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Azadirachtin-induced effects on various life history traits and cellular immune reactions of *Galleria mellonella* (Lepidoptera: Pyralidae)

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Abstract: The effects of the botanical insecticide azadirachtin were examined on the life history traits, fecundity and immune parameters of *Galleria mellonella* L. (Lepidoptera: Pyralidae). We determined that for the topical application of azadirachtin, the LC_{50} was 16.564 ppm; at 100 ppm the adult emergence time was prolonged, however the longevity of adults remained unchanged above sublethal concentrations. The mean number of healthy eggs and the fecundity of adults decreased, whereas the number of defective eggs increased with azadirachtin treatment. At concentrations >50 ppm female *G. mellonella* adults laid no eggs. Azadirachtin reduced total hemocyte counts at 24 and 48 h posttreatment, however the alterations in differential hemocyte counts were only significant at 100 ppm. Laminarin-induced nodulation response and the spreading ability of hemocytes were also suppressed with azadirachtin treatment. Our results suggest that azadirachtin, as a good candidate for integrated pest control, has the capability to affect the biological parameters and cellular immunity of the model insect *G. mellonella*.

Key words: *Galleria mellonella*; azadirachtin; fecundity; hemocyte count; nodule

INTRODUCTION

Because of the rising environmental problems associated with the dominant use of synthetic pesticides, researchers have found new methods of dealing with insect pests, ranging from usage of predators, parasitoids and biopesticides. Unique among these biopesticides, azadirachtin, a tetranortriterpenoid compound extracted from the seeds of neem tree (*Azadirachta indica* A. Juss.), has attracted great interest since it possesses anti-insect properties that manifest on a wide range of agricultural, medical and veterinary pests [1,2]; it has also been shown to have little or no toxicity on beneficial insects and vertebrates, including humans [3-5].

As a biodegradable natural insecticide, azadirachtin, which possesses antifeedant, repellant, growth regulatory and sterility properties against various insect pests, has been well documented [6-9]. Though there is a large amount of data on the effects of azadirachtin, the precise mode of action was poorly understood at the physiological and molecular level. It is generally accepted that azadirachtin acts as an insect growth regulator by blocking the biosynthesis of insect hormones such as ecdysteroids and inhibiting the development of reproductive organs and vitellogenesis that reduce the fecundity and fertility of insects [10,11]. In addition, recent works demonstrated that azadirachtin has antiproliferative effects by arresting the cell cycle and inducing apoptotic effects in insect cell lines [12-14]

Insects resist external materials with their highly effective immune system that relies on cellular and humoral components. Humoral immune reactions include the production of antimicrobial peptides, intermediates of nitrogen, oxygen and cytokines, as well as the prophenoloxidase (PO) cascade that regulates melanization of hemolymph [15]. Cellular immune reactions include phagocytosis, nodulation and encapsulation that are carried out by various types of hemocytes in circulation [15]. Both encapsulation and nodulation response end with the melanization of hemocyte clusters into blackened nodules because of phenoloxidase activation [15,16]. The immune system of insects is linked with the functions of various systems within the organism and has been found to be

susceptible to different environmental factors such as temperature, insecticides or plant-derived compounds [17,18]. Therefore, the immune function of an insect could be used as a reliable biomarker of the systemic toxic effects of biopesticides [19]. Also, the suppressed immunity of insects upon exposure to biopesticides represents a critical developmental phase when insects may become more susceptible to infection [16]. Besides the known multiple effects, it is possible that azadirachtin can exert adverse effects on the immune system of insects. However, to our knowledge there is little information about the potential of azadirachtin to affect the immunity of insects. It has been reported that treatment with azadirachtin interferes with various responses of humoral and cellular immunity in *Rhodnius prolixus* Stål (Hemiptera: Reduviidae) [20,21], *Spodoptera litura* Fabricius (Lepidoptera: Noctuidae) [22] and *Spodoptera littoralis* Boisduval (Lepidoptera: Noctuidae) [23].

