Ekoloji 23, 92, 9-18 (2014)
doi: 10.5053/ekoloji.2014.922

In vivo Genotoxicity of Four Synthetic Pyrethroids with Combinations of Piperonyl Butoxide (PBO) Using the *Drosophila* SMART Assay

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Abstract
In this study, the genotoxic effects of four synthetic pyrethroids (cypermethrin, cyphenothrin, deltamethrin, and permethrin) alone and their combinations with different rates of piperonyl butoxide (PBO) were studied using the wing somatic mutation and recombination test (SMART) of the *Drosophila melanogaster*. In the first stage, lethal concentration values (LC_{25} or LC_{50}) of the synthetic pyrethroids and concentrations of PBO used for the synthetic pyrethroids were determined. Then, *Drosophila* larvae were exposed to lethal concentrations of synthetic pyrethroids and combinations with different rates of PBO (1:0.25, 1:0.5, 1:0.75, 1:1, and 1:2). According to the obtained results, alone and with the PBO of the mixtures of the four synthetic pyrethroids are not genotoxic when compared with the negative control. In addition, the PBO when used alone demonstrated negative results when exposed to 1, 5, and 25 ppm concentrations, while demonstrating positive result when exposed to 50 ppm concentration. However, the PBO did not show any co-genotoxic activity with the four tested synthetic pyrethroids. Results of this study will take an important place in human and environmental health with the new results for the PBO ratios in insecticide formulations.
Keywords: *Drosophila melanogaster*, SMART, piperonyl butoxide (PBO), and synthetic pyrethroids.

***Drosophila* SMART Testinde Piperonil butoksid (PBO) Kombinasyonları ile Dört Sentetik Piretroitin In vivo Genotoksisitesi**

Özet
Bu çalışmada, *Drosophila melanogaster* kanat somatik mutasyon ve rekombinasyon testi (SMART) kullanılarak dört sentetik piretroitin (sipermetrin, sifenotrin, deltametrin, permetrin) tek ve onların piperonil butoksid (PBO)'in farklı oranları ile kombinasyonlarının genotoksik etkileri çalışıldı. Birinci aşamada, sentetik piretroitler için kullanılan PBO konsantrasyonları ve sentetik piretroitlerin letal konsantrasyon değerleri (LC_{25} veya LC_{50}) belirlendi. Daha sonra, sentetik piretroitlerin letal konsantrasyonları ve PBO'nun farklı oranları (1:0.25, 1:0.5, 1:0.75, 1:1 ve 1:2) ile kombinasyonları *Drosophila* larvalarna maruz bırakıldı. Elde edilen sonuçlara göre, dört sentetik piretroit karışımı negatif kontrol ile karşılaştırıldığında tek başlarına ve PBO ile birlikte uygulandığında genotoksik değillerdir. Buna ek olarak, PBO'nun tek başına kullanıldığı 1, 5 ve 25 ppm konsantrasyonları negatif sonuçlar gösterirken 50 ppm konsantrasyonu pozitif sonuç göstermiştir. Fakat PBO, test edilen dört sentetik piretroit ile birlikte herhangi bir genotoksik aktivite göstermemiştir. Bu çalışmanın sonuçlarının insektisit formulasyonlarında kullanılan PBO oranlarının insan ve çevre sağlığı açısından yeni sonuçları ile literatürde önemli bir yer alacağı düşünülmektedir.
Anahtar Kelimeler: *Drosophila melanogaster*, SMART, piperonil butoksid (PBO), sentetik piretroit.

Demir E, Kaya B, Kocaoglu Cenkci S, Cetin H, Marcos R (2014) In vivo Genotoxicity of Four Synthetic Pyrethroids with Combinations of Piperonyl Butoxide (PBO) Using the *Drosophila* SMART Assay . Ekoloji 23(92): 9-18.

INTRODUCTION

Synthetic pyrethroids, an important class of insecticides, are derivatives from the plant Chrysanthemum, which is a genus in the family Asteraceae, native to North Eastern Europe and Asia. These pyrethroids are widely used as

Received: 15.10.2012 / Accepted: 20.02.2014

insecticide for a variety of crops in agriculture, ectoparasitic diseases, and disinfection (Heudorf and Angerer 2001, Fishel 2005). There are classified as synthetic pyrethroids referred to as pyrethroids I and pyrethroids II. Type I pyrethroids induced prostration and whole body tremor while type II pyrethroids induce writhing, dermal tingling, and salivation (Wolansky and Harrill 2008). Also, many pyrethroids during acute poisoning and chronic exposure act as endocrine disruptors (Perry et al. 2007). Some pyrethroids have elevated the estrogenic activity and the increased estrogen level has been implicated in the development of human malignant breast tumors (Kasat et al. 2002).

