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Somatic mutations and recombination test in *Drosophila melanogaster* used for investigating the genotoxicity of some food additives

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Abstract

In the present study, the effects of several food colorings, namely (Ponceau 4R (E 124), Tartrazine (E 102), and Pea green (E 102-E 133), were investigated in vivo using the wing spot test, SMART (somatic mutation and recombination test), in *Drosophila melanogaster*. Food colorings are the food additives, which are used for improving the appearance of food and beverages. In SMART, multiple wing hair (*mwh*), flare (*flr*^{3}), and beaded serrate (*BdS*) marker genes on the third-largest chromosome of *Drosophila* are used. The genotoxic effects of the food colorings on the imaginal disc cells that will develop into the wing spot cells during the embryonic development of *Drosophila* heterozygous larvae and the genotypic changes caused by mutation or recombination in somatic cells also play a role in the formation of mutant spots in the wings. Classes by mutant clones are as follows: small single spots containing 1-2 *mwh*, large single spots containing ≥3 *mwh* or ≥4 *flr*^{3}, and twin spots containing adjacent *mwh* and *flr*^{3} cells (GRAF et al., 1984). Negative control medium was prepared with distilled water, while positive control medium was prepared with 1 mM EMS (ethyl methane sulfonate). According to results obtained from SMART, Ponceau 4R, Tartrazine, and Pea green demonstrated significant results in trans-heterozygous flies (*mwh*/*flr*^{3}) for inducing the mutant wing spots compared to control groups at 25 mg/ml, 50 mg/ml, and 75 mg/ml exposure concentrations. On the other hand, Ponceau 4R, Tartrazine, and Pea green yielded significant results for inducing the mutant wing spots in balancer-heterozygous flies (*mwh*/*TM3*) at 25 mg/ml, 50 mg/ml, and 75 mg/ml exposure concentrations. The numbers of mutant wing spots were increased by all three colorings depending on the concentration (X^2= df=3, P<0.001). It was also determined that these numbers were significantly higher than the flies in the negative control medium and it suggests that these food colorings have genotoxic effects. However, the numbers of mutant wing spot were less than the flies in the positive control medium; this finding indicates that genotoxic effects of the food colorings were not as much as the EMS.

Keywords: Ponceau 4R, Tartazine, Pea green, *Drosophila melanogaster* Meigen, SMART

Introduction

Nutrition is one of the most important factors ensuring the continuity of life. Nowadays, it is seen that people's eating habits significantly differ as a result of the changes in lifestyle and economic development. Although the food additives play a significant role in the food industry, their effects on human health constitute an important issue. As a result of the increase in ready-made food consumption together with urbanization, potential health risks occur with the body's exposure to more additives (Akbulut, 2011).

Synthetic food colorings, which are commonly used class food additives, can create genotoxic effects, as well as health problems such as allergic reactions, skin rashes, asthma, hyperactivity, and concentration disorder when not used within

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the legal restrictions (Yentür et al., 1996). It is also stated that the food coloring agents cause hypersensitivity, migraine, preterm delivery, salicylate sensitivity, and cancer (Maier et al., 2010). With genotoxicity tests and epidemiological studies on *Drosophila*, mouse, rat, and bacteria, when not used within the limits specified in regulations, the synthetic food colorings have been reported to be carcinogenic when used in order to imitate the high quality (Yentür et al., 1996; Sarıkaya et al., 2010). It has been determined that the level of food colorings used in studies on this subject in our country is well above the statute limits and may have harmful effects in terms of public health (Yentür et al., 1996; Topsoy, 1990; Yaman, 1996).

Many studies on human diseases have shown that *Drosophila melanogaster* Meigen, a down-organized eukaryotic organism, can be used instead of mammals. Because, the biological properties of the imaginal disc cells in *Drosophila* larvae undergoing proliferation and differentiation to form body parts in the adult stage are similar to many cancer-sensitive mammalian cells, and more than 60% of genes identified in human genetic diseases are common with the genes in *Drosophila* genome. *Drosophila* is frequently included as a model organism in SMART test. This test is suitable for detecting mutagenic and recombinogenic activities that are the results of genotoxic and anti-genotoxic effects in somatic cells induced by chemicals. When compared with the other tests, SMART is a quite fast, precise, and economical option (Bernards & Hariharan, 2001). Moreover, this in vivo test method in *Drosophila* may be associated with in vivo genotoxicity tests in mammals (Graf et al., 1984; Graf & Wurgler, 1996). Mutations induced by chemicals in somatic cells of *Drosophila* larvae are transferred to daughter cells through the several cell divisions in wing SMART. Genotypic changes caused by a mutation or recombination in somatic cells may be seen as mutant spots in the wings (Graf et al., 1984; Tripathy et al., 1989).

