



MUTAGENICITY OF SYNTHETIC FOOD COLOURANTS AND EXPLOITATION OF BEET ROOT PIGMENTS IN CONFECTIONARIES

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ABSTRACT

Food colourants play a vital role in marketing various confectionary products like biscuits, cakes, jams, jellies and so on. Colour added to these products is believed to improve the appetite and also it makes the food items look attractive. Almost all the colours used in these food products are synthetic so the health aspect is always questionable. The most commonly used synthetic food colourants like apple green, kesar colour and strawberry pink have been tested for their toxicity through AMES test using the tester strain TA 98 and TA 1535 and the results analysed statistically. Natural food colour yielding plants are screened and pigments from beet root are extracted and processed to suit various confectionary utility. The AMES test revealed the mutagenic potential of selected synthetic food colourants and such potential is absent in the natural food colourants. This study has proved the beneficial aspects of using a natural colourant in the food industry as compared to using a synthetic food colourant which is found to be very harmful to health.

KEY WORDS: Natural food colourants, Beet root, Betalain, Confectionery, genotoxic, Synthetic colourants.

INTRODUCTION:

Food products are being added with various food colourants to make the food products more attractive and appealing. Moreover, food colourants are believed to improve the aesthetic value of the food. The colourants added to the food products give an artificial freshness to the food products. The food industry now made the usage of food colourants indispensable in order to market their products. They relate the flavor of food products to the colour. For instance, strawberry flavor is always correlated with pink colours, pista with green colour and so on. The history of usage of these food colourants goes back to 1500 BC, but they preferred to use only natural food colourants. By 1800 BC the food industry began to use artificial food colourants as the natural food colourants could not be used in large scale. Moreover, natural food colourants are expensive and only limited colour availability. Indigo, carmoisine, erythrosine, brilliant blue, tartrazine, fast green, sunset yellow and ponceau 4R are the eight synthetic food colourants which are permitted by the Food and Drug Administration (FDA) to be used in various food items¹. The most commonly used synthetic food colourants are sunset yellow, apple green and strawberry pink. These colours are used invariably in products like biscuits, cakes, jams, jellies etc. These colourants are believed to be toxic and can cause various health hazards. The synthetic food colourants may show additive, synergistic or even antagonistic effects². Methyl yellow causes testicular damage in the gametogenic element to arrest spermatogenesis in guinea pigs, rats and mice³, whereas the reproductive and neurobehavioral toxicity of tartrazine in mice has been reported⁴. The mutagenic potential of apple green synthetic colourant has been proved⁵. Sunset yellow is reported to induce immune suppression and also alters the relative expression of surface receptors in T & B cells⁶. In contrast, the natural food colourant, in addition to the colouring property, they have numerous health benefits. For example, carotene is an essential vitamin source. Betalains are the sources of an amino acid. Anthocyanins are quality control markers of food stuffs. Flavonoids are colourants with high pharmacological potential. The demand for natural colour is increasing day by day, the market for natural food colour is estimated to increase by approximately 10 percent annually. Developing countries like India and China may play a major role in supplying natural colours either in processed forms or as raw materials to the EU markets. The natural colour extraction from various plant sources is being reported by various researchers as follows: Annatto extract⁷, Dehydrated red beet extract⁸, Canthoxanthinalgal extract⁹, Carmine biocolourant- Magneta red¹⁰, Cotton seed meal - yellow¹¹, Grape skin extract - Reddish purple¹², Paprika - carotene¹³. In our study, the three commonly used synthetic colourants like Sunset yellow, Apple green and Strawberry pink are analyzed by AMES test. The beet root is chosen to extract natural colouring pigment and processed to suit the confectionary utility.

MATERIALS AND METHODS

Tester strains used were *Salmonella* histidine auxotrophs TA98 and TA1535¹⁴. Specific genotypes of these strains are described below (Table 1). *Salmonella* tester strains were grown on minimal bottom agar plates supplemented with histidine/biotin and growth would be observed after 24 hours. A lawn culture was made and crystal violet disc was placed on the culture plate and observed the plate for 24 hours to depict rfa Cell wall mutation.

