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EVALUATION OF GENOTOXICITY OF THREE FOOD PRESERVATIVES IN *DROSOPHILA MELANOGASTER* USING SMART AND COMET ASSAYS

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ABSTRACT

The continuously growing food and beverage industry relies on food additives as a main component in their products. Such increased reliance on processed food, lead to neglectance of the harmful effects of the food additives on human health; among these are hypersensitivity, allergic reactions, genotoxicity, mutagenicity and more. This study investigates genotoxic effects of three food preservatives commonly consumed in daily meals; sodium sulphite, boric acid, and benzoic acid using the somatic mutation and recombination test (SMART) and comet assay in *Drosophila melanogaster* system. All of the tested compounds showed significantly high levels of tumor induction and frequency compared to a negative control in SMART assay. They also showed high amount of DNA damage in the comet assay indicating their high potential of being genotoxic materials.

Keywords: mutagenic, genotoxic, comet, Drosophila, food additives

INTRODUCTION

Food additives are substances frequently added to food to enhance the quality of the final product in several aspects; extending life time by retarding or inhibiting growth of microorganisms, colouring, sweetening, flavouring and thickening **(Rekha and Dharman, 2011)**. For a long time, no observed adverse effects had been proven on the basis of toxicological studies. However, several studies had proven that the consumption of some additives in processed food might have increased the risk of human cancer despite the respected legal limits of these additives by the manufactures. The carcinogenic risk of food additives can be attributed to various factors; interaction of additives with some food ingredients, chemical formula of food additives might be changed during food processing to a carcinogenic formula, a negative synergistic effects when combined with other additives, unsuitable storage conditions, and unknown carcinogenic by-products occurring during the food processing **(Gülsoy *et al*., 2015)**.

Sodium sulphite, a preservative used to stop the browning and further ripening of fruits, was found to induce inhibition of DNA synthesis in *Vicia faba* root, bridges in anaphase and chromatin erosion of interphase nuclei **(Njagi and Gopalan, 1982)**.

According to **(Olorunfemi et al., 2012),** tests carried out at cytogenetic anomalies *Allium cepa* reveals a decrease of the mitotic index caused by treatments applied. Mitosis analysis indicates the development of a number of structural chromosomal aberrations and interphasis aberrations identified in the different stages of mitosis, the process of cell division being significantly affected.

Benzoic acid, a commonly used preservative as antimicrobial substance in many food products, was found to cause a weak positive increase in chromosomal aberration test in CHO cells **(Ishidate *et al*., 1984).** It also induced somatic mutations in *Drosophila* SMART test **(Sarikaya and Solak, 2003)** and increased the chromosomal aberration, sister chromatid exchange, and micronucleus frequency in human peripheral lymphocytes **(Yilmaz *et al*., 2009; Al-Tai *et al*., 2014)**.

Boric acid, an effective preservative against yeast and bacteria, inhibited the proliferation of prostate cancer cell lines, DU-145, and LNCaP, in a dose-dependent manner. It also inhibited non-tumorigenic prostate cell lines, PWR-1E, and RWPE-1, and the cancer cell line PC-3, but required higher concentrations than observed in human blood levels. It stimulated cell death independent proliferative inhibition, with little effect on cell cycle stage distribution and mitochondrial function in DU-145 cell line, **(Barranco and Eckhert, 2004)**. Borax, a salt of boric acid, had an inhibitory effect on HepG2 cell growth and induced apoptosis in a concentration-dependent manner **(Wei *et al*., 2016)**.

Among the most accepted tests that are applied to assess the carcinogenic potential of a given substance is the Somatic Mutations and Recombination Test (SMART) carried out in *Drosophila melanogaster* **(Demir *et al*., 2013)**. This assay uses tumor suppressor gene *warts* which is a homolog to the mammalian tumor suppressor gene *LATS* **(Nepomuceno, 2015; Vasconcelos *et al*., 2017)**. The evolutionary conservation of tumor suppressor genes among Drosophila and mammals has prompted studies of tumor induction in *Drosophila*, such studies has contributed to the understanding of cancer in human **(Potter et al., 2000; Eeken et al., 2002)**. Homozygosity loss of the *warts* gene induced by mitotic recombination in somatic cells leads to the formation of greatly overgrown cell clones that can be easily detected as tumors on fly body **(justice et al., 1995)**. SMART is a rapid, very sensitive to different classes of agents and inexpensive assay which is able to evaluate the carcinogenic activity of single compounds as well as complex mixtures. It also allows various protocols for the application of the test materials as single, combined or sequential treatments of the larvae. Factors capable of Inducing tumors in *Drosophila* instead of marker clones might directly adverse the risk of these factors for inducing cancer in humans **(Sidorov *et al*., 2001)**. In flies heterozygous for the *wts* gene, the genetic events that can lead to the tumor appearance and hence can be detected by SMART include; gene mutations in the *wts* gene, multilocus-deletions (partial), chromosomal loss and somatic recombination collectively referred to as loss of heterozygosity **(Eeken *et al*., 2002).**

