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Do synthetic food additives possess higher genotoxic effect than natural ones?

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Many health risks occur as a result of the use of synthetic food additives in industry on a large scale that was illustrated by a lot of health organizations and cancer organizations. So this study was investigated the genotoxicity of nine food additives that include different classes of synthetic and natural additives (Colors, Preservatives, Antioxidants and Sweeteners) on rat lymphocyte cells. The genotoxicity was estimated by using alkaline comet assay while the cytotoxicity was determined by Trypan blue exclusion method and the IC_{50} was calculated for each compound. Significant genotoxicity by comet assay appeared as tail % and tail moment for DNA strand breaks and migration of its fragmentations from the head (chromosomes in nuclease) by electrophoretic mobility on low melting agarose gel. Most of natural food additives showed no genotoxicity at the tested concentrations, while most of the corresponding synthetic food additives gave significant genotoxicity at the same concentrations. However carminic acid as natural color showed genotoxic effect similar to its corresponding synthetic one (Erythrosine). Isoamyl acetate and Butylated hydroxyl anisole (BHA) as synthetic additives have no genotoxic effect. Aspartame at 6 µg/ ml and 10 µg/ ml revealed a higher significant ($p<0.05$) DNA damage.
Synthetic food additives (Erythrosine, Potassium sorbate and Aspartame) and Carminic acid as natural food color cause DNA strand breaks of lymphocyte cells that leads to dangerous health effects.

Keywords: Synthetic and natural food additives, cytotoxicity, genotoxicity, alkaline comet assay.

INTRODUCTION

Erythrosine (disodium salt of tetra iodofluorescein) an artificial red dye (cherry-pink) vastly used in food, pharmaceutical and cosmetic colouring made from coal tar (Combes and Haveland-Smith, 1982). According to the World Health Organization iodine may be affect thyroid which leads to thyroid tumors. Erythrosine gives positive results with chromosomal aberration test and negative results with Ames test **(**Ishidate et al., 1984). In recent studies there is a great interest in published genotoxicity data on erythrosine which proved that erythrosine induces DNA damage in stomach, colon, lung and bladder (Sasaki et al., 2002)**.** On the contrary, Carminic acid has negative result as genotoxic compound when it was tested by in vitro micronucleus test on human peripheral lymphocyte cells with and without the post-mitochondrial fraction (S9) (Geissel, 2014).

Potassium sorbate is one of most used food preservatives in juices, cakes, wine and personal care products. Potassium sorbate gives positive result with *in vitro* chromosomal aberration test when treated a Chinese hamster fibroblast cell line at 4mg/ml for 48h (Ishidate et al., 1984). It reduces mitotic index to more than 50% of control value at concentration 0.04M, it also cause sister chromatid exchange and chromosomal aberration at concentration 0.005M (Abe and Sasaki, 1977).

Additionally potassium sorbate causes DNA strand breaks in blood lymphocytes by using alkaline comet assay (Mamure et al., 2010). Eugenol is the major component of clove oil that reduces the genotoxic effect of cyclophosphamide, procarbazine,N-methyl-N-nitro-N-nitrosguanidine and urethane by conducting *in vivo* micronucleus assay on bone marrow cells. There is no genotoxicity when eugenol is administered to mice alone (Abraham, 2001). Furthermore clove oil has not genotoxicity on human embryo lung cells (HEL12469) after 24h (Puskarova et al., 2017). However, Isoamyl acetate (Banana oil) does not induce chromosomal aberrations at concentration 2mg/ml for 48h on a Chinese hamster fibroblast cell line (Ishidate et al., 1984).

