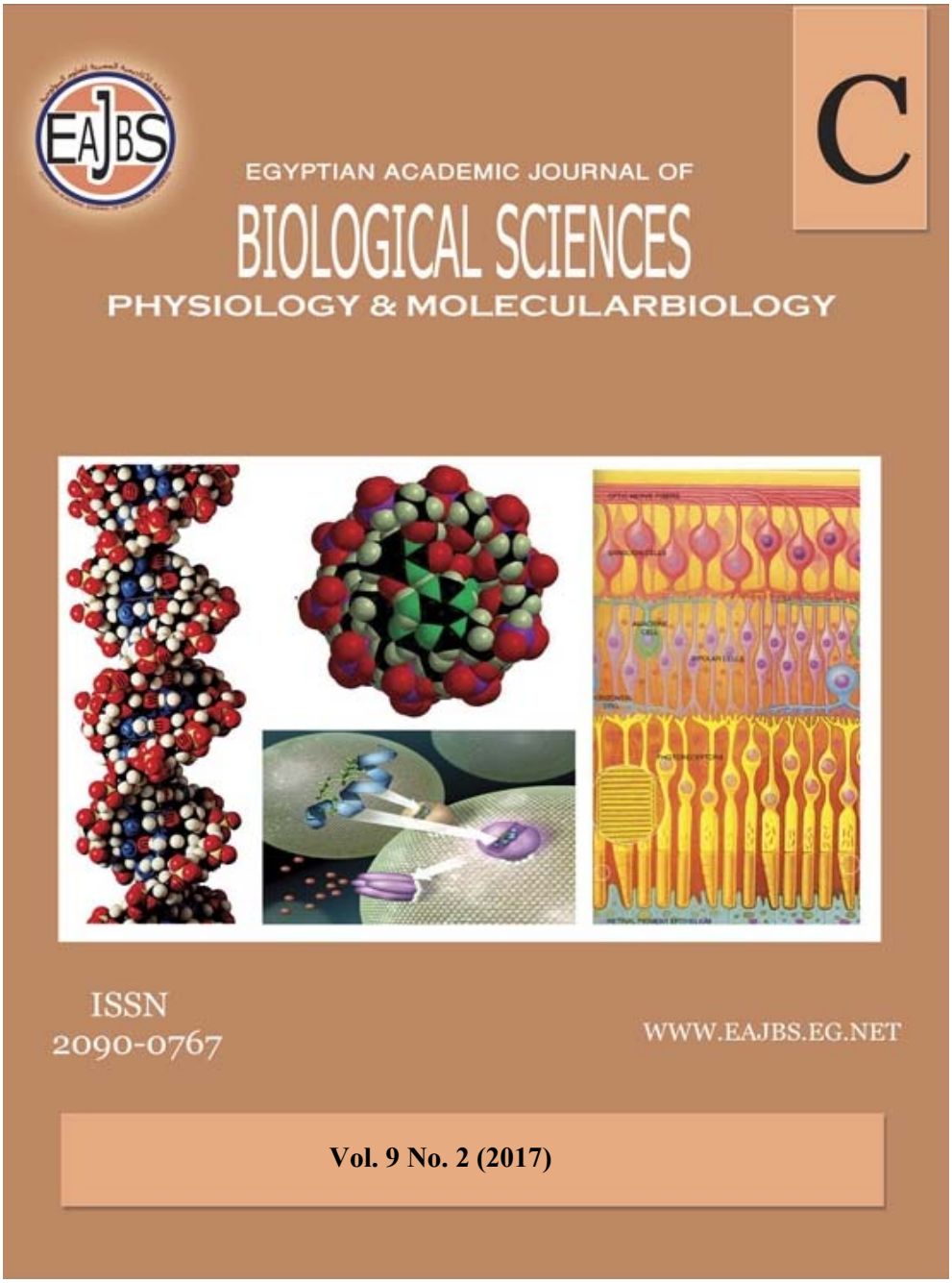

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Carcinogenic and Cytotoxic Effect of Some Food Additives on *Drosophila melanogaster* and Human Cell Lines

