



## Genotoxicity of azadirachtin on *Galleria mellonella* L. (Lepidoptera: Pyralidae)

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### Abstract

In this paper, genotoxic effects of the pure azadirachtin (AZA) on the larval hemocytes of model insect and storage pest *Galleria mellonella* L. (Lepidoptera: Pyralidae) was investigated. The comet assay was performed to measure and analyze DNA damage in larval hemocytes at Anadolu University (Eskişehir Technical University) between 2017 and 2018 years. For this purpose, sublethal AZA doses (0.5, 1, 1.5, and 2 µg/larva) given to *G. mellonella* larvae via insect force feeding method were used to monitor tail intensity, tail moment and tail migration the commonly known comet parameters. These DNA damage indicators were analyzed in hemocytes obtained from larvae at 24 and 72 h post force feeding. All comet parameters at all doses of AZA increased in comparison with negative and positive control at 24 and 72 h. At 72 h post force feeding with median lethal dose of AZA, a significant increase in DNA damage indicators was observed in larval hemocytes as compared with untreated groups. Consequently, this study showed that AZA caused significant damage in the genome of *G. mellonella* larvae even at sublethal doses and the comet assay was useful in the monitoring of in vivo genotoxicity of AZA in larva hemocytes.

**Key words:** azadirachtin, COMET, *Galleria mellonella*, genotoxicity, hemocytes

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### Azadirachtin'in *Galleria mellonella* (Lepidoptera: Pyralidae) üzerindeki genotoksitesitesi

#### Özet

Bu çalışmada, azadirachtin (AZA)'ın model böcek ve depo zararlısı olan *Galleria mellonella* L. (Lepidoptera: Pyralidae)'nın larval hemositleri üzerindeki genotoksik etkileri araştırılmıştır. Larval hemositlerdeki DNA hasarının analiz edilmesi ve ölçülmesi comet analizi kullanılarak, 2017-2018 yılları arasında Anadolu Üniversitesi (Eskişehir Teknik Üniversitesi)'nde gerçekleştirilmiştir. Bu amaçla, hemositlerdeki kuyruk yoğunluğu, kuyruk momenti ve kuyruk göçü gibi önemli comet parametrelerinin analiz edilmesi için *G. mellonella* larvalarına, böcekler için ağızdan besleme yöntemiyle verilen subletal AZA dozları (0.5, 1, 1.5 ve 2 µg/larva) kullanılmıştır. Ağızdan besleme uygulamasından 24 ve 72 saat sonra larvalardan elde edilen hemositlerde bu DNA indikatörleri analiz edilmiştir. AZA uygulanmasından 24 ve 72 saat sonra, larval hemositlerde belirlenen tüm comet parametrelerinde negatif ve pozitif kontrol grubuna kıyasla hem doza hem de zamana bağlı olarak artış meydana geldiği görülmüştür. Özellikle, ortalama letal doz AZA uygulamasından 72 saat sonra, larval hemositlerdeki DNA hasarı indikatörlerinde kontrol gruplarına kıyasla önemli bir artış gözlenmiştir. Sonuç olarak, bu çalışma AZA'nın subletal dozlarda bile *G. mellonella* larval genomunda önemli oranda hasara neden olduğunu ve larval hemositlerinde AZA'nın genotoksitesitesinin *in vivo* değerlendirilmesinde comet analizinin kullanışlı olduğunu göstermiştir.

