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Assessment of cytotoxicity and mutagenicity of insecticide Demond EC25 in *Allium cepa* and Ames Test

Arzu Özkara

Department of Molecular Biology and Genetic, Faculty of Arts and Sciences, Afyon Kocatepe University, Afyonkarahisar/TURKEY
E-mail: ozkara@gmail.com

Abstract. The mutagenicity and cytotoxicity of Demond EC25, a synthetic pyrethroid insecticide, was assessed using two standard genotoxicity assays of the *Salmonella typhimurium* mutagenicity assay (Ames test) and *Allium cepa* test. Cytogenetic effects of Demond EC25 were evaluated in the root meristem cells of *Allium cepa*. The test concentrations of compounds were selected by determining EC_{50} of the *Allium* root growth and onion seeds were exposed to Demond EC25 (50, 100, and 200 ppm) for 24, 48, and 72 hours. The concentrations Demond EC25 was compared with the value for the negative control using Dunnet-t test, 2 sided. The results indicated that mitotic index was clearly decreased with increasing the concentration of Demond EC25 in each treatment group as compared to the controls. Demond EC25 was tested for mutagenicity in bacterial reversion assay systems with two strains (TA98 and TA100) of *Salmonella typhimurium* absence and presence of S9 fraction. The doses of Demond EC25 were 50, 100, 200, 400, 800 µg/plate and test materials were dissolved in DMSO. Our results show that Demond EC25 was found to be mutagenic in 800 and 400 µg/plate doses of TA98 in the without S9 mix and 800 µg/plate in the with S9 mix. In TA100, Demond EC25 was found to be mutagenic only 800 µg/plate doses without S9 mix. The other doses of this insecticide was not found to be mutagenic in both test strains.

Keywords. Allium test, Ames test, cytotoxicity, Demond EC25, mutagenicity, pesticide.

INDRODUCTION

Pyrethroids are among the most commonly used insecticides in agriculture; they are also widely used indoors in pet shampoo, lice treatment, and even insect repellent (Saillenfait et al. 2015). They are therefore frequently present in food, air and dust of dwellings and thus can lead to both dietary and non-dietary exposure (Morgan 2012). Pyrethroids are botanical insecticides which are synthetic derivatives of pyrethrins and have been used for many years. However, most of pyrethroids are defined as moderately hazardous (Class II) by the World Health Organization (WHO 2009) (Jensen et al. 2011). The residues of pyrethroids have been detected in fruits, vegetables,

tea, pasteurized milk and porcine muscle (Nakamuraet al. 1993). Wider use of pyrethroids posed a serious risk to environment and human. Therefore, it may be an urgent need to evaluate the possible adverse effects of their use (Miao et al. 2017)

Pyrethroid pesticides disrupt the nervous system of insects and, to a lesser degree, of mammals, and thus raise human health concerns. (Oulhote and Bouchard 2013; Viel et al. 2015). Pyrethroid residual insecticides exert their toxic effects by targeting the nervous system of insects. Pyrethroids interfere with sodium channels in nerve fiber membrane and organophosphates bind to inhibit the activity of AChE found in the synaptic junction. Both actions result in continued nerve signaling and over-stimulation of nerve cells. Poisoned insect exhibits tremors and convulsions, eventually leading to death (ATSDR 2003; Valles and Koehler 2003). It is essential to carefully study and analyze the hazards of pyrethroids on human health including their genotoxic and cytotoxic properties. Hereby, it can be take adequate measures to prevent humans from potential mutagenic and carcinogenic effects. (Nagy et al. 2014).

Deltamethrin is a synthetic pyrethroid insecticide, sold by Safa Tarım Limited with trade names Demond EC 25 in local market. To our knowledge, there is no study mutagenicity of Demond EC 25 except in the present paper. The aim of this experiment was to evaluate both the mutagenic and cytotoxic effects of different doses of Demond EC 25 by the bacterial reverse mutation assay in *S. typhimurium* TA98 and TA100 strains with or without S9 mix and *Allium cepa* test, respectively.