The greater wax moth *G. mellonella* is a serious insect pest in beehives because it feeds on wax, honey and pollen. It is frequently used as a model organism to test the effects of toxic substances as the larvae of *G. mellonella* present several technical advantages such as easy cultivation in the laboratory, large size for *in vivo* physiological investigations and a sufficient amount of hemolymph [24-26]. Hence, *G. mellonella* was selected to investigate the effects of azadirachtin in the current paper. We defined for the first time the suppression of cellular immune parameters such as total and differential hemocyte counts, nodulation, melanization and spreading of hemocytes in *G. mellonella* by topical application of azadirachtin. We also studied the azadirachtin-induced effects on mortality, adult emergence time and longevity as developmental indicators, and the fecundity of *G. mellonella* adults as a reproductive indicator.

MATERIALS AND METHODS

Insects

Laboratory colonies of the greater wax moth *G. mellonella* were established from adults that were collected from several beehives located near Balıkesir, Turkey. Insect cultures were held in an incubator at 29±1°C, 60±5% RH, and a photoperiod of 12:12h (L:D). Insects were kept in 1-L jars and fed with natural blackened honeycomb to maintain conditions similar to their natural media in beehives [26]. Honeycomb was also used as an egg deposition substrate for adult insects. In all experiments 7th instar larvae of *G. mellonella* were used and larvae were weighed before treatments in order to apply the same concentration to each specimen.

Toxicity analysis

A commercial formulation of azadirachtin (NeemAzal-T/S, Trifolio-M GmbH, Germany, 10 g/L) was used for experimental analysis. The liquid preparation of azadirachtin was diluted with distilled water to nine concentrations (1, 5, 10, 50, 100, 500, 1000, 3000, 10000 ppm) that were tested for insecticidal activity and evaluation of lethal concentrations (LC), along with a control group. Five µL of each concentration were applied topically (from head to abdomen) to the freshly molted final instar larvae (0.18±0.1 g). Control groups consisted of 15 untreated larvae for toxicity analysis. Control and azadirachtin-treated experimental groups were held in sterile Petri dishes (60x15 mm) in an incubator in the same abovementioned conditions and observed daily to determine the percentage of mortality. Blackened and inert larvae and pupae that did not respond to mechanical stimulus were referred to as dead. According to mortality data, some chosen LC_x values (LC_{30}, LC_{50}, LC_{70}, LC_{95} and LC_{99}) of azadirachtin with associated 95% confidence levels ($P<0.05$) were determined using probit analysis in SPSS software (version 18.0 for Windows, SPSS Science, Chicago, IL). The experimental design was completed with 15 randomly chosen larvae for each experimental and control concentration and replicated three times.

Development and fecundity

The emergence time and longevity of adults after treatment with different concentrations of azadirachtin were determined as developmental indicators. According to probit results, all individuals were dead within the range of 500 to 10000 ppm azadirachtin. For this reason, we did not test these concentrations for adult emergence time, longevity and fecundity of adults. Azadirachtin was tested in a series of concen-

trations above and below the LC_{50} values (1, 5, 10, 50 and 100 ppm) in developmental experiments. Controls consisted of two groups as untreated and larvae treated with distilled water in the development studies and all subsequent experiments. Five µL of each concentration was applied to 7th instars topically. Controls and treated larvae in Petri dishes were transferred into an incubator adjusted to 29±1°C and 60±5% RH, and a photoperiod of 12:12h (L:D) and observed daily until adult emergence. Time taken to reach the adult stage from azadirachtin application to last instars was recorded as adult emergence time. Newly emerged *G. mellonella* adults from the azadirachtin-treated experimental groups were transferred into sterile Petri dishes to determine adult longevity. The Petri dishes were observed daily until all individuals died, and the elapsed time from adult emergence until death was recorded as adult longevity. The term fecundity in this study refers to the total number of eggs laid per adult female. To determine the fecundity of azadirachtin-treated adults, the bottom part of the Petri dish was covered with gauze and a piece of paper was placed between the gauze and the lid of the Petri dish as a substrate for deposition. The papers on which the experimental females laid their eggs were removed and the total number of eggs was counted daily using an Olympus SZ51 (Olympus, Japan) stereo microscope until the individuals die. The observed spherical and transparent eggs were considered as healthy, while wrinkled and dry ones were considered damaged eggs. In each experiment, 15 randomly chosen larvae (0.18±0.1 g) were tested in three replicates for each concentration and control groups.