Butylated hydroxyanisol (BHA) at concentration 1×10^{-4}M reduces mitotic index to more than 50% of control value and at 0.001M lead to stop mitosis and causes sister chromtid exchange at concentrations 1×10^{-6}M ,1×10^{-5}M and1×10^{-4}M (Abe and Sasaki, 1977). On the other hand, BHA has negative result in chromosomal aberration test on chinese hamster cells *in vitro* (Ishidate and Odashima, 1977). Also, BHA makes chromosome aberrations in Chinese hamster ovary (CHO) cells when the S9 (metabolic activator) incubates with it but BHA at concentrations do not exceed 300 µM, it does not affect the chromosomes of CHO cells (Philips et al., 1989). In the other hand, Vitamin C is an antioxidant and free radicals scavenger (Shamberger, 1984 and Anderson, 1996). Its usefulness as antioxidant is important for protection of DNA from oxidative damage by various agents (Stich et al., 1979). Also it has antitumor effect against many clastogenic agents like cyclophosphamide (Gebhart et al., 1984 and Ghaskadbi et al., 1992). Vitamin C at 10 and 100 µg/ml does not alter the mitotic index of human lymphocytes but cisplatin is anti-tumor agent which used to investigate the anti-clastogenic effect of vitamin C at 0.5 µg/ml reduces the mitotic index and induces chromosomal aberrations. While inhibitory effect of cisplatin on the mitotic activity and chromosomal aberrations reduce in pre-treatment, simultaneous and post-treatment of vitamin C (Nefic, 2001).

Aspartame is a methyl ester of a dipeptide made out of aspartic and phenylalanine. Aspartame causes a chromosomal aberrations in human lymphocytes at (500, 1000 and 2000 ppm) after 24h and 48h and it doesn't induce sister chromatid exchange (Rencüzoğulları et al., 2004). Sorbitol has anticancer activity mentioned in a lot of studies, one of these examinations is that sorbitol induces apoptosis in human colon cancer cell line (HCT116) (Lu et al., 2014).

MATERIALS AND METHODS

Chemicals

Medium RPMI 1640 and Ficoll for cell separation were purchased from Lonza Company, Belgium. Fetal bovine serum was bought from life sciences Company (Brazil). Chemicals of alkaline comet assay were gotten from sigma. Potassium sorbate and L-ascorbic acid was obtained from ALPHA CHEMIKA Company, Cairo, Egypt. Isoamyl acetate (Banana oil) with purity degree 98% was purchased from LOBA Chemie Company. Clove oil was brought from Becht Company. BHA was brought from Moly CHEM, Mumbai, India. Aspartame was purchased as product which is called diet sweets from New Emad pharmacy, Giza, Egypt and Sorbitol (70%) was purchased from El-Gomhoria Chemical Company, Cairo, Egypt.

Materials concentration, Erythrosine and Carminic acid were tested at the same concentrations 25, 50 and 100 µg/ml. Also Potassium sorbate, Isoamyl acetate and Clove oil were used at 200 and 250 µg/ml, as well Potasssium sorbate was tested at 300 µg/ml. BHA and Vitamin C were investigated at 5 and 10 µg/ml as well Vitamin C was used at 50 µg/ml and Aspartame was tested at 4, 6 and 10 µg/ml while Sorbitol was used at 200, 300 and 500 µg/ml. Further positive control was Doxorubicin at 1 µg/ml.

Isolation of blood lymphocyte cells from male rats

Cells were separated using gradient centrifugations by centrifuging blood with specific liquid with specific density (ficoll) at 2000 rpm/ 20 min. Buffy coat layer was appeared and isolated then washed by phosphate buffer saline (PBS) (Boyum, 1968).

Viability evaluation

The viability of lymphocyte cells was measured in both normal and treated cells using the Trypan blue exclusion method (Cavalcanti, 2008).

In vitro alkaline comet assay

The alkaline comet assay was evaluated according to Singh et al., (1988) with little adjustments and following suggestions as described in Tice et al., (2000). After cells were

isolated from blood, cells were incubated with different concentrations of different food additives for 12h under proper sterilized conditions 5% CO_2 and suitable humidity. Cells were washed by PBS then cells were re-suspended 0.75-1% low dissolving agarose and they were spreaded onto slides that were covered by 1% typical agarose. Agrose was leaved for solidify at 40C for 5min then slides were placed in lysis buffer (2.5 M NaCl, 10 mM Tris, 100 mM EDTA, 1% Triton X-100 and 10% DMSO, pH=10) at 40ºC overnight. Slides were placed in unwinding buffer (300 mM NaOH and 1mM EDTA, pH>13) at 40ºC for 40mins then electrophoresis was occurred for 20 mins at 25V (1.0 V/cm), and 300 mA . After electrophoresis step, slides were neutralized (0.4 M Tris, pH=7.5) then were stained with ethidium bromide 20 µg/ml. Furthermore, the tail intensity % and tail moment were assessed by utilizing Comet examine IV software.