MATERIAL METHOD

Chemicals

The test substance Demond EC25 was purchased from a local market in Afyonkarahisar/Turkey and dissolved in sterille distilled water. *Allium cepa* onion bulbs, 25–30 mm diameter, were obtained from a local market without any treatments. The other chemicals were obtained from Merck and Riedel.

Test strains

The LT-2 TA98 and TA100 histidine demanding auxotrophs of *S. typhimurium* were kindly obtained from Prof. B.N. Ames (University of California, Berkeley). These strains were incubated for 16h in liquid nutrient broth and kept at -80°C. Their genetic markers and other properties, such as the numbers of spontaneous revertants and responses to positive controls, were controlled as described by Maron and Ames (1983).

Allium Test

EC_{50} determination and mitotic index analysis

The procedure of the root inhibition test as described by Fiskesjo (1985) was followed with some modifications. The *Allium* root inhibition test was carried out to determine suitable concentrations for the genotoxicity assay. The outer scales of the bulbs and the dry bottom plate were removed without destroying the root primordia. The onions were grown in freshly distilled water for the first 24h and afterwards exposed for 96h to the Demond EC25 solutions (12.5, 25, 50, 100, and 200 ppm, respectively). In order to determine the EC_{50} values, the roots from each bundle were cut off on the fifth day and the length of each root was measured from both the Demond EC25 exposed bulbs and the control group. The EC_{50} value was considered as the concentration which retards the growth of the root 50% less when compared to the control group.

The EC_{50} value for Demond EC25 was approximately 100 ppm. In order to demonstrate possible concentration-dependent effects of this pesticide, the root tips were treated with 50 ppm (EC_{50}/2), 100 ppm (EC_{50}), 200 ppm (EC_{50}x2) concentrations of Demond EC25, and all application groups were tested 24, 48, and 72h treatment periods. Additionally we also used positive control group by using methyl methanesulfonate (MMS). After the treatment, the roots were washed in distilled water and fixed in 3:1 ethanol: glacial acetic acid for 24h and then the roots were transferred into 70% alcohol and stored at +4°C. The root tip cells were stained with Feulgen and five slides were prepared for each test group.

Ames *Salmonella*/Microsome Assay

The mutagenicity of the Demond EC25 was determined using the standard plate incorporation assay. *Salmonella typhimurium* strains TA98 and TA100 were used with or without S9 mix in this test (Ames et al. 1975; Maron and Ames 1983). The tester strains were tested for the presence of the strain-specific markers as described by Maron and Ames (1983). The cytotoxic doses of the Demond EC25 (800, 400, 200, 100, 50 µg/plate) were determined by the method of Dean et al. (1985). The stock solutions of the test materials were dissolved in sterile distilled water and stored at 4°C. The *S. typhimurium* strains were incubated in nutrient broth at 37°C for 16h with shaking. The positive controls were

4-nitro-o-phenylenediamine (NPD) for the TA 98 and sodium azide (SA) for the TA100, used without metabolic activation, and 2-aminofluorene (AF) for TA 98 and 2-aminoanthracene (2AA) for the TA 100 used with metabolic activation.

The test plates for the assays without the S9 mix were prepared by adding 0.1 ml of the test suspension for each concentration, 0.1 ml bacterial suspension from an overnight culture, and 0.5 ml phosphate buffer to 2 ml top agar (kept in 45°C water bath). The mixture was shaken for 3 s using a vortex mixer and then poured into the minimal agar. The test plates with the S9 mix were prepared by adding 0.5 ml of S9 mix instead of the phosphate buffer. All the test plates were incubated for 72h at 37°C, and then the revertant colonies on each plate were counted. The experiments were run in triplicate for each concentration and all the results from the two independent parallel experiments were used for the statistical analysis.

Statistical analysis

The data obtained for the root length, MI, and mitotic phases were expressed as percentages. The levels of difference in the treatment groups were analyzed statistically by using the SPSS 15.0 version for Windows. In the analyses, the Dunnett-t test (2 sided) was performed on both the *Allium* and Ames tests.

Table 1. Allium root growth inhibition test.

