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Original article (Orijinal araştırma)

Mortality, developmental biology and cellular immunity in *Achroia grisella* (Fabricius, 1794) (Lepidoptera: Pyralidae) larvae exposed to azadirachtin[1]

Azadirachtine maruz kalan *Achroia grisella* (Fabricius, 1794) (Lepidoptera: Pyralidae) larvalarında ölüm oranı, gelişim biyolojisi ve hücresel bağışıklık tepkileri

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Abstract

Azadirachtin, obtained from neem trees, can be a robust alternative to synthetic pesticides for the control of agricultural pests with no resistance problems. Azadirachtin-induced influences on mortality, life history traits and cellular immunity indicators of the lesser wax moth *Achroia grisella* (Fabricius, 1794) (Lepidoptera: Pyralidae) were evaluated. The experiments were conducted under controlled laboratory conditions at Balıkesir University. The topical application of azadirachtin gave an LD_{50} of 0.02 mg/ml whereas the PD_{50} (deaths without pupation) was 0.05 mg/ml. The prolongation of the larval stage and adult emergence time was significantly increased at 0.05 mg/ml and 0.1 mg/ml while the duration of the pupal stage was only significant at 0.1 mg/ml. Adult emergence ratios and longevity were reduced at all doses. Topical application of azadirachtin caused a marked decrease in the number of circulating hemocyte counts and spreading ability 24 and 48 h after treatment, however, the variations in plasmatocyte and granulocyte counts were not significant. Although azadirachtin has potential effects in the control of *A. grisella*, its effects on biological control agents such as parasitoids and predators must be determined to recommend its safe use in agroecosystems.

Keywords: *Achroia grisella*, azadirachtin, hemocyte count, toxicity

Öz

Neem ağaçlarından elde edilen Azadirachtin, direnç sorunu olmayan ve tarımsal zararlıların kontrolü için sentetik pestisitlere güçlü bir alternatif oluşturmaktadır. Küçük mum güvesi *Achroia grisella* (Fabricius, 1794) (Lepidoptera: Pyralidae)'da ölüm, gelişim biyolojisi ve hücresel bağışıklık göstergeleri üzerindeki azadirachtin kaynaklı etkiler değerlendirilmiştir. Denemeler kontrollü laboratuvar ortamında Balıkesir Üniversitesi'nde gerçekleştirilmiştir. Azadirachtinin topikal uygulamasına bağlı olarak LD_{50} 0.02 mg/ml bulunurken, PD_{50} (pupa dönemine geçmeden ölümler) 0.05 mg/ml olarak tespit edildi. 0.05 mg/ml ve 0.1 mg/ml'de larva dönemi ve ergin çıkış süresi, önemli ölçüde artarken, pupa dönemindeki uzama sadece 0.1 mg/ml'de önemli bulunmuştur. Ergin çıkış oranları ve ergin yaşam süresi, kullanılan tüm dozlarda azalmıştır. Azadirachtinin topikal uygulaması, uygulamadan 24 ve 48 saat sonra dolaşımdaki hemosit sayılarında ve hemosit yayılma davranışında önemli bir azalmaya neden olurken, plazmatosit ve granülosit sayılarındaki varyasyonlar istatistiksel olarak anlamlı bulunmamıştır. *Achroia grisella* ile mücadelede azadirachtinin potansiyel etkileri olmakla birlikte, agroekosistemlerde güvenli kullanımının önerilmesi için parazitoitler ve predatörler gibi biyolojik kontrol ajanları üzerindeki etkilerinin belirlenmesi önem arz etmektedir.

Anahtar sözcükler: *Achroia grisella*, azadirachtin, hemosit sayısı, toksisite

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Introduction

Concerns about the adverse effects of synthetic insecticides used in combating insects that damage agriculture, forestry and stored products continue to increase on the environment and human health. Short and long-term disadvantages of conventional pesticides have led to the emphasis on alternative control methods with natural origin, especially plant-derived compounds. One of the most well-known examples of biopesticides used as insect growth disruptors is azadirachtin, a limonoid tetranortriterpenoid obtained from the neem tree, *Azadirachta indica* A. Juss. (Sapindales: Meliaceae) (Mordue, 2004; Dorrah et al., 2019). Bioinsecticides with the active ingredient of azadirachtin are widely used in agriculture and integrated control programs within the scope of biological control against pests, which significantly reduce agricultural productivity (Bezzar-Bendjazia et al., 2017). The outstanding feature of these bioinsecticides depends on multiple anti-insect modes of action with no resistance problems (Mordue et al., 2005). Also, azadirachtin has also been defined as harmless for non-target organisms; however, this previous understanding has been recently reinterpreted, especially concerning pollinators, predators and parasitoids (Barbosa et al., 2015; Xavier et al., 2015; Bernardes et al., 2017).

