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Genotoxicity Assessment of Amaranth and Allura Red Using *Saccharomyces Cerevisiae*

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Abstract Amaranth (E123) and Allura red (E129), very important food azo dyes used in food, drug, paper, cosmetic and textile industries, were assessed for their genotoxic potential through comet assay in yeast cells. Comet assay was standardized by with different concentration of H_2O_2. Concentrations of Amaranth and Allura red were maintained in sorbitol buffer starting from 9.76 to 5,000 μg/mL and 1×10^4 cells were incubated at two different incubation temperatures 28 and 37°C. Amaranth (E123) and Allura red (E129) were found to exhibit their genotoxic effect directly in *Saccharomyces cerevisiae*. No significant genotoxic activity was observed for Amaranth and Allura red at 28°C but at 37°C direct relation of Amaranth concentration with comet tail was significant and no positive relation was seen with time exposure factor. At 37°C the minimum concentration of Amaranth and Allura red at which significant DNA damage observed through comet assay was 1,250 μg/mL in 2nd h post exposure time. The results indicated that food colors should be carefully used in baking products as heavy concentration of food colors could affect the fermentation process of baking.

Keywords Amaranth · Allura red · Comet assay · Genotoxicity · *Saccharomyces cerevisiae*

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Color additives are extensively used in food, cosmetics, drugs and certain medical devices such as contact lenses (Macioszek and Kononowicz 2004). Some commonly used color additives are rose bengal, amaranth, erythrocine, allura red, tartrazine, new coccine and phloxine). Among them tartrazine, new coccine, allura red and amaranth have been evaluated as most genotoxic (Sasaki et al. 2002).

The embryo genotoxicity of amaranth was assessed and then its genotoxic effects were reported as positive (Collins and McLaughlin 1972, 1973), but further studies reported negative results and the effect was reproducible (Collins et al. 1976; Flint et al. 1984; Larsson 1975; Piersma et al. 1995).

In United States, the food additive allura red which is regarded as non-genotoxic is permitted for commercial application (Combes and Haveland-Smith 1982). According to the U.S. National Toxicological Program allura red food color is non mutagenic in *Salmonella* (Anonymous 2000). Allura red is not carcinogenic in rats (Borzelleca et al. 1989) and also in mice. The red food dye was reported as non-teratogenic in one review (Collins et al. 1989).

There is controversy in views about genotoxicity of amaranth, it was reported that amaranth having genotoxic potential in one study (Combes and Haveland-Smith 1982) and was not classified as genotoxic in another (Chung and Cemiglia 1992).

Comet assay is a sensitive and rapid technique and have been used to determine the genotoxicity of industrial chemicals, biocides and pharmaceuticals (Singh et al. 2010). The single cell gel electrophoresis is capable of detecting single strand breaks in deoxyribose nucleic acid (DNA) (Tice et al. 2000).

In the present communication, comet assay was applied to *S. cerevisiae* as test model being fast growing organism

and its cultivation is easy. Moreover, its molecular mechanisms including transcription, translation and DNA damage having striking similarities with higher eukaryotes (Terziyska et al. 2000).

As the food coloring agents are arbitrarily used in our food and little is known about their genotoxicological effects, so the preliminary study has recorded the direct genotoxic levels of amaranth and allura red in *S. cerevisiae*.

Materials and Methods

Amaranth and Allura red were procured (Rainbow dye tech. PVT Ltd) from local market. These dyes were dissolved in sorbitol buffer by making different concentrations ranging from 9.76 to 5,000 μg/mL.

Saccharomyces cerevisiae culture was procured from the Molecular Diagnostic Laboratory, Institute of Microbiology, University of Agriculture, and Faisalabad Pakistan.

