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Mutagenic and cytotoxic activity of insecticide Napoleon 4EC in *Allium cepa* and Ames test

DILEK AKYIL

Department of Molecular Biology and Genetic, Faculty of Arts and Sciences, Afyon Kocatepe University, Afyonkarahisar/Turkey

E-mail: dilekakyil9@gmail.com

Abstract. The objective of this study was to explore the mutagenic and cytotoxic effects of Napoleon 4EC pesticide used in Turkey to control insect pest by using two standard assays. The *Allium cepa* test was used for determined the cytotoxic effects of this pesticide. For this test, onion seeds were exposed to Napoleon 4EC (100, 200, and 400 ppm) for 24, 48, and 72 hours. For each test group root tip cells were stained with Feulgen and five slides were prepared for each concentration and counted microscopically. The concentrations Napoleon 4EC 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 Napoleon 4EC in each treatment group as compared to the controls. The percentage of mitotic phases has been markedly impacted. Five different doses of the pesticide (50, 100, 200, 400, 800 µg/plate) were examined with Ames test using *Salmonella typhimurium* strains TA98 and TA100 with and without S9 metabolic activation for mutagenic activity. Ames test results showed a dose dependent effect, but not twice the negative control for *S. typhimurium* TA98 and TA100, with or without S9 mix except 800 µg/plate doses. In 800 µg/plate doses, colony numbers are two-fold increase according to colony number of control group. So, this places the this compound as a weak mutagen according to the parameters.

Keywords: Allium test, Ames test, cytotoxicity, mutagenicity, Napoleon 4EC.

INTRODUCTION

The identification of chemicals capable of inducing mutations has become an important procedure in safety estimation and such substances can potentially induce to fertility problems in future generations. Mutagenic materials are also capable of inducing cancer, and this problem has been increased of the importance the mutagenicity testing systems (Kumar et al. 2013). Earlier studies have showed that some pesticides are clastogenic and mutagenic in different biological test systems (Siroki et al. 2001; Celik 2003; Stivaktakis et al. 2010; Moulas et al. 2013; Akyil et al. 2014; Akyil and Konuk 2015; Özkara et al. 2015a). Organophosphorus pesticides (OPs) are one of the most important groups of pesticides which have been broadly used in industry, hygiene and agriculture (Bello-Ramírez et al. 2000; Ballesteros and Par-

rado 2004; Wu et al. 2007). Napoleon 4EC is also an OPs which has an important role to control crops.

OPs are strong inhibitors of cholinesterase enzymes and they were improved to replace organohalide pesticides in the late 1950's due to relatively easier to degrade via microbial or environmental processes (Obare et al. 2010). On the other hand, these substances or their derivatives can accumulate in the living organisms and induce mutagenicity, teratogenicity, immunotoxicity and carcinogenicity.

Short-term test methods have been used for many years from past to present to identify the genotoxic activities of pesticides (Miao et al. 2017; Özkara 2017; Khallef et al. 2018; Özkara 2019; Akyil 2019). The short-term test systems can detect different types of genetic DNA damage: (i) gene or point mutations; (ii) primary DNA damage and repair; (iii) chromosomal alterations.

One of these test systems is the Ames test which is used to evaluate the mutagenic activity of chemicals; it is a short-term bacterial reverse mutation assay (Mortelmans and Zeiger 2000; Liman et al. 2010; Arriaga-Alba et al. 2013; Escobar et al. 2013). The other test system is the Allium test which is one of the well-known and reliable test systems to determine the toxicity in the laboratories (Konuk et al. 2007; Liman et al. 2010; Özkara et al. 2015; Bonciu et al. 2018). To analyze the effects of different substances, higher plants (*Vicia faba*, *Tradescantia paludosa*, *Pisum sativum*, *Hordeum vulgare*, *Crepis capillaris* and *Allium cepa*, etc.) have proven to be useful when used as bioindicators (Enan 2009; Siddiqui and Al-Rumman 2020). However, *Allium cepa* has an advantage due to its large chromosomes, easily observed with a light microscope; also in relation to the features that may reveal an effect even at relatively low level of interaction of the tested substance with the genetic material, besides its long history of use as cytotoxicological test. As it is an *in vivo* test, the data can be used for assessment of genotoxicity on plants and even for eukaryotes in general, including humans (Bonciu et al. 2018).

