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Investigation of Mutagenic Effects of Synthetic Acidic Textile Dyes by Umu-Test (*Salmonella thyphimurium* TA1535/pSK1002)-a Short Term Bacterial Assay

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Abstract

In this study, genotoxic properties of some synthetic acidic dyes were researched by *umu*-test (*Salmonella thyphimurium* TA1535/pSK1002) which is a short term bacterial test. The study analyzed genetoxic activity of Acid Blue 127, Acid Orange 51, Acid Black 63, Acid Yellow 17 and Acid Blue 113 synthetic acidic dyes in presence and absence of S9 fraction used in Textile industry. Solutions of dyes at concentrations of 400 µg/ml, 120 µg/ml, 40 µg/ml and 4 µg/ml were prepared; and biotransformation effects of dyes that undergo chemical modifications on organisms were examined by measuring betagalactosidase activity in presence of liver enzymes by using rat S9 fraction. At the mentioned concentrations the absorbance values of betagalactosidase activity were measured for 5 synthetic acidic dyes and none of them showed mutagenic effect either in presence or absence of S9 fraction. In addition, these results mean that these synthetic acidic dyes are not metabolized with liver enzymes.

Keywords: Genotoxicity, mutagenic, *Salmonella thyphimurium*, synthetic acidic dyes, Umu-Test

1. Introduction

All living things in the nature are exposed to natural or artificial chemicals. It has been a matter of discussion that some of such chemicals which are released to environment in a controlled or uncontrolled manner named as pollutants might be mutagenic or carcinogen (Elisangela et al., 2009; He, Hu, & Li, 2004). Such substances that are released to nature by humans and constitute the main effect of pollution cause numerous diseases. Some of them are heart diseases, premature aging, cataract, inherited and developmental birth disorders (Ames, Lee, & Durston, 1973; Mix, 1986).

Rapid and sensitive bioassay systems have been developed to detect environmental genotoxins as a whole based on the ability of these substances to cause DNA damage (Monarca et al., 2004). Among different bioassays for the detection of environmental mutagens, the SOS/umu test, which monitors the expression of one of the SOS genes (umuC gene) by measuring b-galactosidase activity (Oda et al., 1985), has attracted vast attention because of its simplicity, short test time and low requirement for disinfection (Hamer et al., 2000). Employing a single Salmonella typhimurium tester strain (S. typhimurium TA1535/pSK1002), this bioassay system provides the detection of SOS mutagenesis after treatment through DNA-damaging agents (Michel, 2005). As the standardized method of ISO (ISO/CD 13829) (ISO2000) and DIN (DIN 38415-3) (DIN 1996), the SOS/umu test has been extensively applied to detect genotoxicity in different media including airborne particles (Oda et al., 2004), tap water (Shen et al., 2003), reclaimed water (Wu et al., 2010) and industrial wastewater (Dizer et al., 2002).

Dye production in Turkey is 12.000 tons and imported amount is 46.009,5 tons (Senel, Demirtas, & Senel, 2014). These figures are almost the same with India being the biggest dye productor with 64000 tonnes per year which is the 6.6% of the world production (Mathur & Bhatnagar, 2007). Textile industry is developing very rapidly in various geographical regions of Turkey, especially in Marmara and Aegean Regions. This development in textile industry aims to meet the demands of the national and international markets. However, this rapid development brings out an uncontrolled growth. Seas, lakes, rivers and drinking water basins are the sources being undouptedly affected by this uncontrolled growth at the highest level. Organisms in these natural habitats are affected by this pollution. Additionally, the materials causing this pollution reach up to human that is on the top of food chain (Chatterjee, Ruj, & Mahata, 2001)

After being applied on fabric in factories, 10-20 percent of dyes is discharged via waste water as a result of the absence of adsorption on the fabric. (Chen, Lin, Liu, Cheng, & Lee, 2001; Chatterjee et al., 2001; Gomez, Larrenchi, & Callao, 2007). It is calculated that nearly 10-15 percent of dyes migrates to waste water stream during dyeing process of textile industy. Per each kilogram of product in textile industry, nearly 40-65 liters of waste water is produced (Manu & Chaudhari, 2002). The photosynthetic activity in aqueous fauna is also negatively affected by high amount synthetic dye content in discharged textile waste waters. As a result of this, light permeability decreases; and such situation causes a very toxic effect on aquatic organisms (Aksu, 2005).