Hemolymph collection, total and differential hemocyte counts

Immune parameters were examined at azadirachtin concentrations above sublethal doses to explore the azadirachtin-induced correlation in hemolymph fluidity and total hemocyte counts (THC). Last instars of approximately the same sizes (0.18±0.1) of *G. mellonella* were administered with different concentrations (100, 500, 1000, 3000 and 10000 ppm) of azadirachtin topically (5 µL). Controls were designed as untreated and treated with distilled water. All control and treated larvae were held in the abovementioned conditions. Twenty-four and 48 h after azadirachtin application larvae were bled with a sterile 19-gauge needle on the first hind leg. Four µL of hemolymph from each experimental and control larvae were collected with a glass microcapillary tube (Sigma, St. Louis, MO) and transferred into a sterile Eppendorf tube containing 36 µL ice cold anticoagulant buffer (98 mM NaOH, 186 mM NaCl, 17 mM Na_2 EDTA, and 41 mM citric acid, pH 4.5). Ten µL of diluted hemolymph suspension were applied to an improved Neubauer hemocytometer (Superior, Germany) after gently mixing by passing through a micropipette, and counted under an Olympus BX51 (Olympus, Japan) microscope. Fifteen larvae were evaluated for each experimental and control group in three replicates.

Differential hemocyte counts (DHC) were evaluated and three selected azadirachtin concentrations (100, 1000 and 3000 ppm) were used to determine the effects of azadirachtin. Larvae were bled as described above 24 h post azadirachtin application and the obtained hemolymph from each experimental and control larva was transferred into ice-cold phosphate buffered saline (PBS) (Sigma, St. Louis, MO) in a sterile Eppendorf tube. An aliquot of 20 µL of diluted hemolymph was applied to a microscope slide. The slides were left in a moist chamber at 29±1°C in the dark for 20 min to facilitate the identification of hemocytes. The slides were examined under an Olympus BX51 microscope (Olympus, Japan) to determine the DHC. Hemocyte types were classified according to Er et al., (2010). Approximately 300 hemocytes from five randomly selected fields of view were counted and the percentage of different hemocyte types was recorded. The percentage of mitotic hemocytes was also recorded. Nine larvae were evaluated for each experimental and control group in three replicates.

Nodulation

Topically azadirachtin-treated larvae (with 100, 1000 and 3000 ppm) along with control groups were maintained in an incubator as described above. A stock solution of laminarin (Sigma, St. Louis, MO) was prepared in PBS at a concentration of 10 mg/mL. Nodulation was induced by injecting 10 µL of laminarin on the first hind leg of last instar larvae 24 h after azadirachtin application. All larvae were anesthetized by chilling on ice for 10 min before injection. Nodula-

tion was assessed 24 h post laminarin injection. The larvae were chilled on ice and dissected under an Olympus SZ51 (Olympus, Japan) stereo microscope. The darkened melanized nodules embedded in fat body, hemolymph and other organs were counted. Fifteen larvae were evaluated for each experimental and control group in three replicates.

Cell spreading

The hemocyte monolayers were prepared using hemolymph samples from topically azadirachtin-treated *G. mellonella* larvae (100, 1000 and 3000 ppm) along with control groups. Four µL of hemolymph were obtained from each experimental and control larva as mentioned in the hemolymph collection section, and transferred into a sterile Eppendorf tube containing ice-cold PBS. Twenty µL of diluted hemolymph suspension were dropped on a sterile microscope slide after gently mixing by passing through a micropipette. The slides were then placed in a moist chamber and incubated at 29±1°C in the dark for 20 min to allow hemocytes to attach to the glass. Subsequently, the slides were overlaid with a cover slip and were examined under an Olympus BX51 (Olympus, Japan) microscope. The relative number of spread hemocytes was observed by counting 300 hemocytes from five randomly selected fields. Nine larvae were evaluated for each experimental and control group in three replicates.

Statistical analysis

Data on the biological parameters, fecundity and hemolymph experiments were tested for normality of data distribution using Levene's test. As all data met the assumptions of the parametric tests, one-way analysis of variance (ANOVA) was used to compare means. Differences were separated by Tukey's honestly significant post hoc test (HSD) according to homogeneity of variances. An arcsine square-root transformation was performed on percentage values before analyses but untransformed means are presented. An SPSS software program (SPSS 18.0 for windows) was used for data analysis. Results were considered statistically significant when $P<0.05$.