The synthetic food colorings used in the present study were preferred because, in the literature, there are very few scientific studies similar to the subject of the present research. There is no study carried out on Ponceau 4R (E 124) and Pea green (E 102-E 133) in *Drosophila* by using SMART, and there is only one previous study of Tartrazine (E 102), (Tripathy et al., 1989). There are few studies carried out on *Drosophila* regarding the genotoxicity of these food colorings.

Using SMART, the present research was conducted in order to determine whether Ponceau 4R, Tartrazine, and Pea green at 25 mg/ml, 50 mg/ml, 75 mg/ml concentrations have a detrimental effect on *D. melanogaster* Meigen's *mwh* and *flr*^{3} lines.

Materials and Methods

Culture of *Drosophila* Lines

D. melanogaster Meigen lines used in the present study were obtained from Trakya University and Akdeniz University, Science Faculty, Department of Biology. *Drosophila* flies were cultured in an incubator, where optimum living conditions (25±1 °C and 40-60% relative humidity) were provided and adjusted to 12 hours light-dark cycle.

Experimental Groups

SMART method developed by (Graf et al., 1984) was used in determining the mutagenic and/or recombinogenic effects of Ponceau 4R, Tartrazine, and Pea green on *D. melanogaster* Meigen lines.

Genetic Structure of *Drosophila* Mutant Strains

In *Drosophila* SMART, the *mwh*, *flr*^{3}, and *BdS* (beaded serrate) marker genes on the third-largest chromosome of *Drosophila* are used. In *Drosophila* wing SMART, *flr*^{3}/*TM3*, *BdS* virgin females and *mwh*/*mwh* males are crossed for normal metabolic activity (Lindsley & Grell 1968; Lindsley & Zimm 1992; Garcia-Bellido & Dapena 1974).

Experiments in the present study were simultaneously conducted in the application groups consisting of experimental and control groups. Distilled water was used in the negative control group and, in literature, 1 mM concentration of EMS (Kasımoğlu & Uysal 2016), which was used in the positive control group, was stated to have a mutagenic effect. All of the food colorings used in the experiment groups dissolved in distilled water. Ponceau 4R, Tartrazine, and Pea green were used at 25‰, 50‰, and 75‰ concentrations for 72±4 hours in the experimental groups for *Drosophila* larvae.

D. melanogaster Meigen lines were fed with a standard *Drosophila* medium when kept in stock and in a cross bottle in order to ensure the fertilization and embryogenesis to obtain heterozygous larvae. *Drosophila* instant medium was used for *Drosophila* larvae for 72±4 hours in the experimental and control groups. *Drosophila* instant medium and the food colorings used in the study were purchased commercially. *Drosophila* instant medium was procured from Carolina Biological Supply Company, Ponceau 4R with Pea green from Roha JJT Group Company, Tartrazine from Parshwanath Colour Chem Group, and EMS from Sigma Aldrich GmbH.

Applications in Experimental Groups

Larvae in groups of 100 were left into falcon tubes, where 1.5 g of *Drosophila* instant media were wetted with 5 ml of solutions containing 25‰, 50‰, and 75‰ concentrations of Ponceau 4R, Tartrazine, and Pea green. While edges of wings of the adult flies developed from trans-heterozygous larvae (*mwh*/*flr*^{3}) at each concentration in experimental groups have normal structure, the edges of wings of the balancer-heterozygous flies (*mwh*/*TM3*) are in the form of serrate. In analyses, 40 wings were used for each concentration in each experimental group. Slides of normal and serrate wings were examined using a light microscope under 40×10 magnification in order to detect the presence of mutant clones (Kaya, 2000). Classes by mutant clones are as follows: small single spots containing 1-2 *mwh*, large single spots containing ≥3 *mwh* or ≥4 *flr*^{3}, and twin spots containing adjacent *mwh* and *flr*^{3}cells (Graf et al., 1984).

Wing spot test (SMART)

The SMART assay principle is based on loss of heterozygosity in recessive marker genes; *mwh* and *flr*^{3} on the third-largest chromosome lead to transformation of imaginal disc cells in *Drosophila* heterozygous larvae into mutant wing spots cell by mutation or recombination (GRAF et al., 1984).

Statistical Analysis

Minitab package program was used in the statistical analysis

of the study results. In the data analysis, it was investigated using the Chi-Square test if the total number of mutant spots in the wings of the flies in the positive and negative control groups was statistically different (df=1). Chi-square test (df=3) was also used in the analyses, where the total numbers of mutant spots in the wings of the flies at three different doses in experimental groups were compared separately with the flies fed in the positive control group and negative control group.

As a result of analyses, the total number of wing mutant spots obtained from the study and presented as Fr (frequency) values and the differences below $P<0.05$ were considered to be statistically significant.

Results and Discussion

When the results of the study were evaluated, the total number of mutant wing spots in normal ($X^2=213.22$, df=1, $P<0.001$) wings (in Table 1) and serrate ($X^2=69.06$, df=1, $P<0.001$) wings (in Table 2) of *Drosophila* flies in the positive control group was found to be much more than those of negative control group.