Azadirachtin has both physiological and behavioral modes of action. The physiological effects are reported as the inhibition of insect growth, development, reproduction and synthesis of juvenile hormone (Chaudhary et al., 2017). Azadirachtin also acts as an ecdysone (molting hormone) antagonist by interacting with neural secretory cells of the insect brain-corpus cardiacum complex (Mordue et al., 2005; Bezzar-Bendjazia et al., 2017). Behavioral effects vary between different insect species defined as repellants or inhibitors of feeding (Schmutterer & Singh, 1995). In addition to these well-known and relevant influences, azadirachtin may also interact with the innate immune system of insects relying on germline-encoded factors to recognize and clear infection that is too often overlooked.

Insects have a highly conserved immune system consisting of humoral and cellular mechanisms. Cellular immune reactions are maintained by the hemocytes that phagocytose or capture non-self-invaders in multicellular layers called capsules and nodules while humoral immunity involves the synthesis of antimicrobial peptides like attacins, defensins and cecropins and a series of enzymatic cascades that regulates melanization (Lavine & Strand, 2002). In many Lepidopteran model insects, granulocytes, plasmatocytes, prohemocytes, spherulocytes and oenocytoids are the main hemocyte types in circulation (Kaya et al., 2021). Granulocytes and plasmatocytes are the most abundant hemocyte types with their ability to phagocytose, spread on foreign materials and capsule forming. Of the remaining hemocyte types that can be present in a small proportion in circulation, oenocytoids contain phenoloxidase precursors, spherulocytes are potential sources of cuticular components and prohemocytes differentiate into other hemocyte types as progenitor cells (Eleftherianos et al., 2021). It is important to point out that exposure to xenobiotics, even those of botanical origin, has the capacity of leading to stress on insects triggering immune defense reactions (Silva et al., 2020). Therefore, immune function in insects can be handled as an effective bioindicator to determine the systemic toxicity of biopesticides. Also, it can be a marker of which stage the insect will be more susceptible to infection. A limited number of studies demonstrated that azadirachtin can impact the immune functions of insect species (Azambuja et al., 1991; Sharma et al., 2003; Er et al., 2017; Silva et al., 2020). However, there appears to be no reports on the influence of azadirachtin on the biology and immune reactions of *Achroia grisella* (Fabricius, 1794) (Lepidoptera: Pyralidae). The lesser wax moth, *A. grisella*, is one of the major pests of beehives that feed on pollen, honey and wax. The pest insect is also a developing model organism frequently used to demonstrate the biological effects of xenobiotics (Uçkan et al., 2011; Çelik et al., 2017). In this study, the effects of topically applied azadirachtin on various biological parameters including mortality, development time and longevity as biological indicators and suppression of insect hemocyte counts and behavior as immune indicators were assessed.

Materials and Methods

Insect rearing

The larvae of the lesser wax moth *A. grisella* were established from adults that were obtained from apicultural regions in Balıkesir, Türkiye. Adult insects were transferred to 1-L jars containing honeycomb as an egg oviposition substrate. Insect cultures were kept in an incubator at 28 ± 2°C, 60 ± 5% RH and 12:12 h L:D photoperiod regime and were fed with blackened honeycomb to sustain their natural habitat in beehives (Er & Keskin, 2016). Last instar larvae of *A. grisella* used in all experiments. The process of establishing successive *A. grisella* cultures was continued throughout the experiments both to ensure the continuation of the culture and to obtain adults that would yield the last instar larvae used in the experiments. The experiments were conducted under laboratory conditions at Balıkesir University.

