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Genotoxic Effects and Liver Toxicity in Swiss Albino Mice Males after Acute and Chronic Exposure of Diacetyl and Butter flavors

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Abstract

Since the mid-1950s, the volatile structure of butter oil and butter were researched, and an exhaustive list of elements has been collected. Diacetyl is an aromatic popular synthetic fragrance that gives food a buttery taste used in ice cream, snacks and potting with butter, strawberry, caramel, or cheese flavor. The chromosomal aberrations and micronuclei are commonly used biomarkers of chromosomal damage, genome stability, and cancer risk assessment. In vivo trials are still important to assess the genetic toxicology of chemical products such as industrial chemicals, pharmaceuticals, and food additives. This study aimed at assessing the potential genotoxic effect of diacetyl and butter flavors on swiss albino mice using alterations in liver function enzymes, micronucleus (MN), and chromosomal aberrations (CA) assays. The results showed that exposure of swiss albino mice males to diacetyl and butter flavors induced (CA) and (MN) in a statistically highly significant manner compared to the control. Meanwhile, the biochemical analysis revealed that these substances caused an exceptional rise in liver function enzymes (AST, ALT, and ALP) activity in serum of treated experimental animals. In conclusion, both tested compounds have increased the chromosomal aberration, micronucleus test, and serum levels of liver function enzymes indicating their high potential of being cytotoxic and genotoxic materials.

Keywords: Diacetyl, Butter flavors, micronuclei, chromosomal aberrations, liver function enzymes.

1 Introduction

As food is one of the essential human needs, artificial flavors and fragrances plays a pivotal role in food Industry (Msagati 2013). In recent decades, the evolution of new technologies in this industry has steadily incorporated chemical additives into daily consumed diets (Sales et al 2018). Unfortunately, the inclusion of additives to maintain or boost various parameters usually ignores their effect on human health. Moreover, their behavior as well as the approaches to diagnose their health threats are subjected for many speculations, contradictions, uncertainty, and are the topic of ample discussion (Carocho et al 2014).

Diacetyl, widely known as 2,3-diketobutane or 2,3-butadione, is a volatile ketone that imparts the odor and flavor of butter to food (Hubbs et al 2002, Harber et al 2006, Whittaker et al 2008). It can be used as a liquid, powder, or paste in additives (Hewitt 2015). It is usually associated with dairy products e.g.,

butter, margarine, yogurt, sour cream, and several cheeses giving it its distinctive buttery aroma (Clark and Winter 2015). Diacetyl is included in the Food and Drug Administration (FDA) Generally Recognized as Safe (GRAS) list and is commonly used at amounts of 6−9 μg / g (Shibamoto 2014).

Excessive use of food additives might affect the liver functions and impose hepatotoxicity. Increases in the activity of both ALT (located in the cytoplasm) and AST (located predominantly in organelles such as mitochondria) suggest damage to both hepatic and mitochondrial cell membranes. (Senthil et al 2003, Amin and Al-Shehri 2018). The activities of hepatic serum enzymes (AST and ALT) increased in rats administrated with food colorants particularly at high doses, suggesting elevated permeability, injuries, and impairment of the hepatic cells (Senthil et al 2003). Moreover, rats treated with a high dose of tartrazine or carmoisine showed significant increase in the enzymatic activities of ALT, AST, and ALP, while the low dose displayed a significant rise in the ALT and alkaline phosphatase activities as compared to the control rats (Amin et al 2010). In another Study sodium nitrite and, sodium benzoate increased the serum levels of aspartate aminotransferase "AST" and, alanine aminotransferase "ALT" in male rats (Radwan et al 2020).

The consumption of processed foods and broth containing some food additives might increase the risk of cancer in humans, despite the allowed limits of such additives in foods. Genotoxicity studies are conducted to detect the risk of gene mutations, change in number or structure of a cell, and chromosomal or DNA damage caused by a medical device, biomaterial, or their extracts (Gultekin et al 2015). Previous studies demonstrated that artificial food flavoring was able to induce chromosomal aberrations in mice bone marrow cells, *Vicia faba*, Allium cepa and Chinese hamsters' cells (Matsuoka et al 1979, Pandey and Upadhyay 2007, Tripathy and Rao 2015, Hassan and Jasem, 2020). several other studies assayed the genotoxicity of food flavorings using micronucleus assay in human lymphocytes, Chinese Hamster Ovary-K1 (CHO-K1) cells, Fetal lung fibroblast cells (MRC-5), normal human corneal epithelial cells (HCEC) and immortalized human keratinocytes cells (HaCaT) (Mamur et al 2012, Lestari et al 2017, Banti et al 2019).

Therefore, this study aimed at assessing the in vivo genotoxicity of the food flavors, diacetyl and butter flavors using liver enzymes along with the micronucleus and chromosomal aberration assays in bone marrow cells of male mice.