SMART data obtained from experimental group studies of Ponceau 4R were compared with results of positive and negative control groups in both Table 1 and Table 2. The total number of mutant wing spots in the normal wings (in Table 1) and serrate wings (in Table 2) of the *Drosophila* flies grown in the medium containing with 25‰, 50‰, and 75‰ concentrations of the Ponceau 4R were higher than those grown in the medium prepared with distilled water (normal wing: $X^2=22.84$, df=3, $P<0.001$; serrate wing: $X^2=29.82$, df=3, $P<0.001$) but it was found to be less than those grown in medium with EMS addition (normal wing: $X^2=330.87$, df=3, $P<0.001$; serrate wing: $X^2=51.64$, df=3, $P<0.001$).

In Tables 1 and 2, the study results obtained in the experimental group by using different concentrations of Tartrazine and in positive and negative control groups are given comparatively. According to the results obtained, the total number of mutant wing spots in the normal wings (in Table 1) and serrate wings (in Table 2) of the *Drosophila* flies grown in the medium containing with Tartrazine at 25‰, 50‰, and 75‰ concentrations were higher than those of the flies grown in the medium prepared with distilled water (normal wing: $X^2=22.42$, df=3, $P<0.001$; serrate wing: $X^2=29.91$, df=3, $P<0.001$) but it was found to be less than those of the medium EMS added (normal wing: $X^2=324.56$, df=3, $P<0.001$; serrate wing: $X^2=65.19$, df=3, $P<0.001$).

The numbers of mutant wing spots and total mutant spots in normal wings (in Table 1) and serrate wings (in Table 2) of *Drosophila* flies grown in the medium prepared with Pea green at 25‰, 50‰, and 75‰ concentrations were higher than those grown in the medium containing with distilled water (normal wing: $X^2=35.85$, df=3, $P<0.001$; serrate wing: $X^2=24.98$, df=3, $P<0.001$) but it was found to be less than those of the flies grown in the medium with EMS (normal wing: $X^2=277.27$, df=3, $P<0.001$; serrate wing: $X^2=40.45$, df=3, $P<0.001$). In Tables 1 and 2, the study results of the experimental group containing with different concentrations of Pea green are compared with positive and negative control groups comparatively.

Table 1. SMART data with *mwh/flr3* wings obtained with the food coloring agents and positive and negative control groups tested.

Application groups/ Concentration	Number of wing	Small single spots (1-2 cell)		Large single spots (>2 cells)		Twin spots		Total mwh spots		Total spots	
		No	Fr	No	Fr	No	Fr	No	Fr	No	Fr
Distilled water	40	3	(0.08)	5	(0.13)	4	(0.13)	3	(0.08)	12	(0.30)
1 mM EMS	40	49	(1.23)	130	(3.25)	68	(1.70)	99	(2.48)	247	(6.18) a*
Ponceau 4R											
25 mg/ml	40	1	(0.03)	37	(0.93)	0	(0.00)	1	(0.03)	38	(0.95) a* b*
50 mg/ml	40	1	(0.03)	41	(1.03)	0	(0.00)	1	(0.03)	42	(1.05) a* b*
75 mg/ml	40	1	(0.03)	49	(1.23)	0	(0.00)	1	(0.03)	50	(1.25) a* b*
Tartrazin											
25 mg/ml	40	0	(0.00)	40	(1.00)	0	(0.00)	1	(0.03)	40	(1.00) a* b*
50 mg/ml	40	3	(0.08)	44	(1.10)	0	(0.00)	5	(0.13)	47	(1.18) a* b*
75 mg/ml	40	4	(0.10)	42	(1.05)	0	(0.00)	4	(0.10)	46	(1.15) a* b*
Pea Green											
25 mg/ml	40	1	(0.03)	40	(1.00)	0	(0.00)	1	(0.03)	41	(1.03) a* b*
50 mg/ml	40	1	(0.03)	57	(1.43)	0	(0.00)	1	(0.03)	58	(1.45) a* b*
75 mg/ml	40	3	(0.08)	59	(1.48)	0	(0.00)	4	(0.10)	62	(1.55) a* b*

No: Number of mutant clones, Fr: Frequency; X^2: In evaluation with Chi-Square test; a: with Distilled water, b: with EMS, *: $P<0.001$

Table 2.SMART results with *mwh/TM3* wings obtained with the food colorings and positive and negative control groups tested.