**Anahtar kelimeler:** azadirachtin, COMET, *Galleria mellonella*, genotoksitesite, hemosit

#### 1. Introduction

Many countries profoundly use chemical control methods to combat pests in agricultural and apicultural systems. Uncontrolled and unconscious use of chemicals such as arsenic, organic chlorides and organic phosphates containing compounds causes resistance in pests and also negatively affects non-target organisms (Nicolopoulou-Stamati et al.,

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2016). Due to the side effects imposed on the local ecosystems by these chemicals, most countries have imposed restrictions and bans on the use of these chemical compounds. For this reason, using of the eco-friendly chemicals such as biopesticides or plant-based insecticides as alternative methods of combating pests while protecting non-target beneficial insects, humans, and other living things has important in integrated pest management (IPM) programs (Olson, 2015; Nicolopoulou-Stamati et al., 2016). In recent years, among these plant-based insecticides, azadirachtin (AZA) formulations which have active ingredients with various insecticidal properties has gained importance among natural chemicals (Sanchez et al., 2010). Earlier experiments revealed that AZA has toxicity on physiological process which change with insect hormones (like juvenile hormone and 20-hydroxyecdysone as growth factors), oocyte structure, fecundity, oviposition, egg viability, immune system, nervous system, antioxidant system and some metabolic pathways in various insects (Qiao et al., 2013; Asaduzzaman et al., 2016; Boulahbel et al., 2015; Dere et al., 2015; Shao et al., 2016; Aribi et al., 2017; Er et al., 2017) but it is non-toxic to vertebrates including mammals (Saxena & Kesari, 2016). However, no studies determining effects of pure AZA on insect genome, in particular, wax moth and model insect *Galleria mellonella* L. (Lepidoptera: Pyralidae) have been found in open literature. For this reason, the deficiency of scientific data on the determination whether the AZA as a destructive effects on the genetic material of hemocytes has been overcome by this study.

The greater wax moth *G. mellonella* is an economic and storage pest worldwide in apiculture. Its adults lay their eggs on the honeycombs and larvae feed on pollen, honey and beeswax (Charriere & Imdorf, 1997). In the protection of honeycombs against *G. mellonella* infestation, various chemical (aluminum phosphide, ethylene dibromide, paradichlorobenzene (Naphthalene), sulfur, carbondioxide), physical (cold-hot) and biological techniques (*B. thuringiensis*) have been employed in various ways (Kwadha et al., 2017). In addition to these applications, eco-friendly pesticides such as AZA could be used as alternative botanical insecticides for the protection of honeycombs against larval infestation. On the other hand, the larval stage of wax moth is considered as a model organism used for immunological and ecotoxicological investigations of various environmental chemicals, and laboratory cultivation is economical, easier and faster (Dere et al., 2015; Altuntaş & Duman, 2017; Kwadha et al., 2017). For this reasons, the identification of genotoxicity of AZA against insects in particular pests was determined using model organism *G. mellonella* with this study.

It is well known that the comet assay also known as single cell gel electrophoresis (SCGE) is a fast, simple, and more susceptible method to assess the genotoxicity of various chemicals in environment, and commonly used for environmental monitoring, and ecogenotoxicology applications (Singh et al., 1988; Collins, 2004; Olive & Banáth, 2006). Previous studies also showed that comet assay is very useful for the biomonitoring studies in human exposed to various genotoxins (Anderson et al., 1997; Martelli et al., 2002; Rajaguru et al., 2002). However, there are limited studies performed on the evaluation of the DNA damaging potential of some chemicals on insects using comet assay (Mukhopadhyay et al., 2004; Maria-Packiam et al., 2015; Qari et al., 2017). Mukhopadhyay et al. (2004) determined the genotoxicity of cypermethrin in *Drosophila melanogaster* using comet assay. In other studies, the genotoxic effects of novel phytopesticide and some plant volatile oils in larval hemocytes of *Helicoverpa armigera* (Maria-Packiam et al., 2015) and *Rhyzopertha dominica* (Qari et al., 2017) was determined with the comet assay. Despite all these studies with various environmental compounds, there has been no study to determine the sublethal effects of pure AZA on larval genome of *G. mellonella*. In the light of these informations, the aim of this study was to examine the genotoxicity of pure AZA on the larval hemocytes of model insect *G. mellonella* by comet assay and provide the new information to the available literature.