Umu-test is a test system used for determining mutagenic effects of various chemicals, metal salts and wastewaters (Wittekindt, Fisher, & Hansen, 2000; Yamamoto, Kohyama, & Hanawa, 2001; Nakamura et al., 1990). *Umu*-test is a test system enabling easy determination of mutagen agents by measuring β-galactosidase activity with spectrophotometer colorometrically. Result is obtained within 3-4 hours after preparation of bacteria for test. Principle of Umu-Test is the recognition of agents damaging DNA and having a carcinogen potential by umu operon. pSK1002 plasmid from *E. coli* CSH26/pSK1002 strain (carries umuC'-'lacZ gene) was prepared and firstly modified into *S. thyphimurium* SJ10002 strain and then transformed into plasmid *S. thyphimurium* TA1535 (hisG46, rfa, uvrB) strain; and thus *S. thyphimurium* TA1535/pSK1002 strain was obtained. TA1535/pSK1002 strain can measure β-galactosidase activity, the level of umu operon expression with a composite gene it produces. In UmuC'-'lacZ composite gene; umu operon is affected by DNA-damage agents and genetically corrected by recA and lexA genes (Ono, Somiya & Oda, 2000).

In this study, genotoxic properties of Acid Blue 127, Acid Orange 51, Acid Black 63, Acid Yellow 17 and Acid Blue 113 dyes were researched at concentrations of 400 µg/ml, 120 µg/ml, 40 µg/ml and 4 µg/ml via *umu*-test (*Salmonella thyphimurium* TA1535/pSK1002) which is a short term bacterial test. Likewise, biotransformation effects of dyes on organisms were observed in presence of liver enzymes using S9 fraction.

2. Materials and Methods

Five different synthetic acidic dyes -Acid Blue 127, Acid Orange 51, Acid Black 63, Acid Yellow 17 and Acid Blue 113- were used in this study. Genotoxic properties of these synthetic acidic dyes were examined through umu-test (*Salmonella thyphimurium* TA1535/pSK1002) system (JIMRO, Gunma, Japan) which is a short term mutagenity test.

2.1 Preparation of Bacteria for Experiment

Salmonella thyphimurium TA1535/pSK1002 strain was found in Umu-test kit (JIMRO, Gunma, Japan) with 1 ml volume in lyophilized state. Bacteria strain had been kept in –20 °C, which provides storage condition, 6 hours before the study. 2 ml TGA (JIMRO, Gunma, Japan) medium was added on it. It was incubated at 37 ºC for 3 hours. 100 µl was taken from bacteria culture and planting was made to 10 ml Nutrient Broth. It was incubated at 37 ºC for 18 hours. Absorbance of the bacteria was set to 0.06 (OD600). Bacteria strain was incubated at 37 ºC for 3 hours by planting 100 µl in 2ml TGA and taken down to -20 °C upon adding DMSO (10%). Absorbance of it was set to 0.06 (OD600), and genetical properties were controlled by using the samples taken from overnight culture of *Salmonella thyphimurium* TA1535/pSK1002 strain (Yamamoto et al., 2001).

2.2 Preparation of Positive and Negative Controls

2-Aminoanthracene (2AA) and Furilfuramide (AF-2) were used for positive controls. These substances are found in the kit with 1 ml volumes per each. 2-Aminoanthracene and Furilfuramide are found in quantities of 300 µg/ml and 9 µg/ml respectively. Positive controls were diluted with DMSO. 10 µg/ml, 5 µg/ml and 1 µg/ml 2-aminoanthracene solutions were prepared. 100 ng/ml, 50 ng/ml and 10 ng/ml solutions of Furilfuramide were prepared. 2-Aminoanthracene was used as positive control in determination of metabolic activity. Furilfuramide was used as a positive control in non-metabolic activity (Yamamoto et al., 2001). Sterile distilled water was used as a negative control. Dye samples were supplied from commercial companies.