Statistical Analysis

Data were showed as means ±SE. Statistical analysis was occurred by using one way analysis of variance (ANOVA) followed by Duncan's multiple range test to get difference between negative control, different treatments and positive control.

RESULTS AND DISCUSSION

Cells viability

The effect of nine food additives on the viability of rat lymphocyte cells were given in figure 1 using Trypan blue exclusion method and the IC_{50} values were illustrated in table 1as follows:

The viability of cells was measured after incubation with the tested food additive for 12h at different concentrations. Results showed in Fig. 1 a clear decreased in viability with increasing of concentration. The cells death % = 50% that is expressed IC_{50} was calculated for each compound by using Graph Pad Prism version 6 (Table 1). Natural food additive Carminic acid has IC_{50} =1438 µg/ml and it is higher than that of erythrosine as synthetic food color (185.6 µg/ml). Vitamin C as natural antioxidant has no cytotoxicity for cells and the IC_{50} was obtained at concentration 1000 µg/ml and the same trend was found by Sorbitol as natural sweetener (possess IC_{50} =1123 µg/ml). Both values are significantly higher than synthetic ones (BHA as synthetic antioxidant and Aspartame as synthetic sweetener). However, Clove oil showed less viability compared with the other natural additives. Therefore, the natural food additives have no effect on the viability of live cells in contrast to the synthetic ones.

Alkaline comet assay

Genotoxicity of Erythrosine and Carminic acid as food colors

DNA damage parameters of rat lymphocyte cells that exposed to two food colors are given in figure 2 using alkaline comet assays.

Rat lymphocytes cells were treated with Erythrosine and Carminic acid for 12h at different concentrations (25, 50 and 100 µg/ml) were illustrated in figure 3. Results analysis showed that Erythrosine at 100 µg/ml gives higher significant ($p<0.05$) for tail intensity % and tail moment of DNA in cells than other concentrations as well as the standard doxorubicin (positive control). The present data agreed with the results of Ishidate et al., (1984) and Sasaki et al., (2002) who detected a genotoxic effect of Erythrosine. However, Geissel (2014) showed no appearance of micronuclei (negative result) by micronucleus assay on human peripheral lymphocytes at 1230, 2460 and 4920 µg/ml of carminic acid for 4h with and without S9 fraction. However, the effect on DNA by the Carminic acid obtained in the present study (Figure 2) may be due to cells from rats instead of human as well as the change in concentration of compound.

Genotoxicity of Potassium sorbate, Isoamyl acetate and Clove oil

Food preservatives and flavors as natural and artificial additives were tested on DNA of lymphocytic cells and the obtained results are illustrated in figure 3. The DNA damage parameters of rat lymphocytes that exposed to food additives showed clear tail intensity and tail moment using alkaline comet assay.

Data given in figure 3 illustrate that potassium sorbate causes DNA damage at 300 µg/ml, which has the significant ($p<0.05$) compared with positive control (Doxorubicin 1µg/ml). Also, potassium sorbate has significant effect at 250 µg/ml and no significant effect at 200 µg/ml that appears via parameters of DNA damage (tail moment and tail %). The present data agreed with the results of Mamur et al., (2010) who represented potassium sorbate causes DNA damaging of peripheral blood lymphocytes by using comet assay.