Ingy M. El Hefny ,Ayman A. Diab ,Walaa G. Hozayen and Neima K. Al-Senosy
Department of Genetics, Faculty of Agric., Ain Shams University, Cairo, Egypt

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ABSTRACT

Some food additives that are commonly used by humans were recently proved to be mutagenic. It is of significant importance to evaluate their genotoxic effects, since they are frequently consumed by humans in their daily meals. In this proposal, we investigated the effects of sodium sulphite, boric acid, and benzoic acid on human cell lines; liver cancer (HepG2), colon cancer (HCT-116), lung cancer (A-459), and normal lung (Wi38) and cells were evaluated using neutral red cytotoxicity assay and assessed using the somatic mutation and recombination test (SMART). These compounds at 100mM concentrations induced tumor induction and increased the frequency compared to a negative control in SMART assay. Also, they reduced the viability of the four examined cell lines cells using different concentrations (75, 150, 300 and 600µg/ml). Boric acid had the highest toxic effect while benzoic had the lowest on the examined cells. The toxicity effect of the tested food additives was higher on normal lung human cells than on lung cancer cells, therefore, these food additives may act as carcinogenic agents

INTRODUCTION

Food additives are substances that are added to food to extend the time of using by retarding or inhibiting growth of microorganisms. Also, they have other uses including coloring, sweetening, flavouring, and thickening (Rekha and Dharman, 2011). For a long time, no observed adverse effects level have been proved on the basis of toxicological studies. Recently, studies proved that the consumption of some processed foods by additives might have increased the risk of cancer in human although the respected legal limits of these additives by the manufactures. The increased carcinogenicity risk caused by food additives can be due to various factors; interaction of food additives with some food ingredients, food processing may change the chemical formula of food additives to a formula like carcinogenic compound, 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 can induce inhibition of DNA synthesis in *Vicia faba* root, moreover, bridges in anaphase and interphase chromatin erosion nuclei ((Njagi and Gopalan , 1982). Benzoic acid did not induce genetic activity in tests of mammalian

cells (chromosome aberrations in Chinese hamster fibroblast cell line (CHL) and Chinese hamster ovarian (CHO) cells, sister chromatid exchange in human lymphocytes) without metabolic activation (Oikawa *et al*., 1980). On the other hand, benzoic acid had a weak positive increase in chromosomal aberration test in CHO cells (Ishidate *et al*., 1984) and increased the somatic mutations in *Drosophila* SMART test (Sarikaya and Solak, 2003). Also, it increased the chromosomal aberration, sister chromatid exchange, and micronucleus frequency (200 and 500 µg/ml) in human peripheral lymphocytes without changing the pH medium in a dose dependent manner. While the mitotic index decreased as benzoic acid concentration increased (Yilmaz *et al.,* 2009; Al-Tai *et al*., 2014).

Boric acid inhibits the proliferation of prostate cancer cell lines, DU-145, and LNCaP, in a dose-dependent manner. Also, it inhibited non-tumorigenic prostate cell lines, PWR-1E, and RWPE-1, and the cancer line PC-3, but required concentrations higher than observed human blood levels. Using DU-145 cells, boric acid stimulated cell death independent proliferative inhibition, with little effect on cell cycle stage distribution and mitochondrial function (Barranco and Eckhert, 2004). Boric acid addition decreased the genotoxicity and cytotoxicity caused by the anticancer drug (paclitaxel) used for treatments of breast, ovarian, and lung cancers (Turkez *et al*., 2010). Borax, a salt of boric acid, had inhibitory effect on HepG2 cell growth and induced apoptosis in a concentration-dependent manner (Wei *et al.*, 2016).