A number of previous studies reported that many synthetic pyrethroids were a genotoxic potential (Carbonell et al. 1989, Puig et al. 1989, Surralles et al. 1990, Barrueco et al. 1992, 1994, Çelik et al. 2003), cause cellular damage (Mukhopadhyay et al. 2006), and impair some developmental stages of the *Drosophila melanogaster* (Karataş and Bahçeci 2009). On the contrary, some pyrethroids did not demonstrate any genotoxicity (Pluijmen et al. 1984, Herrera and Laborda 1988). Pyrethroids act as neurotoxins on the sodium channels in the central neurons of insects and human nervous system (Narahashi et al. 1998, Vais et al. 2001). There have been related symptoms found in humans when induced or exposed to them. The most common symptoms are headache, fatigue, nausea, convulsions, coma, anorexia, and muscular fasciculation (He et al. 1989). Also, synthetic pyrethroids can cause contact dermatitis and asthma-like reactions (Penagos et al. 2001). Epidemiological data and investigations in rodents have shown that pyrethroids undergo metabolism by carboxylesterases and cytochrome P450 enzyme systems (Anand et al. 2006, Godin et al. 2006, Ross et al. 2006, Crow et al. 2007, Godin et al. 2007). Hydrolysis of pyrethroids is generally considered a detoxification process (Casida et al. 1983, Cantalamessa 1993).

Piperonyl butoxide (PBO) is often used as an organic insecticide synergist with synthetic pyrethroids that inhibits the mixed-function microsomal monooxygenases system, thereby slowing the oxidative break down of synthetic pyrethroids (Kumar et al. 2002). According to carcinogenicity evidence, PBO has been classified by the U.S. Environmental Protection Agency (Anonymous 1995) as a possible human carcinogen. However, PBO has shown negative results in genotoxicity studies (Beamand et al. 1996).

In the present study, we used the *Drosophila* wing somatic mutation and recombination test (SMART) as described by Graf et al. (1984). The SMART assay in *D. melanogaster* has been designed to detect the genotoxic damages in a rapid and inexpensive way in one generation. The importance of the SMART assay is in the in vivo system and the metabolic machinery of *Drosophila* cells which are similar to mammalian cells (Vogel 1987). Moreover, >60% of genes associated with human disease have *Drosophila orthologues* (Bernards and Hariharan 2001). The aim of this study was to evaluate, for the first time with this assay, the genotoxic activity of the lethal concentration values of synthetic pyrethroids (cypermethrin, cyphenothrin, deltamethrin, and permethrin) and PBO. In addition, the possible genotoxic effects of the four synthetic pyrethroids combinations with PBO were also evaluated. Since these synthetic pyrethroids and PBO are widely used, there is a need for more data on the genotoxicity in order to assess their potential hazards to human and environmental health.

MATERIAL AND METHODS

Chemicals

Cypermethrin (92% purity; CAS No. 52315-07-8), cyphenothrin (96% purity; CAS No. 39515-40-7), deltamethrin (98% purity; CAS No. 52918-63-5), permethrin (95% purity; CAS No. 52645-53-1), ethyl alcohol (≥99.5% purity; CAS No. 64-17-5), acetone (≥99.5% purity; CAS No. 67-64-1), and ethyl methanesulfonate (EMS, 99% purity; CAS No. 62-50-0) were obtained from Sigma-Aldrich. Prior to use, the synthetic pyrethroids (cypermethrin, cyphenothrin, deltamethrin, and permethrin) were dissolved in 3% acetone while the PBO (Polisan A.Ş. Turkey) was dissolved in 0.5% ethyl alcohol. Different concentrations of PBO (1, 5, 25, and 50 ppm) were determined based on preliminary studies.

Drosophila Strains

Two *D. melanogaster* strains were used: the multiple wing hairs strain with genetic constitution of mwh/mwh and flare-3 strain with the genetic constitution of flr^3/In (3LR) TM3, Bd^s. More detailed information on all the genetic markers is given by Lindsley and Zimm, 1992.