Comet assay (also called, single cell gel electrophoresis, SCGE) is an assay used over the last few decades to detect any prospective damage for DNA after certain treatments. The assay is able to detect DNA strand breaks and alkali labile sites by measuring the migration of DNA from immobilized nuclear chromatin. This assay is one of the most widely accepted tests for detection of DNA damage as it offers several advantages over the other tests, these include: (1) damage to the DNA in individual cells is measured; (2) only small number of cells are needed to carry out the assay (<10,000); (3) the assay can be performed on virtually any eukaryotic cell type; (4) and it is faster and more sensitive than the alkaline elution method for detecting DNA damage DNA **(Singh *et. al*, 1988)**. Comet assay is a useful tool for the evaluation of local genotoxicity, particularly organs or cell types, which can hardly be evaluated with other standard tests **(Brendler *et al.*, 2005)**. Thist assay has become the prime choice in the assessment of DNA

damage and genotoxicity testing considering that it is an easy to perform, short time and low cost test that requires small numbers of cells/sample. Moreover, it is sensitive for detecting low levels of DNA damage. Alkaline comet assay (pH>13), the most commonly used version, is able to detect all possible kinds of DNA damage **(Tice *et al*., 2000)**. In recent years, the comet assay has been adapted to use *in vivo* in *Drosophila* **(Mukhopadhyay et al., 2004; Shukla et al., 2011)**, to combine its advantages with those well-established of this fly.
The objective of this study was to evaluate the genotoxic and carcinogenic effects of three food additives; sodium sulphite, boric acid, and benzoic acid using SMART and comet assays in *Drosophila*.

MATERIAL AND METHODS

Somatic Mutation and Recombination Test (SMART) in *D. melanogaster*

Drosophila Mealnogaster strains

Two different Drosophila strains were used in this study; wild type strain and a strain that carries *wtsMT*$^{4\text{-}1}$, a lethal warts allele balanced on TM3, characterized by multiple inversions and marked by the dominant mutation stubble according to **Eiken et al., (2002)** and **fly base (2006).** The genetic structure of this strain is; *st* p in *ri wtsMT4-1/ TM3 Sb*, which was abbreviated wts/TM3. Details about the various markers and the balancer chromosome can be found in **Lindsley and Zimm (1992).**

Crosses and treatments

The *wts*/TM3 females were crossed to wild type males resulting in two genotypes offspring, wts/+ and TM3, Sb wts$^{+/+}$. After 2 days, the parental flies were removed and 56-68 hours old larvae were washed with 20% glycerol, then collected using a fine mesh sieve and transferred to five different vials representing the five test groups. For food additives treated groups (Sodium sulphite, Boric acid, and Benzoic acid); the flies were transferred to a standard *Drosophila* medium to which a 100 mM of each food additive powder was added and properly dissolved at 50ºC. The larvae were submitted to chronic treatment for approximately 48 h., then they were transferred to standard Drosophila medium. The positive control group was transferred to a vial where 20 µg/ml of an appropriate Mitomycin C (MMC) solution was mixed with a standard *Drosophila* medium, kept for 24 hours, then they were transferred to standard *Drosophila* medium. Negative control group was directly transferred to a standard *Drosophila* medium. Afterwards, larvae of all groups were left to feed on the medium until completion of their development when they leave the medium and pupate. All *Drosophila* stocks and crosses were maintained at 25ºC. Only adult flies, without the chromosome balancer (TM3, Sb) with no truncated bristles were analysed.

Scoring of *Warts*:

After metamorphosis, the adult flies were transferred to flasks containing 70% ethanol. Flies were analysed for tumor presence using a Leica stereomicroscope used at a standard magnification of 25 X and an entomological tweezers. Only tumors that were large enough to be unequivocally classified are recorded **(Eeken et al., 2002)**.

Statistical Analysis

The tumor frequency was calculated as the number of tumors/number of *wts* $^{+}$/+ flies **(Eeken *et al*., 2002)**, while tumor induction was calculated as Number of tumors/ Number of tumor flies. The statistical assessment of the gentotoxic potential from tested compounds was identified by the Mann, Whitney and Wilcoxon nonparametric *U* test, using α=0.05 level of significance.