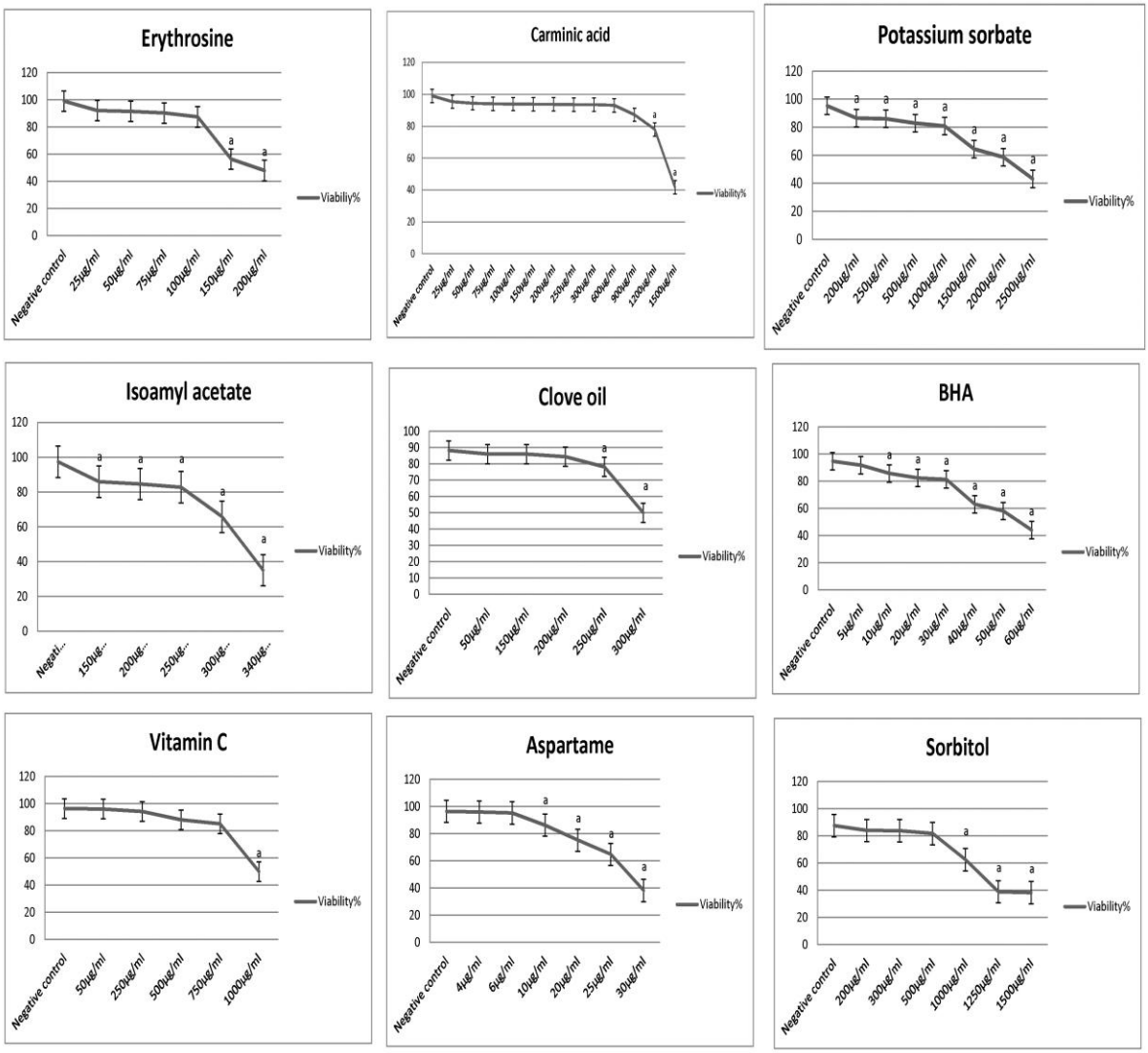

Figure 1 Cell viability % of nine food additives (synthetic and natural compounds)
[a]: Significant different from negative control at *p<0.05*.

Table 1 IC_{50} of the tested food additives

	Food additive	IC_{50} (µg/ml)
1	Erythrosine	185.8
2	Carminic acid	1438
3	Potassium sorbate	2436
4	Isoamyl acetate	320.7
5	Clove Oil	299.0
6	Vitamin C	1000
7	BHA	53.59
8	Aspartame	28.16
9	Sorbitol	1123