The culture was propagated in Yeast Peptone D-glucose (YPD) broth containing 1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) glucose. The culture was incubated at 30°C for 18 h as recommended (Novick and Bostein 1985). The suspension was confirmed through microscopic examination for the typical oval shaped budding cells and it was centrifuged at $300\times g$ for 3 min in order to collect the yeast cells and was resuspended in sorbitol buffer (1 M sorbitol, 25 mM $KHPO_4$ pH 6.5). Yeast cells were counted by Breed's smear method and standard count of cells suspension was maintained to 1×10^4/mL and kept under refrigeration temperature.

Saccharomyces cerevisiae standard suspension was treated with each diluted solution of the dye and incubated at temperature 28°C and 37°C as explained (Jabar Al-Mossawi 1983). Briefly, the triplicate (5 μL) sample from treated and control suspension were collected after every hour interval till 4 h from each dye solution kept at 28 and 37°C for comet assay.

Rough (frosted) microscopic slides were coated with four layers of normal and low melting point agarose as described (Lah et al. 2004). Briefly, *S. cerevisiae* cells were treated with hydrogen per oxide (H_2O_2), in order to standardize the technique. Yeast cells were exposed to different concentrations of H_2O_2 from 0.01, 0.05 and 0.1 mM for 5, 10 and 15 min. Yeast cell were embedded in third layer of low melting point agarose and four layered slides were treated with above mentioned concentrations of H_2O_2.

First layer of 1 % normal melting point agarose up to 400 μL was applied on the frosted surface of slides. Second layer of 0.6 % normal melting point agarose applied as supportive layer and was solidified on ice. Dye treated suspension (50 μL) was mixed with 350 μL of low melting point agarose and was spread on rough microscopic slide as third layer. In this third layer Amaranth and Allura red treated cells were immobilized. Fourth layer of 0.5 % low melting point agarose up to 500 μL was applied to prevent yeast DNA escape during incubation in different buffers like lysing buffer and electrophoresis buffer. Four layered microscopic slides were prepared from both Amaranth and Allura red treated cells and control suspension. The alkaline version of the comet assay was adopted. Four layered slide were incubated in lysing buffer (30 Mm NaOH, 1 M NaCl, 0.1% *N*-laurylsarcosine, 100 Mm DMSO and 1% Triton-x 100) for 75 min. Slides were rinsed three times for 20 min in electrophoresis buffer (30 Mm NaOH, 2 mM EDTA, pH 12.5). Electrophoresis was conducted at 25 V and 300 mA for 15 min (pH 12.5) in the same buffer. Following electrophoresis the gels were neutralized in buffer (400 Mm Tris Hcl pH 7.5) for 15 min. The gels were analyzed after staining with ethidium bromide (20 μg/mL).

ANOVA test was applied (Sokal and Rohlf 1981). DNA damage in relation to concentration and time exposure was determined by using two way ANOVA.

Results and Discussion

The cells treated with H_2O_2 were analyzed under fluorescent microscope. The detailed length of damaged DNA fragments tail was recorded and quantified as given in Table 1.

Amaranth and Allura red was treated with *S. cerevisiae* cell suspension in different concentrations as described earlier. At 28°C no significant DNA damaging effects were observed with any concentration of Amaranth and Allura red even after 4 h exposure. Whereas, at 37°C significant DNA damage was calculated starting from 1,250 μg and observed up to 5,000 μg concentration both in case of Amaranth and Allura red as described in Tables 2, 3, 4 and 5. Damaged DNA moved toward anode during electrophoresis and forms an image of a comet. Three nuclei were focused per slide and comets were measured through Image J software.

Saccharomyces cerevisiae cells were directly exposed to food colors and performed in vivo assay. The analysis of variance showed, the effect of H_2O_2 treatment to *S. cerevisiae* was significant at $p \leq 0.05$. Hydrogen per oxide showed dose dependant DNA damage in yeast cells. Yeast cells showed comet tails by DNA damaging agents at ten time's lower concentration than in mammalian cells (Horvathova et al. 1998).