2 Materials and Methods

2.1 Chemicals

Diacetyl (2,3-butadione) and butter flavor (Diacetyl, 4-Methyl –2-pentanol, Ethyl butyrate, Butyric acid, Hexanoic acid, Octanoic acid, Δ- Decalactone,Δ- Dodecalactone) were kindly provided by Life Essence, (ElAsher of Ramadan industrial city, Egypt). The dose was prepared shortly before administration (50 ppb) in (1ml) corn oil and orally administered using two treatment strategies, acute (for one week) and chronic (for four weeks). Mitomycin-C was purchased from Sigma-Aldrich Chemical Co. (St. Louis MO, USA).

2.2 Animals

Eighty swiss Albino male mice (20-25 g) were procured from National Cancer Institute – Egypt (NCIE) and checked by a vet. Each Five males were randomly housed in a cage. Mice were left to acclimate in laboratory environment with free access to food and water for one week before the onset of the experiment. Light-dark photoperiod was maintained at 12:12 h in a controlled environment temperature of 25±2 ℃ and a relative humidity of 40±5% according to (Jedlicka et al 2018).

The Medical Research Ethics Committee (MREC) reviewed and accepted the animal-based Animal Procedures Act (1986) and related recommendations, EU Animal Test and

Animal Use Directive 2010/63/EU and was applied in compliance with the World Medical Association Code of Ethics for animal experimentations. The national regulations on animal treatment and use were compiled by (Ball 1986).

2.2.1 Experimental design

The mice used in the current work were randomly divided into four groups.

- Group1 (G1): (n=10) this group was treated with 1 ml of distilled water by gavage as a control.
- Group 2(G2): (n=15) This group was treated with an acute dose of diacetyl (for one week) ، butter flavor and corn oil (50 ppb in 1ml corn oil) by gavage.
- Group3(G3): (n=15) This group was treated with a chronic group dose of diacetyl (for four weeks) ، butter flavor and corn oil (50 ppb in 1ml corn oil) by gavage
- Group4(G4): (n=5) This group was used as positive control group mice(24h) mice were treated with positive control dose of MMC (0.5 mg/kg) by intraperitoneal injection.

2.3 liver Enzymes

Determination of aspartate aminotransferase (AST) activity and alanine aminotransferase (ALT) activities in serum was carried out according to (Bergmeyer et al 1978). Determination of alkaline phosphatase (ALP) activity in serum was done according to the method of demonstrated by (Duncan et al 1984, Moss 1982).

2.4 Bone marrow chromosomal aberration assay

Well spread metaphase bone marrow cells were prepared for examination for chromosomal aberration according to the procedure described by (Preston et al 1987). Cells were examined using a vertical fluorescence microscope (Leica DM2500) equipped with a cooled digital color camera (Leica DFC340FX).

2.5 Micronucleus assays using bone marrow

Slides of bone marrow were prepared and stained for micronucleus assay according to the method described by (Krishna and Hayashi 2000, Agarwal and Chauhan 1993).

The examination was performed using a vertical fluorescence microscope (Leica DM2500) equipped with a cooled digital color camera (Leica DFC340FX).

2.5 Statistical analysis

Data was gathered, coded, revised, and included in version 23 of the statistical Package for Social Science software (SPSS). The quantitative data were presented as mean, standard deviations and ranges, and the comparison between groups was applied using the One-Way ANOVA Analysis of Variance test followed by a post hoc analysis with the LSD test. The confidence interval was set to 95% and the margin of error accepted was set to 5%. and the p-value was considered significant at $p < 0.05$.

3 Results and Discussion

The investigation of the genotoxic effects of Diacetyl and butter flavors in male mice was evaluated by the detection of liver function enzymes, micronucleus frequency in Polychromatic erythrocyte (PCEs), and chromosomal aberrations in the bone marrow. Sign of sickness indicated by increased activity in mice (Hyperactivity), no mortality was observed with the rats used during the study.

3.1 Liver Enzymes

Results in **Table 1** showed that there was a highly significant change in the ALT, AST, and ALP activity in mice treated with diacetyl, and significant butter flavors compared with between the control group male mice.

After one-week (acute exposure), ALT activity was elevated in male mice exposed to diacetyl in the acute exposure study group 61.90 ±2.16 U/L and 45.20 ±1.24 U/L of butter flavors male mice group compared with the control group 26.96±0.95 U/L. While AST activity was with significant changes between the male mice in the control group 37.32±1.07 U/L and the male mice in the acute study group

Table 1. Effect of diacetyl and butter flavors on serum alkaline phosphatase ALP activity, serum alanine amino transferase ALT activity and serum aspartate amino transferase AST activity in male mice for acute treatment and chronic treatment