## 2. Materials and methods

### 2.1. Insect rearing

Laboratory cultivation of *G. mellonella* was reared in an insectarium (D51-41) in the animal physiology laboratory at Eskisehir Technical University, Turkey. Photoperiodical conditions in insectarium were maintained at 28±2°C temperature, 60±5 % relative humidity (RH) and in continuous darkness to ensure stock and experimental culture continuity. The semi-artificial diet including 340 g of bran, 20 g of pollen, 75 ml of honey, 100 g of dark honeycomb, 150 ml of glycerol and 75 ml of bidistilled water as described by Dere et al. (2015) was used to feeding the larval instars.

### 2.2. Force feeding assay

AZA (Sigma, St. Louis, MO, 2 mg/ml) to be used in the study was obtained as a pure powder. In a previous study by Dere et al. (2015), median lethal dose (LD<sub>50</sub>) and lethal dose (LD<sub>99</sub>) values of AZA, administered by force feeding assay to *G. mellonella* larvae, were reported to be 2.1 and 4.6 µg/larva respectively. For this reason, stock AZA was dissolved in 10 % ethanol and diluted to form solutions in sublethal doses at 0.5, 1, 1.5, and 2 µg/larva. Healthy larvae weighing 0.17±0.01 g selected from the stock culture were given 5 µl of the different prepared doses of AZA via force feeding method (Dere et al., 2015; Altuntaş et al., 2016). Prior the insect force feeding assay, all selected larvae were starved for 4 hours after which, they were kept on ice for 2 minutes to be anesthetized. Then 5 µl of AZA was

administered orally to each larva through the esophagus using a 10  $\mu$ L hamilton injector (26 g gauge). For the negative and positive control groups, 5  $\mu$ l of distilled water and 10 % ethanol solution were given to each larva, respectively. Each of the treated larvae was maintained in a 2 g artificial diet containing sterile plastic box (50 ml, OrLab) at  $28\pm 1^\circ\text{C}$ ,  $60\pm 5$  % RH. At 24 and 72 h after force feeding treatment, hemolymph samples were collected from each larvae to analyze the changes in the DNA. For each of the doses and control groups, 45 larvae in total three replicate were used.

### 2. 3. Comet assay

The comet assay was carried out according to Singh et al. (1988) using larval hemocytes. At 24 and 72 h post AZA treatment, all force-fed larvae were sterilized with 70 % ethanol then, the third proleg of each larva was pierced with microscissors and five microliters of hemolymph were collected. The collected hemolymph (5  $\mu$ l) samples were mixed in 1 % low melting agarose (95  $\mu$ l) in PBS ( $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  free). The hemocyte suspension was applied to the surface of a microscope slide which was precoated with 1 % normal melting point agarose and covered with a cover glass which was removed after 15 minutes at  $4^\circ\text{C}$  to form a microgel. After this process, slides were kept in lysis buffer (100 mM EDTA, 10 mM Tris-HCL, 2.5 M NaCl, 1 % TritonX-100, 10 % DMSO, pH = 10.0) for 1 h at  $4^\circ\text{C}$  in darkness. Subsequently, slides were transferred to electrophoresis tank containing alkaline buffer (200 mM EDTA, 10 N NaOH, pH > 13) for 45 minutes to facilitate DNA unwinding after which electrophoresis was run for 30 min (20 Volt, 300 mA). Then slides were washed in a neutralization (0.4 M Tris-HCL, pH = 7.4) solution and bidistilled water, respectively for 5 min. All slides were left to dry overnight at room temperature. After electrophoresis, slides were stained with SYBR Green I (1:10.000) (Sigma-Aldrich, Taufkirchen, Germany) overnight and washed with bidistilled water to remove excess stains then maintained to dry at room temperature. To analyze the stained DNA in the hemocytes, slides were examined under a Leica DM6000 B model fluorescent microscope at blue filter. Tail intensity, tail moment and tail migration were measured via software Comet Assay IV imaging system (Perceptive Instruments Ltd, UK-Italy) to determine DNA damage in larval hemocytes. One hundred randomly selected hemocytes were analyzed per larvae.