No significant genotoxic activity of Amaranth and Allura red was observed at 28°C but at 37°C these dyes caused DNA damage at different concentrations and with different

Table 1 Different treatment regimes of H_2O_2 from 0.01 Mm to 0.1 Mm for 5 to 15 min duration with *S. cerevisiae* culture and control groups

Time of exposure (min)	Comet tail length mean ± SE (μm) at different concentration of H_2O_2 in mM				Control group
	0.01	0.05	0.1	Overall mean	
5	6.86 ± 0.15	7.52 ± 0.13	7.79 ± 0.12	7.39 ± 0.15	1.33 ± 0.20
10	8.01 ± 0.09	8.56 ± 0.15	8.83 ± 0.21	8.46 ± 0.14	1.41 ± 0.30
15	8.58 ± 0.05	8.80 ± 0.06	11.36 ± 0.73	9.58 ± 0.49	1.69 ± 0.16

Means sharing similar superscripts do not differ significantly at $p \leq 0.05$

Table 2 Treatment regimes of various dye concentrations of Amaranth treated with *S. cerevisiae* cells and control group incubated at 37°C

Food dye	Samples μg/mL	Comet tail length mean ± SE (μm) at different time interval			
		1 h	2 h	3 h	4 h
Amaranth	9.76	1.54 ± 0.30	2.70 ± 0.86	1.43 ± 0.10	0.76 ± 0.35
	19.52	2.86 ± 0.46	1.00 ± 0.69	1.93 ± 0.56	1.81 ± 0.84
	39.06	1.30 ± 0.56	1.90 ± 0.87	1.40 ± 0.41	1.33 ± 0.63
	78	1.54 ± 0.70	3.19 ± 0.93	1.30 ± 0.14	1.75 ± 0.33
	156	1.41 ± 0.30	1.60 ± 0.15	2.52 ± 0.76	1.69 ± 0.16
	312	1.33 ± 0.20	2.16 ± 0.33	1.45 ± 0.74	2.5 ± 0.47
	625	1.90 ± 0.87	2.34 ± 0.76	1.75 ± 0.33	1.54 ± 0.30
	1,250	2.86 ± 0.45	7.84 ± 0.10	7.85 ± 0.10	8.08 ± 0.58
	2,500	2.73 ± 0.65	8.25 ± 0.06	8.83 ± 0.30	8.86 ± 0.32
	5,000	4.78 ± 0.56	8.58 ± 0.12	8.60 ± 0.09	8.71 ± 0.12
Control group		1.9 ± 0.70	1.54 ± 0.30	1.41 ± 0.30	1.75 ± 0.33

Two way ANOVA was applied to determine the significance effect of time exposure and concentration. Collective effect of concentration and time exposure is not significant. Statistical analysis indicates that time exposure effect was also not significant while concentration effects were highly significant at $p \leq 0.01$ and behaved independently

Table 3 Analysis of variance of amaranth treated *S. cerevisiae*, showing comet tail length in different concentrations and time

Source	Degree of freedom	Sum of squares	Means squares	F-value
Concentration (C)	2	3.683	1.841	21.196^{**}
Hours (H)	2	0.331	0.166	1.907^{NS}
C × H	4	0.404	0.101	1.162^{NS}
Error	18	1.564	0.087	
Total	26	5.982		

NS non-significant

** Highly significant at $p \leq 0.01$

time exposure. The possible reason of it could be that Amaranth and Allura red has optimal action on yeast cells at 37°C (Jabar Al-Mossawi 1983).

The effect of treatment of Amaranth in relation to concentration was highly significant at $p \leq 0.01$. Direct relation of Amaranth concentration with comet tail was observed as concentration increases tail length also increases. However, any positive relation with time exposure factor was not observed. Moreover, no significant interaction was found between concentration and time exposure. The effect of Allura red treatment in relation to concentration was observed significant at $p \leq 0.05$. In the study, there was observed significant relation with concentration of Allura red and tail length as concentration increases tail length also increases. Whereas, the effect of Allura red in relation to time exposure was highly significant at $p \leq 0.01$. As time exposure increases highly significant increase in tail length was found and vice versa.