Table 1: Tester Strain Genotypes

Tester Strain	his/trp Mutation	Additional Mutations		Plasmid
		Repair	LPS	
TA98	hisD3052	uvrB	Rfa	pKM101
TA1535	hisG46	uvrB	Rfa	-

For pKM101 Plasmid also lawn culture was made and ampicillin disc was placed on the culture plate and observed the plate for 24 hours. The optical density of the overnight cultures were determined at 650 nm in a spectrometer and found to be within the range of 0.04 to 0.06, which demonstrates the cultures were in late exponential or early stationary phase with 10⁹ cells/ml. The sunset yellow (sample 1), apple green (sample 2), strawberry pink (sample 3) purchased in local market in Chennai was dissolved in sterile distilled water. Dose formulations were prepared on the day of use. Dose formulation concentrations of 0.312, 0.625, 1.25, 2.5 and 5.0 mg/ml of samples were prepared by serial dilution for the study. The mutagenicity assays were performed using tester strains TA98 and TA1535 in the absence of S9, positive controls were also evaluated. All test and control items doses were evaluated using plates. Tester strains were exposed to the samples via the plate incorporation methodology¹⁴. This methodology has been shown to detect a wide range of classes of chemical mutagens. In the plate incorporation methodology, the tester strain, samples, are combined in molten top agar, which is then overlaid onto a minimal bottom agar plate. This plating procedure was used in the dose range-finding and mutagenicity assays. Each plate was labeled with the samples, test phase, tester strain, activation condition, and dose. Treatments in the absence of S9 were performed by adding 100 l tester strain and 100 l test or control article to 2.5 ml molten diluted top agar (maintained at 45 °C). The mixtures were vortexed and overlaid onto the surface of bottom agar dishes. After the overlay solidifies; the plates were inverted and incubated for 72 hrs at 37 °C. After incubation the plates were evaluated for the condition of the background lawn for the evidence of mutagenicity and samples precipitate in comparison with the control and the plates were evaluated for the number of revertant colonies. Results of statistical analysis were reported in the form of Mean ± SD. The revertant colony count of all tester strains with and without metabolic activation were analyzed as described below using licensed copies of Graph pad Prism 5.0 statistical package.

EXTRACTION OF COLOURING PIGMENT FROM BEET ROOT:

To extract natural pigments, the beet roots are freshly purchased from markets and they are thoroughly washed in running water for about 5-10 minutes later the skin is removed with the help of a sterile knife then the beet root is scrapped and scrapped beet roots are shade dried to remove moisture then they are again dried in hot air oven at various temperatures (Room temperature, 50°C, 60°C, 80°C). Then the dried beet root scrap is powdered finely in a mixer and it dissolved in water and ethanol separately at various concentrations and their O.D is marked using spectrophotometer. Jelly was prepared in laboratory by adding different levels of red beet pigments i.e.-0.10, 0.20, 0.30, 0.40 and 0.50% w/w in laboratory using the traditional procedure. Jellies were wrapped by polyethylene and aluminum foil and packed in carton bags and were stored at room temperature 25±5°C. The control jelly was prepared with 0.10% synthetic colour (carmoisine). Sensory evaluation was carried out by ten panelists. The panelists

were asked to evaluate color, taste, odor and overall acceptability for prepared jelly according to the Standard method¹⁵. The OD values are tabulated and mean, standard deviation and variance is calculated for all concentrations for both the solvents namely water and ethanol.