RESULTS

Toxicity of azadirachtin

The cumulative percentage mortality of *G. mellonella* after exposure to different concentration of azadirachtin is shown in Fig. 1. The highest percentage mortality was observed at 500-10000 ppm of azadirachtin. Mortality data can be analyzed using the log-Probit program [27]. This program tests the linearity of concentration mortality curves and provides statistical endpoints (LCx). Table 1 revealed that LC_{99} was 3191.307 ppm (95% confidence limits,

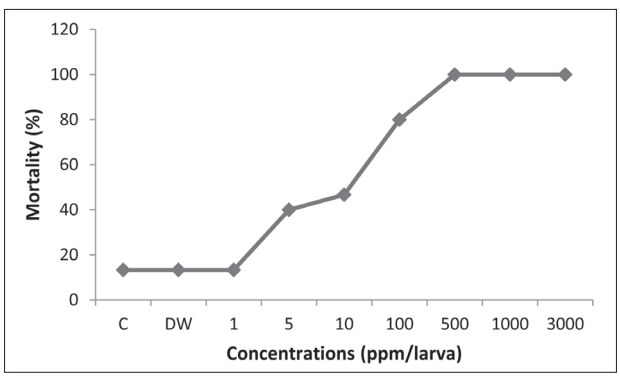

Fig. 1. Mortality of *G. mellonella* after exposure to different concentrations of azadirachtin (ppm).

Table 1. Some chosen LC_x values of azadirachtin applied to last instars of *G. mellonella*.

Treatment				Lethal concentrations (ppm/larva) [b]			
	N[a]	X^2 (df)	Slope±SE	LC_x values (95% CL)		Lower bound	Upper bound
				LC_{30}	5.060	0.669	14.194
				LC_{50}	16.564	4.517	46.428
AZA	150	11.604 (7)	1.018±0.162	LC_{70}	54.226	19.987	231.529
				LC_{95}	683.356	175.844	19557.633
				LC_{99}	3191.307	541.489	354112.773

[a]Total number of insects used for the bioassay.
[b]Values are displayed with the lower and upper confidence limits. AZA – azadirachtin, CL – confidence limits. Y(Probit) = _1.241+1.018 (log dose)

541.489-354112.773 ppm) while the LC_{50} value for larvae was 16.564 ppm (95% confidence limits, 4.517-46.428 ppm). According to probit analysis, all doses of azadirachtin, even the lower ones, showed insecticidal activity on *G. mellonella* larvae. Meanwhile, larvae displayed unusual spinning covering the surface of the Petri dishes at all concentrations ≥LC_{30}.

Effects of azadirachtin on adult emergence time, longevity, and fecundity

The effects of azadirachtin on adult emergence time and longevity of *G. mellonella* are presented in Fig. 2. Azadirachtin application caused significant differences in emergence time (F=5.789; df=6, 82; P=0.000). The adult emergence time was increased in experimental groups >1 ppm, however the extension was only statistically important at 100 ppm compared to controls and in samples exposed to 1, 5 and 50 ppm. Azadirachtin treatment reduced the adult longevity at all concentrations but the decline of life span was considerable at 1, 5, and 10 ppm with respect to the controls (F=6.254; df=6, 59; P=0.000)

The total average number of eggs laid by a single female was 256.75±17.39 in the untreated control groups. Azadirachtin application caused an important decrease in egg numbers at all concentrations compared to untreated larvae (F=25.690; df=5, 38; P=0.000). Similarly, the number of healthy eggs was significantly reduced in all experimental groups with respect to untreated larvae (F=37.116; df=5, 38; P=0.000). On the other hand, the increase in damaged egg number was only significant at 50 ppm compared to both control groups (F=5.260; df=5, 38; P=0.001) (Fig. 2). The number of damaged eggs was about 2% for both control groups, while in the experimental groups it was about 38% (F=10.587; df=5, 38; P=0.000) (Table 2).