Application groups/ Concentration	Number of wing	Small single spots (1-2 cell)		Large single spots (>2 cells)		Twin spots		Total mwh spots		Total spots	
		No	Fr	No	Fr	No	Fr	No	Fr	No	Fr
Distilled water	40	1	(0.03)	6	(0.13)	0	(0.00)	1	(0.03)	7	(0.18)
1 mM EMS	40	22	(0.55)	66	(3.25)	0	(0.00)	71	(1.78)	88	(2.2) a*
Ponceau 4R											
25 mg/ml	40	1	(0.03)	24	(0.93)	0	(0.00)	2	(0.05)	25	(0.63) a* b*
50 mg/ml	40	0	(0.00)	30	(1.03)	0	(0.00)	1	(0.03)	30	(0.75) a* b*
75 mg/ml	40	3	(0.08)	44	(1.23)	0	(0.00)	3	(0.08)	47	(1.18) a* b*
Tartrazin											
25 mg/ml	40	1	(0.03)	18	(1.00)	0	(0.00)	2	(0.05)	16	(0.48) a* b*
50 mg/ml	40	0	(0.00)	26	(1.10)	0	(0.00)	0	(0.00)	26	(0.65) a* b*
75 mg/ml	40	4	(0.10)	40	(1.05)	0	(0.00)	6	(0.15)	44	(1.10) a* b*
Pea Green											
25 mg/ml	40	4	(0.10)	32	(1.00)	0	(0.00)	4	(0.10)	36	(0.90) a* b*
50 mg/ml	40	1	(0.03)	31	(1.43)	0	(0.00)	1	(0.03)	32	(0.80) a* b*
75 mg/ml	40	5	(0.13)	38	(1.48)	0	(0.00)	5	(0.13)	43	(1.08) a* b*

No: Number of mutant clones, Fr: Frequency; X^2: In evaluation with Chi-Square test; a: with Distilled water, b: with EMS, *: P<0.001

Figure 1 illustrates the study data with the types of mutant wing spots and total mutant spots in the Ponceau 4R at 25‰, 50‰, and 75‰ concentrations in experimental groups and in the positive and negative control groups in the normal wings of the *Drosophila* flies.

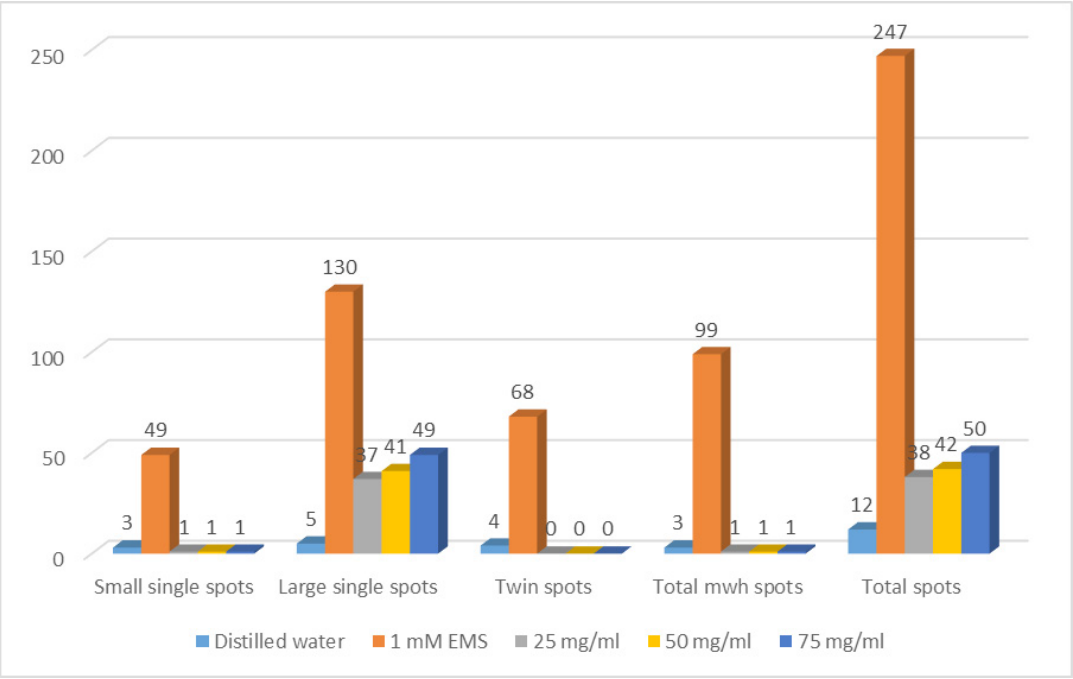

Figure 1. Classification of mutant clones with Normal wings obtained with Ponceau 4R

Figure 2 illustrates the study results with the types of mutant wing spots and total mutant spots in the Ponceau 4R experimental groups in 25‰, 50‰, and 75‰ concentrations and in the positive and negative control groups in the serrate wings of the *Drosophila* flies.