The Somatic Mutations and Recombinations Test (SMART) in *Drosophila melanogaster* is used successfully to detect carcinogenic compounds. This assay is preferable to researchers because it is rapid, inexpensive, and sensitive to different classes of agents. It uses tumor suppressor gene *warts* which is a homolog to the mammalian tumor suppressor gene *LATS* (Nepomuceno, 2015; Vasconcelos *et al*., 2017). Induction of tumors in *Drosophila* instead of marker clones may 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 include gene mutations in the *wts* gene, multilocus-deletions (partial), chromosomal loss, and somatic recombination collectively referred to as loss of heterozyygosity (Eeken *et al*., 2002).

Fekrazad *et al*., (2017) mentioned that neutral red uptake assay is used for *in vitro* assessment of cytotoxicity of infectious agents, food additives, and pharmaceuticals. This assay has some advantages; it is rapid, economical, semi-automated, and can be used with a variety of cell types to provide quantitated data that can be used to rank test agents according to their potencies (Babich and Borenfreund, 1990).

The objective of this study was to evaluate the genotoxic effect of food additives such as, sodium sulphite, boric acid, and benzoic acid on somatic cells of *Drosophila* and human cell lines.

MATERIALS AND METHODS

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

Drosophila Crosses and Treatments:

For Detection of epithelial tumors in *D. melanogaster*, the *wts*/TM_3 females, the genetic structure of this strain is; *st* p in *ri wtsMT4-1/ TM3 Sb*,. Details about the various markers and the balancer chromosome can be found in Flybase (1999) and Lindsley and Zimm (1992). These *wts*/TM3 females crossed to wild type males. After 2 days, the parental flies were removed and 56-68 hours old larvae were transferred to a standard *Drosophila* medium containing 20 µg/ml

of an appropriate Mitomycin C (MMC) solution for 24 hours, then they were transferred to standard *Drosophila* medium. For food additives (Sodium sulphite, Boric acid, and Benzoic acid) treatments; concentration of 100 mM powder was dissolved in standard *Drosophila* medium at 50^0C. All *Drosophila* stocks and crosses were maintained at 25^0C.

Scoring of *Warts:*

To score tumor of *warts*, later the males and females of the (wts^+/+) genotype, which was wild type to be analyzed for tumor (*wart*) presence. Only tumors that were large enough to be unequivocally classified are recorded. The flies were observed using a Leica stereomicroscope used at a standard magnification of 25 X. Tumors were only included when large enough to be classified unambiguously.

Statistical Analysis:

The tumor frequency was calculated as the number of tumors/number of *wts* $^+$/+ flies (Eeken *et al*., 2002) and Tumor induction = Number of tumors/ Number of tumor flies. The carcinogenic potential from compounds is identified by the Mann, Whitney, and Wilcoxon nonparametric *U* test, using α=0.05 level of significance, to evaluate the significant of difference between negative control and other treatments.

Neutral Red Cytotoxicity Assay:

Neutral red cytotoxicity assay based on the initial protocol described by Borenfreund and Puerner (1984) and modified by Fotakis and Timbrell (2006) was carried out. The cells from mother flasks were seeded in a 24-well microtitre plate (Corning) (1X106) cell/well. The plates were incubated at 37ºC in 5% CO2 for 24 hrs to achieve monolayer confluence. Culture medium containing different concentrations of each chemical compounds (75, 150, 300 and 600 µg/ml) were added in triplicate. Medium without chemical compounds served as untreated control. The dye-medium was removed and the plates were washed with formol-calcium (10 ml 40% formaldehyde, 10 ml 10% anhydrous calcium chloride, and 80 ml water). Five hundred µl of acetic acid-ethanol (one ml glacial acetic acid in 100 ml 50% ethanol) was added and the plates were kept for 15 min at room temperature to extract the dye. Plates were then shacked for a few seconds, so that complete dissolution was achieved. The absorbance of the extracted dye was measured by spectrophotometric reading (Spectra max 190-Molecular devices) using with 540 nm filter. The mean of three measurements for each concentration was determined (n=6). The viability % was calculated for having the concentration of the test chemical reflecting the half maximum concentration of the cell proliferation (IC50).