The use of insecticides has become increasingly widespread throughout the world so additional studies are necessary to determinate the potential toxic risk of insecticides on non-target organisms through bacterial mutational/ames test and Allium test, a plant assay (Yaduvanshi et al. 2012). To our knowledge, there is no study on the cytotoxicity and mutagenicity of Napoleon 4EC except in the present paper. The aim of this experiment was to evaluate both the mutagenic and cytotoxic effects of different doses of Napoleon 4EC by the bacterial reverse mutation assay in *S. typhimurium* TA98 and TA100 strains with or without S9 mix and Allium cepa test, respectively.

MATERIALS AND METHODS

Chemicals and 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 16 h 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). The test substance Napoleon 4EC was purchased from a local market in Afyonkarahisar/Turkey and dissolved in sterile 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.

Ames plate incorporation test

The mutagenicity of the Napoleon 4EC was determined using the standard plate incorporation assay. *Salmonella typhimurium* strains TA98 and TA100 were used with or without S9 mix in this test (Maron and Ames 1983). The tester strains were tested for the presence of the strain-specific markers as described by Maron and Ames (1983). Before the experiment, determination of the cytotoxic doses of the substance to be used during the experiment is carried out as a preliminary step. Cytotoxic doses of Napoleon 4EC (10,000, 1,000, 100, 10, 1 and 0.1 µg/plate) were determined by the method of Dean et al. (1985). The strains were selected according to the strategies of Mortelmans and Zeiger (2000). 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. A specific positive control was always used to test the experimental defect, if any, for each tester strain. Positive controls were 4-nitro-o-phenylenediamine (NPD) for TA98 and sodium azide (SA) for TA100, applied without metabolic activation, and 2-aminofluorene (AF) for TA98 and 2-aminoanthracene (2AA) for TA100 used with metabolic activation.

Determination of cytotoxic doses

For the test of cytotoxic doses were prepared by adding 0.1 ml of the test suspension for each concentration and 0.1 ml bacterial suspension of TA100 from an over-

night culture to 2 ml top agar which kept in 45°C water bath. The mixture was shaken for 3 s with a vortex mixer, and added into the nutrient agar. All test plates were incubated for 24 h at 37°C and then the revertant colonies were counted for each plate and determined toxic and non-toxic doses which used in the experiment.

For the test of without 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 which kept in 45°C water bath. The mixture was shaken for 3 s with a vortex mixer, and added into the minimal agar. For the test plates with S9 mix were prepared by adding 0.5 ml of S9 mix instead of the phosphate buffer. All test plates were incubated for 72 h at 37°C and then the revertant colonies were counted for each plate. Samples were evaluated on triplicate plates in two independent parallel experiments and all results of the experiment were analysed by the statistical analysis.

Allium test

The root inhibition test procedure was performed as described by Fiskesjo (1985). Preliminary experiments were conducted to determine the concentrations of each pesticide to be used in the actual cytotoxicity experiments. The pesticides were dissolved in distilled water. Outer scales of the bulbs and the dry bottom plates were removed without damage the root primordia. The onions were germinated in freshly distilled water for the first 24 h and then exposed for 96 h to the different doses of Napoleon 4EC (25, 50, 100, 200 and 400 ppm, respectively). The roots from each group including control group were cut off on the fifth day and length of each root was measured in order to determine the EC_{50} values. EC_{50} value was determined as the concentration which retards the growth of root 50% less when compared with the control group.

Root Growth Inhibition Test (EC_{50} determination)

The onions were grown in freshly made distilled water for 24 h and then exposed for four days to the control group and other concentrations of extracts. In order to determine efficient concentration (EC_{50}) values, ten roots from each onion were cut off at the end of the treatment period, and the root's length was measured. The concentration that decreased root growth about 50% when compared to the negative control group (distilled water), was accepted as EC_{50} value. To determine the possible toxic effects on roots, $EC_{50}/2$, EC_{50}

and $EC_{50}\times 2$ concentrations of root were used in *Allium* mitotic index test.