2.3 Preparation of Test Samples

Quantity of test samples and controls to be used in Umu-test is 10 µl. Therefore, error margin was meant to be reduced by using 100 ml volumes while preparing different concentrations of dyes. 40 mg of dye weighted with precision scale was added to containers filled with 100 ml distilled water and mixed thoroughly. It was sterilized for 20 minutes in autoclave at 121 °C. 10 ml was put in sterile tubes with a volume of 15 ml. Thus, 400 µg/ml concentration was obtained as the first step. Other concentrations were obtained by using 400 µg/ml concentration. The first, second and third tubes were filled with 7 ml, 9 ml and 9 ml distilled water, respectively. 3ml volume was taken from 400 µg/ml concentration and added to the first tube and 120 µg/ml concentration was obtained. 1 ml volume taken from 400 µg/ml concentration was added into the second first tube and 40 µg/ml concentration was obtained. 1 ml volume was taken from the second tube and added into the third tube and 4 µg/ml concentration was obtained.

2.4 Cytotoxicity Research

Cytotoxicities of dyes were examined. 10 µl was taken from different concentrations of the samples, genotoxicities of which will be studied and placed in wells. 100 µl from bacteria culture absorbance of which was set to 0.06 (OD600) was added. It was incubated at 37 °C for 2 hours. Upon waiting period, 100 µl ONPG was added and incubated at 37 °C for 1 hour for the second time. 100 µl DMSO was added to stop the reaction. Absorbance was measured at 620 nm. They were compared with negative control group. S-9 Rat liver enzymes were supplied ready (JIMRO, Gunma, Japan).

2.5 Experiment Procedure

96 well sterile disposable well plates were taken for *Umu*-test. A1- A5 wells were assigned to determine the mutagenity of a dye in metabolic activity for S-9 fraction; and A6-A10 wells were assigned to determine mutagenity in an environment where there is no metabolic activity. Thus, wells were assigned for a concentration of 5 different dyes. Other wells were assigned for positive and negative controls. 10 µl dyes, positive and negative controls were placed in assigned wells in order. 100 µl bacteria, absorbance of which was set to 0.06 (OD600), were added in each well. Dyes which are added with bacteria were incubated at 37°C for 2 hours. 100 µl O-Nitrophenil-β-Dgalactopyranoside was added on bacteria-test mixture that is taken out upon the waiting period. It was incubated at 37 °C for another 1 hour. 100 µl DMSO was added to each well upon second waiting period. Enzymatic reaction was stopped by DMSO. Absorbance measurement was done at 620 nm. Volume of the mixture obtained from the test was 310 µl. Since the volume of the mixture obtained from Umu-test was 310 µl, volume was completed to 2 ml for making a more reliable measurement. 1.69 ml distilled water was placed in wells of spectrophotometer. 310 µl mixtures were added on that. Total volume was completed to 2 ml. Absorbance of each mixture was read at 620 nm and recorded. Umu-test was repeated for 3 times with one-week intervals for specified concentrations of the dye and mean values were taken.

3. Results

In this test system, we determined the status of these 5 synthetic acidic dyes after being metabolized in a living body with the assistance of S9 rat liver enzymes. Therefore, it was determined that whether dyes have genotoxicity characteristics directly, if so, whether they lose their genotoxicity characteristics with the treatment of enzymes in living bodies and whether they gain genotoxicity characteristics with the treatment of enzymes in living bodies if they do not have genotoxicity.

The strain TA1535/pSK1002 monitors the levels of umu operon expression by measuring the betagalactosidase (β-galaktosidase) activity with the compound produced. The β-galaktosidase activity is proportional to the mutagenic effects. β-galaktosidase activity was measured three times for each test and control groups in a spectrophotometer at 620 nm. The Umu-test results of positive, negative controls and test groups are shown at the Table below and were compared with h SPSS.

β-galaktosidase activity was calculated by following equations;

$$\text{β-galaktosidase activity of Test Groups} = 6.451\ (T_1+T_2+T_3)_{620\,nm} / 3 \pm SD \quad (1)$$

$$\text{β-galaktosidase activity of Negative Control} = 6.451(K_1+K_2+K_3)_{620\,nm} / 3 \pm SD \quad (2)$$