### 2. 4. Statistical analysis

All data were identified as mean $\pm$ standard error (Mean $\pm$ SE). The SPSS software program (version 18.0 for Windows, Chicago, IL) was used for statistical analysis. Dose-dependent changes in the means of tail intensity, tail migration and tail moment were verified to be normally distributed. To compare means, ANOVA (one-way analysis of variance) and to determine the significant differences LSD-post hoc tests (Least Significant Difference) were conducted. A t-test was carried out to analysis the significance of the effects of the sublethal AZA doses on DNA structure in response to time interval (24 and 72 h). The results obtained in the experiments were evaluated as being statistically significant at a 95 % confidence interval with  $P < 0.05$ .

## 3. Results

The comet assay parameters, namely tail intensity (%), tail migration ( $\mu\text{m}$ ), and tail moment (arbitrary units) were used to determine DNA damage in larvae exposed to  $\leq \text{LD}_{50}$  doses of AZA. All results obtained in the study showed statistically significant a dose-time dependent increases in DNA damage of hemocytes post AZA treatment (Figure 1). Furthermore, a statistically significant elevation was evident in DNA damage indicators analyzed in hemocytes of forced larvae at  $\text{LD}_{50}$  doses of AZA with respect to control groups at 72 hours post treatment (Figure 1).

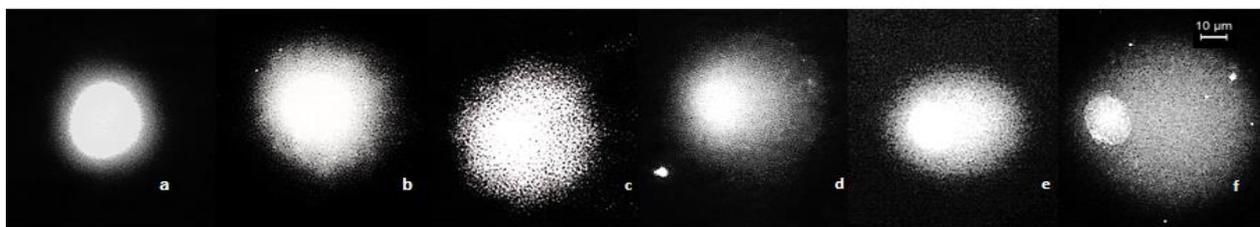


Figure 1. The comet assay images obtained in hemocytes of *G. mellonella* larvae at 72 h post force feeding. a) Negative control, b) Positive control, c) 0.5  $\mu\text{g}/\text{larva}$  AZA, d) 1  $\mu\text{g}/\text{larva}$  AZA, e) 1.5  $\mu\text{g}/\text{larva}$  AZA, f) 2  $\mu\text{g}/\text{larva}$  AZA

A significant increase in tail intensity (% DNA) of larval hemocytes was detected in all doses at 24 and 72 hours post AZA treatment when compared with control groups ( $F_{24} = 20.042$ ;  $df_{24} = 5, 2994$ ;  $P_{24} = 0.000$ ;  $F_{72} = 78.676$ ;  $df_{72} = 5, 2994$ ;  $P_{72} = 0.000$ ). A drastic increase in tail intensity was however observed at 2  $\mu\text{g}/\text{larvae}$  ( $\text{LD}_{50}$ ) with respect to controls and other sublethal doses of AZA (Figure 2).