Effects of azadirachtin on THC and DHC of *G. mellonella*

The number of circulating hemocytes of untreated last instars normally displayed 26.45 and 21.05x10^6 cells/mL at 24 and 48 h post-treatment, respectively. Hemocyte number decreased significantly among treatments in a concentration-dependent manner at 24 h

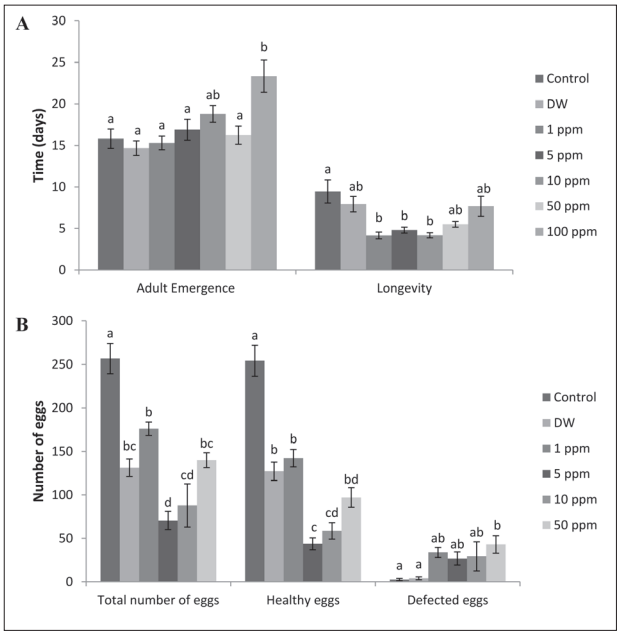

Fig. 2. Azadirachtin-related changes in adult emergence time and longevity (days) (**A**) and fecundity (**B**) of *G. mellonella* when topically applied to last instar larvae. Each bar represents the mean±standard error of three replicates. Significant differences are indicated by different letters (a-d) (P<0.05; Tukey's HSD test).

Table 2. Azadirachtin-related changes in percentages of defective eggs of *G. mellonella* after irs topical application to last instar larvae.

AZA (ppm)	Percent defective eggs (%)
Control	1.06±0.58 a
DW	3.39±1.58 a
1	19.71±3.43 ab
5	37.07±5.72 b
10	26.05±8.65 b
50	30.67±6.16 b

Each represents the mean±standard error of three replicates.
The numbers in columns (a-b) followed by the same letter are not significantly different (P>0.05; Tukey's HSD test).

(F=26.049; df=6, 98; P=0.000) and 48 h (F=45.808; df=6, 98; P=0.000) after azadirachtin treatment (Fig. 3). The minimum count of 5.65x10^6 cells/mL was observed at 48 h after azadirachtin application at the highest concentration of azadirachtin 10000 ppm, whereas a maximum count of 17.18x10^6 cells/mL was determined at the lowest concentration of 100 ppm at 24 h. We observed that the hemolymph fluidity was highly increased at azadirachtin concentrations that lead high mortality rates in *G. mellonella* according to probit analysis, showing correlation with a reduced number of total hemocytes.

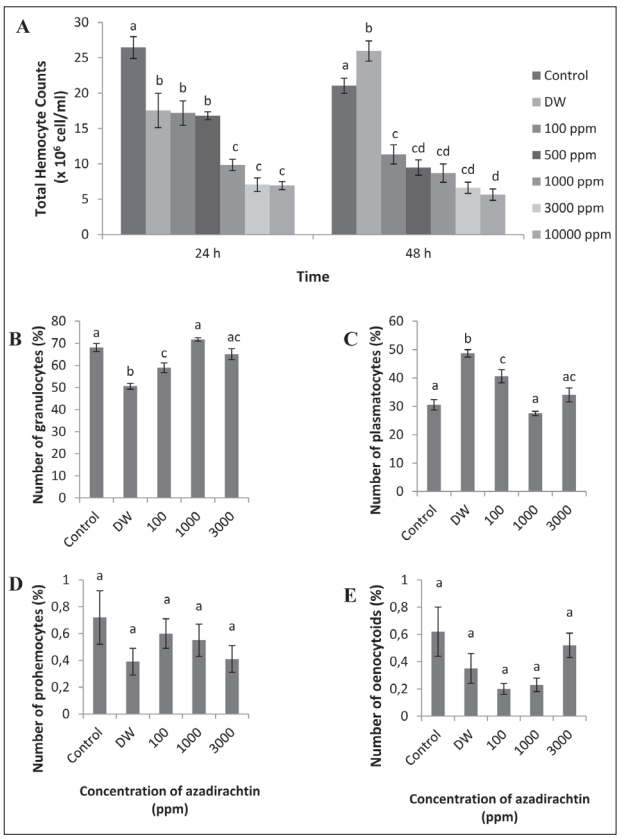

Fig. 3. Total hemocyte counts (x10^6 cell/ml) (**A**), granulocyte (**B**), plasmatocyte (**C**), prohemocyte (**D**) and oenocytoid (**E**) counts (cells/100) of *G. mellonella* larvae treated with different concentrations of azadirachtin. Each bar represents the mean±standard error of three replicates. Significant differences are indicated by different letters (a-d) (P<0.05; Tukey's HSD test).