Experimental Procedure

The Somatic mutation and recombination test (SMART) on the wings of *D. melanogaster* is based on the loss of heterozygosity (LOH) for the two recessive markers mwh (3-0.3) and flr (3-38.8) (Graf et al. 1984). This test is able to detect a wide spectrum of alterations, including the point mutations, deletions, mitotic recombination, chromosomal loss, and non-disjunction (Würgler and Vogel 1986). The trans-heterozygous larvae were obtained by parental crosses between flare-3 virgin females and mwh males and that only the trans-heterozygous individuals were analysed. The eggs were collected from this cross in 8-h periods. The third instar larvae were floated off with tap water then transferred into plastic vials containing 4.5g dry *Drosophila* Instant Medium (Carolina Biological Supply Company Burlington, NC, USA) re-hydrated with 9 ml of the freshly prepared respective test solutions (PBO, synthetic pyrethroids and synthetic pyrethroids + PBO and distilled water, and 0.5% ethyl alcohol or 3% acetone for the negative controls). The larvae were fed with different concentrations of the test compound. Feeding ended with pupation of the surviving larvae. All experiments were performed at 25±1°C and 70% relative humidity. Concentrations of the PBO were: 1, 5, 25, and 50 ppm. For the determination of lethal concentration values (LC_{25} or LC_{50}) (Cetin et al. 2010), the three-day-old (72±4 h) trans-heterozygous larvae were exposed to eleven different concentrations (0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50, 100, 150, and 200 ppm) of the synthetic pyrethroids. The number of emerging flies was recorded 10 days after treatment and the larvicidal activity was recorded as the percentage of used larvae that were unable to develop into adults. Twenty-five larvae and four replicates were used for each treatment. The experiment was repeated three times on subsequent days. The lethal concentration values (LC_{50}) of cyphenothrin, deltamethrin, and permethrin in combinations with PBO resulted in the death of all the flies. Therefore, the synthetic pyrethroids used the LC_{25} combinations with the PBO. The LC_{25} or LC_{50} of the synthetic pyrethroids are as follows: LC_{50}=4 ppm for cypermethrin, LC_{25}=15 ppm for cyphenothrin, LC_{25}=7.5 ppm for deltamethrin, and LC_{25}=22.5 ppm for permethrin. The distilled water, ethyl alcohol (0.5%), and acetone (3%) served as a negative control and 1 mM EMS was used as a positive control.

Preparation and Microscopic Analysis of the Wings

After metamorphosis, the trans-heterozygous flies were collected, and then stored in a 70% ethanol solution at +4°C. Afterwards, the wings, using a Nikon SMZ645 model stereo microscope, were removed and mounted on slides in Faure's solution (30g of Gum arabic, 30 mL of Glycerol, 50g Chloral hydrate, and 50 mL Distilled water). Next they were scored using a Nikon YS100 model light microscope at 400 X magnification for the presence of clone cells showing malformed wing hairs. Wings were scored for (1) small (1-2 cells) single spots, (2) large (>2 cells) single spots, both with mwh or flr^3 phenotype, and (3) twin spots (phenotypes mwh and flr3 in adjacent clone) (Graf et al. 1984). While single spots originate from recombination, non-disjunction, deletion, and point mutation, twin spots originate from recombination.

Statistical Analysis

The data was evaluated according to the multiple-decision procedure of Frei and Würgler (1988, 1995). These methods tests two alternative hypotheses: (1) the mutation frequency in the treated group is no higher than the mutation frequency in the control group; and (2) the frequency in the treated group is no less than m times as high as the observed spontaneous mutation frequency in the control group. For the statistical calculations, the conditional binomial test according to Kastenbaum and Bowman (1970) was used at 5% significance levels. The statistical approach used was ANOVA followed by the StatPlus computer probit analysis program according to Finney (1971), which was used to evaluate the lethal concentration rates (LC_{50} or LC_{25}). On the other hand, the rates of the viability (%) of the flies was determined following the Duncan Multiple Range Test (DMRT) ($p<0.05$) with SPSS 10.0.