Ttreatment	**ALT level**	**AST level**	**ALP level**
	(Mean ± SEM)	(Mean ± SEM)	(Mean ± SEM)
Control	26.96 ±0.95	37.32 ±1.07	227.10 ±6.63
Diacetyl (acute treatment)	61.90 ±2.16**	63.84 ±1.65**	352.25 ±11.23**
Butter flavors (acute treatment)	45.20 ±1.24*	50.88 ±1.20*	280.20 ±9.48*
Control	26.25 ±1.00	37.67 ±1.08	234.21 ±7.65
Diacetyl (chronic treatment)	69.65 ±1.75**	70.78 ±1.27**	384.12 ±7.04**
Butter flavors (chronic treatment)	51.18 ±1.38*	63.84 ±1.65*	296.52 ±8.53*

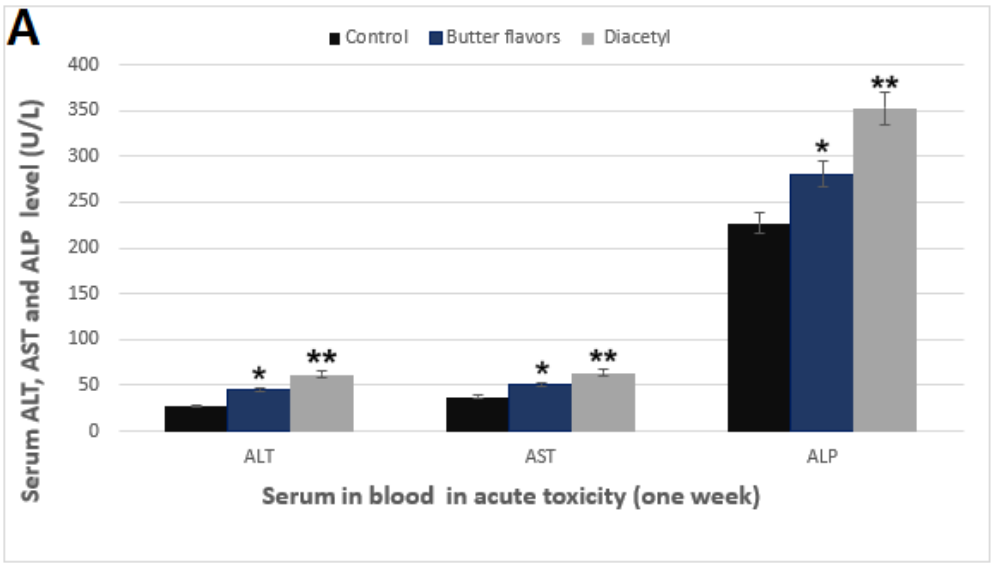
*Significant difference from the control group (*P < .05). **Hight Significant difference from the control group (**P < 0.01).

63.84 ±1.65 U/L and 50.88 ±1.20 U/L. Also, ALP activity increased with significant changes between the male mice in the control group 227.10±6.63 U/L and the male mice in the acute study groups 352.25 ±11.23 U/L and 280.20 ±9.48 U/L show **Fig 1, A.**

Furthermore, **Fig 1, B** clarifies the effect of diacetyl and butter flavors for four weeks (chronic treatment), ALT activity elevated with significant changes between the male mice in the control group 26.25 ±1.00 U/L and the male mice in the chronic study group 69.65 ±1.75 U/L and 51.18 ±1.38 U/L. While AST activity was elevated with significant changes between the male mice in the control group 37.67 ±1.08 U/L and the male mice in the chronic study group 70.78 ±1.27 U/L and 63.84 ±1.65 U/L. Also, ALP activity was elevated with significant changes between the male mice in the control group 234.21±7.65 U/L and the male mice in the acute study groups 384.12 ±7.04 U/L and 296.52 ±8.53 U/L.

3.2 Chromosomal aberrations assay (CA)

Different kinds of chromosomal aberrations, including structural and numerical aberrations, were observed in the metaphase stage of bone marrow cells. In bone marrow cells, the structural aberrations included ring chromosomal, deletion, gaps, fragment, acentric and, centromeric fusion (**Fig. 2 B, C, D, E, F, and G**). Chromatid gaps and breaks were noted to be more frequent than other aberrations in acute and chronic diacetyl and butter flavors treatments. Polyploidy is the observed numerical aberrations that include variations in the number (**Fig. 2 H)**. The increased frequency of chromosomal aberrations (CA) with increasing the time of the treatments compared with the positive control was observed**.** MMC stimulated a highly significant increase of chromosomal aberrations (34 ± 2.9) after 24 h administration of MMC (**Table 2**). Also, the results showed that treatment of diacetyl refers to a height significant increase in the mean values of the individual and total chromosomal aberration. The mean values after acute treatment at one week was (39.000 ± 3.2) and at chronic were (70.333± 4.1). While the mean values of acute and chronic treatment of butter flavors (35.667 ± 2.2, 70.333± 6.9 respectively) show (**Fig. 3 A, B**).