Test Substance	Concentrations (ppm)	Mean of root length±SD
Negative Control	-	3.57±0.24
Positive Control	-	1.03±0.15*
Demond EC25	12.5	3.12±0.42*
	25	2.02±0.15*
	50	1.68±0.41*
	100	1.45±0.23*
	200	1.12±0.22*

*Significantly different from negative control (p<0.05 Dunnet-t test, 2-sided), SD: Standart deviation.

RESULTS

Allium root growth test results are summarized in Table 1 and Table 2 gives the effect of Demond EC25 on MI and mitotic phase in the root meristematic cells of *A. cepa* treated for 24, 48 and 72h. The effective concentration (EC_{50}) was determined as 100 ppm in *Allium* test. At all concentrations treated in the incubations of root decreased MI compared to negative control at all exposure time. The reduced of MI results (p<0.05) were found statistically significant with all concentrations and all treatment time. All doses of Demond EC25 applied in the experiment caused changes in the percentage of particular phases' distribution in comparison to the control.

Table 2. The effects of Demond EC25 on MI and mitotic phases in the root cells of *A. cepa.*

Concentration (ppm)	Treatment Time	Counted Cell Number	Mitotic Index ± SD	Mitotic Phases (%) ± SD			
				Prophase	Metaphase	Anaphase	Telophase
Negative control	24 hour	4965	82.45±6.71	79.12±9.42	1.80±0.32	1.12±0.32	0.92±0.57
Positive control		5001	68.78±5.46*	34.24±4.62*	0.48±0.70*	0.49±0.54*	0.52±0.81
50		4889	51.48±4.09*	34.05±2.17*	1.00±0.72*	0.79±0.21*	1.10±0.62
100		4963	46.25±4.74*	32.54±4.20*	0.94±0.42*	0.68±0.34*	1.03±0.42
200		5013	45.21±2.69*	30.21±2.54*	0.82±0.40*	0.52±0.21*	0.59±0.74
Negative control	48 hour	5007	67.28±3.47	70.11±6.74	1.62±0.21	1.27±0.24	1.21±0.26
Positive control		4997	63.11±3.14*	31.75±3.45*	0.45±0.31*	0.52±0.16*	0.65±0.13
50		5101	52.25±3.45*	30.26±3.35*	0.71±0.92*	0.62±0.21*	1.19±0.21
100		5051	42.42±3.65*	28.45±2.70*	0.68±0.40*	0.54±0.02*	1.06±0.32
200		5113	36.20±1.25*	24.45±2.92*	0.52±0.32*	0.45±0.18*	0.89±0.14
Negative control	72 hour	5142	38.21±2.65*	57.52±3.41	1.43±0.41	1.21±0.21	1.09+±0.52
Positive control		5123	26.36±3.02*	29.04±2.28*	0.31±0.43*	0.60±0.42*	0.68±0.32
50		5263	19.23±1.75*	25.45±3.85*	0.58±0.23*	0.52±0.45*	0.49±0.41
100		5047	14.12±2.42*	21.42±2.56*	0.49±0.41*	0.46±0.71*	0.50±0.72
200		4985	13.21±2.21*	16.47±2.31*	0.34±0.42*	0.39±0.43*	0.56±0.61

* Significantly different from negative control (p< 0.05 Dunnet-t test. 2-sided) SD: Standart deviation.

Table 3.The mutagenicity assay results of Demond EC25 for *S. tyhimurium* TA98 and TA100 strains

Test Substance	Concentration (µg/plate)	No of His+ revertants/plate, mean±SD			
		TA98		TA100	
		- S9	+ S9	- S9	+ S9
Demond EC25	800	95.32±5.41*	116.42±5.52*	206.45±9.44*	215.52±12.85
	400	88.04±4.13*	102.21±3.96	178.42±7.45	203.12±10.25
	200	68.12±4.63	92.54±4.25	142.45±6.74	184.32±9.54
	100	52.09±3.86	78.09±4.52	121.22±6.61	168.35±8.65
	50	47.31±3.38	56.24±4.45	102.10±5.08	123.09±6.85
Neg. Control	100	36.07±3.36	49.14±3.70	90.10±13.42	114.23±7.38
SA	10			2965.56±56.35*	
2AA	5				2628.42±60.41*
2AF	200		1002.40±16.65*		
NPD	200	1575.50±24.56*			

*Mean statistically significant at p<0.05 (Dunnett t-test), SA:Sodium azide, NPD: 4-nitro-o-phenylendiamine, 2AF: 2-aminofluorene, 2AA: 2-aminoanthracene, SD: Standard deviation, Negative control: distilled water.