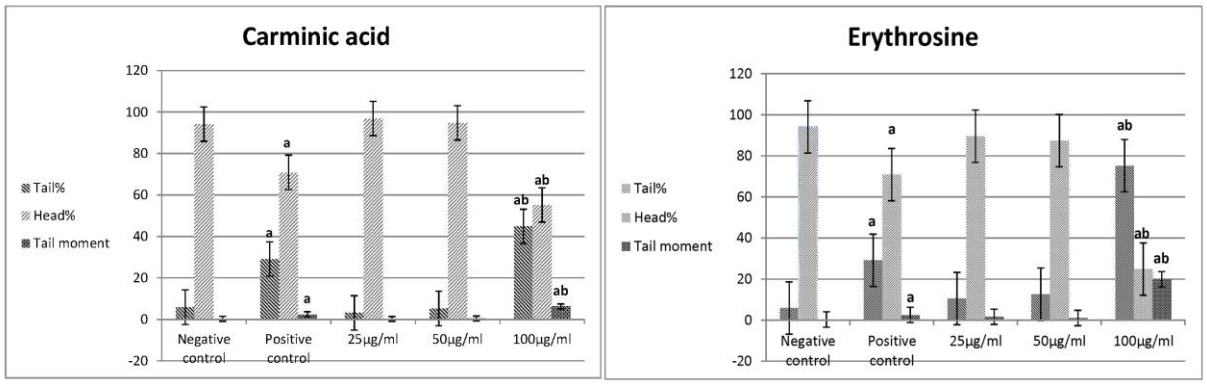

Figure 2 Comet analysis of lymphocyte cells exposed to different concentrations of Erythrosine and Carminic acid at different concentrations

(Differences between treatment and control were conducted with Duncan's multiple range test after one-way ANOVA. Negative control was PBS and Positive control was Doxorubicin 1µg/ml)
[a]: Significant different from negative control at *p<0.05*.
[b]: Significant different from positive control at *p<0.05*.

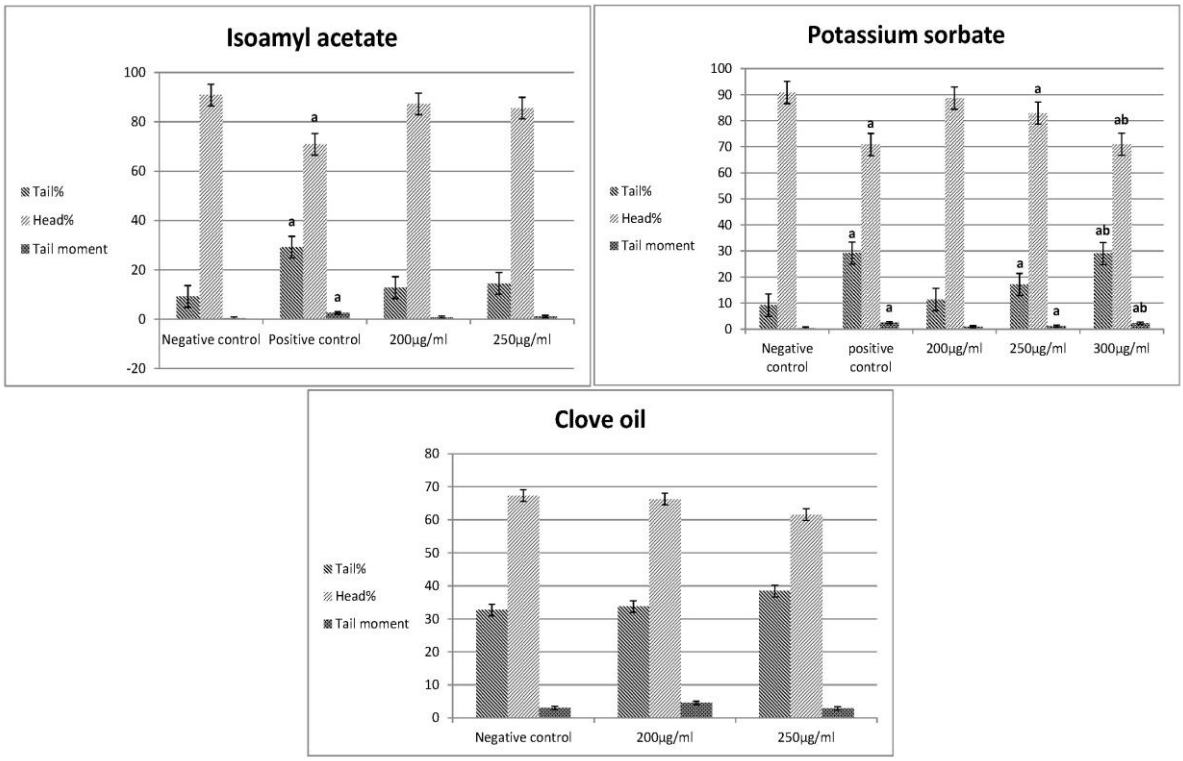

Figure. 3 Comet analysis of lymphocyte cells exposed at different concentrations of Potassium sorbate, Isoamyl acetate and clove oil