DNA fragmentation by comet assay (single cell gel electrophoresis, SCGE)

Extent of DNA strand breaks in all types of cells of the isogenic strain w^{1118} of *Drosophila melanogaster* were assessed using the alkaline comet assay in adult flies developed from both the untreated (control) and the 2nd instar larvae treated for 24 h with the three tested compounds, basically as described by **Singh *et al*. (1988).** Around 100 adult flies frozen in liquid nitrogen were gently homogenized into powder, and then an alkaline comet assay as described by **Tice *et al*., (2000)** was utilized. 1 g of crushed samples were transferred to 1 ml ice-cold PBS. This suspension was stirred for 5 min and filtered. Cell suspension (100 µ1) was mixed with 600 µ1 of low-melting agarose (0.8% in PBS). 100 µ1 of this mixture was spread on pre-coated slides. The coated slides were immersed in lyses buffer (0.045 M TBE, pH 8.4, containing 2.5% SDS) for 15 min. The slides were placed in electrophoresis chamber containing the same TBE buffer, but devoid of SDS. The electrophoresis conditions were 2 V/cm for 2 min and 100 mA. Staining with ethidium bromide 20µg/m1 at 4ºC. The observation was with the samples still humid, the DNA fragment migration patterns of 100 cells for each dose level were evaluated using Axio fluorescence microscope (Carl Zeiss, Germany) with an excitation filter of 524 nm and a barrier filter of 605 nm. The comets tails lengths were measured from the middle of the nucleus to the end of the tail with 40x increase for the count and measure the size of the comet. Komet 5 image analysis software developed by Kinetic Imaging, Ltd. (Liverpoo1, UK) linked to a CCD camera was used to assess the quantitive and qualitative extent of DNA damage in the cells by measuring the length of DNA migration and the percentage of migrated DNA. Finally, the program calculates tail moment. Generally, 50 to 100 randomly selected cells are analyzed per sample.

RESULTS AND DISCUSSION

Detection of mutagenic Agents Using Somatic Mutation and Recombination Test (SMART) in *D. melanogaster*

The F1 generation of the crossed flies was divided into five treatment groups: a negative control group transferred to basic drosophila medium, positive control group transferred to a medium containing 20µg/ml MMC) and three treatment groups each was transferred to a medium containing 100mM of one of the tested compounds; sodium sulphite, boric acid and benzoic acid.
The frequency of tumors in *wts*/+ negative control flies was 0.07 i.e., 7 flies with one warts per each 100 scored flies. Tumor induction in the negative control was also low (1.1). On the other hand, MMC treatment recorded the highest frequency 1.33 associated with the highest tumor induction (2.18). These tumors were detected in every part of the examined flies.
Comparing the effect of the tested food additives to the negative control showed that: both sodium sulphite and benzoic acid showed statistically significant increase in the frequency of *warts* tumor, while Boric acid treatment showed highly significant increase in tumor frequency Table (1). Tthe tumor induction of spontaneous and induced *warts* Epithelial tumors in +/*wts* flies after treatments can be arranged in descendant manner as Mitomycin C (MMC), Boric acid, Sodium sulphite and Benzoic acid (Fig. 1).

Table 1 Frequencies of induced tumor in trans-heterozygous (*wts*/+) after larvae feeding treatments with three concentrations of Sodium sulphite, Boric acid, and Benzoic acid compared with the MMC as a positive control and negative control.

Treatments	Total No. of Fly Scored	No. of Fly Scored with Tumor	No. of Tumor Scored	Tumor Induction	Frequency (No. of Tumors/fly ± S.D.) [(b)]
Negative Control	950	61	69	1.1	0.07±0.06
MMC 20µg/ml	765	465	1016	2.18	1.33±0.45
Sodium Sulphite 100mM	682	305	474	1.55	0.69±0.11*
Boric Acid 100mM	601	291	486	1.67	0.8±0.14**
Benzoic Acid 100mM	800	490	590	1.2	0.73±0.18*

[(b)]*and ** significant, highly significant difference from the negative control at $P<0.05$ using Mann, Whitney and Wilcoxon nonparametric *U* test. Frequency (No. of Tumor/fly) = Number of tumors/Total number of tested flies. Tumor induction = Number of tumors/ Number of tumor flies.