The EC_{50} value was approximately 200 ppm for Napoleon 4EC. In order to show possible concentration-dependent effects of this pesticide, the root tips were treated with 100 ppm ($EC_{50}/2$), 200 ppm (EC_{50}) and 400 ppm ($EC_{50}\times 2$) concentrations of Napoleon 4EC and all application groups were performed 24, 48 and 72 h treatment periods. After treatment, the roots were washed in distilled water and fixed in 3:1 ethanol:glacial acetic acid for 24 h and then roots were taken in 70% alcohol and stored +4°C. Feulgen was used for staining root tip cells. Slides were randomly coded and scored blindly. For mitotic index (MI), the different stages of mitosis were counted in a total of 5000–6000 cells (1000 cells/slide) per concentration, and expressed as a percentage.

Statistical Analysis

Root length and MI datas were given as percentages. The levels of difference in treatment groups were analyzed statistically by SPSS 15.0 version for Windows. Dunnett-t test (2 sided) was used on both the *Allium* and Ames tests in the analyses.

RESULTS

Ames test results was carried out for mutagenicity determination of the tested material. For this test histidine mutant strains of *S. typhymurium*, TA98 and TA100 were used, and control group colony numbers were compared with the test material. The concentrations which caused two-fold increase in the colony number of control group were accepted as mutagenic ones. A compound is considered a weak mutagen if it produces a reproducible, dose-related increase in the number of revertant colonies in one or more strains but the number of revertants is not double the background number of colonies (Mortelmans and Zeiger 2000).

It was found that only 1000 $\mu\text{g}/\text{plate}$ concentration was cytotoxic against *S. typhymurium* strains among six tested concentrations for cytotoxicity tests (1000, 800, 400, 200, 100, 50 and 25 $\mu\text{g}/\text{plate}$). So this toxic concentration was not applied in Ames test. In Ames test positive controls were 4-nitro-o-phenylenediamine (NPD) for TA98 and sodium azide (SA) for TA100, used without metabolic activation, and 2-aminofluorene (AF) for TA98 and 2-aminoanthracene (2AA) for TA100 used with metabolic activation, while distilled water was used as a negative control group. Most of the results, whether

Table 1. The mutagenicity assay results of Napoleon 4EC for *S. typhimurium* TA98 and TA100 strains.

Test Substance	Concentration ($\mu\text{g}/\text{plate}$)	No of His+ revertants/plate, mean \pm SD			
		TA98		TA100	
		- S9	+ S9	- S9	+ S9
Napoleon 4EC	800	70.20 \pm 4.14*	83.24 \pm 6.25	110.54 \pm 6.04	197.54 \pm 11.58
	400	52.10 \pm 3.21	83.40 \pm 3.46	102.21 \pm 4.54	168.21 \pm 6.52
	200	45.21 \pm 3.56	76.45 \pm 4.32	90.54 \pm 6.87	154.31 \pm 10.25
	100	40.90 \pm 3.68	65.20 \pm 6.25	91.21 \pm 9.62	142.30 \pm 11.54
	50	31.62 \pm 1.83	46.70 \pm 5.54	83.02 \pm 7.04	138.12 \pm 12.26
Neg. Control	100	32.70 \pm 4.68	45.21 \pm 2.57	81.21 \pm 11.24	126.32 \pm 5.83
SA	10			2824.56 \pm 68.31*	
2AA	5				2468.24 \pm 70.15*
2AF	200		988.50 \pm 17.57*		
NPD	200	1445.60 \pm 22.23*			

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

Table 2. Allium root growth inhibition test.

Test Substance	Concentrations (ppm)	Mean of root length \pm SD
Negative Control	-	3.86 \pm 0.41
Positive Control	-	1.12 \pm 0.14*
Napoleon 4EC	25	3.41 \pm 0.26*
	50	2.20 \pm 0.14*
	100	1.94 \pm 0.31*
	200	1.62 \pm 0.21*
	400	1.27 \pm 0.14*

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

increasing or decreasing relative to the negative control group, were not statistically significant at $P < 0.05$ (Dunnett-t test, 2 sided) except for in the 800 $\mu\text{g}/\text{plate}$ doses of the Napoleon 4EC in the TA98 without S9 mix.