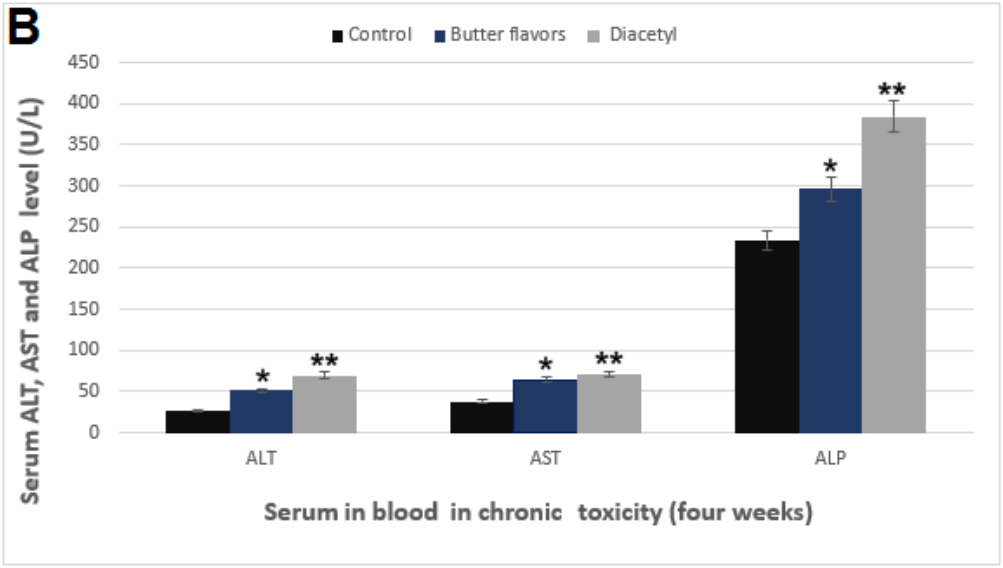


Fig 1 A. Effect of diacetyl and butter flavors on serum alkaline phosphatase ALP activity, serum alanine amino transferase ALT activity and serum aspartate amino transferase AST activity in male mice for one week (acute toxicity). **(B)** Effect of diacetyl on serum alkaline phosphatase ALP activity, serum alanine amino transferase ALT activity and serum aspartate amino transferase AST activity in male mice for four weeks (chronic toxicity). Using One Way ANOVA analysis using LSD test $P > 0.05$: Non-significant, *$P < 0.05$: Significant, **$P < 0.01$: Highly significant from the control group.

Table 2. Chromosomal aberrations of male mice in bone marrow cells treated with diacetyl, butter flavors, and MMC.

			Percentage of types of chromosomal aberration										
Time	Treatment	Total no. of division cells	Gaps	Breaks	acentric	chromosmal ring	chromatid break	sister chromatid union	Centromeric fusion	Fragments	Deletion	Polyploidy	Total abnormal Metaphases Mean ±SEM
24h	MMC	300/3	±0.6 5.000	±0.6 5.000	±0.6 2.000	±0.5 3.000	±1.8 2.667	±0.3 1.667	±1.2 3.333	±0.9 3.333	±0.3 5.333	±1.5 3.000	±2.9** 34.333
one week	Control	300/3	±0.3 1.333	±0.3 1.333	±0.3 0.667	±0.7 0.667	±0.7 1.667	±0.6 1.000	±0.3 1.333	±0.9 1.33	±0.9 1.667	±0.6 1.000	±2.1 12.000
	Oil	300/3	±0.6 2.000	±0.6 2.000	±0.6 1.000	±0.7 0.667	±0.3 1.667	±0.3 0.667	±1.2 2.000	±0.7 1.667	±0.9 1.667	±0.7 1.667	±2.1 15.000
	Butter Flavors	300/3	±1.2 6.000	±1.2 6.000	±0.9 2.333	±0.7 1.667	±0.3 2.333	±0.6 2.000	±0.6 4.000	±1.2 3.667	±0.7 4.667	±0.6 3.000	±2.2** 35.667
	Diacetyl	300/3	±0.9 7.333	±0.9 7.333	±0.6 2.000	±0.6 2.000	±0.9 3.333	±0.7 2.333	±0.6 3.000	±0.9 4.667	±0.9 4.333	±0.3 2.667	±3.2** 39.000
24h	MMC	300/3	±0.6 5.000	±0.6 5.000	±0.6 2.000	±0.5 3.000	±1.8 2.667	±0.3 1.667	±1.2 3.333	±0.9 3.333	±0.3 5.333	±1.5 3.000	±2.9** 34.333
four weeks	Control	300/3	±0.9 1.333	±0.9 1.333	±0.6 1.000	±0.9 1.333	±0.3 1.667	±0.3 1.333	±0.7 0.667	±0.3 1.333	±0.9 1.667	±0.3 0.667	±0.7 12.333
	Oil	300/3	±1.2 3.000	±1.2 3.000	±0.7 1.667	±0.6 2.000	±0.9 1.667	±0.9 1.333	±0.7 1.333	±0.6 2.000	±1.8 2.667	±0.7 2.333	±2.5 21.000
	Butter Flavors	300/3	±2.7 12.667	±2.7 12.667	±1.2 3.667	±1.8 5.333	±0.6 4.000	±1.2 5.333	±2.4 6.333	±1.2 6.667	±1.5 7.667	±1.5 6.000	±6.9** 70.333
	Diacetyl	300/3	±1.2 10.000	±1.2 10.000	±0.9 6.667	±1.5 5.667	±1.5 7.000	±1.5 5.333	±0.9 5.333	±0.9 7.667	±1.5 7.667	±2.1 5.000	±4.1** 70.333

*Significant difference from the control group (*P < .05). **Hight Significant difference from the control group (**P < 0.01).