RESULTS

The results obtained after the treatment with synthetic pyrethroids (cypermethrin, cyphenothrin, deltamethrin, and permethrin), PBO, and synthetic pyrethroids + PBO combinations in the *Drosophila* wing spot test are shown in Tables 1-6. Our previous studies showed that the mixture of pyrethroids and PBO caused an increase in the mortality and knock down values and decreased knock down times more than the pyrethroids alone, on the *D. melanogaster* in

a dose dependent manner up to a certain dosage (Cetin et al. 2010). EMS (1 mM), which was used as a positive control for the *Drosophila* wing somatic mutation and recombination test (SMART), induced all kinds of spots. The data demonstrated the strong mutagenic and recombinogenic activity of EMS (Graf et al. 1984).

In the first group, at 72 ± 4 h, the *Drosophila* larvae were exposed to lethal concentration values of the four synthetic pyrethroids. For the tested four synthetic pyrethroids the results obtained from the trans-heterozygous wings (mwh/flr^3) demonstrated either negative or inconclusive results for all categories when compared with the 3% acetone used as the control (Table 1). In other words, the lethal concentration values of synthetic pyrethroids are not an indication of genotoxicity in *D. melanogaster*.

In the second group, 72 ± 4 h, the *D. melanogaster* larvae were exposed to four different concentrations of PBO. The tested PBO demonstrated inconclusive results for all categories in 1, 5, and 25 ppm exposure concentrations while demonstrated clearly positive results when exposed to 50 ppm concentration for small single spots, total mwh spots, and total spots (Table 2). The highest concentration of PBO showed genotoxic effects in the *Drosophila* (Table 2). However, the concentration of PBO used in our study, even at the rate of positive results observed, the concentration of PBO is lower than 50 ppm.

In the third group, 72 ± 4 h, the *Drosophila* larvae were exposed to the combinations of cypermethrin and PBO. The combination at the rate of 1:2 caused death for all flies. Therefore, this combination rate could not be evaluated. According to our results, the combinations demonstrated either negative or inconclusive results for all categories when compared with cypermethrin used alone as the control (Table 3). In this experiment, the combination of cypermethrin with PBO was not genotoxic. In addition, the rates of viability observed were from 2 to 54% for the larvae (Table 3).

In the fourth group, 72 ± 4 h, the *D. melanogaster* larvae were exposed to lethal cyphenothrin and PBO combinations. In *Drosophila* SMART assay, the tested combinations demonstrated either negative or inconclusive results for all categories obtained from the trans-heterozygous wings (mwh/flr3). In other words, the combinations of cyphenothrin with PBO did not show genotoxic activity in *Drosophila*. Nevertheless, it showed an increase of clone frequencies results for the two highest concentrations of PBO (1:1 and 1:2) when compared with the control. As well as, the rates of viability observed were from 4 to 20% for the larvae (Table 4).

In the fifth group, 72 ± 4 h, the *D. melanogaster* larvae were exposed to lethal deltamethrin combinations with PBO . According to the results obtained, all treatments were not genotoxic in *Drosophila*. On the other hand, looking at the results of the rates of viability obtained, they were from 4 to 20% for the larvae (Table 5).

In the last group for the tested permethrin combinations with PBO the data demonstrated inconclusive results for only twin spots while demonstrated clearly negative results for exposure to all concentrations for small single spots, large single spots, total mwh spots, and total spots. The rates of viability observed were from 60 to 86% for the larvae (Table 6). The combination of Permethrin and PBO was not genotoxic as in the other three synthetic pyrethroids.

DISCUSSION

In our study, no genotoxic effects of the four synthetic pyrethroids alone and with PBO were detected in the *D. melanogaster* SMART assay. As well as, the four synthetic pyrethroids in combinations with PBO did not induce genotoxicity in *Drosophila*. However, previous studies on the genotoxicity and insecticidal activity of our tested synthetic pyrethroids (cypermethrin, cyphenothrin, deltamethrin, and permethrin) and PBO products have shown quite different results, depending on the genetic system, on the compound or the assay used (Wickham 1998, Osaba et al. 1999, Patel et al. 2006).

PBO is a cytotoxic found in the Chinese hamster ovary cells (Tayama 1996), mutagenic in cultured human RSa cells (Suzuki and Suzuki 1995), and genotoxic in mucosal epithelial cells from human tonsil tissue (Tisch et al. 2007). PBO also increased organ weights, biochemical, hematologic, and histopathologic changes with reduced food consumption and body weights (Yavuz et al. 2010). Some negative results were found for PBO and its genotoxic activity assayed with different organisms. PBO showed negative results in the Salmonella/microsome mutagenicity test (Ames)

Table 1. Genotoxic effects of the lethal concentration values of Cypermethrin, Cyphenothrin, Deltamethrin, and Permethrin. Results obtained with the mwh/flr3 of the wings.