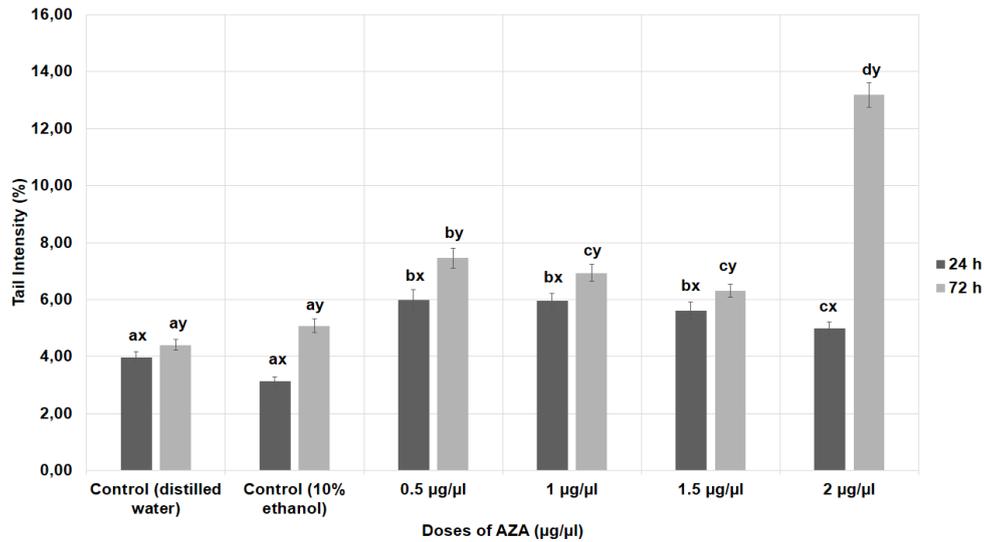


Figure 2. The tail intensity (Tail DNA %) results obtained from the hemocytes of *G. mellonella* larvae at 24 and 72 h  
 \* All data represents as means ± standard error. Each column indicated by the different letter (a-d, LSD test) or between black and grey column indicated by the different letter (x-y, t-test) are significant statistically (P < 0.05)

Tail migration in hemocytes of *G. mellonella* larvae increased in both dose and time related manner with respect to negative and positive control groups (P < 0.05, Figure 3). At 24 and 72 hours post-AZA treatment, tail migration in larval hemocytes showed significant increases at all doses except at 0.5 µg/larva AZA dose compared to the results of the control groups (F<sub>24</sub> = 67.03; df<sub>24</sub> = 5, 2994; P<sub>24</sub> = 0.000; F<sub>72</sub> = 66.212; df<sub>72</sub> = 5, 2994; P<sub>72</sub> = 0.000). Similar to tail intensity, the highest tail migration level were detected in larvae treated with LD<sub>50</sub> dose of AZA at 72 hours (Figure 3).

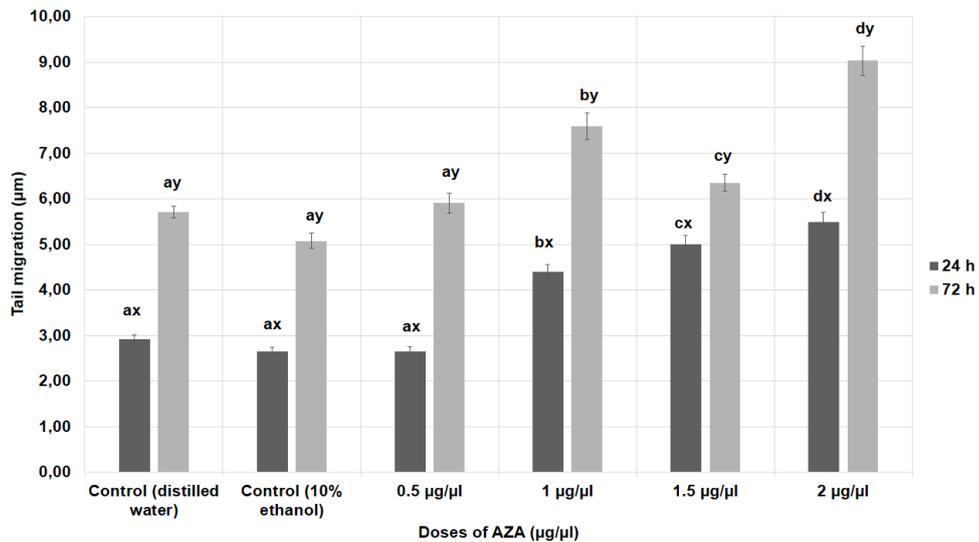


Figure 3. The tail migration (µm) results obtained from the hemocytes of *G. mellonella* larvae  
 \* All data represents as means ± standard error. Each column indicated by the different letter (a-d, LSD test) or between black and grey column indicated by the different letter (x-y, t-test) are significant statistically (P < 0.05)