Azadirachtin treatment and toxicity bioassays

Azadirachtin used in the experimental analyses was purchased as a commercial product (NeemAzal-T/S, 10 g/L, Trifolio-M GmbH, Lahnau, Germany). Azadirachtin was diluted with distilled water to three concentrations (0.01, 0.05 and 0.1 mg/ml), accompanied by a control group was tested to search for the influences on biological and immunologic parameters. Five µl of diluted azadirachtin concentrations were applied topically (head to the abdomen) to 30 final instar larvae of *A. grisella* in three replicates for toxicity analyses and life-history traits. Control groups consisted of 30 untreated larvae. Azadirachtin-treated experimental groups along with control groups were transferred to incubators under the same conditions in Petri dishes and observed daily to determine the total and cumulative mortality. The larva that did not respond to mechanical stimulation and darkened in color were considered as dead. Based on obtained mortality data some chosen LDx and PDx concentrations were generated with probit analyses calculated by SPSS software (SPSS 22, IBM, Armonk, NY, USA).

Developmental biology

Measurement of the larval duration, the ratio of pupation (%), pupal period, duration and the ratio of adult emergence (%) was determined after azadirachtin treatment to the last instars of *A. grisella* at the same concentrations. Five µl of each concentration of azadirachtin were applied topically to *A. grisella*. Treated and control larvae were transferred to an incubator adjusted to 28 ± 2°C, 60 ± 5% RH, 12:12 h L:D photoperiod and monitored daily until adult emergence. Freshly emerged *A. grisella* adults obtained from the experimental sets were located in Petri dishes and observed daily until the adults die to record adult longevity. All the developmental indicators were determined for each replicate. For each experimental and control group, 10 arbitrarily selected last instar larvae were selected and evaluated by three replicates (n = 30).

Indicators of cellular immunity

Total and differential hemocyte counts (THC and DHC, respectively), and hemocyte spreading experiments were performed on each larva in all control and experimental groups. To determine the effects of azadirachtin on total and differential hemocyte counts and cell spreading, 5 µl of each azadirachtin concentration (0.01, 0.05 and 0.1 mg/ml) were applied topically to the last instars of *A. grisella*. Hemolymph was collected from the last instar larvae 24 and 48 h after azadirachtin treatment. *A. grisella* last instars were pierced with a 19-gauge sterile needle on the first hind leg and the hemolymph was pooled using a glass microcapillary tube (Sigma, St. Louis, MO, USA).

Total and differential hemocyte counts

To clarify the influence of azadirachtin on THCs, 4 µl hemolymph from control and azadirachtin treated groups were pooled in 36 µl ice-cold anticoagulant buffer (17 mM Na2 EDTA, 98 mM NaOH, 41 mM citric acid, 186 mM NaCl, pH 4.5) into an Eppendorf tube. Ten µl of the obtained cell suspensions were loaded

to a Neubauer hemocytometer (Neubauer Improved Hemocytometer; Superior Marienfeld, Lauda-Königshofen, Germany) and then examined under an Olympus BX51 microscope. Ten arbitrarily selected last instar larvae were assessed for every experimental and control group in three replicates (n = 30).

In order to determine DHCs, azadirachtin-treated and control last instars were bled with the aid of a 19-gauge needle and the hemolymph transferred into an Eppendorf tube containing phosphate-buffered saline (PBS, Sigma) kept on ice and mixed. Twenty µl of this dilute hemolymph was drawn onto a microscope slide and incubated in a humid chamber (Sigma) for 30 min. The hemocyte layers were examined and classified according to Er et al. (2009) under an Olympus BX51 Phase-contrast microscope (Olympus, Tokyo, Japan). Granulocytes and plasmatocytes were calculated from five arbitrarily selected fields (300 cells) and expressed as the ratio of total hemocytes.

Hemocyte spreading

Due to define the effects of azadirachtin on the spreading behavior of *A. grisella* hemocytes, 4 µl of hemolymph from control and azadirachtin-treated larvae were transferred in an Eppendorf tube containing PBS and gently mixed. Twenty µl of this suspension were spread on a slide and kept for 30 min in a humid chamber at 29 ± 1°C to allow the hemocytes to attach to the slide. Following the protocol, a total of 300 cells were considered from five arbitrarily determined fields under a light microscope and relative numbers of spreading hemocytes from 15 larvae in three replicates were recorded.