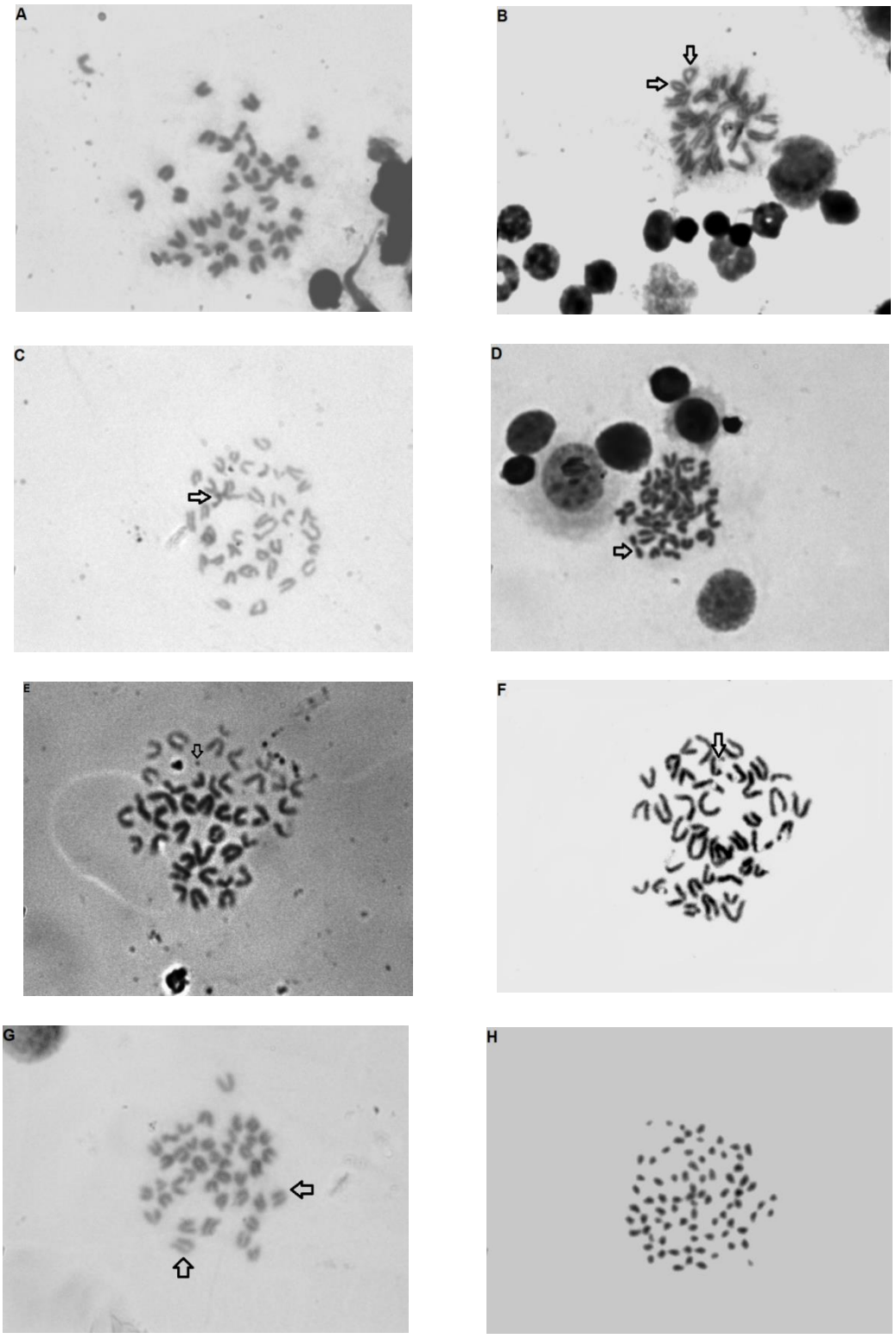
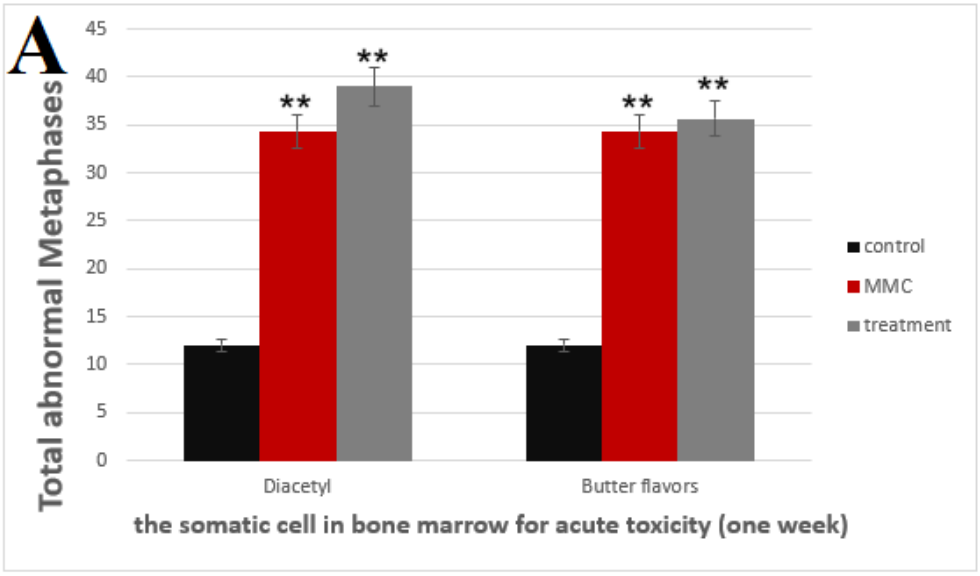