Calculations and Statistics:

Cytotoxicity assay was measured as optical density at 540 nm. Dose-response curves were plotted, and 50% inhibitory concentrations of plants extracts (IC50) were calculated through Graph Pad Prism software program. Data are presented as mean ± SD. For statistical analysis of data, multiple comparisons were performed using one-way analysis of variance (ANOVA) followed by the LSD test for post hoc analysis. Statistical significance was accepted at a level of $P < 0.05$. Data were analyzed using SPSS.

RESULTS

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

The SMART assay in Drosophila melanogaster has been widely used with many different objectives. In the present study, we evaluated the carcinogenic potential of some food additives such as, sodium sulphite, boric acid, and benzoic acid. In the treatment procedure of that test, the larvae are treated for 24 h in vials containing medium with the test

compound at a certain concentration. Then they were transferred to standard Drosophila medium. The larvae feed on this medium until completion of their development when they leave it and pupate.

Data in Table (1) showed that investigation of five groups of crosses as following: a negative control group, MMC 20µg/ml, sodium sulfite 100mM, boric acid 100mM, and benzoic acid 100mM. The frequency of tumors in *wts*/+ negative control flies was 0.07 i.e., 7 flies with one warts in 100 flies scores nearly. Tumor induction in a negative control was 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.

Concerning the effect of sodium sulphite on the frequency of *warts* tumor was statistically significant, showed increase (0.69), where the tumor induction was (1.55) as shown in Table (1). Boric acid treatment highly increased in tumor frequency (0.8) and was statistically highly significant, which showed (1.67) tumor induction. In case of benzoic treatment, the frequency of tumor and tumor induction were 0.73 and 1.2, respectively. 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 as observed in (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 Tumor/fly)
Negative Control	950	61	69	1.1	0.07
MMC 20µg/ml	765	465	1016	2.18	1.33
Sodium Sulphite 100mM	682	305	474	1.55	0.69*
Boric Acid 100mM	601	291	486	1.67	0.8**
Benzoic Acid 100mM	800	490	590	1.2	0.73*

*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.

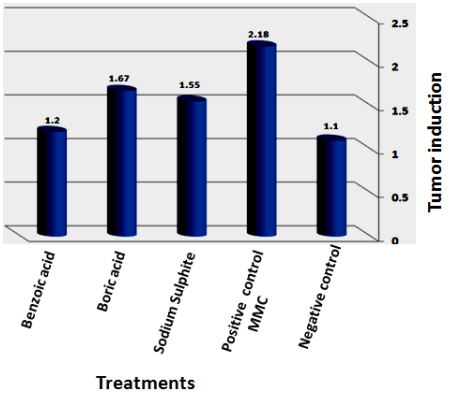

Fig. (1): Diagram represents the tumor induction of spontaneous and induced warts Epithelial tumors in +/*wts* flies after treatments with Mitomycin C (MMC), Sodium sulphite, Boric acid, and Benzoic acid.

***In vitro* Assay for Cytotoxic Activity Human Cell Lines (Neutral Red Assay):**

The effects of four different concentrations (600, 300, 150 and 75 µg/ml) of the three food additives; sodium sulphite, boric, and benzoic on the proliferation of colon cancer cells in comparison to a positive control (3µg/ml) were determined using the neutral red cytotoxic assay.

In general, the cell viability was decreased gradually as the concentration of the three tested food additives increased as illustrated in Table (2). The cytotoxicity and cell viability of sodium sulphite, boric, and benzoic with the concentrations (75, 150, 300 and 600 µg/ml) and a positive control 3µg/ml were evaluated *in vitro* against human liver cell lines (hepatoma cells HepG2). The viability of positive control was 62.85%, and the viability of HepG2 was reduced as the concentration increased of the three tested food additives, but the reduction was non-significant in sodium sulphite and benzoic. The significant reduction in the viability was observed in boric at 300 and 600 µg/ml. The Dose inducing 50% cell growth inhibition (IC50) against hepatoma cell line cells (HepG2) is presented in Table (2) and Dose-response curves for cell viability in Figure (2).