Test Compounds and Conc. (ppm)	Number of wings (N)	Small single spots (1-2 cells) (*m*=2)			Large single spots (> 2 cells)(*m*=5)			Twin spots (*m*=5)			Total *mwh* spots (*m*=2)			Total spots (*m*=2)			Frequency of clone formation per 10^5 cells
		No	Fr	D	No	Fr	D	No	Fr	D	No	Fr	D	No	Fr	D	
Distilled water	80	20	(0.25)		1	(0.01)		0	(0,00		21	(0.26)		21	(0.26)		1.08
1 mM EMS	80	102	(1.28)	+	27	(0.34)	+	7	(0.09)	+	132	(1.65)	+	136	(1.70)	+	6.76
3% Acetone	80	30	(0.38)	i	3	(0.04)	i	1	(0.01)	i	34	(0.43)	i	34	(0.43)	i	1.74
Cypermethrin LC_{50}=4	80	15	(0.19)	-	6	(0.08)	i	1	(0.01)	i	22	(0.28)	-	22	80.28)	-	1.13
Cyphenothrin LC_{25}=15	80	30	(0.38)	i	1	(0.01)	-	0	(0.00)	i	31	(0.39)	-	31	(0.39)	-	1.58
Deltamethrin LC_{25}=7.5	80	37	(0.46)	i	2	(0.03)	-	4	(0.05)	i	43	(0.54)	i	43	(0.54)	i	2.20
Permethrin LC_{25}=22.5	80	42	(0.53)	i	5	(0.06)	i	0	(0.00)	i	46	(0.58)	i	47	(0.59)	i	2.15

No: number, Fr: frequency, D: statistical diagnosis according to Frei and Würgler (1988, 1995), +: positive, -: negative, i: inconclusive, m: multiplication factor, probability levels $\alpha = \beta = 0.05$.

Table 2. The Genotoxicity of the Piperonyl butoxide (PBO) in the Drosophila wing spot test. The results obtained with the mwh/flr3 of the wings.

Test Compounds and Conc. (ppm)	Number of wings (N)	Small single spots (1-2 cells) (*m*=2)			Large single spots (> 2 cells) (*m*=5)			Twin spots (*m*=5)			Total *mwh* spots (*m*=2)			Total spots (*m*=2)			Frequency of clone formation per 10^5 cells
		No	Fr	D	No	Fr	D	No	Fr	D	No	Fr	D	No	Fr	D	
Distilled water	80	20	(0.25)		1	(0.01)		0	(0.00)		21	(0.26)		21	(0.26)		1.08
Ethanol (0.5%)	80	22	(0.28)	-	2	(0.03)	-	0	(0.00)	i	24	(0.30)	-	24	(0.30)	-	1.23
1	80	23	(0.29)	i	2	(0.03)	i	0	(0.00)	i	25	(0.31)	i	25	0.31)	i	1.28
5	80	25	(0.31)	i	1	(0.01)	i	0	(0.00)	i	26	(0.33)	i	26	0.33)	i	1.33
25	80	32	(0.40)	i	4	(0.05)	i	0	(0.00)	i	36	(0.45)	i	36	0.45)	i	1.84
50	80	47	(0.59)	+	4	(0.05)	i	1	(0.01)	i	51	(0.64)	+	52	0.65)	+	2.61

No: number, Fr: frequency, D: statistical diagnosis according to Frei and Würgler (1988, 1995), +: positive, -: negative, i: inconclusive, m: multiplication factor, probability levels $\alpha = \beta = 0.05$.

Table 3. Wing spot test data obtained after treatment with lethal concentration value of Cypermethrin in combination with Piperonyl Butoxide (PBO). Results obtained with the mwh/flr3 of the wings.