Statistics

Concentration-dependent changes in immune and developmental parameters of *A. grisella* due to azadirachtin treatment were tested for normal distribution using Levene's test. As all data were normally distributed and one-way analyses of variance were used for comparing experimental mean data. To determine the significant differences Tukey HSD test was conducted. Data for percentage values were Arcsine transformed before analyses. All statistical tests were performed with the SPSS version 22.0 software program (IBM SPSS Statistics for Windows, Armonk, NY, USA) and results were evaluated as significant when $P < 0.05$.

Results

Toxicity of azadirachtin

Mortality rates in *A. grisella* were evaluated in two categories as larval death and total death. Larval mortality rates increased from 7.5% at the lowest tested concentration of azadirachtin to 85% at 0.1 mg/ml in a dose-dependent manner. Similar results were also obtained in total mortality rates (Table 1). The topical application of azadirachtin presented an LD_{50} of 0.02 mg/ml whereas the PD_{50} (deaths without pupation) was 0.05 mg/ml (Table 2).

Table 1. Effects of topically applied azadirachtin concentrations (mg/ml) on larval and total mortality of *Achroia grisella*

Azadirachtin (mg/ml)	Larval mortality (% ± SE) *	Total mortality (% ± SE) *
0 (control)	5.0 ± 0.3 a	10.0 ± 0.8 a
0.01	7.5 ± 0.9 a	37.5 ± 3.5 a
0.05	37.5 ± 3.6 b	57.5 ± 4.2 b
0.1	85.0 ± 8.3 c	87.5 ± 7.5 c

* Groups marked with different letters are significantly different ($P < 0.05$; Tukey's HSD test). Larval mortality, $F = 17.2$, $df = 3,16$, $P = 0.001$; and total mortality, $F = 37.6$, $df = 3,16$, $P < 0.001$.

Table 2. LD (lethal doses) and PD (deaths in larval stage without pupation) of azadirachtin concentrations (mg/ml) administered to the last larval instars of *Achroia grisella*

Treatment	N#	X^2 (df)	Slope ± SE	Lethal doses (mg/ml)	
				LD (%95 CL)	
				LD_{10}	0.003
				LD_{30}	0.001
	200	8.24 (2)	1.60 ± 0.2	LD_{50}	0.020
				LD_{75}	0.050
				LD_{90}	0.130
Azadirachtin				PD (%95 CL)	
				PD_{10}	0.016
				PD_{30}	0.030
	200	8.35 (2)	2.77 ± 0.3	PD_{50}	0.047
				PD_{75}	0.082
				PD_{90}	0.136

*Total number of insects sampled in the bioassay.

Developmental biology and longevity

Duration of the larval stage was significantly elevated at 0.05 and 0.1 mg/ml azadirachtin treatment compared to control and 0.01 mg/ml doses (Table 3). Azadirachtin also prolonged the pupal period in a dose-dependent mode however the increase was only significant at 0.1 mg/ml (Table 3). Adult emergence time of azadirachtin-treated individuals increased with controls; however, significant prolongations were only observed at 0.05 and 0.1 mg/ml (Table 3). The longevity of azadirachtin-treated *A. grisella* adults was reduced significantly at all doses in comparison with the control group (Table 3).

Table 3. Effects of topically applied azadirachtin concentrations on larval, pupal period and adult emergence time of *Achroia grisella*

Azadirachtin (mg/ml)	Larval period (days ± SE) *	Pupal period (days ± SE) *	Adult emergence time (days ± SE) *	Longevity (days ± SE) *
0 (control)	7.6 ± 0.2 a	6.4 ± 0.2 a	14.1 ± 0.2 a	10.2 ± 0.6 a
0.01	7.2 ± 0.4 a	7.1 ± 0.3 a	14.2 ± 0.5 a	5.3 ± 0.4 b
0.05	14.1 ± 0.9 b	7.7 ± 0.5 ab	22.5 ± 1.0 b	3.6 ± 0.6 b
0.1	14.7 ± 1.1 b	9.2 ± 0.5 b	25.0 ± 0.7 b	4.8 ± 0.3 b

* Groups marked with different letters are significantly different ($P < 0.05$; Tukey's HSD test). Larval period, $F = 30.2$, df = 3,47, $P < 0.001$; pupal period, $F = 188$, df = 3,8, $P < 0.001$; adult emergence time, $F = 57.4$, df = 3,47, $P < 0.001$; and longevity, $F = 27.0$, df = 3,48, $P < 0.001$.

