and LD_{90} values.

3. Results and Discussion

3.1. Single dose contact toxicity screening tests

All tested plant extracts caused some contact toxicity to larvae of *L. decemlineata*, ranging from 1.5% to 100%. Among the tested plant extracts, *H. lupulus* showed the greatest contact toxicity to 1st instar larvae with 97.8% mortality 24 HAT. *Heracleum platytaenium* was the second most effective extract with 94.0% mortality rate. Mortality rates significantly between the treatments ($F= 86.87$; $df= 7, 16$; $P<0.05$). Unlike 1st instar larvae in 2nd instar larvae, the greatest mortality was observed when treated with *H. platytaenium* followed by *H. lupulus*. After 24 hours, mortality rate was 100% in case of *H. platytaenium* extract followed by 89.8% mortality recorded in case of *H. lupulus* extract (Table 2).

Insecticidal activities of the plants belonging to *Heracleum* genus against important insect pest species were previously reported by other researchers. Metspalu et al (2001) tested *Heracleum sosnowskyi* and *A. millefolium* against different stages of *L. decemlineata* larvae under laboratory conditions. They reported that the greatest contact toxicity was seen in *H. sosnowskyi* extract with 80% mortality. However their findings were not comparable to our studies possibly due to variation in way of extraction of plant extracts and polarity of solvents used for extractions (Ghosh et al 2012).

Chemical analysis of plants belonging to *H. platytaenium* genus showed that the leaves contained intensive secondary metabolite compounds such as octyl acetate, octyl butyrate, (z)-4-octenyl

Table 2- Contact toxicity of the plant extracts (15% w v^{-1}) on various development stages of *Leptinotarsa decemlineata* larvae after 24 hours

Çizelge 2- Bitki ekstraktlarının (% 15 w v^{-1}) Leptinotarsa decemlineata'nın farklı gelişme dönemleri üzerine 24 saat sonundaki kontakt toksisiteleri

	% Mortality±SD*			
Treatment	*1.instar*	*2. instar*	*3. instar*	*4. instar*
Control	0.00±0.00 b[1]	0.00±0.00 c	0.00±0.00 c	0.00±0.00 c
Acanthus dioscoridis	1.49±1.12 b	1.49±1.12 c	0.00±0.00 c	1.49±1.12 c
Achillea millefolium	2.18±1.79 b	4.32±0.20 c	1.49±1.12 c	0.00±0.00 c
Bifora radians	1.49±1.12 b	1.49±1.12 c	0.00±0.00 c	0.00±0.00 c
Heracleum platytaenium	94.00±4.76 a	100.00±0.00 a	100.00±0.00 a	3.33±0.00 c
Humulus lupulus	97.82±1.79 a	89.74±1.57 b	95.68±0.20 b	48.90±0.93 b
Phlomoides tuberosa	1.49±1.12 b	1.49±1.12 c	1.49±1.12 c	0.00±0.00 c
Spinosad	99.63±1.12 a	94.82±0.63 ab	87.10±0.84 b	90.77±1.41 a

[1], different letters following means in the same column indicate statistical significance from each other (Anova P<0.05, Tukey test); *, standard deviation

acetate**,** (z)-4-octenyl butyrate**,** octyl 3-methyl butyrate (=octyl isovalerate)**,** octyl hexanoate**,** octyl octanoate**,** hexyl 2-methylbutyrate**,** hexyl 3-methylbutyrate (=hexyl isovalerate)**,** decyl acetate and many others. Among these elements, octyl acetate and octyl butyrate have a major share (Iscan et al 2004) and both are very important essential oils (Carroll et al 2000) thus playing role in insect-pests' management (Koul et al 2008). In *H. lupulus*; humulene, caryophyllene and myrcene are the major constituents which are terpenes in nature thus playing significant role in insect-pests' management (Bernotienë et al 2004; Koul et al 2008). These chemicals could play an important role in toxicity of this plant species to CPB larvae.

Contact toxicity of *H. lupulus* extract was also very high and the mortality rate of 3rd instar larvae was treated with this extract was 95.7% 24 HAT. Similar activity with *H. lupulus* extract on the 3rd instar larvae was also reported by Gökçe et al (2007) who observed 91% mortality on their study.