Test Compounds and Conc. (ppm)	Number of wings (N)	Small single spots (1-2 cells) (*m*=2)			Large single spots (> 2 cells) (*m*=5)			Twin spots (*m*=5)			Total *mwh* spots (*m*=2)			Total spots (*m*=2)			Frequency of clone formation per 10^5 cells	Rates of viability (%) *
		No	Fr	D	No	Fr	D	No	Fr	D	No	Fr	D	No	Fr	D		
Cypermethrin LC_{50}=4 ppm	80	15	(0.19)		6	(0.08)		1	(0.01)		22	(0.28)		22	(0.28)		1.13	
Cypermethrin: PBO values																		
1: 0.25	80	10	(0.13)	-	3	(0.04)	-	0	(0.00)	i	13	(0.16)	-	13	(0.16)	-	0.67	52
1: 0.50	80	13	(0.16)	-	1	(0.01)	-	0	(0.00)	i	13	(0.16)	-	14	(0.18)	-	0.67	54
1: 0.75	80	17	(0.21)	i	1	(0.01)	-	1	(0.01)	i	19	(0.24)	-	19	(0.24)	-	0.97	3
1: 1	80	13	(0.16)	-	1	(0.01)	-	0	(0.00)	i	14	(0.18)	-	14	(0.18)	-	0.72	2

No: number, Fr: frequency, D: statistical diagnosis according to Frei and Würgler (1988, 1995), +: positive, -: negative, i: inconclusive, m: multiplication factor, probability levels $\alpha = \beta = 0.05$. * Calculated using these results with Duncan Multiple Range Test (DMRT) ($p<0.05$).

using Salmonella typhimurium strains TA98, TA100, TA1535, TA1537, and TA1538, the chromosomal aberrations in Chinese hamster ovary cells, and the unscheduled DNA synthesis (UDS) assay in rat liver primary cell cultures (Butler et al. 1996). In addition, Osaba et al. (1999) found negative result for PBO with the high bioactivation cross and the standard cross in *D. melanogaster*. Similarly, they were obtained as non-genotoxic in the *Drosophila* wing spot test (Tripathy et al. 1990). In our study, no genotoxic effects of the PBO were detected in the *D. melanogaster*. PBO demonstrated inconclusive results for all categories in 1, 5, and 25 ppm exposure concentrations while demonstrated clearly positive results exposure to 50 ppm concentrations for small single spots, total mwh spots, and total spots. In other words, a 50 ppm concentration of PBO showed genotoxic effects in

Table 4. Wing spot test data obtained after treatment with a lethal concentration of Cyphenothrin in combination with Piperonyl Butoxide (PBO). Results obtained with the mwh/flr3 of the wings..

Test Compounds and Conc. (ppm)	Number of wings (N)	Small single spots (1-2 cells) (*m*=2)			Large single spots (> 2 cells) (*m*=5)			Twin spots (*m*=5)			Total *mwh* spots (*m*=2)			Total spots (*m*=2)			Frequency of clone formation per 10^5 cells	Rates of viability (%) *
		No	Fr	D	No	Fr	D	No	Fr	D	No	Fr	D	No	Fr	D		
Cyphenothrin LC_{25}=15 ppm	80	30	(0.38)		1	(0.01)		0	(0.00)		31	(0.39)		31	(0.39)		1.58	
Cyphenothrin: PBO values																		
1: 0.25	80	28	(0.35)	-	4	(0.05)	i	3	(0.04)	i	35	(0.44)	i	35	(0.44)	i	1.79	20
1: 0.50	80	16	(0.20)	-	4	(0.05)	i	2	(0.03)	i	22	(0.28)	-	22	(0.28)	-	1.12	17
1: 0.75	80	30	(0.38)	i	4	(0.05)	i	0	(0.00)	i	34	(0.43)	i	34	(0.43)	i	1.74	14
1: 1	80	36	(0.45)	i	4	(0.05)	i	1	(0.01)	i	41	(0.51)	i	41	(0.51)	i	2.10	5
1: 2	80	40	(0.50)	i	3	(0.04)	i	1	(0.01)	i	44	(0.55)	i	44	(0.55)	i	2.25	4

No: number, Fr: frequency, D: statistical diagnosis according to Frei and Würgler (1988, 1995), +: positive, -: negative, i: inconclusive, m: multiplication factor, probability levels $\alpha = \beta = 0.05$. * Calculated using these results with Duncan Multiple Range Test (DMRT) ($p<0.05$).

Table 5. Wing spot test data obtained after treatment with a lethal concentration of Deltamethrin in combination with Piperonyl Butoxide (PBO). Results obtained with the mwh/flr3 of the wings.