The results of the Ames test are shown in Table 3. In this experiment, first, the cytotoxic doses of Demond EC25 were determined. As seen in Table 3, spontaneous revertants were within the normal values in all the strains examined. All of the doses with and without S9 mix in TA98 and TA100 slightly increased when compared to the negative control. On the other hand, the plates containing positive control mutagens displayed very significant increases in the spontaneous mutation rate in two strains tested. Most of the results, whether increasing or decreasing relative to the negative control group, were not statistically significant at P<0.05 (Dunnett-t test, 2 sided) in the examined strains, except for in the 800 and 400 µg/plate doses of the Demond EC25 in the TA98 without S9 mix and 800 µg/plate doses with S9 mix. Additionally it was obtained mutagenic in the TA100 without S9 mix 800 µg/plate doses.

DISCUSSION

Pyrethroid insecticides are commonly used in agriculture, veterinary medicine, and to control insect pests in human dwellings because of their high selective toxicity for insects and relatively low acute toxicity to mammals (Casida and Quistad 1998). These insecticides are favored because of their effective role and have replaced organophosphorus pesticides in many areas of applications (Ministry of the Environment in Japanese 2011).

Because of their advantages, pyrethroid insecticides including Demond EC25 are becoming widespread and, therefore, studies on the biological effects of these pesticides are of immediate concern. Numerous studies on their toxicity, both in insects and mammals, have been reported in the literature. Although pyrethroid insecticides have consistently shown negative results in microbial genotoxicity tests, the outcome of other assays has been variable and it has not been possible to draw definite conclusions about the genotoxicity of this group of pesticides (Grossman 2007; Surralles et al. 1995).

In determining mutagenicity of chemicals, the Ames test has shown a variety of chemicals to be either mutagenic or anti-mutagenic, and has been shown to be over 90% accurate in predicting genotoxicity (Weisburger 2001). In the Ames test, *S. typhimurium* strains that have a mutation in the *his*-operon are used to detect the mutagenicity of chemicals (Maron and Ames 1983). In the present study, Demond EC25 was studied for its mutagenic activity with the Ames test and results can be concluded that Demond EC25 induced mutations in the 800 and 400 µg/plate doses of the TA98 without S9 mix and 800 µg/plate doses with S9 mix and in the TA100 without S9 mix.

Under our experimental conditions, Demond EC25 showed to produce point mutations in the Ames test, both in the absence and presence of the S9 metabolic activation system in high concentrations of both test strains. In order to characterize the possible mechanism of mutagenicity, the important bacterial strains, sensitive to different mutational events due to their specific genotypes, were used.

Particularly, *S. typhimurium* TA98 is characterized by the -1 frameshift deletion hisD3052, which affects the reading frame of a nearby repetitive –C–G– sequence

and can be reverted by frameshift mutagens. TA100 contains the marker hisG46, which results from a base-pair substitution of a leucine (GAG/CTC) by a proline (GGG/CCC): this mutation is reverted by mutagens causing base substitutions at G-C base pairs (Di Sotto et al. 2008). Taking into account these bacterial features, our results highlighted that the Demond EC25 mutagenicity, in the absence and presence of S9 in TA98, was likely due to frameshift mutations, and in the absence S9 in TA100 due to base-change mechanisms.

The data reported on the genotoxicity of synthetic pyrethroids are rather controversial, depending on the genetic system used (Akintonwa et al. 2008; Saleem et al. 2014). Studies have shown an important relationship between a substance's chemical structure and its biological activity (Oztas¸ 2005) chlor. Several factors, including rings, the functional groups, and the positions of binding locations in the chemical structure may affect a chemical's binding ability.