K: Dilution coefficient =6.451,

X: Average of absorbance values,

SD: Standard deviation

Table 1. Umu-test result values of positive, negative controls and test groups

Acidic Dyes	Concentrations	Absorbance Values (OD_{620})	
	(μg/ml)	K* S-9 (+)	K* S-9 (-)
Acid Blue 127	400	0,204	0,253
	120	0,183	0,164
	40	0,128	0,124
	4	0,105	0,149
Acid Orange 51	400	0,118	0,090
	120	0,119	0,116
	40	0,110	0,135
	4	0,058	0,062
Acid Black 63	400	0,145	0,174
	120	0,097	0,142
	40	0,129	0,097
	4	0,092	0,079
Acid Yellow 17	400	0,145	0,164
	120	0,132	0,135
	40	0,110	0,078
	4	0,071	0,058
Acid Blue 113	400	0,187	0,235
	120	0,152	0,176
	40	0,135	0,077
	4	0,077	0,058
2- Aminoantrasen	10	0,545	-
	5	0,355	-
	1	0,223	-
Furylfuramid	100 ng/ml	-	0,281
	50 ng/ml	-	0,220
	10 ng/ml	-	0,133
Negative Control (Distelled water)	10 ml	0,122	0,127

In a certain concentration of a substance to be called mutagen, the following equation must be obtained.

$$\beta\text{-galaktosidase activity of Test Group} \geq 2 \times \beta\text{-galaktosidase activity of Negativ Controls} \quad (3)$$

In order to consider a substance as a mutagen in different concentrations too, as the concentration of a substance increases, β-galactosidase activity of this substance should be in parallel with the increase level in positive control (Yamamoto et al., 2001).

The absorbance values (OD_{620}) of the metabolic and non-metabolic *umu*-test findings based on the concentrations of acid dyes are analyzed. In accordance with these findings; it is defined that Acid Blue 127, Acid Orange 51, Acid Black 63, Acid Yellow 17 and Acid Blue 113 dyes do not have mutagenic effect when they are metabolized or not metabolized with liver enzymes.

4. Discussion

In this study, mutagenic properties of some synthetic acidic dyes which are used in high quantities in Turkish Textile Industry were researched via *Umu*-test (*Salmonella thyphimurium (*TA1535 /pSK1002) which is a short term mutagenity test.

The mutation in *Salmonella* short-term test systems is determined according to the number of the histidine prototroph. Unlike that procedure, it is possible to determine the activity level of β-galactosidase protein produced by bacteria in *umu*-test. Although this data is an important criterion for demonstration of the mutagenic potentials of chemicals; however, it can be affected by various parameters. It is very difficult to find the same data results in different laboratories or in the same laboratory in different times for a study. These parameters are as follows: the type of minimal medium, glucose-6-phosphate concentration, phosphate buffer concentration (Boath, Welch, & Garner, 1980), the number of cells planted in petri plates, humidity rate in drying oven (Belser et al., 1981).

The umu-test uses a fusion operon which responsible for producing β-galactosidase, a protein which degrades lactose under the control of the umu-related proteins. The β-galactosidase activity can be measured quantitatively through spectrophotometry.

The results of colorimetric measurements of five acidic textile dyes showed us that the β-galactosidase activity values of these dyes were not 2 times bigger than the β-galactosidase activity values of negative control. Besides, the increase of β-galactosidase activity values of dyes at mentioned concentrations were not parallel as values of positive controls. The β-galactosidase activity values of 2-aminoantrasen are 0.223, 0.355 and 0.545 at 1 µg/ml, 5 µg/ml and 10 µg/ml respectively. Also, the β-galactosidase activity values of furylfuramid are 0.133, 0.220 and 0.281 at 10 ng/ml, 50 ng/ml and 100 ng/ml respectively which are increasing proportional according to the concantrations. So, the β-galactosidase activity values of each acidic dyes are increasing proportional (except Acid orange 51 and Acid blue 127 at the absence of S9) too. It is an indicator of a genotoxicity to specify a substance but it is not sufficient by itself. In addition, the β-galactosidase activity values of each acidic dye must be at least 2 times bigger than the β-galactosidase activity values of negative control is 0.122 at 620 nm at presence of S9 and 0.127 at teh absence of S9. But none of the β-galactosidase activity values of 5 acidic dyes are 2 times bigger than the values of negatiive control. Because of that reason none of these 5 acidic textile dyes are genotoxic.