In this study, the DHC was expressed in relative numbers of granular cells, plasmatocytes, prohemocytes and oenocytoids in this study. The major hemocyte type was the granular cells that comprised 68.09 and 50.59% in the total hemocyte population of untreated and distilled water-treated individuals, respectively. Plasmatocytes with 30.55% (untreated) and 48.65% (distilled water-treated) were the second highest group of cells in the total number of hemocytes (Fig. 3). Azadirachtin application caused alterations in the number of both granular cells (F=20.374; df=4, 40; P=0.000) and plasmatocytes (F=20.751; df=4, 40; P=0.000). The reduction in granulocyte and increase in plasmatocyte ratios were only significant at 100 ppm with regard to untreated larvae, whereas no differences were observed in prohemocyte (F=0.748; df=4, 40; P=0.565) and oenocytoid (F=2.032; df=4, 40; P=0.108) ratios.

The percentage of mitotic values decreased in a concentration-dependent manner, but the decline ratio was only significant at 1000 and 3000 ppm compared to untreated controls (F=3.602; df=4, 40; P=0.013) (Fig. 4).

Effects of azadirachtin on nodulation of *G. mellonella*

Nodule formation was assessed 24 h post injection of laminarin at all concentrations and control groups. Large melanized nodules could be seen attached to the fat body and the other organs. The nodule counts re-

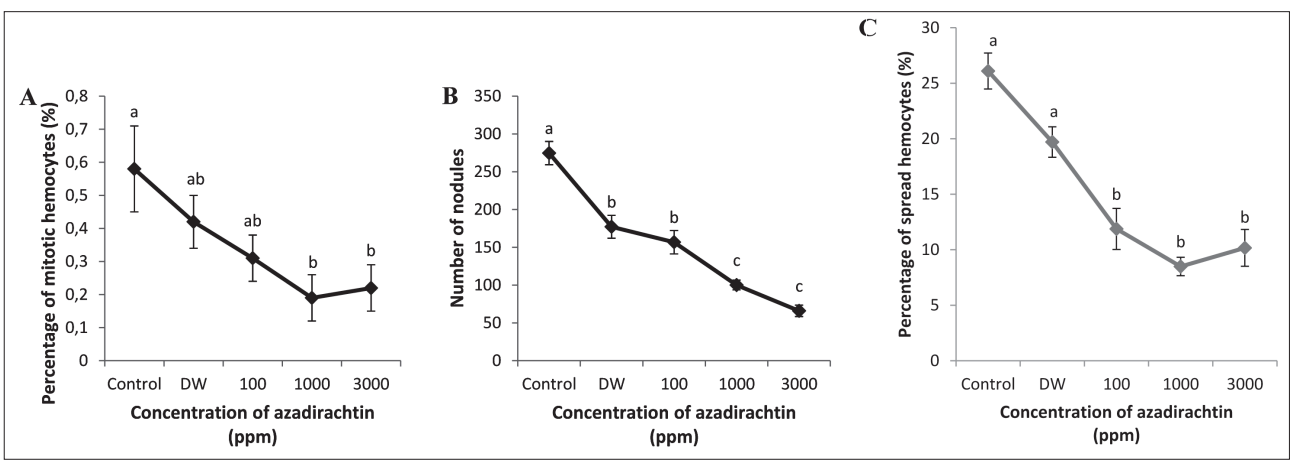

Fig. 4. Effects of different azadirachtin concentrations on mitosis frequency (%) (A), number of nodules (B) and hemocyte-spreading behavior (C) of *G. mellonella* larvae. Each bar represents the mean±standard error of three replicates. Significant differences are indicated by different letters (a-c) (P<0.05; Tukey's HSD test).

sulting from azadirachtin application are summarized in Fig. 4. The number of nodules was 274.67±15.47 in larvae injected with laminarin only. Larva treated with azadirachtin-laminarin gave a concentration-dependent decrease in the level of nodule formation (F=40.053; df=4, 70; P=0.000). The reduction ratio of nodules rose to 76% at 3000 ppm compared to the laminarin-injected control.