Fig 2. Bone marrow cells of mice treated with diacetyl at metaphase showing different types of chromosomal aberrations **(A)** Normal **(B)** ring **(C)** Centromeric fusion **(D)** Gap **(E)** Fragment **(F)** Deletion **(G)** Acentric **(H)** Polyploidy



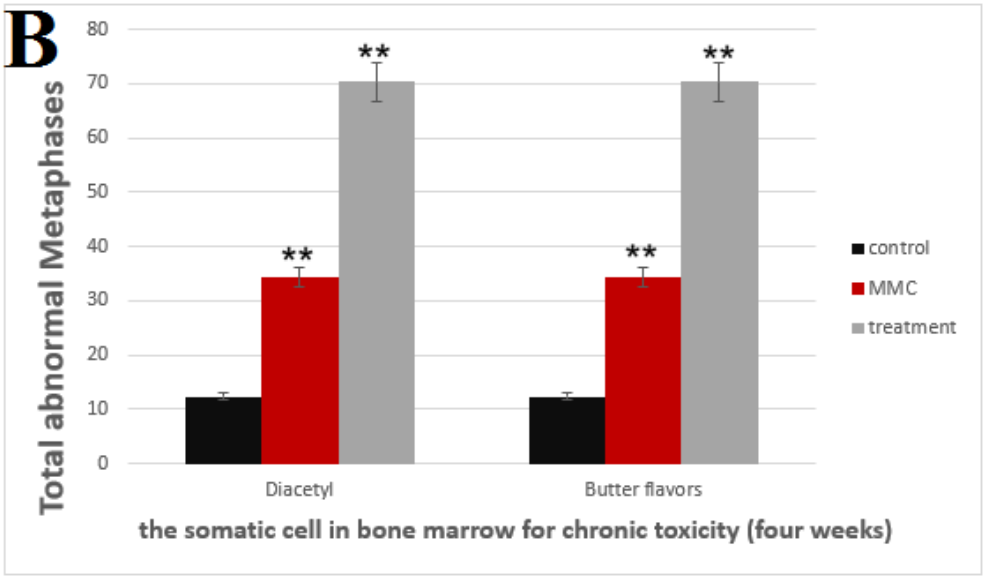


Fig 3 A. Total aberrations in diacetyl and butter flavor after acute treatment. (**B**) Total aberrations in diacetyl and butter flavor after chronic treatment. Using One Way ANOVA followed by Post Hoc analysis using LSD test P > 0.05: Non-significant, *P < 0.05: Significant, **P < 0.01: Highly significant from the control group. MMC is a positive control.

3.3 Micronucleus assay (MN)

The examination of bone marrow slides of male mice exposed to diacetyl and butter Flavors revealed the high frequency of occurrence of micronuclei **Fig 4**. In comparison with their respective controls, the PCE/total erythrocytes ratio exhibited high significant differences diacetyl acute and chronic treatment (22.667± 2.6, 30 ± 5.5 respectively). On the other hand, the mean frequency of micro-nucleated cells appeared with butter flavors in acute and chronic treatment (21 ± 2.1, 27± 3.2 respectively) **Fig 5 A, B**. While the mean value of MMC was (17.000± 3.2) (**Table 3**).