Percent pupation in control *A. grisella* larvae was 99% (Table 4). Pupation ratios decreased in all azadirachtin concentrations in a dose-related manner but were only significant at 0.05 and 0.1 mg/ml (Table 4). Similar dose-dependent reductions were also detected in adult emergence ratios. The percent adult emergence rates were 53.3, 26.6 and 5.0% at 0.01, 0.05 and 0.1 mg/ml azadirachtin treatments, respectively. The calculated adult emergence ratios were significant at all applied doses of azadirachtin (Table 4).

Table 4. Effects of topically applied azadirachtin concentrations (mg/ml) on pupation ratio (%) and adult emergence ratio (%) of *Achroia grisella*

Azadirachtin (mg/ml)	Pupation (% ± SE) *	Adult emergence (% ± SE) *
0 (control)	99.7 ± 0.5 a	99.7 ± 0.5 a
0.01	91.1 ± 7.7 a	53.3 ± 6.6 b
0.05	56.6 ± 1.3 b	26.6 ± 1.4 c
0.1	12.3 ± 1.7 c	5.0 ± 0.3 d

* Groups marked with different letters are significantly different ($P < 0.05$; Tukey's HSD test). Pupation ratios, $F = 188$, df = 3,8, $P < 0.001$; and adult emergence ratios, $F = 74.7$, df = 3,8, $P < 0.001$.

Total and differential hemocyte counts

THC in the hemolymph samples of last instar *A. grisella* larvae were 32.5 and 32.8 x 10^6 cell/ml at 24 and 48 h, respectively (Table 5). Topical application of azadirachtin caused a remarkable decrease at all doses compared to control at 24 and 48 h. The minimum count of 18.5 x 10^6 cell/ml was detected at 48 h after azadirachtin treatment at the maximum dose of 0.1 mg/ml.

Table 5. Effects of topically applied azadirachtin concentrations on total hemocyte count (x10^6 cell/ml) of *Achroia grisella*

Azadirachtin (mg/ml)	Total hemocyte count (x10^6 cell/ml) (mean ± SE) *	
	Time after treatment	
	24 h	48 h
0 (control)	32.5 ± 1.7 a	32.8 ± 0.9 a
0.01	23.6 ± 0.7 b	20.4 ± 1.5 b
0.05	23.5 ± 1.1 b	23.6 ± 1.5 b
0.1	23.1 ± 1.0 b	18.5 ± 0.8 c

* Groups marked with different letters are significantly different ($P < 0.05$; Tukey's HSD test). 24 h, $F = 14.5$; df = 3,116, $P < 0.001$; and 48 h, $F = 25.8$, df = 3,116, $P < 0.001$.

In this study, DHC was expressed as relative numbers of granulocytes and plasmatocytes that are the most prominent cell types classified in the hemocyte population of *A. grisella*. Plasmatocytes comprised 57.5 and 55.2% of the total hemocyte population of *A. grisella* last instars at 24 and 48 h control groups, respectively (Table 6). Granulocytes were the second-highest group of hemocytes in circulation with 42.0% and 44.1% in 24 and 48 h control groups. Topical application of different doses of azadirachtin caused alterations in both granulocyte and plasmatocyte ratios at 24 and 48 h after treatment however the changes were not significant ($P > 0.05$).

Table 6. Effects of topically applied azadirachtin concentrations (mg/ml) on differential hemocyte count (%) of *Achroia grisella*

Azadirachtin (mg/ml)	Granulocyte (% ± SE) *		Plasmatocyte (% ± SE) *	
	Time after treatment		Time after treatment	
	24 h	48 h	24 h	48 h
0 (control)	42.0 ± 1.7 a	44.1 ± 1.3 a	57.5 ± 1.7 a	55.2 ± 1.3 a
0.01	37.1 ± 3.2 a	46.0 ± 4.1 a	61.5 ± 3.2 a	52.1 ± 4.1 a
0.05	33.2 ± 3.1 a	45.8 ± 2.5 a	66.8 ± 3.1 a	53.4 ± 2.6 a
0.1	36.8 ± 2.6 a	39.7 ± 2.3 a	63.4 ± 2.6 a	56.7 ± 2.7 a