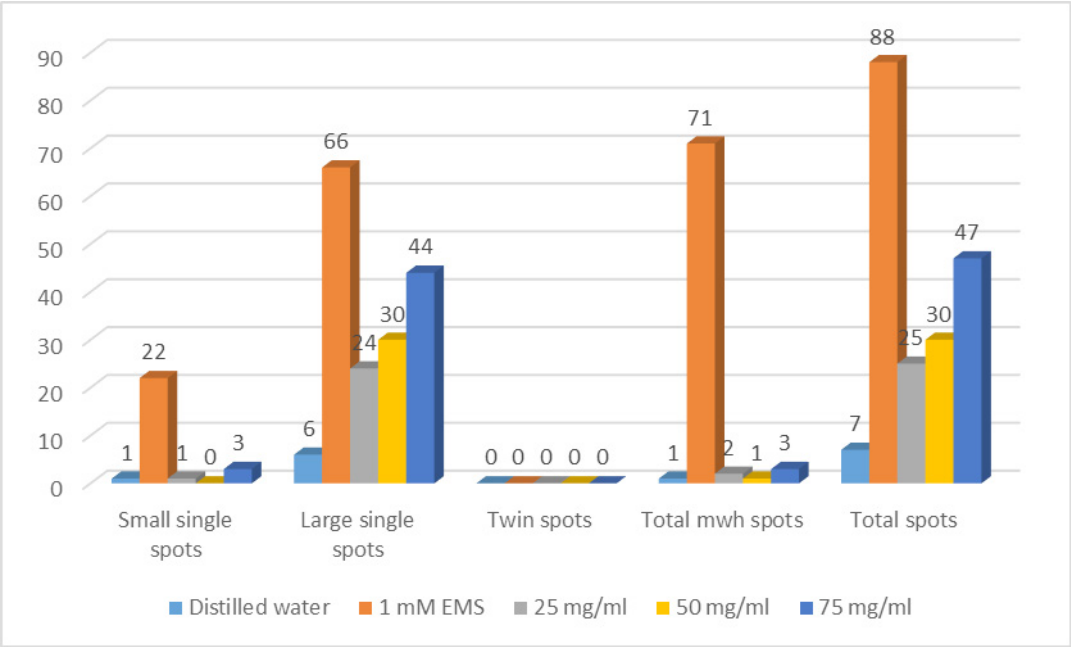

Figure 2. Classification of mutant clones with Serrate wings obtained with Ponceau 4R

The types of mutant wing spots and total mutant spots in the normal wings (in Figure 3) and serrate wings (in Figure 4) of the *Drosophila* flies in the experimental group with 25‰, 50‰ and 75‰ concentrations of Tartrazine and those of the flies in the positive and negative control groups were observed.

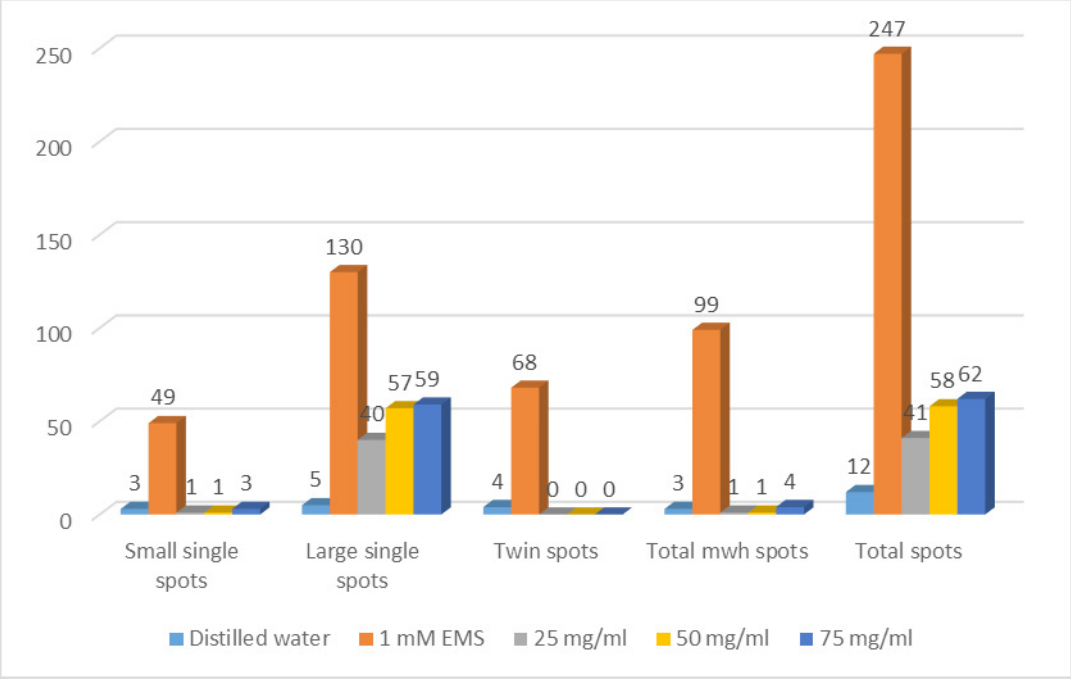

Figure 3. Classification of mutant clones with normal wings obtained with Tartrazine