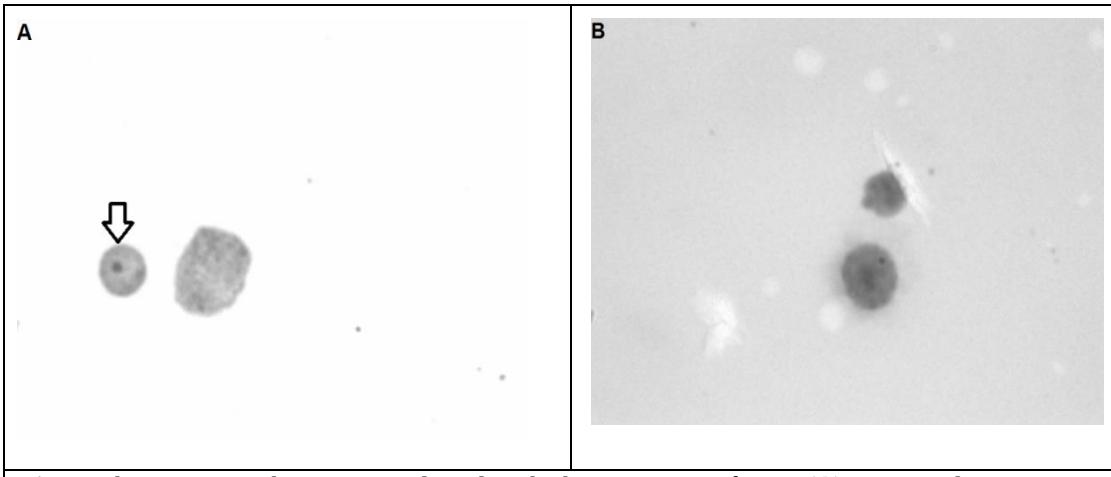
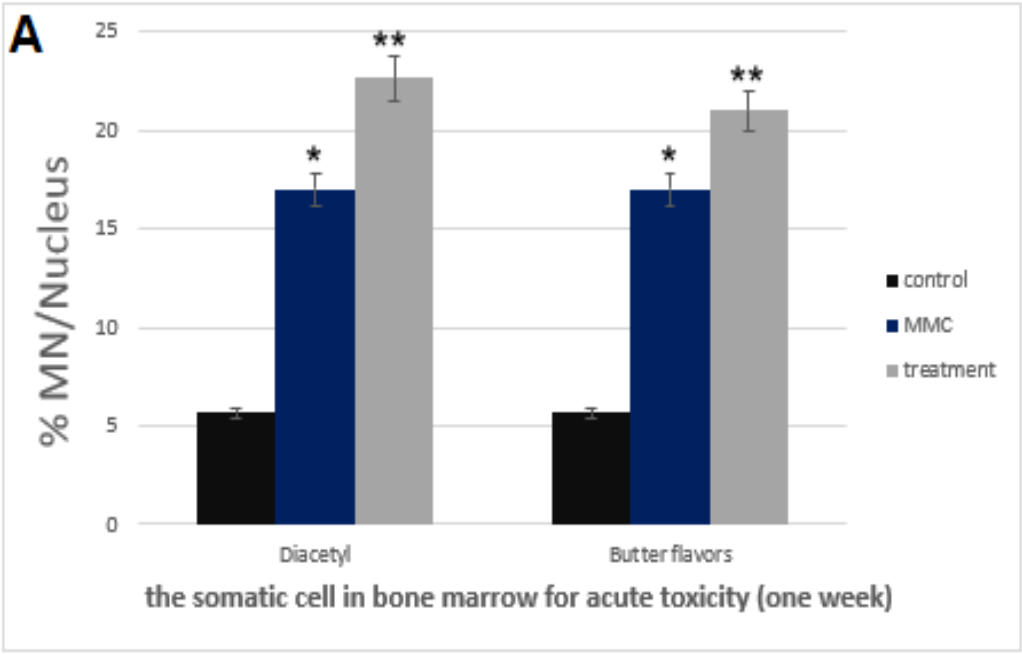


Fig. 4. Photomicrograph at micronucleated in the bone marrow of mice **(A)** Micronucleus in a PCE **(B)** NO micronucleus in a PCE.