Test Compounds and Conc. (ppm)	Number of wings (N)	Small single spots (1-2 cells) (*m*=2)			Large single spots (> 2 cells) (*m*=5)			Twin spots (*m*=5)			Total *mwh* spots (*m*=2)			Total spots (*m*=2)			Frequency of clone formation per 10^5 cells	Rates of viability (%) *
		No	Fr	D	No	Fr	D	No	Fr	D	No	Fr	D	No	Fr	D		
Deltamethrin LC_{25}=7.5 ppm	80	37	(0.46)		2	(0.03)		4	(0.05)		43	(0.54)		43	(0.54)		2.20	
Deltamethrin: PBO values																		
1: 0.25	80	31	(0.39)	-	3	(0.04)	i	1	(0.01)	-	35	(0.44)	-	35	(0.44)	-	1.79	30
1: 0.50	80	30	(0.38)	-	1	(0.01)	i	1	(0.01)	-	32	(0.40)	-	32	(0.40)	-	1.63	12
1: 0.75	80	25	(0.31)	-	2	(0.03	i	0	(0.00)	-	27	(0.34)	-	27	(0.34)	-	1.38	11
1: 1	80	50	(0.63)	i	3	(0.04)	i	1	(0.01)	-	53	(0.66)	i	54	(0.68)	i	2.71	7
1: 2	80	24	(0.30)	-	1	(0.01)	i	2	(0.03)	-	27	(0.34)	-	27	(0.34)	-	1.38	4

No: number, Fr: frequency, D: statistical diagnosis according to Frei and Würgler (1988, 1995), +: positive, -: negative, i: inconclusive, m: multiplication factor, probability levels $\alpha = \beta = 0.05$. * Calculated using these results with Duncan Multiple Range Test (DMRT) ($p<0.05$).

Table 6. Wing spot test data obtained after treatment with a lethal concentration of Permethrin in combination with Piperonyl Butoxide (PBO). Results obtained with the mwh/flr3 of the wings.

Test Compounds and Conc. (ppm)	Number of wings (N)	Small single spots (1-2 cells) (*m*=2)			Large single spots (> 2 cells) (*m*=5)			Twin spots (*m*=5)			Total *mwh* spots (*m*=2)			Total spots (*m*=2)			Frequency of clone formation per 10^5 cells	Rates of viability (%) *
		No	Fr	D	No	Fr	D	No	Fr		No	Fr	D	No	Fr	D		
Permethrin LC_{25}=22.5 ppm	80	42	(0.53)		5	(0.06)		0	(0.00)		46	(0.58)		47	(0.59)		2.15	
Permethrin: PBO values																		
1: 0.25	80	25	(0.31)	-	4	(0.05	-	0	(0.00)	i	29	0.36)	-	29	(0.36)	-	1.28	86
1: 0.50	80	20	(0.25)	-	4	(0.05)	-	1	(0.02)	i	25	0.31)	-	25	(0.31)	-	1.02	79
1: 0.75	80	26	(0.33)	-	1	(0.01)	-	1	(0.01)	i	28	0.35)	-	28	(0.35)	-	1.33	73
1: 1	80	29	(0.36)	-	4	(0.05)	-	1	(0.01)	i	34	0.43)	-	34	(0.43)	-	1.49	62
1: 2	80	24	(0.30)	-	1	(0.01)	-	1	(0.01)	i	26	0.33)	-	26	(0.33)	-	1.23	60

No: number, Fr: frequency, D: statistical diagnosis according to Frei and Würgler (1988, 1995), +: positive, -: negative, i: inconclusive, m: multiplication factor, probability levels $\alpha = \beta = 0.05$. * Calculated using these results with Duncan Multiple Range Test (DMRT) ($p<0.05$).

Drosophila (Table 2).

In experimental systems, cypermethrin has induced oxidative stress and generation of reactive oxygen species (ROS) (Giray et al. 2001). Genotoxic properties of the cypermethrin were evaluated using the alkaline Comet assay (Mukhopadhyay et al. 2004, Patel et al. 2006), the chromosomal aberrations, sister chromatid exchange, and the micronucleus in mouse bone marrow and spleen (Amer et al. 1993, Anonymous 2003, Giri et al. 2003, Chauhan et al. 2005), the micronucleus assay in whole blood and isolated human lymphocyte cultures (Surrallés et al. 1995b), in the somatic cells of *Hordeum vulgare* L. (Singh et al. 2008), and the sister chromatid exchange, chromosomal aberrations, and micronucleus formation in human