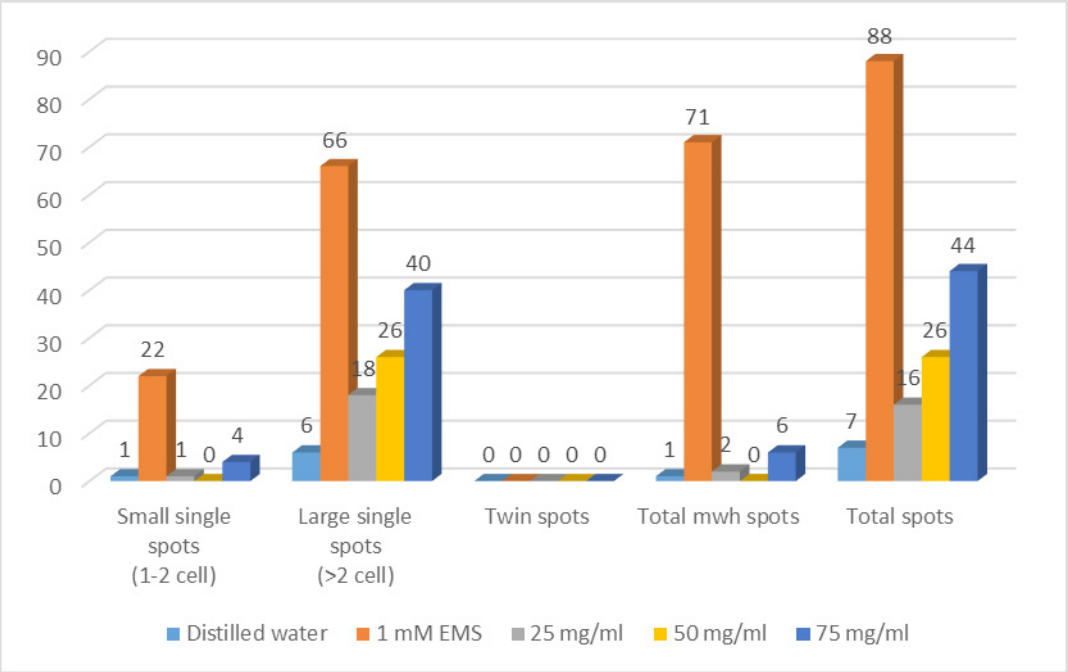

Figure 4. Classification of mutant clones with serrate wings obtained with Tartrazine

Figure 5 shows SMART results with the types of mutant spots and total mutant spots in normal wings of the flies in the Pea green experimental group at 25‰, 50‰, and 75‰ concentrations with those in the positive and negative control groups.

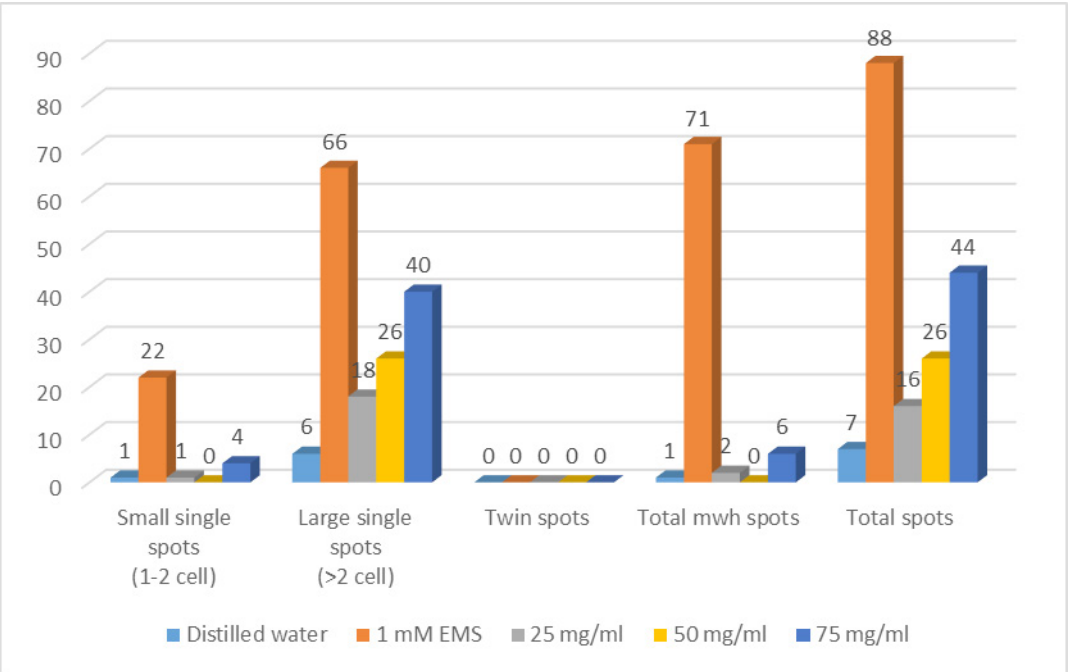

Figure 5. Classification of mutant clones with normal wings obtained with Pea Green

Figure 6 illustrates SMART data with the types of mutant spots and total mutant spots in serrate wings of fruit flies in the Pea green experimental group at 25‰, 50‰, and 75‰ concentrations with those in the positive and negative control groups.