In human colon cell lines (HCT 116), a positive control attained 44.8% of viability. Boric was the most effective on the viability reduction among the three tested food additives where the all concentrations had significant effect while benzoic was the lowest without significant change. The IC50 values were 1219, 870.6, and 1986 in sodium Sulphite, boric, and benzoic, consequently, as shown in Table (2) and illustrated in Figure (3).

Lung cancer (A-459) treated with different concentrations (75, 150, 300 and 600µg/ml) of sodium sulphite, boric, and benzoic in addition to positive control (3µg/ml) were checked on the viability as shown in Table (2). The viability affected by positive control was 53.8%. The viability reduced significantly by sodium sulphite at 300 and 600µg/ml while benzoic, the viability slightly decreased without significant change. Using boric, the viability decreased significantly as the concentration increased except at the lowest concentration (75µg/ml). As mentioned above, boric acid gave the highest toxic effect among tested food additives. IC50 were 3637 in sodium sulphite, 1617 in boric, and 3289 in benzoic as observed in Table (2) and Dose-response curves for cell viability in Figure (4).

Data in Table (2) showed that cell survival rate of normal lung cell line (Wi38) was affected by sodium sulphite, boric, and benzoic with different concentrations and positive control (3µg/ml). The viability affected by positive control was 61.8%. Normal cell line (Wi38) viability did not induce significant change with 75 and 150µg/ml of the three tested food additives. Like A459, the viability in Wi38 was affected negatively with the concentration of the three tested food additives but the reduction in viability was higher than those in A-459. Dose-response curves for cell viability of human normal ling cell line treated with sodium sulphite, boric acid, and benzoic acid using a typical neutral red cytotoxicity, as shown in Figure (5).

Table (2): The cell viability percentage and IC50 of human cell lines tested by Sodium sulphite, Boric acid, and Benzoic acid compared with Positive control using neutral red cytotoxicity assay.

Human Cell Line	Concentration By µg/ml	Viability %			Positive Control 3 µg/ml
		Sodium Sulphite	Boric Acid	Benzoic Acid	
Liver Cancer (Hep G2)	75	101	98.8	100	62.85
	150	100	81.5	98	
	300	95.3	76.6*	97.6	
	600	86.5	71.5*	92.0	
IC50		**1532**	**1209**	**3079**	
Colon Cancer (HCT 116)	75	92.9	80*	99	44.8
	150	89*	77*	97.16	
	300	83.96*	73.9*	91.7	
	600	75.94*	66*	90	
IC50		**1219**	**870.6**	**1986**	
lung Cancer (A459)	75	100	93.4	100.4	53.8
	150	95.3	86.7*	98.6	
	300	92.8*	81.2*	94.3	
	600	85.5*	77*	90	
IC50		**3637**	**1617**	**3289**	
Normal Cell Line (Wi38)	75	101	104	100	61.8
	150	95.6	96.9	96.9	
	300	78*	76*	86.9*	
	600	72.4*	64*	84.5*	
IC50		**1523**	**1240**	**2500**	

* Significant difference from the negative control at $P<0.05$ using one-way analysis of variance (ANOVA).

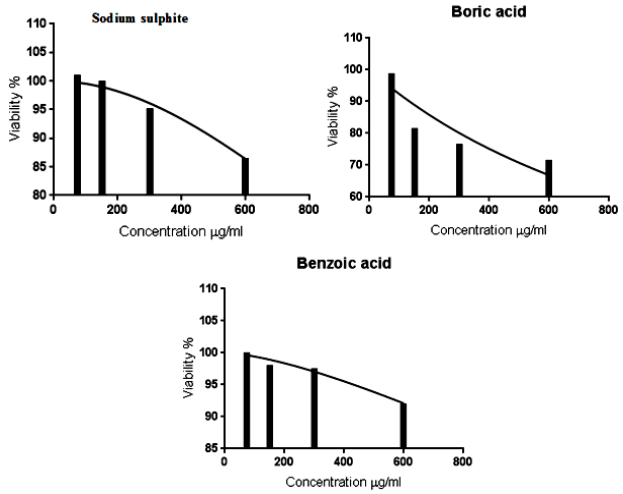

Fig. (2): Dose-response curves for cell viability of human liver cancer (Hep G2) cell lines treated with sodium sulphite, boric acid, and benzoic acid using a typical neutral red cytotoxicity.