* Groups marked with different letters are significantly different ($P < 0.05$; Tukey's HSD test). Granulocyte 24 h, $F = 12.9$, df = 3,36, $P = 0.526$; granulocyte 48 h, $F = 21.9$, df = 3,36, $P = 0.210$; plasmatocyte 24 h, $F = 17.1$, df = 3,36, $P = 0.32$; and plasmatocyte 48 h; $F = 24.2$, df = 3,36, $P = 0.125$.

Hemocyte spreading

The ability of insect hemocytes to spread on a glass surface is commonly used as an indicator of immune fitness. Here in this study, the ratio of spreading hemocytes was 39.8% and 35.7% in control *A. grisella* larvae at 24 and 48 h period (Figure 3). The percentage of hemocytes exhibiting spreading behavior was reduced at all treated doses of azadirachtin compared to control at 24 and 48 h after treatment Table 7).

Table 7. Effects of topically applied azadirachtin concentrations on hemocyte spreading ability (%) of *Achroia grisella*

Azadirachtin (mg/ml)	Spread hemocytes (% ± SE)*	
	Time after treatment	
	24 h	48 h
0 (control)	39.8 ± 3.2 a	35.7 ± 2.3 a
0.01	23.6 ± 3.7 b	21.3 ± 3.1 b
0.05	20.5 ± 3.2 b	27.0 ± 2.0 b
0.1	21.7 ± 2.4 b	17.3 ± 2.1 b

* Groups marked with different letters are significantly different ($P < 0.05$; Tukey's HSD test). 24 h, $F = 7.89$, df = 3,36, $P < 0.001$; and 48 h, $F = 10.4$, df = 3,36, $P < 0.001$.

Discussion

Azadirachtin is one of the prominent botanical biopesticides used for agricultural pest control worldwide with more than 20 commercial products (Kilani-Morakchi et al., 2021). Existing literature reveals that the toxicity of azadirachtin varies in different insect species due to different penetration rates of pests and their physiological status. Slight to moderate toxicological effects have been reported on mortality rates (Er et al., 2017; Zhong et al., 2017; Amaral et al., 2019), growth inhibition and retardation of developmental time (Zhao et al., 2019), antifeedant activity (Qin et al., 2020), prevention of fecundity and egg viability (Amaral et al., 2018; Ferdenache et al., 2019). Also, azadirachtin is also a possible candidate to use in synergy with other microbial biocontrol agents and botanical compounds against insect pests (Konecka et al., 2019). As a basis for future studies involving such a combination with microbial control agents, more information is needed on the systemic impact of azadirachtin on the physiological state of pests, especially on insect immunity.

Topical application to last instars gave an LD_{50} of 0.02 mg/ml and led to concentration-related mortality. This finding is in accordance with a previous study demonstrating the effects of azadirachtin on the greater wax moth *Galleria mellonella* (L., 1758) (Lepidoptera: Pyralidae) (Er et al., 2017). High and dose-dependent mortality rates of azadirachtin as a bioinsecticide have also been documented in various Lepidopteran species containing *Helicoverpa armigera* (Hübner, 1808) (Lepidoptera: Noctuidae), *Spodoptera litura* Fabricius, 1775 (Lepidoptera: Noctuidae), *Schistocerca gregaria* (Forskål, 1775) (Orthoptera: Acrididae), *Tirathaba rufivena* Walker, 1864 (Lepidoptera: Pyralidae) and *Plutella xylostella* (L., 1758) (Lepidoptera: Plutellidae) (Schmutterer & Singh, 1995; Zhong et al., 2017). However, its effectiveness mostly depends on the doses, application methods and stages of insects. In a study on *T. rufivena* larvae, the contact effect of azadirachtin was found to be greater than the ingestion effect (Zhong et al., 2017). The influence of azadirachtin on insect developmental biology is due to diverse modes of action in insects (Scudeler & dos Santos, 2013). Delayed adult emergence due to azadirachtin treatment has been documented earlier in numerous lepidopteran species (Jagannadh & Nair, 1992; Adel & Sehnal, 2000; Tunca et al., 2012; Er et al., 2017). Similar results of larval, pupal and adult emergence time prolongation and high inhibition of pupal molting and adult emergence were also obtained in this study. In insects, growth and developmental processes are highly regulated by hormonal homeostasis of juvenile hormone (JH) and 20-hydroxyecdysone (Bensebaa et al., 2015; Kilani-Morakchi et al., 2021). Azadirachtin is considered to interfere with the hormonal balance by suppressing and modifying hemolymph JH and ecdysteroid titers leading to reduced pupation, failure of adult emergence, malformations and incomplete ecdysis (Bezzar-Bendjazia et al., 2017; Kilani-Morakchi et al., 2021). In a recent study, Shu et al. (2021)