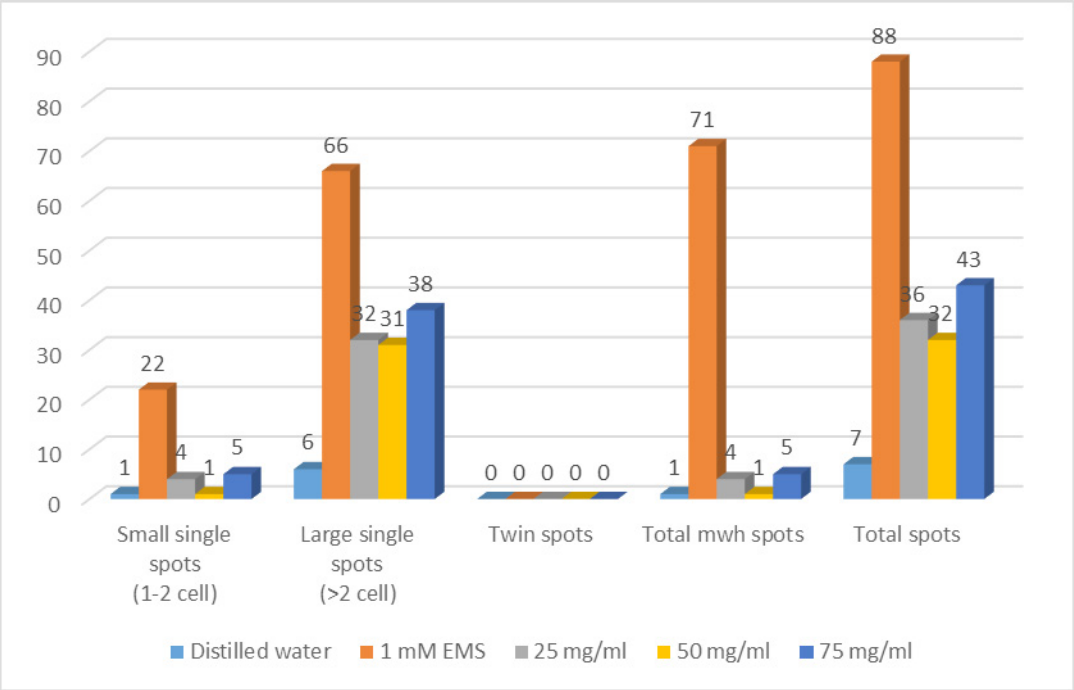

Figure 6. Classification of mutant clones with serrate wings obtained with Pea Green

Discussion

In addition to the nutritional values of consumed food, its safety and effects on human health are nowadays widely discussed (Alkan & Anlas, 2015). With the new technologies used in the food industry and the increasing variety of products, the interest in ready-made foods is increasing day-by-day with consumer demand (Akbulut, 2011). Food additives are frequently found in ready-to-eat foods and are among the potentially important genotoxic agents (Vural, 2005; Alkan & Anlas, 2015).

SMART plays an important role in the present study for examining the synthetic food colorings that cause genotoxic damage on the model organism, *D. melanogaster* Meigen. When the present study data are evaluated using SMART, it becomes clear that Ponceau 4R has a mutagenic effect in normal and serrate wings of *Drosophila* flies in studied concentrations. This finding clearly indicates that the food coloring agent has a genotoxic effect in the imaginal disc cells that will transform into the wing spot cells during the embryonic development of *Drosophila* heterozygous larvae and the genotypic changes caused by mutation or recombination in somatic cells also play a role in the formation of mutant spots in the wings. Serrate wings of the fruit flies contain only clones resulting from mutation, while mutant clones resulting from both mutation and recombination can be seen in normal wings (Kaya et al., 1999; Zordan et al., 1994). The mutant spots induced in *Drosophila* flies with Ponceau 4R are a result of the genotoxic activity of this genotoxic agent depend on its concentration. The data are similar in genotoxicity to the following studies on Ponceau 4R. Uysal & Semerdöken (2011) showed increased toxicity in *Drosophila* larvae at 72±4 hours due to the concentration increase of Ponceau 4R. Semerdoken (2012) reported that it showed an increase in mortality rate of Oregon R wild and Vestigial mutant strains of *Drosophila* larvae (72±4 hours) as the application concentration of Ponceau 4R rise. Turkoglu et al. (2015) published that the longevity of *Drosophila melanogaster* decreased significantly, depending on the different concentrations of Ponceau 4R. Uysal et al. (2017) notified that maximum mean life of Oregon R wild of *D. melanogaster* larvae at 72±4 hours decreased in different concentrations of Ponceau 4R depend on feeding. The researches data are compatible and supports the results of this study.