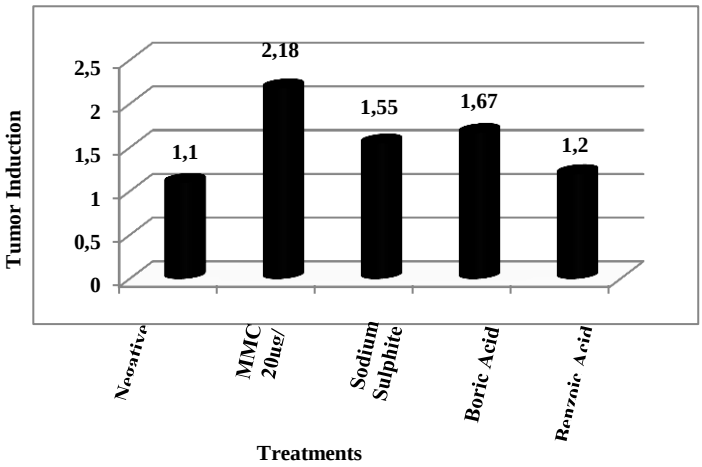

Figure 1 Diagram represents the tumor induction of spontaneous and induced warts Epithelial tumors in +/*wts* flies after treatments with Mitomycin C (MMC), Sodium sulphite, Boric acid, and Benzoic acid.

DNA fragmentation by comet assay (single cell gel electrophoresis, SCGE)

DNA damage was assessed in adults of the homogenic *Drosophila* strain w^{1118} emerged from 2nd larval instar exposed to three tested compounds. According to DNA damage parameters; tailed%, untailed %, tails length, tail DNA% and tail moment, all of the three tested salts caused significant DNA damage Table(2). Moreover, an increase in tail length was observed in sodium sulphite, boric acid and benzoic acid treated groups as compared to control group (Fig. 2), which is a clear indication on DNA degradation and strand breaks. Migration length is considered to be directly related to fragment size and proportional to the level of single stranded breaks and alkali-labile sites **(Tice *et al*., 2000).**

Benzoic Acid had significantly higher deleterious effect on DNA of *D. melanogaster* (8.5±0.58 µm compared with control 1.29±0.10) than Sodium Sulphite (6.90± 0.50 µm tails length) and Boric Acid (5. 34±0.19 µm tails length). Tail DNA percentage was 1.45% in control, 7.11% in Benzoic Acid treatment, 5% in Sodium Sulphite treatment and 4.15% in Boric Acid as recorded in Table (2).

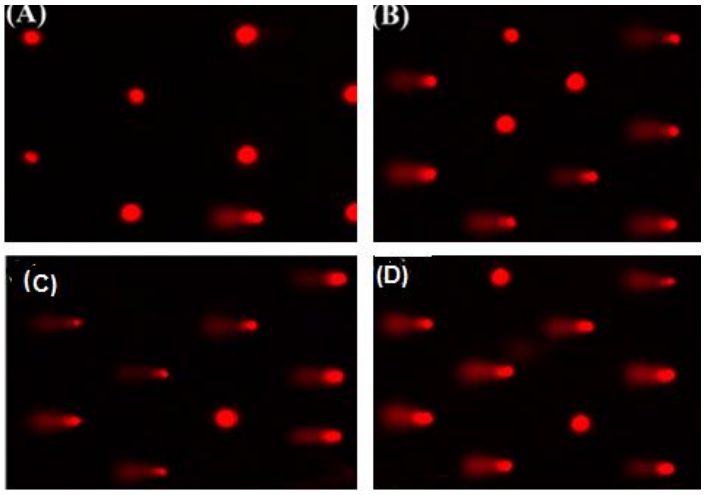

Figure 2 DNA damage in adult *Drosophila* whole body cells, Comet images of DNA strand breaks of control (A), (B) and (C) and (D) DNA damaged after exposure to sodium sulphite, boric acid and benzoic acid respectively.

Table 2 Detection of DNA damage by the comet assay, assessed as tail moment (TM) in whole body cells of white eye adult *Drosophila* treated with the sodium sulphite, boric acid and benzoic acid.