Ames test results showed a dose dependent effect, but not twice the negative control for *S. typhimurium* TA98 and TA100, with or without S9 mix except 800 $\mu\text{g}/\text{plate}$ doses. In 800 $\mu\text{g}/\text{plate}$ doses, colony numbers are two-fold increase according to colony number of control group. So, this places the this compound as a weak mutagen according to the parameters. When S9 was added, revertant colony numbers in TA98 and TA100 became stronger and Ames test data's is summarized in Table 1.

Table 2 are summarized in Allium root growth test results. The effective concentration (EC_{50}) was determined as 200 ppm. Table 3 gives the effect of Napoleon 4EC on MI and mitotic phase in the root meristematic

cells of *A. cepa* treated for 24, 48 and 72 h. At all concentrations treated in the incubations of root diminished MI compared to negative control at each exposure time. The highest values were showed from 24 h examination of 100 ppm, and the lowest one in 72 h application of 400 ppm concentrations of Napoleon 4EC. The reduced of MI indicates statistically significant results ($p < 0.05$) all concentrations and all treatment time. All doses of Napoleon 4EC applied in the experiment caused changes in the percentage of particular phases' distribution in comparison to the control.

DISCUSSION

While the use of pesticides are planned to eliminate pests and develop the quality and quantity of yield in agriculture, there is concern about their use because some have cytotoxic/mutagenic and carcinogenic effects and harm non-target organisms (Asita and Mokobo 2013). Earlier studies have reported that some pesticides have mutagenic and clastogenic activities in several biological test systems (Yaduvanshi et al. 2012; Topcu et al. 2013; Asita and Mokobo 2013; Özkara et al. 2015b; Karaismailoğlu 2016; Khallef et al. 2017).

The bacterial reverse mutation test uses amino-acid requiring strains of *S. typhimurium* to identify point mutations, which include substitution, deletion or addition of one or a few DNA base pairs. The cause of many human genetic diseases are originated point mutations and their occurrence in oncogenes and tumor suppressor genes of somatic cells are caused in tumor formation

Table 3. The effects of Napoleon 4EC on MI and mitotic phases in the root cells of *A. cepa*.

Concentration (ppm)	Treatment Time	Counted Cell Number	Mitotic Index \pm SD	Mitotic Phases (%) \pm SD			
				Prophase	Metaphase	Anaphase	Telophase
Negative control	24 hour	5102	87.40 \pm 9.68	83.21 \pm 10.66	1.86 \pm 0.23	1.01 \pm 0.45	0.88 \pm 0.75
Positive control		4802	43.36 \pm 4.76*	38.27 \pm 4.26*	0.62 \pm 0.17*	0.55 \pm 0.54	0.50 \pm 0.28
100		4685	41.45 \pm 4.06*	41.10 \pm 2.71*	1.02 \pm 0.27*	0.71 \pm 0.22	1.05 \pm 0.46
200		5002	38.21 \pm 4.25*	38.44 \pm 4.02*	0.98 \pm 0.24*	1.00 \pm 0.53	1.27 \pm 0.24
400		5012	39.26 \pm 2.85*	36.11 \pm 2.45*	0.87 \pm 0.14*	0.45 \pm 0.12	0.62 \pm 0.47
Negative control	48 hour	5045	78.34 \pm 4.54	75.21 \pm 6.47	1.70 \pm 0.12	1.34 \pm 0.42	1.26 \pm 0.18
Positive control		5065	38.22 \pm 3.12*	36.85 \pm 3.54*	0.60 \pm 0.19*	0.64 \pm 0.31*	0.72 \pm 0.31
100		5145	31.54 \pm 3.65*	37.62 \pm 3.53*	0.79 \pm 0.49*	0.76 \pm 0.12*	1.21 \pm 0.24
200		5214	28.42 \pm 3.06*	32.54 \pm 2.08*	0.62 \pm 0.14*	0.60 \pm 0.20*	1.02 \pm 0.23
400		5162	23.40 \pm 1.48*	30.54 \pm 2.78*	0.45 \pm 0.23*	0.51 \pm 0.11*	0.92 \pm 0.41
Negative control	72 hour	5252	65.45 \pm 2.23	62.25 \pm 3.14	1.54 \pm 0.41	1.50 \pm 0.24	0.94 \pm 0.25
Positive control		5265	31.12 \pm 3.16*	32.02 \pm 3.69*	0.42 \pm 0.24*	0.86 \pm 0.24*	0.85 \pm 0.23
100		5189	28.17 \pm 5.44*	30.27 \pm 3.58*	0.65 \pm 0.19*	0.66 \pm 0.54*	0.52 \pm 0.14
200		4989	25.01 \pm 2.14*	24.24 \pm 2.45*	0.53 \pm 0.14*	0.62 \pm 0.27*	0.54 \pm 0.47
400		4980	18.17 \pm 2.25*	17.45 \pm 2.07*	0.38 \pm 0.47*	0.49 \pm 0.14*	0.61 \pm 0.51