The 4th instar larvae are the most destructive stages of CPB and cause serious damages on green parts of the plant (Wale et al 2008). The chemical standard spinosad as expected was the most effective treatment against this larval stage. Among the plant extracts, the most effective was *H. lupulus* with 48.9% mortality 24 HAT but this rate was lower than the mortality rates seen in the first three stages. Similarly, Gökçe et al (2006) reported that the first three larval stages were more sensitive than 4th instar larvae and adult insects. Scott et al (2003) tested plant extracts belonging to *Piperaceae* on CPB adults and larvae and they concluded that last stage larvae, pupae and adults were less sensitive than early stage larvae were. The results of the above studies are in accordance with our results. Varying contact toxicity effects of the plant extracts to CPB larvae could be related with physiological changes in developing larvae (Karakoç & Gökçe 2012).

3.2. Dose-response bioassay

Treatment of larval stages of CPB with various concentrations of *H. platytaenium* and *H. lupulus* extracts produced different LD_{50} and LD_{90} values. For 1st instar larvae, 0.126 µL insect^{-1} LD_{50} was calculated in case of *H. platytaenium* extract while that obtained with *H. lupulus* extract was 0.150 µL insect^{-1} (Table 3). There was no significant difference among the treatments (P<0.05). The LD_{90} values were 0.274 and 0.345 µL insect^{-1} for *H. lupulus* and *H. platytaenium* extracts, respectively. For the 2nd instar larvae, similar results were observed among treatments i.e. LD_{50} values were i.e. 0.168 µL insect^{-1} and 0.204 µL insect^{-1} for *H. lupulus* and *H. platytaenium* (P<0.05).

Additionally, no significant difference was also observed among LD_{90} values of these plant extracts. In the 3rd instar larvae, calculated LD_{50} was 0.206 µL insect^{-1} for *H. platytaenium* extract and 0.149 µL insect^{-1} for *H. lupulus* extracts with no significant difference among the treatments (Table 3). These results showed that LD_{50} and LD_{90} values increased according to developmental stages of larvae as expected. This could be related to morphological and physiological changes in the beetle larvae as there is a considerable size difference especially between 1st and 3rd instars. Therefore, more plant extract is required to produce 50% or 90% mortality in the tested larvae, which leads to bigger LD_{50} or LD_{90} values. Similarly, Gökçe et al (2006) stated that LD_{50} and LD_{90} values increased according to larval stages of CPB. Dose-response bioassay with *H. platytaenium* extract on 4th stage larvae showed that LD_{50} and LD_{90} values were 0.458 and 0.566 µL insect^{-1}, respectively.

Table 3- Results of dose-response bioassays of *Heracleum platytaenium* and *Humulus lupulus* extracts on various development stages of *Leptinotarsa decemlineata* larvae after 24 hours

Çizelge 3- Heracleum platytaenium ve Humulus lupulus ekstraktlarının 24 saat sonunda Leptinotarsa decemlineata'nın farklı gelişim dönemleri üzerindeki doz-etki denemeleri sonuçları

Plant	*Larval term*	*Slope±SD**	*LD_{50} (µL insect^{-1}) (Fudicial limit)*	*LD_{90} (µL insect^{-1}) (Fudicial limit)*
H. platytaenium	1st instar larvae	2.927±0.234	0.126 (0.087-0.190)	0.345 (0.220-0.928)
	2nd instar larvae	5.710±0.460	0.204 (0.154-0.285)	0.342 (0.256-1.073)
	3rd instar larvae	7.443±0.578	0.206 (0.189-0.226)	0.402 (0.358-0.461)
	4th instar larvae	14.034±1.733	0.458 (0.438-0.485)	0.566 (0.524-0.660)
H. lupulus	1st instar larvae	4.901±0.405	0.150 (0.137-0.164)	0.274 (0.242-0.324)
	2nd instar larvae	4.853±0.426	0.168 (0.152-0.185)	0.308 (0.267-0.378)
	3rd instar larvae	2.767±0.243	0.149 (0.118-0.189)	0.433 (0.311-0.776)

*, standard deviation

4. Conclusions

Evaluation of the plant extracts contact toxicities against the most destructive larval stages of CPB showed that especially *H. platytaenium* and *H. lupulus* were as effective as the chemical standard, spinosad, up to 4th instar larvae, and that the extracts obtained from those plants could be used in the control of Colorado potato beetle. This research is a core study; therefore it is considered that the study will become more significant with the help of other disciplines, which enable the purification and characterization of the active compound(s). That will definitely help further development of these plant extracts by the industry.

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