identified the azadirachtin-respondent genes in *Spodoptera frugiperda* Smith & Abbot, 1797 (Lepidoptera: Noctuidae) and reported that the genes interrelated in chitin biosynthesis were mostly down-regulated by azadirachtin. The authors speculate that azadirachtin-induced suppressed expression of these genes is the molecular basis for prolonged larval molt and development inhibition (Lai et al., 2014; Shu et al., 2021). Elongated adult occurrence time of insects in the agricultural systems may give rise to greater pest mortality ratios owing to biotic and abiotic factors such as multiplied exposure to predators and pathogens (Akthar et al., 2012).

Topical application of azadirachtin on the last instars of *A. grisella* reduced adult longevity at all azadirachtin doses compared to control. The effects of azadirachtin on adult longevity have been demonstrated in a diverse array of pest insects, including *Anopheles gambiae* Giles, 19002 (Diptera: Culicidae), *Ceratitis capitata* (Wiedemann, 1824) (Diptera: Tephritidae), *Zabrotes subfasciatus* (Boheman, 1833) (Coleoptera: Bruchidae), *Amphiareus constrictus* (Stål, 1860) (Heteroptera: Anthocoridae), *G. mellonella* (Okumu et al., 2007; Silva et al., 2013; Vilca Malqui et al., 2014; Gontijo et al., 2015; Er et al., 2017). It was also mentioned that sublethal doses of azadirachtin-induced hormesis effect on adult longevity of greater wax moth *G. mellonella* (Er et al., 2017), however here in the current work longevity was decreased at all doses and not in line with the previous study. Shu et al. (2021) demonstrated azadirachtin-sensitive genes in *S. frugiperda* and postulated that there is an effect of azadirachtin on regulation of longevity. It is a known phenomenon that stress responses in insects are energetically-demanding events (Uçkan et al., 2011) and it was reported that insecticide stress although of botanical origin may cause decreases in hemolymph components such as free amino acids, protein, lipid and carbohydrates associated with energy metabolism (Sharma et al., 2012; Altuntaş et al., 2014). Previous work has demonstrated that the botanical insecticide azadirachtin also affects energy reserves, metabolism and biochemical processes of various insect species by interfering with protein synthesis and reducing protein, lipid and carbohydrate concentrations (Li et al., 1995). Most probably the reduction in energy reserves of insects terminating from azadirachtin-related stress may lead to delays in growth and development processes and also a decline in adult longevity. The shortened longevity of *A. grisella* adults may cause decreased fecundity of females in a shortened lifespan and reduce the pest abundance in subsequent generations.

The current study also demonstrated that azadirachtin interacts with the cellular immune system of *A. grisella*. Hemocytes are key components of the insect immune system and it was found that topical application of *A. grisella* last instars with azadirachtin led to a decrease in total hemocyte counts of larval hemolymph at 24 and 48 h after treatment even at low doses. The decline in the total circulating hemocyte numbers recorded in the current study has also been mentioned in other insect pests exposed to azadirachtin via various treatment methods (Azambuja et al., 1991; Sharma et al., 2003; Pandey et al., 2008; Pandey & Tiwari, 2011; Er et al., 2017; Duarte et al., 2020). Pandey et al. (2008) discussed that the decline in total hemocyte numbers as a result of azadirachtin treatment may be associated with the clustering of hemocytes in one region, the toxicological effects of azadirachtin and their inhibitor effects on endocrine glands. The most profound effect of azadirachtin at the physiological level is the inhibition of the synthesis and release of ecdysteroids from the prothoracic gland, leading to incomplete ecdysis in immature insects (Isman, 2006). It is most likely that the reduction in THC is a result of endocrine regulation of azadirachtin because it was recently reported that the cellular immunity in insects is influenced by the hormones circulating in the hemolymph, including ecdysteroids (Nunes et al., 2021). An alternative explanation could be that the reductions in THC may be due to inhibition of hematopoietic function in larvae or declined mitotic division as alterations in hemocyte counts are also influenced by these factors (Gardiner & Strand, 2000; Rajak et al., 2015). Previous studies have demonstrated the cell cycle arrest and antimitotic effects of azadirachtin in insect cell lines (Salehzadeh et al., 2003; Huang et al., 2011). To prove this hypothesis, studies of azadirachtin-induced effects on *A. grisella* hemocyte division need to be conducted. In a recent study, Zhao et al. (2019) reported that genes related to apoptosis were up-regulated in