(Differences between treatment and control were conducted with Duncan's multiple range test after one-way ANOVA. Negative control was PBS and Positive control was Doxorubicin 1µg/ml)
[a]: Significant different from negative control at *p<0.05*.
[b]: Significant different from positive control at *p<0.05*.

On the other hand, Isoamyl acetate as synthetic flavor and Clove oil natural preservative and flavor are not genotoxic at tested concentrations (200 and 250 µg/ml) also Isoamyl acetate has no significant harmful effect by comparing with Doxorubicin (1µg/ml) as a Positive control. Ishidate et al., (1984) revealed that Isoamyle acetate does not induce any chromosomal aberrations at concentration 2000 /ml for 48h on a Chinese hamster fibroblast cell line likewise Puskarova et al., (2017) revealed that Clove oil has not genotoxicity on human embryo lung cells (HEL12469) after 24h at 0.0025-0.2 ppm.

Genotoxicity of BHA as synthetic antioxidant and Vitamin C as natural antioxidant

DNA damage parameters of rat lymphocyte cells that exposed to two food antioxidants, Vitamin C and BHA are given in figure 4 using alkaline comet assay.

Butylated hydoxyanisole has no significant genotoxic effect at 5 and 10 µg/ml when compared with Doxorubicin at 1µg/ml which possesses high significant effect (Figure 4). The same negative results appear with Vitamin C at 5 µg/ml, 10 µg/ml and 50 µg/ml. These results do not agree with the data showed by Abe and Sasaki (1977) and Ramadan and Takayoshi (2012) who revealed the genotoxicity of BHA the conflicting may back to the difference of tested concentrations and tested cells type. While data of Vitamin C in the present study agree with Nefic (2001) who illustrated that the vitamin C does not alter the mitotic index of human lymphocytes. However, it reduces the clastogenic effect of cisplatin which reduces the mitotic index and induces chromosomal aberrations by pre-treatment, simultaneous and post-treatment of vitamin C.

Genotoxicity of Aspartame as synthetic sweetener and Sorbitol as natural sweetener

The DNA damage parameters of rat lymphocyte cells that exposed to two food sweeteners, Aspartame and Sorbitol are given in figure 5 using alkaline comet assay.

In the present study, Aspartame has high significant ($p<0.05$) genotoxic effect at 10 µg/ml compared with negative control and positive control .Also another concentration 6 µg/ml gives significant genotoxic effect compared with negative control but tested concentration 4 µg/ml has no significant effect (Fig. 5). These data confirmed with the results of Rencüzoğulları et al., (2004) who showed that Aspartame decreases the mitotic record at 500, 1000 and 2000 µg/ml after 48h and induces micronuclei at 2000 µg/ml after 24h and 48h. Sorbitol results do not possesses genotoxicity at 200, 300 and 500 µg/ml while positive control has a higher significant ($p<0.05$) at tested concentration of 1 µg/ml.