The mutagenic potential of five synthetic acidic textile dyes which is mentioned in this paper, is lower at the concentration of 400µg/ml when compared with various synthetic textile dyes as Reactive Black 5, Reactive Yellow 84 and Direct Blue 200. Also only Direct Blue 200 showes a higher mutagenic effect at the concentration of 200 µg/ml (Senel & Demirtas, 2012). At the other concentrations mutagenic potential levels of discussed dyes are closed to each other. In addition, the mutagenic potential of Dispers Red 74 was compared in following figure that has not mutagenic effect at any concentration which are studied (Senel, Demirtas, & Senel, 2014).

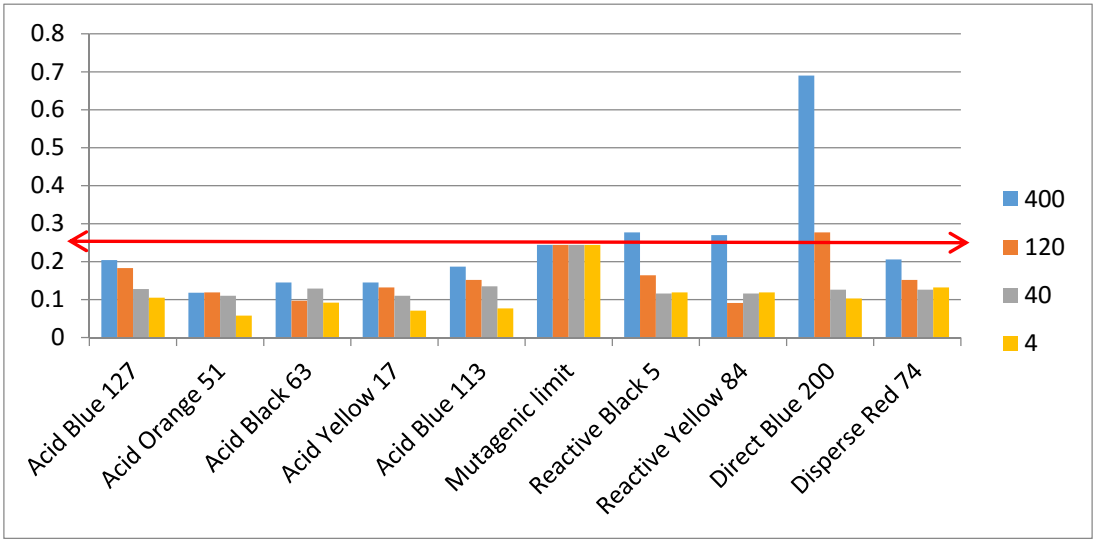

Figure: Comparison of mutagenic potential of Acidic synthetic textile dyes with various textile dyes at 400 µg/ml, 120 µg/ml, 40 µg/ml and 4 µg/ml concentrations

Umu-test has many advantages in terms of the results and application. These can be stated as; it has high level of sensitivity in genotoxic researches for pure substances and environmental samples and in the analysis of complex mixtures; and it shows high level of correlation with the results obtained in bacterial genotoxicity test systems (Wittekindt et al., 2000).

Biodegredation of chemicals as textile dyes can be measured or examined via umu-test and also FTIR max and HPLC. The FTIR spectrum of the AB-24 dye suggests the presence of azo bond (–N=N–) peak at –1 1618.28 cm. Absence of the azo bond in the degraded sample spectrum indicates biodegradation of the dye (Prasad & Rao, 2014).

In order to minimize the errors resulting from the abovementioned parameters, *Umu*-test kit (JIMRO, Gunma, Japan) is used. The nutrient medium, buffers, and control materials to be used in the experiments were prepared previously and stocked. In addition, a bacteria plantation was made after each study for the next day in order to keep the number of the bacteria to be used in the test as fixed, and their genetic properties were controlled. After these controls, the absorbance was adjusted to 0.06 at 600 nm (Yamamoto et al., 2001).

5. Conclusion

Nowadays, companies do extensive and expensive research about products of factories for their damages on environment and human health. The result of these researches quide the companies to decide what they will do in the production of materials. So, this study verified that the mentioned 5 synthetic acidic dyes which Acid Blue 127, Acid Orange 51, Acid Black 63, Acid Yellow 17 and Acid Blue have not genetoxic activity so they are suitable to produce for a company.

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