The genotoxic mechanism of food colors may be due to their conversion into aromatic amines which are nucleophilic (Anonymous 1996) and forming covalent bonds with DNA. During replication, DNA broken down into fragments due to aromatic amine—DNA adducts resulting as comet tail in the present technique.

Amaranth and Allura red are red dyes belongs to same azo dye group so it could be the reason that both dyes start DNA damage at same concentration that is 1,250 μg/mL. Moreover, during first hour no effect was been observed, the reason behind that yeast cells have absorptive mode of metabolism and releases enzymes out side the cellular body and then absorb nutrition after its digestion therefore, the minimum time of 1 h have shown the DNA toxicity after absorption of azo dyes from the environment of medium.

Comet assay was selected because; it is simple, sensitive and rapid method to determine genotoxic potential of food colors. It is semi quantitative technique which quantifies the DNA damage for individual cells i.e. indicate per cell damage.

Table 4 Treatment regimes of various dye concentrations of allura red from 9.76 µg/mL to 5,000 µg/mL treated with *S. cerevisiae* (culture) and control group incubated at 37°C

Food dye	Samples µg/mL	Comet tail length mean ± SE (µm) at different time interval			
		1 h	2 h	3 h	4 h
Allura red	9.76	1.90 ± 0.87	2.52 ± 0.76	1.45 ± 0.74	0.96 ± 0.60
	19.52	2.5 ± 0.47	1.75 ± 0.33	1.69 ± 0.16	3.01 ± 0.79
	39.06	1.54 ± 0.30	2.70 ± 0.86	1.43 ± 0.10	0.76 ± 0.35
	78	1.33 ± 0.20	1.90 ± 0.87	2.86 ± 0.45	2.73 ± 0.65
	156	1.75 ± 0.33	1.00 ± 0.69	1.60 ± 0.15	2.85 ± 0.86
	312	1.69 ± 0.16	1.90 ± 0.87	1.75 ± 0.33	1.54 ± 0.30
	625	2.5 ± 0.47	3.19 ± 0.93	3.76 ± 0.46	2.70 ± 0.86
	1,250	1.90 ± 0.87	8.15 ± 0.10	8.22 ± 0.10	8.26 ± 0.13
	2,500	2.85 ± 0.66	7.44 ± 0.6	7.69 ± 0.3	8.48 ± 0.20
	5,000	1.43 ± 0.50	8.15 ± 0.10	8.22 ± 0.10	8.26 ± 0.13
Control group		1.54 ± 0.30	1.41 ± 0.30	1.75 ± 0.33	1.9 ± 0.70

Significant at $p \leq 0.05$ in relation to concentration

Highly significant at $p \leq 0.01$in relation to time exposure

Table 5 Analysis of variance of allura red treated *S. cerevisiae* showing comet tail length in different concentration and time

Source	Degree of freedom	Sum of squares	Means squares	F-value
Concentration (C)	2	3.785	1.892	6.050*
Hours (H)	2	3.200	1.600	5.116**
C × H	4	1.348	0.337	1.077NS
Error	18	5.630	0.313	
Total	26	13.962		

NS non-significant

* Significant at $p \leq 0.05$; ** Highly significant at $p \leq 0.01$

Direct effect of Amaranth and Allura red to *S. cerevisiae* was seen. The results showed that the yeast cells were affected by genotoxic agents. *Saccharomyces cerevisiae* have ability to biodegrade the food colors (Amaranth and Allura red) up to 0.1 % and above this concentration food colors can produce genotoxic effects in *S. cerevisiae*. As, *S. cerevisiae* is a baker's yeast, so heavy concentration of food colors should be avoided in baking products.

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