The tail moment results for hemocytes exposed to ≤ LD<sub>50</sub> doses of AZA and the control groups are given in Figure 4. As is evident in figure 4, unlike data obtained from other comet parameters, tail moment increased only at 1.5 and 2 µg/larvae after 24 hours post-AZA treatment with respect to control and other experimental groups (F = 20.268; df = 5, 2994; P = 0.000). At 72 hours post AZA treatment however, dose dependent increase in tail moment was observed (F = 46.892; df = 5, 2994; P = 0.000) with values of up to 1.85 ± 0.12 at 2 µg/larva (Figure 4).

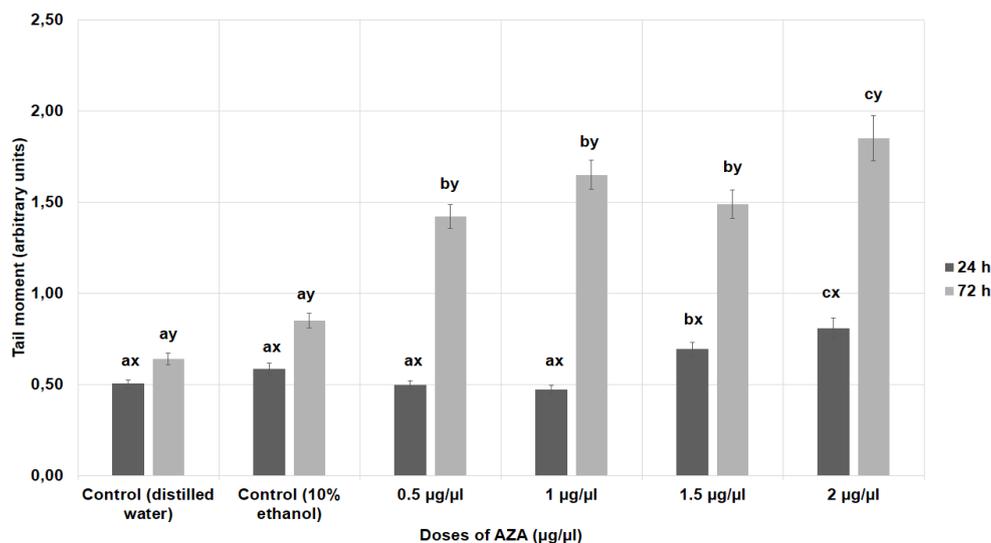


Figure 4 The tail moment ratio obtained from the hemocytes of *G. mellonella* larvae at 24 and 72 h.

\* All data represents as means  $\pm$  standard error. Each column indicated by the different letter (a-c, LSD test) or between black and grey column indicated by the different letter (x-y, t-test) are significant statistically ( $P < 0.05$ )

#### 4. Conclusions and discussion

All data obtained from this study revealed that sublethal doses of pure AZA caused genomic damage in the hemocytes of model insect *G. mellonella* larvae. In a previous study, it was argued that using the comet parameters allows determining DNA damage in eukaryotic cells for biomonitoring and detection of genotoxic chemicals or physical agents in terrestrial ecosystems (Zhang et al., 2000). Therefore, the genotoxic effects of pure AZA on the larval hemocyte determined using comet assay within the scope of the present work.