As a result of Tartrazine application in the experimental groups, it was clearly seen that this synthetic food coloring has a mutagenic effect. It is clearly understood that Tartrazine is effective as a mutagen in the development of wing spot cells during the embryonic development of *Drosophila* larvae and plays a role in increasing the number of mutant spots in the wings. Tartrazine got almost the same results like those of Ponceau 4R. The data obtained show, when exposure concentrations of Tartrazine are applied to the fruit flies, its mutagenic effect is so strong that the numbers of mutant spots in the wings of the flies increases. The data obtained in the present study, which clearly show the genotoxicity of Tartrazine, are supported by the following researches on the genotoxic, histopathological, and carcinogenic effects of this food coloring reported in the literature. Tripathy et al. (1989) reported that Tartrazine had genotoxicity in *Drosophila melanogaster* Meigen at 0.6‰ and 1.2‰ concentrations using the eye mosaic test and the wing spot test, whereas Poul et al. (2009) using micronucleus test revealed that Tartrazine was cytotoxic and not genotoxic in mice. Sasaki et al. (2002) determined that Tartrazine caused DNA damage in the stomach, colon, and bladder of mice depend on the dose, Mpountoukas et al. (2010) published that Tartrazine was cytotoxic for human lymphocyte cells at 1 and

2 mM concentrations, and Paterson & Butler (1982) concluded that Tartrazine applied to Muntiacus muntjac cells between mammalian fibroblast cells at 5-20 µg/ml concentrations caused chromosomal disorders. The researches on the harmful effects of Tartrazine, confirm the results of this study.

Results obtained from Pea green experimental group were very similar to experimental data of Ponceau 4R and Tartrazine. When Pea green applications were evaluated using SMART in normal and serrate wings of *Drosophila* flies, it was clearly seen that this coloring agent has a mutagenic effect. It was understood that Pea green has genotoxic effects in the imaginal disc cells that will develop the wing spot cells during embryonic development of the *Drosophila* heterozygous larvae and the genotypic changes caused by mutation and/or recombination in the somatic cells also play role in increasing number of mutant spots in wings. The present study result is consistent with the researches below, where the toxic effects of Pea green (Tartrazine-E 102 and Brilliant Blue FCF-E 133 combination) are examined. Uysal & Semerdöken (2011) reported increased toxicity in Oregon R of *Drosophila* larvae (72±4 hours) depending on the increase in concentration of Pea green, whereas Semerdoken (2012) issued that Tartrazine increased the mortality rate in Oregon R wild and Vestigial mutant strains of *Drosophila* larvae at 72±4 hours and Uysal et al. (2017) reported that, according to larval mortality and life span, Tartrazine had the highest toxicity in Oregon R wild of *D. melanogaster* larvae at 72±4 hours among other food dyes, Turkoglu et al. (2015) notified that Brilliant Blue FCF caused the biggest decreased in life span of *Drosophila melanogaster* among other food colorants and Kumar et al. (2019) revealed that on exposure to Brilliant Blue, larvae and pre-adult stages were prone to developmental toxicity.

Conclusion

It is thought that the present study with the synthetic food colorings can contribute to various scientific researches on toxicological, histopathological, carcinogenic, and teratogenic effects in/on various experimental organisms and may be a source for similar experiments will be done later. The results obtained from the present study are noteworthy because SMART can be applied in vivo (Graf et al., 1984; Graf & Wurgler, 1996) and down-organized eukaryotic *D. melanogaster* Meigen can be used as a model organism to investigate the genotoxic effects of different chemicals. In addition, the biological properties of the imaginal disc cells in *Drosophila* larvae undergo proliferation and differentiation to form body parts in the adult stage are similar to many cancer-sensitive mammalian cells, and more than 60% of genes identified in human genetic diseases are common with the genes in *Drosophila* genome (Bernards & Hariharan, 2001). These expressions increase the value of the results of the present study even more. As shown in similar studies on food additives, the disuse of the food colorings in the present study in accordance with the regulations may cause toxic effects on people. For this reason, the food additives should be consumed in a more controlled manner in terms of public health. When considering the effects of the synthetic food colorings used in the study, public health should be tried to be protected by changing the nutrition habits. Future studies on these food colorings will be of great importance for the continuity of healthy lives in the coming years.

Compliance with Ethical Standards

Conflict of interest

The authors declared that for this research article, they have no actual, potential or perceived conflict of interest.

Author contribution

The contribution of the authors to the present study is equal. All the authors read and approved the final manuscript. All the authors verify that the Text, Figures, and Tables are original and that they have not been published before.

Ethical approval

Not applicable.

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Data availability

Not applicable.

Consent for publication

Not applicable.

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