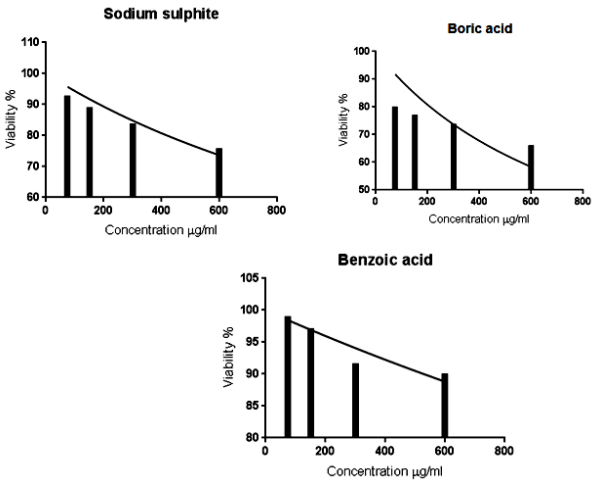

Fig.(3): Dose-response curves for cell viability of human colon cancer (HCT-116) cell lines treated with sodium sulphite, boric acid, and benzoic acid using a typical neutral red cytotoxicity.

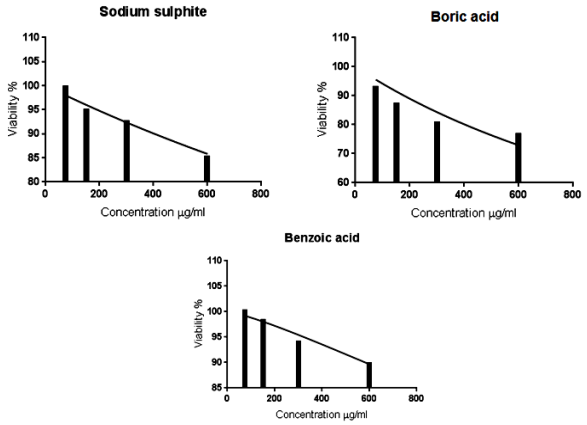

Fig. (4): Dose-response curves for cell viability of human lung cancer (A459) cell lines treated with sodium sulphite, boric acid, and benzoic acid using a typical neutral red cytotoxicity.

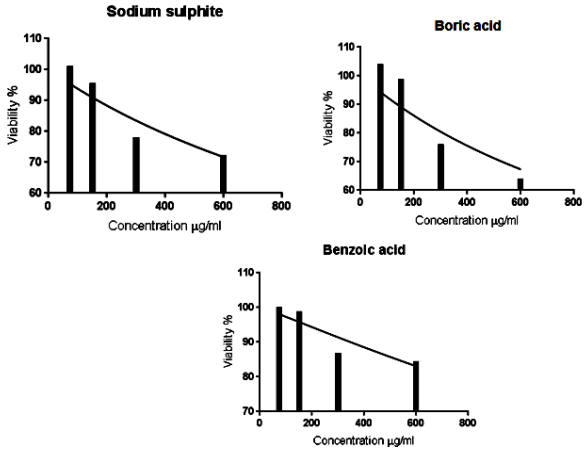

Fig. (5): Dose-response curves for cell viability of human normal lung (Wi38) cell lines treated with sodium sulphite, boric acid, and benzoic acid using a typical neutral red cytotoxicity.