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

in experimental animals and humans (Malev 2012).

The *Salmonella*/microsome assay is broadly used for evaluating the mutagenicity of chemicals including pesticides (Yaduvanshi et al. 2012). In the present studies, Ames plate incorporation assay with the different concentrations of Napoleon 4EC showed a mutagenic response only TA98 tester strain. In order to characterize the possible mechanism of mutagenicity, the important bacterial strains, sensitive to different mutational events due to their specific geno-types, were used. There are much reports on the mutagenic effects of OPs determined with the Ames test (Aufderheide and Gressmann 2007; Coral et al. 2009; Wu et al. 2012; Akyil and Konuk 2014). However, no study has yet reported on the *in vitro* mutagenicity of Napoleon 4EC using the Ames assay.

S. typhimurium TA98 strain is defined by the -1 frameshift deletion hisD3052, which effects the reading frame of a close by repetitive -C-G- bases and can be reverted by frameshift mutagens. TA100 contains the marker hisG46, which causes 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 sequences (Di Sotto et al. 2008). In this context these bacterial properties, our results can be said that Udimo 75 WG mutagenicity in TA98 strain is caused by frameshift mutations and that of TA100 strain is due to base change (Di Sotto et al. 2008).

All concentrations of this pesticide were weak mutagenic in the TA98 and TA100 strains, with or without the S9 fraction except 800 μ g/plate doses of the Napo-

leon 4EC in the TA98 without S9 mix. In 800 μ g/plate doses, colony numbers are two-fold increase according to colony number of control group. Exposure to the pesticide induced G-C base pair mutations (Maron and Ames, 1983) causing a frameshift reversion of the histidine-dependent tester strain (TA98) to the wild type (his^+). However, the addition of S9 mixture resulted in a reduction of the mutagenic effect of Napoleon 4EC but not significant according to negative control. Due to biotransformation, a compound that is active biologically can be changed to an inactive metabolite. Similarly an inactive compound can be changed to an active metabolite (Paolini and Forti 1997). In other words, the presence of an eukaryote enzyme in S9 fraction resulted in eliminate of the mutagenic activity of the tested substance. Therefore, it is vital to use the S9 fraction in the Ames test.

Yaduvanshi et al. (2012) reported that negative results for chlorpyrifos-an organophosphate pesticide-with all three tester strains (TA97, TA98, TA102) of *Salmonella* used in the presence or absence of metabolic activation. However, chlorpyrifos was toxic in TA98 tester strain at the dose of 5000 μ g/plate in absence of metabolic activation while reduction in toxicity was seen on addition of S9 mixture. In another study, five different concentrations of the Chlorthiophos were tested by Ames test using *Salmonella typhimurium* strains TA97, TA98, TA100, and TA102, with and without S9 metabolic activation. No concentrations of Chlorthiophos showed mutagenic activity on the TA97, TA100, and

TA102 strains, with and without S9 fraction, but were all mutagenic to the TA98 strain without S9 (Akyıl and Konuk 2014). Many researchers who have studied Ames test systems with OPs also reported mutagenic or non-mutagenic result (Aiub et al. 2002; Kumar et al. 2013; Arroyo et al. 2015; Akyıl et al. 2017). Chemicals which tested with different test methods can be genotoxic or not genotoxic depending on a number of factors such as chemical structure, biological activity, the positions of the binding location and having rings in the structure (Kutlu et al. 2011). Furthermore, it might be related to differences in test conditions, such as exposure time, concentrations of substances, the dispersal of the materials in the cell and physico-chemical characteristics of the chemicals (Ema et al. 2012; Kaur et al. 2014). Therefore, it could be explained why some studies find an increase of genetic damage while in others result as negative.