The most commonly used DNA damage indicators by comet assay are tail migration, tail intensity (DNA percentage in tail) and tail moment (Knopper, 2005). Knopper (2005) suggest that tail migration can be used to measure of the DNA damage level in regards with the ratio of DNA fragmentation, while tail intensity (% DNA) and moment can be used to determine the severity of DNA damage when exposed to a chemical agent. According to the results from the present study, AZA caused important damage in DNA of *G. mellonella* larvae as indicated by the increase in tail migration at subLD<sub>50</sub> doses at the two time points. These results also concur with those of Qari et al. (2017) who reported increased DNA damage in *Rhyzopertha dominica* exposed to LC<sub>50</sub> of the different plant volatile oils. These observed time and dose dependent changes in the DNA damage may be due to the inhibition of the DNA repair mechanism whose occurrence is dependent on AZA doses and exposure period. For this reason, it was observed that there is reduced DNA repair activity in larval hemocytes exposed to lower doses of AZA following 72 hours of exposure due to increased DNA fragmentation.

Results obtained from comet parameters showed that AZA application at 2 µg/larva to larvae caused severe DNA damage and this severity increased with exposure periods. Consistent with our results, a previous study about genotoxic effects of PONNEEM, a newly developed phytopesticide including karanjin and AZA, on midgut cells of the green worm *Helicoverpa armigera* (Lepidoptera: Noctuidae), reported dose dependent increases in tail moment, tail length and tail DNA (%). Authors also emphasized that PONNEEM at concentrations above 10 ppm caused genotoxicity on midgut cells because it contains active principles such as AZA and karanjin (Maria-Packiam et al., 2015). However, Muangphra and Gooneratne (2011) showed that LD<sub>50</sub> (3.79 and 3.33 µg cm<sup>-2</sup>) doses of commercial NEEM extract (containing AZA) had cytotoxic effects on the soil worm *Pheretima peguana* coelomocytes according to micronucleus analysis but no DNA damage according to the comet assay (Muangphra & Gooneratne, 2011). These differences between the studies suggest that the variations in the genotoxic potential of AZA on different organisms was depends on its formulation, concentrations and exposure time.

Genotoxicity of AZA on *G. mellonella* larvae could be explained by the induction of the autophagic or apoptotic pathways causing the cell death (Er et al., 2017) since AZA is known to reduce protein synthesis, inhibit cell division (Rembold & Annadurai, 1993), and induce apoptosis and autophagy in insect cells (Huang et al., 2011; Shu et al., 2015). Also, in previous studies, technical AZA has been reported to cause formational abnormality and oxidative stress by suppressing the antioxidant system (Dere et al., 2015), reduce cellular immunity, prolong adult emergence time and decrease number of eggs and productivity (Er et al., 2017) in the larvae of *G. mellonella*. Considering all results obtained from the present and earlier studies, it was concluded that increased DNA damage in hemocytes at 72 h post AZA application is associated with cytotoxicity arising from apoptosis. Moreover, several investigators have described apoptotic cells as large fan-like tails and small heads in comet assay due to extensive formation of double strand breaks in DNA during apoptosis (Fairbairn et al., 1996; Olive & Banáth, 2006). Similar cellular appearances were also observed

in comets at doses of 2 µg/larva AZA following a 72 h exposure of hemocytes of the *G. mellonella* larvae in this study. This indicates that the genotoxicity of AZA on hemocytes of *G. mellonella* larvae may be conducted by apoptotic pathways in cells. To prove our suggestions, more detailed investigations need to be carried out about the metabolic effects of pure AZA on cell death pathways.

In conclusion, the present study shows the time dependent genotoxic effects of AZA on insects and provides useful ecotoxicological information for safety data sheet of pure AZA and thus suggesting the use of AZA at low doses in IPM investigations as a phytopesticide for the control of *G. mellonella* and/or other Lepidopterous insect pests instead of harmful chemicals to protect the environment. And the results from the comet assay also demonstrated that hemocytes are useful in screening DNA damage caused by genotoxic xenobiotics.

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