peripheral blood lymphocytes (Kocaman and Topaktaş 2009). All cypermethrin tests yielded genotoxic results. Also, Mukhopadhyay et al. (2006) showed that cypermethrin induced cellular damage in the Hsp70 gene, as a marker of cellular damage in the reproductive tissues of *D. melanogaster*. In contrast to the positive results, the cypermethrin was also analysed by the Salmonella/microsome mutagenicity test (Ames) using *S. typhimurium* strains, TA98 and TA100 in the presence or absence of a rat-liver activation system (S-9) (Pluijmen et al. 1984), the micronucleus assay in human lymphocytes (Surralles et al. 1995a), the alkaline Comet assay (Gabbianelli et al. 2004), and in the *Neurospora crassa* test system (Keskin et al. 2003) demonstrated negative results. These results support our results of cypermethrin in combination with PBO being non genotoxic (Table 3).

Limited data is available on the genotoxicity of cyphenothrin. It causes nerve membrane depolarization and nerve block leading to paralysis of the animal. Both types of action were ascribed to the modifications of the nerve membrane sodium channels (Narahashi 1982). Erkmen et al. (2000) and demonstrated the histopathological activity of cyphenothrin. It also has low mammalian toxicity (Anonymous 1997). In the present study, we found that in combination with PBO and alone, a lethal concentration value of cyphenothrin did not show genotoxic activity in *Drosophila* (Table 4).

Deltamethrin induced DNA damage in human peripheral leucocytes as measured with the Comet assay (Villarini et al. 1998) and increased chromosome damage and mitotic index in the Chromosomal Aberrations Test and in the Micronucleus Assay (Gandhi et al. 1995, Anonymous 2003, Chauhan et al. 2007). This chemical is known to generate ROS, which cause peroxidative damage to both lipids and proteins (Parvez and Raisuddin 2005). In addition, Ansari et al. (2009) showed a dose-dependent increase in the frequencies of micronucleus and nuclear abnormalities in Channa punctata (Bloch 1793). On the contrary, deltamethrin has not shown induction of micronuclei and sister chromatid in human peripheral blood leukocytes (Villarini et al. 1998, Dolara et al. 1992). It was also, found not to be mutagenic in the S. typhimurium strains TA98 and TA100 in the presence or absence of metabolic activation (Pluijmen et al. 1984). Our data indicates that all treatments of deltamethrin in combination with PBO were not genotoxic in *Drosophila*. Our findings in this study for potential genotoxicity of deltamethrin are in terms of negative results in good agreement with the previous reports on the genotoxic potential of deltamethrin (Table 5).

Permethrin induced DNA damage in the Comet assay (Tisch et al. 2002, Gabbianelli et al. 2004, Ündeğer and Başaran 2005), to chromosome damage in the chromosomal aberrations test (Anonymous 2003) and in the sister chromatid exchange, and micronucleus in cultured human peripheral blood lymphocytes (Barrueco et al. 1992). Besides, permethrin induced oxidative stress which consequently leads to biochemical and functional changes (Gabbianelli et al. 2009). On the other hand, permethrin was found as non-mutagenic in the S. typhimurium strains TA98 and TA100 with or without metabolic activation (Pluijmen et al. 1984, Herrera and Laborda 1988). Permethrin was also not mutagenic in the *D. melanogaster* sex-linked recessive lethal test (Gupta et al. 1990). These findings were similar to those of the present study. Looking at the results obtained from our study permethrin in combination with PBO was not genotoxic (Table 6).

In conclusion, our data indicates that synthetic pyretroids (cypermethrin, cyphenothrin, deltamethrin and permethrin) alone and their combinations with PBO in the *D. melanogaster* SMART assay are not genotoxic. Our results contribute to the increase in the genotoxicity database of the synthetic pyretroids and PBO. The results showed that the *Drosophila* wing somatic mutation and recombination test is suitable especially to detect the genotoxicity of some pesticide and synergist combinations. Moreover, it supports the usefulness of this and different studies with *Drosophila* as an in vivo model (Graf et al. 1984, Mukhopadhyay et al. 2004, Ayar et al. 2009, Cetin et al. 2010, Demir et al. 2013). Like this method, new additional genotoxicity studies are required and should be performed to better understand this matter.

ACKNOWLEDGEMENTS

This investigation was supported by The Scientific and Technical Research Council of Turkey (TUBITAK) (Project ID: TBAG-107T189), Ankara, Turkey and the Management Unit of Research Project of Akdeniz University (Project ID: 2008.01.0105.005), Antalya, Turkey.

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