Effects of azadirachtin on cell spreading

The ratio of hemocytes showing spreading behavior is given in Fig. 4. The number of spreading hemocytes was crucially decreased at all azadirachtin concentrations with respect to untreated and distilled water-treated groups (F=20.689; df=4, 40; P=0.000). The relative spread-cell number of untreated and distilled water-treated control groups was 26.10% and 19.70%, respectively. The spreading ratio decreased by >61% at 1000 and 3000 ppm.

DISCUSSION

The results of this study demonstrate that azadirachtin leads to disruption in the developmental, reproductive and immune processes when applied topically to the pest model insect *G. mellonella*. The impact and use of azadirachtin as a bioinsecticide is well documented in earlier investigations. However, considering previous studies, its effects depend on the species, stages of the insect, concentration and the method of application (contact, ingestion and injection) [1,28]. Although a limited number of studies have been conducted indicating the developmental and survival effects of azadirachtin on *G. mellonella* [25,29-31], no data are available about its potential effects on reproductive and immune parameters of the model pest. Initially, we determined LC_{50} and LC_{99} values of azadirachtin to observe lethal and sublethal effects on *G. mellonella*. Topical application of azadirachtin to last instars gave LC_{50} and LC_{99} values as 16.564 and 3191.307 ppm, respectively. There are numerous studies demonstrating the lethal effects of azadirachtin on different insect species [1,25]. However, to our knowledge, this paper demonstrates for the first time the LC values of azadirachtin following topical application on *G. mellonella* last instars. Our data also showed that azadirachtin caused a concentration-dependent mortality. This result is in line with previous studies conducted with pure azadirachtin or neem extracts applied by different ingestion experiments to *G. mellonella* [25,32]. It is quite evident from previous studies and our findings that azadirachtin is highly toxic to insects, even at lower concentrations.

We observed that azadirachtin application on the last instars of *G. mellonella* interferes with adult emergence time. The concentrations below LC_{70} of topically applied azadirachtin induced slight alterations on adult emergence time. However, at 100 ppm the elongation is highly significant. Extension of adult emergence period depending on azadirachtin application has been previously reported in various lepidopteran species [33-35]. It seems that the increase in adult emergence time is based on the strong insect growth-regulating activity of azadirachtin, probably due to interference with the ecdysteroid metabolism in insects [1,10,34]. Delayed adult emergence time in the field may cause higher mortality rates due to abiotic and biotic components such as increased exposure to pathogens and predators, and suppressed immunity [36]. In contrast to our findings, Gelbic and Nelmec [30] stated that azadirachtin had no effect on the adult emergence time of *G. mellonella*. We believe that the variations between our and their findings lie in the applied concentration and formulation of azadirachtin.

On the other hand, azadirachtin applied topically onto the last instars of *G. mellonella* caused a significant decrease in adult longevity at sublethal concentrations, but displayed no effects at concentrations greater than LC_{50}. We suggest that sublethal concentrations of azadirachtin may stimulate a hormesis effect on adult longevity that is widely reported in insect species exposed to insecticides and toxicants. Hormesis is defined as low-dose stimulation of toxic materials at sublethal concentrations that is not revealed by higher doses [37]. Previous studies indicated that sublethal concentrations of azadirachtin and imidacloprid have hormetic effects on the esterases, juvenile hormone levels, fecundity and other parameters of some pest insects [38-40]. The adverse influence of azadirachtin on adult longevity has been investigated in *Anopheles gambiae* sensu lato (Diptera: Culicidae), *Zabrotes subfasciatus* Boheman (Coleoptera: Bruchidae), *Amphia-*

reus constrictus Stål (Heteroptera: Anthocoridae) and *Ceratitis capitata* Wiedemann (Diptera: Tephritidae) [40-43]. The reduced longevity of adults may reduce the pest population in next generations by decreasing the number of eggs in a shortened lifespan.