DISCUSSION

This study evaluated the mutagenicity and carcinogenicity of three food additives commonly used in food industry by testing SMART of *D. melanogaster* and cytotoxicity of these food additives on some human cell lines cells. Loss of heterozygosity (LOH) in somatic cells is a major step involved in the formation of tumors can be determined by SMART assay in *Drosophila*. Not only does the mechanism involve mutation, but also chromosome loss and somatic recombination. The genetic events leading to LOH can be induced in cells of the imaginal disks of *Drosophila* larva and the resulting changes scored as clones of mutant cells in the adult. It is known to us the cell cycle in imaginal disk cells is very similar to that in somatic mammalian cells, and many of the regulation genes are participated between human and *Drosophila*. So, we cannot ignore the negative effect of these compounds with that concentration on the human health. From the results, it was obvious that the cytotoxicity of sodium sulphite, boric, and benzoic at 100mM concentration on *D. melanogaster*. In this trend, Sarikaya and Solak (2003) evaluated the genotoxicity of benzoic acid with 50, 75 and 100mM in the wing SMART of *D. melanogaster*. They found a positive correlation between total mutation and the number of mutated wings. Also, Demir *et. al*,. (2008) evaluated the genotoxicity of four benzyl derivatives; benzaldehyde, benzyl acetate, benzyl alcohol, and benzoic acid at different concentrations (0.1, 0.5, 1, 10, 25 and 50mM) used as flavor ingredients in the wing SMART of *D. melanogaster* 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.

Regarding human cell lines cells, for benzoic, the concentration 75- 600 µg/ml have no dangerous effect on all human cell lines checked except at 300 and 600 µg/ml on Wi38. Also, sod. sulphite is not toxic on Hep-G2 till 600µg/ml concentration. Moreover, the concentrations 75 and 150µg/ml of sod. sulphite are not toxic on the four examined cells except HCT 116, the 150µg/ml is toxic. The toxicity of sod. sulphite starts from higher than 150 µg/ml. The toxicity degree among the four checked cell lines can be ranked in decreasing order as colon cancer (HCT166), Wi38, A459, and Hep-G2. The genotoxicity of sod. benzoate on human lymphocytes was studied by (Patel and Ramani, 2017) using chromosomal aberration and sister chromatid exchange assay. They concluded that sod. benzoate can induce chromosomal aberration and sister chromatid exchange at 0.5, 1 and 1.5 mg/ml concentrations. Also, it can decrease the cell cycle proliferation index. Benzoic at 500 µg/ml decreased the mitotic index and increased the frequency of chromosomal aberration in human lymphocytes (Murli, 2003; Al-Tai, 2014). The impact of sod. metabisulphite and boric 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. Sod. metabisulphite stimulated a significant decrease in mitotic index in human lymphocytes (Meng and Zang, 1992; Rencuzogullari *et al*., 2001).

Boric and its derivatives like borax are used as preservatives in foods and medicines but these compounds became of harmful effect on human health at high consumption (See *et al*., 2010). The

possible lethal doses in babies are in range of 3-6g, whereas 15-20g in adults (Litovitz *et al*., 1988**).** In our present studies, the concentration 75 µg/ml of boric is not dangerous on Hep-G2, A459 and Wi38. The HCT 116 scored the highest toxicity where IC50 was 870 as affected by boric. This indicates that boric hasn't only have an effect on cancer human cells but also on normal cells. In addition to that, boric can cause tumor in normal lung cell line (Wi38) rather than antitumor in lung cancer (A459) cells. In this field, Hep-G2 cells were affected by borax lead to inhibit proliferation and promotion of apoptosis, using MTT and annexin V/P1 staining, respectively, (Weil *et al*., 2016). Another study, Centurk *et al*., (2016) evaluated the effect of boric on an acute leukemia cell line (HL-60) and healthy human lymphocytes using MMT, Neutral Red, transmission microscope, and flow cytometry methods. They noticed that boric at 500µM concentration caused double nucleus and micronucleus formation in both HL-60 cells and lymphocytes in addition to appearance of an expansion in mitochondrial dimension and deformation in cristas. Kumar and Srivastava (2011) observed that boric at (0.25, 0.5, 0.75 and 1%) used for 3 hours produced mitoinhibitory effect and increase in chromosomal aberrations in root tips of *Trigonella foenum-graecum*. The most observed aberrations were stickiness at metaphase and anaphase, scattering at metaphase and bridges at anaphase.