Bactrocera dorsalis Hendel, 1912 (Diptera: Tephritidae) after azadirachtin treatment, including genes encoding cathepsins. In addition to the azadirachtin-induced factors given above, the reduced number of THC after azadirachtin treatment could also be related to apoptotic death of hemocytes non-selective to a single type of hemocyte.

Despite the decreased total hemocyte count in the current study, no significant change was observed in the ratio of granulocytes and plasmatocytes. Previous studies reported significant changes in differential hemocyte counts caused by azadirachtin in various insect species (Dorrah et al., 2019, Pandey et al., 2008, Er et al., 2017). It has been reported that the variation in granulocyte and plasmatocyte numbers may be due to the transformation of some hemocyte types into other types for the phagocytic function, combatting against abiotic and biotic factors and foreign invaders or apoptotic bodies (Dorrah et al., 2019). However, in this study, the suppression of the spreading behavior of hemocytes instead of gaining phagocytic activity as a result of azadirachtin treatment seems to have eliminated the necessity of transformation of hemocytes. Previous reports in the literature strongly suggest that the differentiation of hemocytes in insects is influenced by the secretion of hormones circulating in the hemolymph including ecdysone (Nunes et al., 2021). The fact that azadirachtin, as an ecdysone antagonist may also negatively affect hemocyte differentiation could be the reason why granulocyte and plasmatocyte ratios remain unchanged in the current study. Combine with the reduction in THC, the same azadirachtin-induced effects are thought to be non-specific to one type of hemocyte in circulation.

Hemocyte spreading behavior is also an indicator of immunity in insects that occurs prior to cellular immune responses like encapsulation, phagocytosis and nodulation as it allows plasmatocytes and granulocytes to adhere to foreign materials (Lavine & Strand, 2002). Here we detected significant reductions in the spreading ability of hemocytes at all doses compared to control 24 and 48 h after azadirachtin treatment. However, the effect was not concentration-dependent and increases in azadirachtin concentration produced no further appreciable decrease in the ratio of spreading hemocytes. Probably, the dose-response relationship for the adverse effects on spreading ability reaches a maximum of 0.01 mg/ml or at concentrations lower than the minimum tested dose. Our results are consistent with previous studies that demonstrated inhibited spreading of hemocytes in the greater wax moth *G. mellonella* on exposure to azadirachtin (Er et al., 2017) and other botanicals (Zibaee & Bandani, 2010; Zibaee et al., 2012). Based on proteomic studies, azadirachtin interfered with the regulation of cell adhesion pathways (Sun et al., 2018) and genes responsible for key steps in hormone biosynthesis (Liu et al., 2019). Considering the potent relationship between hormone signaling and the behavior of hemocytes (Nunes et al., 2021), the reduced ratio of spreading hemocytes in the current study could be related to azadirachtin-induced changes in ecdysone titers related to the regulation of immunity.

We conclude that topical application of azadirachtin has detrimental impacts on mortality, developmental biology and the cellular immune function of A. *grisella* larvae. In combination with the previous studies demonstrating the hormonal regulation of azadirachtin in insects, the current findings reveal that azadirachtin can also act as an immunotoxic agent. The interrelation of azadirachtin-like phytochemicals with insects, through regulation of hemocyte counts and immune defenses, may provide opportunities for newer methods of pest control in agroecosystems.

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