It is well known that plants are direct recipients of toxins and the *Allium cepa* assay is one of the plant assay method used broadly to study the genotoxicity of pesticides (Fernandes et al. 2007). Also, *Allium cepa* showed a good correlation with the results from other established test systems using eukaryotic as well prokaryotic cells (Yıldız et al. 2009).

Mitotic index is a parameter that allows to estimate the frequency of cellular division (Marcano et al. 2004) and the reduction of mitotic activities has been used frequently to determine the cytotoxicity (Linnainmaa et al. 1978). Many investigators have reported the change mitotic index following the treatment of test organisms with pesticides (Panda and Sahu 1985; Amer and Farah 1974). In this experiment, mitotic index mostly decreased with increase Napoleon 4EC concentrations at each treatment times in comparison with control groups ($p < 0.05$). When the phase frequencies are compared with control in different treatment groups, significant outcomes were obtained statistically ($p < 0.05$). The percentages of the mitotic phases were clearly influenced in totally almost all applications ($p < 0.05$).

Chlorpyrifos, the active ingredient in Napoleon 4EC, was shown to a dose-dependent increase in DNA damage in the liver and brain of rats using the single cell gel electrophoresis (or comet) assay (Mehta et al. 2008). Chlorpyrifos is known to generate oxidative stress, induce lipid peroxidation, and cause depletion of reduced glutathione (GSH), increase in oxidized glutathione (GSSG), and decrease in the ratio of GSH/GSSG in rat erythrocytes and tissues (Gultekin et al. 2001; Verma and Srivastava, 2003). Chlorpyrifos exposure causes inhibition of antioxidant enzyme activities and increase in the levels of hydrogen peroxide (H_2O_2) in rat brain

and liver (Gultekin et al. 2001; Verma and Srivastava 2003). Additionally, Chlorpyrifos was not mutagenic in the Ames *Salmonella* mutagenicity assay and mammalian cell cultures (CHO/HGPRT assay), cytogenetic abnormalities in mammalian cells both *in vitro* (rat lymphocyte chromosomal aberration test, RLCAT) and *in vivo* (mouse bone marrow micronucleus test) and induction of DNA damage and repair in rat hepatocytes *in vitro* (Gollapudi et al. 1995). Dursban 4 (Chlorpyrifos-ethyl) was decreased mitotic index and induced chromosome aberrations in the root meristem cells of *Allium cepa* (Topcu et al. 2013).

In the present study with *Allium cepa* root tip meristem cells, the three doses of Napoleon 4EC tested induced cytotoxicity thus corroborating the findings of these studies but not the *in vitro* studies with *S. typhimurium* cited above. There are some possible mechanisms for chemically decreased MI in plant cells. 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 dose, it could be 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 Napoleon 4EC 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).

The safety evaluation of a fragrance material includes a broad range of toxicological information, both for the substance 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).

In conclusion, Napoleon 4EC was determined to be cytotoxic due to reducing of MI in *Allium* test and weak mutagenic in Ames test. For this reason, further inves-

tigations 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 determine a compound whether it is toxic or non-toxic. In this study we performed two different test methods. However, for the reliable results, additional mutagenicity studies should be conducted. For example, the umu test may be used in conjunction with the Ames test, as it indicates carcinogens as those substances that induce the expression of the umu operon (Reifferschied and Heil 1996). The other *in vivo* or *in vitro* tests would help confirm the findings of the results and give predictions for the chemical's effect in an organism. Accordingly in order to healthy future generation, the unavoidable use and prescribed precautions be adhered to by the farmers being non target organism so as to minimize the possible health risks associated with the extensive use of pesticides.

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