The mechanism of boric and borax genotoxicity was assessed on zebrafish *Denio rerio* for 24, 48, 72 and 96-hours acute exposure level; 1, 4, 16, 64mg/l in semi-static bioassay experiment. Peripheral erythrocytes drawn from caudal vein were used and Comet assay was applied to assess genotoxicity. The genotoxicity for boric was found as concentration dependent and borax as concentration and time dependent manner. The highest damage in DNA was at 96h for borax and 24h for boric concentrations in peripheral blood of *D. rerio*. For boric, the maximum increase in % tail DNA at 24h and reduction at 48, 72 and 96h were seen. However, the reduction in 96h values, it was still higher than negative control level at all doses. This limited decrease indicated that cytoprotective and tolerant mechanisms or repair of damaged DNA in the cell (Gülsoy *et al*., 2015). On the other hand, Murmu *et al*., (2002) reported that the two new boron compounds, guanidine biboric acid and dihydroxy boron hydrochloride monohydrate adduct have antitumor effect. Also, Gallordo-Williams *et al.*, (2003) observed that mice receiving 1.7 or 90mg/kg daily dosage of boric produced a decrease in tumor size by 38% and 25%, respectively.

It could be concluded that boric acid has the highest toxic effect among the three studied compounds on *Drosophila* SMART, Hep-G2, HCT116, A459, and Wi38 human cell lines. These compounds induce higher toxic effect on normal lung (Wi38) cells than in lung cancer (A459) cells; this indicates that they have cytotoxic activity. However, sodium sulphite, benzoic, and boric are genotoxic in the other short-term genotoxicity tests. For this reason, it is necessary to be careful when using these substances in food and cosmetics as additives.

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ARABIC SUMMARY

السميه الوراثيه لمضافات الغذاء علي بعض الكائنات النموذجية التأثيرات

انجي الحفني، ايمن دياب، ولاء حزين، ونعمه السنوسي
١- قسم الوراثة – كلية الزراعة – جامعة عين شمس

بعض المضافات الغذائية المستخدمة لها تأثيرات مطفرة. لذلك، كان من الضروري تقييم تأثيرات السمية الوراثية لها لانها تستهلك بواسطة البشر. وفي هذا البحث تم تقييم سلفيت الصوديم ، حمض البوريك و حمض البنزويك علي بعض خطوط خلايا الإنسان المسرطنة؛ مثل خلايا سرطان الكبد (HepG2)، خلايا سرطان القولون (HCT-116)، خلايا سرطان الرئة (A-459) و الخلايا الطبيعية للرئة (Wi38) وتقييم السمية الخلوية تم بإختبار neutral red cytotoxicity. علاوة على ذلك، تم تقييم القدرة على إحداث التسرطن بإستخدام إختبار قياس الطفرات الجسمية والعبور الوراثي (SMART) في الدروسوفيلا. أظهرت النتائج إستحثاث تكوين الأورام وزيادة في معدلها مقارنتا بالكنترول السالب وذلك عند تركيز 100mM في إختبار SMART. وأيضا ، حدث خفض في حيوية الأربعة أنواع من خلايا الإنسان المستخدمة وذلك عند تركيزات مختلفه (75, 150, 300 and 600µg/ml). ولكن ، أظهر حمض البوريك تأثير سمي وراثي ومسرطن أعلي بينما البنزويك أقلهم تأثيرا على الخلايا. التأثيرات السمية الوراثية للمضافات الغذائية المختبرة كانت عالية علي خلايا الرئة الطبيعية أكثر من خلايا الرئة المسرطنة ، ولذلك، هذه المضافات الغذائية ربما تكون عوامل مسرطنة.