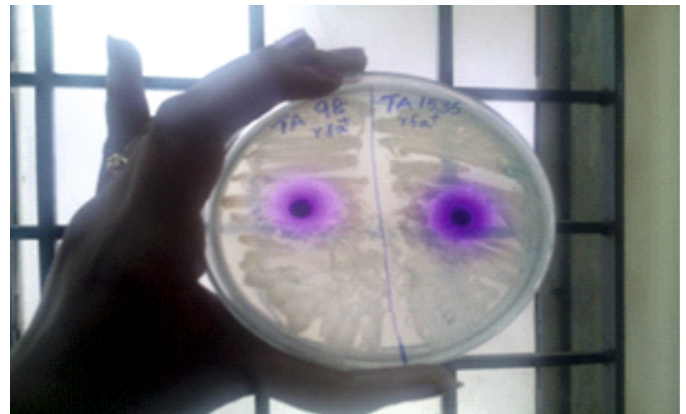
RESULTS

No growth was observed in minimal glucose agar due to the presence of histidine. An inhibition of growth around the crystal violet was observed in all *Salmonella* tester strains thus exhibiting the presence of *rfa* mutation. (Figure2). Growth was observed around the ampicillin disc of the *Salmonella* tester strains TA98, thus exhibiting the presence of pKM101 plasmid and inhibition of *Salmonella* growth in tester strains TA1535 demonstrating the absence of plasmid, pAQ1. The organism *Salmonella typhimurium* used in this experiment were grown as overnight cultures for 24 hours in nutrient broth. Then its density was determined at 650 nm in a spectrometer and found to be within the range of 0.04 to 0.06, which demonstrates the cultures were in late exponential or early stationary phase with 10⁹ cells / ml. The mutagens (positive controls) treated without metabolic activation system showed a 3 fold increase of average revertant colonies per plate when compared with that of concurrent vehicle controls, thus exhibiting the ability to identify the mutagen by the tester strains. The mutagenicity assay was performed with five dose levels (0.312, 0.625, 1.25, 2.5 and 5.0 mg/ml) in the absence of metabolic activation system. Inhibition of background growth of non-revertant bacteria was not found at any of the five dose levels. The average revertant colonies per plate treated with the control in the absence of metabolic activation system were found to be within the acceptance limits of the spontaneous revertant control values of respective *Salmonella* strains. Based on the above results, it is concluded that the kesar colour (sample 1), apple green (sample 2), strawberry pink (sample 3) is moderately mutagenic at the dose levels ranging from 312 to 5000 µg/plate by the AMES bacterial reverse mutation assay in the absence of S9 mix under the conditions of the test employed.(Table 2).

The Effect of temperature on various concentrations of the extracted pigment is analyzed by dissolving in two different solvents namely water and ethanol and observing the OD values (Table 3). In all temperatures chosen to dry, the

scraped beet roots and at all concentrations the OD values are found to be high in aqueous solvents than ethanol. With respect to aqueous solvent OD values are found to be maximum at 4 % of the sample concentrations. Sensory properties of jelly prepared with adding different concentrations of Betalain extracted from red beet as natural colorants is compared with the jellies prepared with 0.10% synthetic red color (carmine). Analysis of variance showed mostly significant differences in colour, taste, odor and overall acceptability for jelly. The addition of natural red color from red beet with different levels significantly affected color, taste, odor and overall acceptability. However, jelly prepared with levels of betalains extracted from red beet in concentrations 0.1 & 0.5 % for jelly received the lowest score in all tested quality attributes. The jelly prepared by adding natural color from red beet at 0.30 % had a highest score of investigated attributes followed by adding 0.4 and 0.2% respectively. In general, consumer perception has been that natural food colorant ingredient would be safe, healthy and considered as potential food colorants for preparing Jellies.

FIGURE 2 DEMONSTRATION OF rfa CELL WALL MUTATION



**TABLE 2
MUTAGENIC POTENTIAL OF DIFFERENT CONCENTRATIONS OF THREE SELECTED SAMPLES IN AMES SALMONELLA MUTAGENICITY TEST**

concentrations (µg /plate)	TA 98 (Mean ± S.E)			TA 1535 (Mean ±S.E)		
	SAMPLE1	SAMPLE 2	SAMPLE3	SAMPLE1	SAMPLE2	SAMPLE3
Positive control SODIUM AZIDE	415±37	415±37	415±37	361±11	361±11	361±11
5000	TNTC	TNTC	204±6.0	200±2.5	223±29	220±10
2500	234±36	274±24	160±10	166±16.0	206±6.0	150±4.5
1250	182±3.5	289±13.5	111±1.0	118±2.5	177±21.0	140±6.5
625	137±13	166±12.5	81.5±3.5	87±8.5	138±27.5	60±5.0
312	89±11	135±15	61±4.0	94±6.0	113±37.5	76±4.0

sample 1: kesar colour
sample 2: apple green
sample 3 :strawberry pink.

**TABLE 3
THE EFFECT OF TEMPERATURE ON OD IN TWO DIFFERENT SOLVENTS**

Temperature	Solvents	OD	Concentrations in %					Statistics		
			0.25%	0.5%	1.0%	2.0%	4.0%	Mean	SD	Variance
(Room Temp)			0.25%	0.5%	1.0%	2.0%	4.0%			
(32°C)	Aqueous	OD value	0.49	0.65	0.81	0.98	1.31	0.848	0.31	0.099
	Ethanol		0.5	0.12	0.15	0.24	0.23	0.248	0.1499	0.022
(50°C)	Aqueous	OD value	0.42	0.66	1.24	1.38	4	1.54	1.43	2.04
	Ethanol		0.04	0.16	0.04	0.01	0.02	0.05	0.06	0.003
(60°C)	Aqueous	OD value	0.42	0.98	1.90	3.35	4	2.13	1.52	2.32
	Ethanol		0.05	0.21	0.27	0.40	0.43	0.27	0.15	0.02
(80°C)	Aqueous	OD value	0.34	0.70	1.70	2.45	4	1.83	1.46	2.15
	Ethanol		0.05	0.12	0.23	0.15	0.42	0.19	0.14	0.02