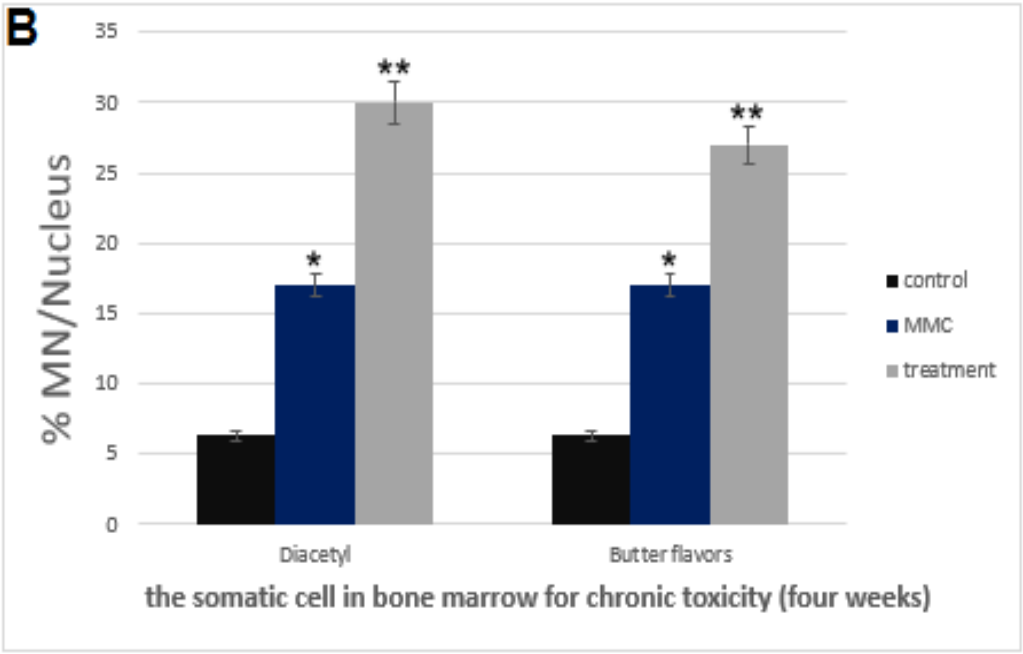


Fig. 5. (A) Micronucleates cell counts at a bone marrow of mice with diacetyl and butter flavor after acute treatment **(B)** Micronucleates cell counts at a bone marrow of mice with diacetyl and butter flavor after chronic treatment. Using One Way ANOVA followed by Post Hoc analysis using LSD test P > 0.05: Non-significant, *P < 0.05: Significant, **P < 0.01: Highly significant from the control group. MMC is a positive control.

Table 3. Genotoxicity evaluation of diacetyl ,butter Flavors and MMC on the micronuclei of bone marrow in the male mice

Time	Treatment	Total no. of studied cells	No. of micronuclei (Mean ±SEM)
24h	MMC	1000	± 3.2* 17.000
one week	Control	1000	± 1.2 5.667
	Oil	1000	± 1.5 6.333
	Butter Flavors	1000	± 2.1** 21.000
	Diacetyl	1000	± 2.6** 22.667
24h	MMC	1000	± 3.2* 17.000
four weeks	Control	1000	± 0.9 6.333
	Oil	1000	± 1.2 6.667
	Butter Flavors	1000	± 3.2** 27.000
	Diacetyl	1000	± 5.5** 30.000

*Significant difference from the control group (*P < .05). **Hight Significant difference from the control group (**P < 0.01).

The aggressive competition in the field of industry introduced new technologies involving the use of artificial flavors without proper approaches to diagnose them rendering much speculation, contradictions, uncertainty, and consequently health threats (Carocho et al 2014). The current study exhibited increases in the levels of liver function enzymes (AST, ALP & ALT), chromosomal structural and numeric aberrations, and micronucleus upon exposure to diacetyl or butter flavors which agreed with Amin and Al- Shehri (2018) who used liver enzymes and hepatotoxicity for the assessment of tartrazine as a synthetic food additive in rats and showed, particularly at high doses, that the activities of hepatic serum enzymes (AST, ALP & ALT) increased, indicating high permeability, injuries, and weakness, of hepatocellular cells. Saxena and Sharma (2015) reported that consumption of food colors including tartrazine significantly increased serum total protein, albumin, ALP, and hepatic MDA level and significantly lowered levels of

SOD, reduced GSH and CAT in the hepatic tissue. These effects suggest that food colors induce hepatic tissue damages in Swiss Albino Rats. In another study, recently, the administration of a combination of food additives produced elevations in liver functions in male albino rats (Raya et al 2020).

According to Llana-Ruiz-Cabello et al (2018), micronucleus assay was applied to evaluate genotoxic effect of oregano essential oil orally administrated to rats and the results showed that PCE/total erythrocytes ratio exhibited significantly different values compared to control. Polat et al (2014) reported that consumption of Butylated hydroxytoluene (BHT) had a cytotoxic effect on bone marrow and the PCE/NCE ratio decreased in all applied BHT doses.

According to Hobbs et al (2017) a chromosome aberration assay and a micronucleus assay were used for assessment of genotoxicity of magnesium stearate on male mice. In another study, Aspartame significantly induced chromosome aberration in male Swiss Albino mice (AlSuhaibani 2010).

4 Conclusion

This study used in vivo chromosomal aberration, micronucleus test, and Serum liver enzymes to assess the genotoxicity of diacetyl and butter flavors on male Swiss albino mice. The exposure to the tested chemicals caused an increase in the formation of chromosomal aberrations, micronuclei, and a rise in liver functional enzymes (AST, ALT, and ALP). Our findings indicate that these compounds have a high potential to exert genotoxic effects and their impact on the cellular level should be studied where caused serious effects of increased level chromosomal aberration assay and micronucleus assay.

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