Mitotic index proved to be a useful parameter that allows one to detect the frequency of the cellular division (Marcano et al. 2004). The estimation of the potential cytotoxicity of the compounds is generally related to the inhibition of the mitotic activities (Smaka-Kincl et al. 1996). In this study, the used concentrations of Demond EC25 also caused significant inhibition of the mitotic index. The significant decline in the mitotic index could be due to the inhibition of the DNA synthesis or the blocking of the G1 suppressing the DNA synthesis or effecting the test compound at the G2 phase of the cell cycle (Sudhakar et al. 2001; Majewska et al. 2003). When a pesticide penetrates the cells and reaches a critical concentration, it could be in an active form, causing lesions during several following cellular cycles (Marcano et al. 2004). The decrease of the mitotic index in our study can be related to this.

In this study, all the concentrations of Demond EC25 caused the changes in the percentage of the particular phases' distribution when compared to the control group. Pesticides accumulate in the cell due to this substance not being able to emerge out of the cell easily after once penetrating the cell and it may be highly toxic in the cell (Antunes-Madeira and Madeira 1979).

Deltamethrin, the active ingredient in Demond EC25 has immunosuppressive (Lukowicz and Krechniak, 1992), reproductive effects on sperm cells (Bhunya and Pati 1990; Carrera et al. 1996) and developmental toxicity (Martin 1990). Deltamethrin is reported to cause chromosomal damage in *Allium cepa* (Chauhan et al. 1986), chromosomal aberrations and micronucleus formation in bone marrow cells of mice exposed *in vivo* (Chauhan et al. 1997; Gandhi et al. 1995). Saxena et al. (2009) evaluated of cytogenetic effects of deltamethrin in root meristem cells of *Allium sativum* and *Allium cepa* and cells analyzed immediately after the exposure showed a significant, concentration-dependent inhibition of mitotic index (MI) and induction of mitotic and chromosomal aberrations in both the test systems. Additionally, i*n vitro* exposure of Deltamethrin is reported to cause DNA damage in Comet assay in human peripheral blood leukocytes (Villarini et al. 1998). In the present study with *Allium cepa* root tip meristem cells however, the three concentrations of Demond EC25 tested induced genotoxicity thus corroborating the findings of these studies.

In contrast to our results, no genotoxic response of Deltamethrin was observed in *Salmonella typhimurium* and V79 Chinese hamster ovary cells (Pluijmen et al. 1984). Data on the genotoxicity and carcinogenicity of Deltamethrin are rather controversial, depending on the genetic system or the assay used (Shukla and Taneja, 2000).

The safety evaluation of a fragrance material includes a broad range of toxicological information, both for the compound itself and for structurally related chemicals belonging to the same chemical group (Bickers et al., 2003). Among toxicological information, genotoxicity is a systemic consideration, as it can be related to carcinogenicity (Di Sotto et al. 2008). Normally, to evaluate a potential genotoxic risk due to a chemical exposition, *in vitro* assays for detecting point mutations (Ames test) and extended treatment (e.g., micronucleus assay, *Allium* test, single cell gel electrophoresis assay or comet assay) are used in the first instance (EMEA 2008; Di Sotto et al. 2013). If the results of these studies are positive, *in vivo* studies, for example a mammalian cytogenetic study, are performed (EFSA 2014).

The tested substances with different test systems can be genotoxic or not genotoxic depending on a number of factors such as chemical structure and biological activity, having rings in the structure and the positions of the binding location (Kutlu et al. 2011). In addition to these, it might be related to differences in test conditions, such as exposure time, cell types, concentrations of substances, the dispersal of the materials and physicochemical characteristics of the compounds (Ema et al. 2012).Therefore, it could be explained why some studies find an increase of genetic damage while in others result as negative.

In conclusion, Demond EC25 was found to be cytotoxic due to decreasing of MI in Allium test and showed mutagenic activity at some doses in the Ames test. Demond EC25 had clear cytotoxic effects and may pose a genotoxic risk for humans. For this reason, further

investigations are needed to determine the toxicity of this compound using other *in vivo and in vitro* biological test systems. A single test system is not enough to determinate a compound whether it is toxic or non-toxic. In this study we performed two different test methods. Further investigations are needed to determine the toxicity of this compound using multiple test systems.

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