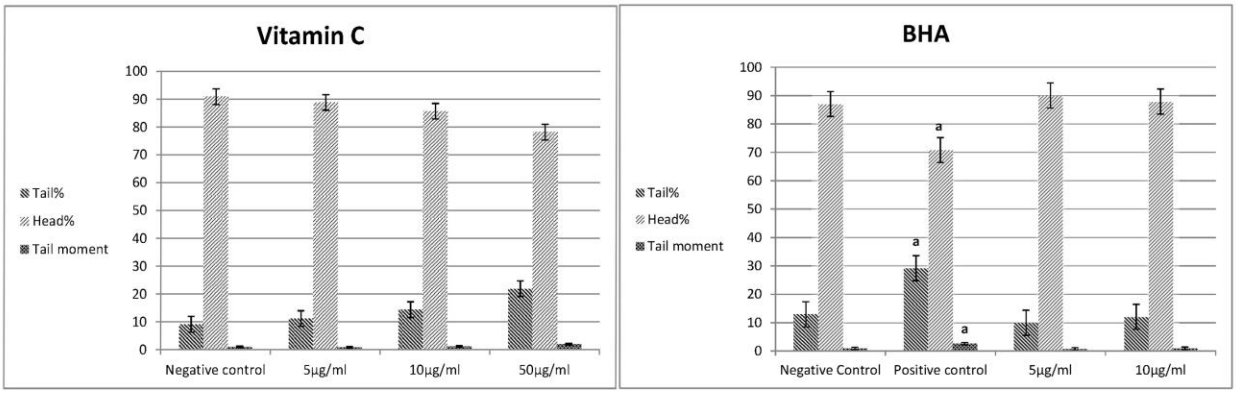

Figure . 4 Comet analysis of lymphocyte cells which exposed to different concentrations of Vitamin C and Butylated Hydroxy Anisol (BHA)

(Differences between treatment and control were conducted with Duncan's multiple range test after one-way ANOVA. Negative control was Tween 80 which is used in emulsification of BHA and Positive control was Doxorubicin 1µg/ml)
[a]: Significant different from negative control at *p<0.05*.
[b]: Significant different from positive control at *p<0.05*.

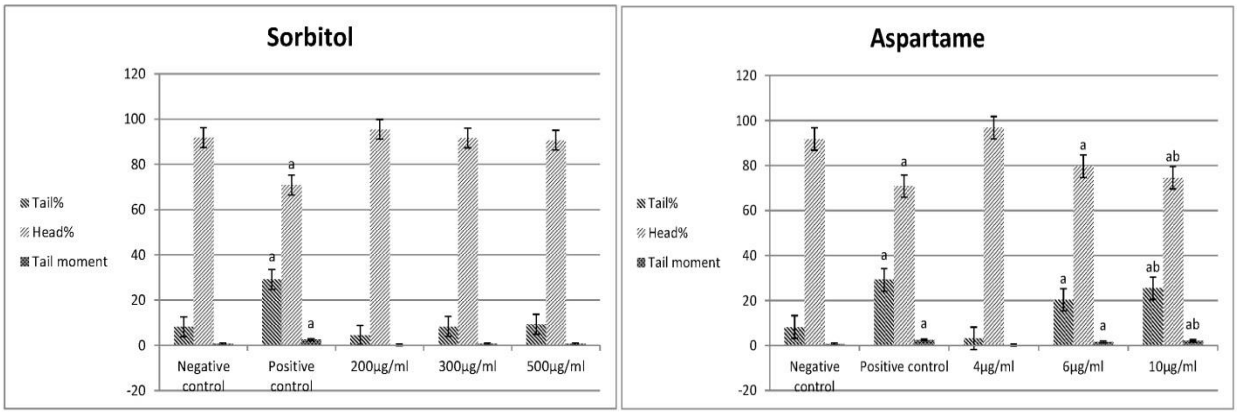

Figure .5 Comet analysis of lymphocyte cells which exposed to different concentrations of Aspartame and sorbitol

(Differences between treatment and control were conducted with Duncan's multiple range test after one-way ANOVA. Negative control was PBS and Positive control was Doxorubicin 1µg/ml)[a]: Significant different from negative control at *p<0.05*.
[b]: Significant different from positive control at *p<0.05*.

CONCLUSION

As a conclusion, the analysis of data that were obtained from sensitive and reliable assay (alkaline comet assay) shows the harmful influence of a lot of synthetic food additives. Erythrosine, Potassium sorbate and Aspartame affect DNA which is very critical molecules in our bodies. It is known that any harmful effect on DNA is usually the main reason of lethal disease such as Cancer. So we must pay great attention to these compounds and try to replace them by natural additives. On the other hand, the other synthetic food additives that appeared safe and non genotoxic need to be supported by testing on another type of cells in addition to *in vivo* studies to be clear and certain they are safe.

CONFLICT OF INTEREST

The authors declared that there no conflict of interest.

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AUTHOR CONTRIBUTIONS

All authors contribute to achieve this work and all authors read and approved the final version.

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