Females of *G. mellonella* that overcame the lethal effects of azadirachtin and emerged as adults exhibited decreased fecundity associated with the reduced number of healthy eggs and increased number of defective eggs. Moreover, the adult females laid no eggs at concentrations >50 ppm. It is likely that azadirachtin had a high level of adverse activity on the reproductive potential of *G. mellonella* as demonstrated in a limited number of lepidopterous pests [6,7,34]. The reproductive disruption of azadirachtin due to interference with vitellogenin synthesis and its uptake into oocytes resulted in failed oocyte growth and maturation that was reported in *R. prolixus* and *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae) [6,44]. Reduction in egg production caused by azadirachtin in *R. prolixus* is also correlated with reduced ecdysteroid levels in ovaries and hemolymph [44]. It is a known phenomenon that interference with the endocrine system may inhibit the maturation of germ cells and deposition of vitellogenin in eggs, resulting in defected eggs [45]. In line with these data, we speculate that azadirachtin may reduce the number of healthy eggs and increase that of defective eggs of *G. mellonella* with the combination of its endocrine regulation and direct effects on reproductive tissues.

In the current study, we also demonstrated for the first time that topically applied NeemAzal, one of the most widely used commercial formulations of azadirachtin, interfered with the cellular immunity of *G. mellonella*. Azadirachtin elicited a sharp decrease in the number of circulating hemocytes at all applied concentrations compared to the control. However, the effect on differential hemocyte counts was less. The reduction in granulocyte and increase in plasmatocyte ratios were only significant at 100 ppm, whereas no differences were observed in prohemocyte and oenocytoid ratios. In agreement with our results, treatment with azadirachtin decreased the number of total hemocytes in *R. prolixus* [20], *S. litura* [22], *Dysdercus koenigii* Fabricius (Heteroptera: Pyrrhocoridae) [46] and *S. littoralis* [23]. Our results are similar to the findings described in studies in which, following topical application of NeemAzal, the decline in total hemocyte counts was 61% in *D. koenigii* [46], and 56 and 59% in *Danaus chrysippus* Linnaeus (Lepidoptera: Nymphalidae) at 24 and 48 h posttreatment, respectively [47]. Azadirachtin influences several physiological pathways due to interference with endocrine physiology [20]. It is possible that the sharp decrease in total hemocyte counts is the result of hormonal regulation of azadirachtin; the relationship between the endocrine and immune systems has been investigated [21]. An alternative explanation could be that azadirachtin decreased the counts of circulating hemocytes due to the induction of autophagic or apoptotic pathways resulting in cell death. It was reported in many studies that azadirachtin leads to apoptosis and autophagy in insect cell lines originated from ovarian tissues [13,14]. To prove this hypothesis, more detailed investigations need to be conducted into the effects of azadirachtin on hemocyte death. In insects, alterations in hemocyte numbers are also influenced by the mitosis of the hemocytes in circulation [48,49]. Azadirachtin application to *G. mellonella* resulted in a decrease in the number of mitotic hemocytes, which could be another possible explanation for reduced hemocyte counts. In line with our findings, the antimitotic effects and cell cycle arrest of azadirachtin have been demonstrated in insect cell lines in other studies [12,13].

One of the most informative ways to define the immune function of an insect is to assess the number of nodules produced in response to the application of a specific antigen to an insect's hemocoel. Spreading is also a hemocytic behavior that occurs during cellular immune responses such as nodulation, encapsulation, and phagocytosis [15]. Our results demonstrated that azadirachtin influenced the immune responses of *G. mellonella* via significant reduction of nodule numbers following a challenge with laminarin, and decreases the number of spreading hemocytes at all concentrations compared to untreated control. We could find only one report that shows a decreased nodulation response after azadirachtin treatment to *R. prolixus* [20]. However, in several studies it has been reported that nodulation and hemocyte spreading are suppressed in response to botanical insecticides, plant products and insect growth regulators [16,17]. The decrease in total hemocyte counts due to treatment with azadirachtin or other botanical applications in the current

and abovementioned studies may be the reason for reduced nodulation and hemocyte spreading under the influence of neuroendocrine effects. Apart from the discussed mode of action, it is obvious from recent studies that azadirachtin also reduces protein synthesis and the expression of some genes related to development, stress and immunity in many insects [11,28,50]. Therefore, we can conclude that azadirachtin treatment on *G. mellonella* may alter the expression profile of immune-related proteins such as plasmatocyte spreading peptide or β-1,3-glucan recognition protein that initiate spreading and nodulation reactions, respectively. Further work is required to understand the molecular regulatory mechanism of azadirachtin on the cellular immune responses of *G. mellonella*.

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