Group	Tailed %	Untailed %	Tails length µm	Tail DNA%	Tail moment
Control	2	98	1.29±0.10	1.45	1.83
P.N P.N Sodium Sulphite	17	83	6.90± 0.50*	5	34.56
S.B Boric Acid	10	90	5. 34±0.19*	4.15	16.12
Benzoic Acid	25	75	8.5±0.58**	7.11	60.15

*and ** significant, highly significant difference from the negative control at $P<0.05$

This study evaluated the potential genotoxicity and carcinogenicity of three food additives commonly used in food industry using SMART test on *Drosophila melanogaster* system and comet assay. The obtained results clearly reveals the genotoxic potential of the tested food preservatives. Formation of tumors in *Drosophila* SMART assay is a strong indication for Loss of heterozygosity (LOH) in somatic cells. The mechanism might involve mutation, chromosome loss or somatic recombination. Further studies will be carried out to investigate the mechanism of LOH. The use of alternative small organisms as models in toxicology has grown tremendously in the last decade. Drosophila has always been a premier model for both developmental biologists and geneticists, however, several recent toxicology studies have used this organism. Currently *Drospohila* is being used in studies of a number of priority environmental contaminants and toxicants **(rand et al., 2015).** The striking resemblance between human and *Drosophila* genes and the presence of numerous highly conserved genes and pathways controlling development of stress response across these two divergent species suggests that the genotoxic potential of the tested might have the same effect on human that cannot be ignored **(Mackay and Anholt, 2006; Misra et al., 2011; Sykiotis and Bohmann,2010)**. In a previous study, **Sarikaya and Solak (2003)** evaluated the genotoxicity of benzoic acid using *D. melanogaster* wing SMART test. They found a positive correlation between total mutation and the number of mutated wings. The genotoxicity of four benzyl derivatives; benzaldehyde, benzyl acetate, benzyl alcohol, and benzoic acid was evaluated by **Demir *et al.* (2008)** using different concentrations (0.1, 0.5, 1, 10, 25 and 50mM) with the aid of the same test. They ordered these compounds according to their genotoxic effect as benzaldehyde, benzyl acetate, benzyl alcohol, and benzoic**. Njagi and Gopalan (1982**) reported that sodium sulphite and sodium benzoate inhibit DNA synthesis and induce the anaphase bridges, chromosome condensation in *Vicia faba* root meristems. The genotoxicity of sodium benzoate on human lymphocytes was studied by **(Patel and Ramani, 2017)** using chromosomal aberration and sister chromatid exchange assay. They concluded that these compounds can induce chromosomal aberration, sister chromatid exchange and decrease the cell cycle proliferation index at 0.5, 1 and 1.5 mg/ml concentrations. Benzoic acid used at a concentration of 500 µg/ml was also found to decrease the mitotic index and increase the frequency of chromosomal aberration in human lymphocytes (**Yelmaz, 2009; Al-Tai, 2014**). The impact of sodium metabisulphite and boric acid on somatic cells of *Vicia faba* L. was studied by **(Pandey and Upadhyay, 2007).** They found a significant decrease in mitotic index and an increase in the abnormality percentage with increasing concentrations. They were also found to stimulate a significant decrease in mitotic index in human lymphocytes **(Meng and Zang, 1992; Rencuzogullari *et al*., 2001).** Results obtained from comet assay have backed up the genotoxic potential of these food additives considering the significant amount of DNA damage in cells treated by the tested compounds compared to negative control. The mechanism of boric acid and borax genotoxicity was assessed in zebrafish *Denio rerio* after 24, 48, 72 and 96-hours acute exposure level to 1, 4, 16, 64 mg/l of each of the tested compounds in semi-static bioassay experiment. Peripheral erythrocytes were drawn from caudal vein and subjected to Comet assay to assess genotoxicity. The amount of DNA damage caused by boric acid was found to be concentration dependent, while that caused by borax was in both concentration and time dependent manner **(Gülsoy *et al*., 2015).** A significant increase in mean tail intensity and mean tail length were observed by **Yilmaz *et al*. (2014)** in human lymphocytes exposed to 50-500 µg/ml concentrations of benzoic acid. In another study, when human male germ cells were exposed to different concentrations 50, 100, 200 and 500 µg/ml of benzoic acid, the results indicated that the concentrations starting from 200 µg/ml showed a significant increase in tail DNA%, tail length and tail moment **(Pandir 2016). Meng *et al*. (2004)** investigated the *in vivo* effects of sodium sulfite and sodium bisulfite on various organs (brain, lung, heart, liver, stomach, spleen, thymus, bone marrow and kidney) of male mice. They found significant increases in DNA damage providing further evidence for a systemic toxic activity of sulfur dioxide derivatives.

CONCLUSION

It can be safely concluded from the present and previous work that all of the tested compounds has a noticeable genotoxic potential that cannot be ignored while using such chemicals in the food industry. Further thorough investigations are recommended before continuing using these substances in food and cosmetics as additives.

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