DISCUSSION:

The aim of the study is to analyse the mutagenicity of three synthetic colourants namely kesar colour, apple green and strawberry pink and the possibility to process a natural colouring pigment to use in various confectionaries. The major ingredients of the selected synthetic food colourants are sunset yellow, tartrazine, brilliant blue, carmoisine and sodium chloride. These ingredients are proved to be mutagenic, cytotoxic and genotoxic but in case of the natural colourant they are proved to be safe and healthy. In our study we observed dose dependant colony increase and it was maximum at 5000µg/plate in the three samples. Among the three samples selected for the test, sample 2 (Apple green) is found to show more number of revertants of 274 for TA 98 and 206 for TA 100 at 5000µg/plate thus depicting that it is more toxic than kesar colour and strawberry pink. Apart from AMES test the genotoxicity of these synthetic colourants is proved by CBMN Assay (cytokinesis block Micronucleus). The genotoxicity of sunset yellow, tartrazine, brilliant blue, carmoisine alone and in combination has been shown and in the same study synthetic food colourants in the permissible limit of 100ppm as per PFA (prevention of food adulteration) Act of India are found to cause genotoxicity in human lymphocytes¹⁶. The cytotoxicity on root meristematic cells of *Allium sp* exposed to sunset yellow and tartrazine has been proved¹⁷. The sunset yellow is proved to cause immunomodulatory effects even at non-cytotoxic dosage¹⁸. Besides the mutagenicity of the colourants the usage limits and the acceptable daily intake (ADI) plays an important role in revealing their harmful effects on sensitive individuals. The beet root pigment is rich in betalains, isobetalains and vulgaxanthins as per the HPLC analysis¹⁹. As per our Hot air treated aqueous and ethanol extraction of beet root pigments, the ideal temperature at which the maximum colour retention was found at 60°C. At this temperature the OD was also found to be maximum in aqueous solvent. The retention of colour is found increase at this temperature along with increase in sample concentrations. As per the Betalain pigment extraction procedure, the colour retention of betalain is found to be maximum at the range of 40°C – 50°C from 70°C the pigment is found to loose colour²⁰. But the extraction procedure standardized by our method proves more colour retention potential of the pigments at 60°C and found to loose colour at 70°C. Moreover the jellies prepared with 0.1% of synthetic colourant carmine produces same colour appearance of the jellies made with 0.2% of natural colourants. To the consumers the above two jellies does not make any physical difference with reference to the colour, even the taste and odour was not found to make any difference. The natural pigment betalain used in this jellies is found to show more antioxidant potential over synthetic antioxidant like BHT: Butylated Hydroxyl Toluene²¹. Betaxanthins is proved to be an essential dietary colourant and also used as a food supplement in order to fortify processed food products with essential amino acids²². A significant suppressive effect of beetroot towards skin and lung cancer in mice has been elucidated²³. The long-term local suppression of liver and skin tumours induced by different chemical carcinogens in mice was demonstrated²⁴. The antiradical scavenging property of betalains was been demonstrated by various in vitro works²⁵. Beet tops among the ten most potent vegetables with respect to their antioxidant activity²⁶. Studies provide evidence that human red blood cells incorporate dietary betalains, which may protect the cells and avoid oxidative hemolysis²⁷. From the above study it was found that natural colourant should be used in double the quantity when compared with that of the synthetic colourant. Although the natural colourant demand more usage the safety and health aspect of natural colourant masks the other hinderance in choosing it as a food colourant. The synthetic food colourant is proved to show various health hazards whereas the natural colourant was proved to be safe and healthy.

CONCLUSION:

The mutagenic effect of three chosen synthetic food colourants kesar, apple green and strawberry pink is tested by AMES proved that all three colours were moderately mutagenic among the colourants. Apple green is found to be more toxic than the other two colourants. The natural colourant extraction was standardized and the colour retention with respect to temperature is analysed spectrophotometrically. From the above study it is evident that natural colourants in addition to the colouring property also serves a variety of health benefits. Hence the usage of the natural pigments in confectionaries should be promoted.

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