

Original Research Article

Validation of Replacement of the Synthetic Food Dye 'Sunset Yellow'-Induced Hepatotoxicity and Genotoxicity with the Nutraceutical 'Curcumin' in Mice

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Abstract

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The widespread use of colours in recent decades, especially the legitimate synthetic Azo dye Sunset Yellow (SY) as an additive to foods, beverages, drugs and cosmetics making them difficult to be indispensable to the modern day consumer. The current study is trying to find a safe alternative crucial colourant (from the aspects of morphological, behavioural, cytotoxicity and genotoxicity influences), by using a natural additive curcumin (CU). The study allocated thirty adult male mice into three groups, assigning as control ones, SY- and CU-groups. The other two groups were fed orally with equivalent (Acceptable Daily Intake) ADI dosage of 30 or 37 mg of SY or CU /kg b.wt./ daily for 30 days, respectively. The results exhibited distinct signs of anorexia and markedly statistical significant decrease ($p<0.05$) in the percentage of body weights of mice together with hair loss, ulcerated or skin rashes post-SY gavage. The ultrastructural observations induced-SY group involved relatively drastic effects in the hepatocytes as manifested by fragmentation of the rough endoplasmic reticulum, lipid droplets and vacuolated mitochondria. Moreover, the SY-group was recorded two main types of chromosomal aberrations, namely: structural (such as deletions/and or fragments, and ring chromosomes) and numerical ones (mainly represented by polyploidy) in a significantly increase ($p<0.05$), when encountered with control- and CU-animals. The frequency of Mitotic Index (MI) of bone marrow cells scored in induced-SY mice a highly significant increase ($p<0.0001$). On contrary, CU-oral feeding revealed a significant decrease ($p<0.05$) in MI. The micronucleated peripheral mature and immature erythrocytes of SY-group demonstrated a significant elevation ($p<0.0001$) in the frequency of micronucleus. This study concluded that the probability of replacement of the ingested synthetic sunset yellow with the nutraceutical curcumin as a food, beverage or drug colourant to avoid its cytotoxicity and genotoxicity.

Keywords: Curcumin, External Impacts, Food Colourants, Genotoxicity, Hepatotoxicity, Mice.

INTRODUCTION

Colour in food is an integral part of our culture during the past several decades and is also indispensable to the modern day consumer (Sharma et al., 2008). The food additives (such as food colouring agents) are considered

any ingredient intentionally added to the foods to modify their physical, chemical, biological, or sensorial characteristics without a nutritional purpose, and to influence the consumer perceptions of the food's flavor

and quality (Gomes et al., 2013; Abbey et al., 2014). Food colours are generally classified as natural and synthetic agents (Harris, 1986; Abbey et al., 2014). The synthetic colours (such as sunset yellow) are added to foods to replace natural colour lost during processing, to reduce batch-to-batch variation and to yield products with consumer appeal where no natural colour exists (Tripathi et al., 2007). The same authors also recorded that in samples of crushed ice and sweets which are preferentially consumed by children population, the presence of sunset yellow (SY) was found to exceed the permissible limit by eight times while in rural areas, and it exceeded the permissible limit by twenty three times. Moreover, Dixit et al. (2013) recorded that the intake of SY saturated the ADI limit to a maximum of 47.8% in Indian children, which is a cause of concern. Also, the dietary intakes of nine synthetic food colours (involving SY) permitted in Korea were measured by Ha et al. (2013), basing on the Estimated Daily Intakes (EDIs) by consumers and compared with the (ADIs) of the colours. This study found that among 704 foods sampled, 471 contained synthetic colours, whereas the EDI of a conservative consumer in the upper 95th percentile reached 37% of the ADI. However, in spite of resistance of some specialists to the ingestion of these additives, the estimate of food dye and pigment production in the world is from 750 to 800 thousand tons a year, and using more and more food additives with the objective of becoming competitive (Marmitt et al., 2010; Gomes et al., 2013).

Sunset Yellow (SY) is an Azo dye authorized as a food additive and from the most synthetic food dyes that are prevalent in soft drinks (Ha et al., 2013; Li et al., 2013). Beside, the main food categories containing colours especially SY were detected in beverages and liquor for adults, and chocolate and for infants and children (Ha et al., 2013; Botelho et al., 2014). Moreover, Hajimahmoodi et al. (2013); Wu et al. (2013); Andrade et al. (2014); Shen et al. (2014); Vasques et al. (2014); Wang et al. (2014) determined the synthetic food colourant 'SY' at high concentrations in cookies, coloured rice, saffron and fruit juice, ice cream, sauces, seasonings, pickles, relishes and chutney besides soft drinks, sugar- and gelatin-based confectionery by using high-performance liquid chromatography. In (2015), Zhu et al. stated that the synthetic dye SY was separated and detected successfully in drinks by poly sodium 4-styrenesulfonate-functionalized paper surface-enhanced Raman spectroscopy substrates. Concerning to the effect of natural sunlight illumination on such Azo dye (SY), Rajamanickam et al. (2015) investigated that the kinetics of SY Azo dye photodegradation was detected under natural sunlight illumination by inducing photocatalytic application.

Abou El-Zahab et al. (1997) found that administration of the synthetic colorants (SY, carmoisine, indigocarmine, brilliant blue and brown chocolate HT) after 60 days induced damage to liver tissue in rats as

evidenced by a significant increase in AST, ALT and ALP in serum of animal groups. The same authors added that the concentrations of ribonucleic acid (RNA) in liver cell homogenates exhibited a highly significant increase ($p < 0.01$) only in rats fed on diet supplemented with blend colour (brown chocolate HT and indigo carmine in addition to small fractions of SY, carmoisine, tartrazine and brilliant blue) for 60 days, whilst the DNA remained unchanged. Haematologically, a significant decrease was recorded in the Hb content, TEC count, haematocrit percentage, MCHC and TLC count in Kesari powder (a blend of SY and tartrazine)-treated female Swiss albino mice (Sharma et al., 2010). Furthermore, Hashem et al. (2011) found that the dosage of 47 mg/kg b.wt. of food colourings 'amaranth and SY' could impair hepatic function and should be avoided during pregnancy in rats.

SY may be responsible for causing an allergic reaction in people with an aspirin intolerance (Ibero et al., 1982), resulting in various symptoms including gastric upset, diarrhoea, vomiting, nettle rash (urticaria) and swelling of the skin (angioedema) (Schultz-Ehrenburg and Gilde, 1987). In (2000), Reus et al. pointed out that the food additives (involving SY) may consider as a cause of some medical symptoms such as asthma and anaphylaxis of abnormal immune responses. Such observations were indicated by the results obtained by Yadav et al. (2013), who suggesting that the non-cytotoxic dose (250 µg/ml) of SY may have immunomodulatory effects and altered functional responses of cultured splenocytes in significantly suppression ($p < 0.05$).

As for cytotoxicity of SY, a study conducted by Poul et al. (2009) demonstrated a lack of genotoxic effect of food dyes SY, amaranth and tartrazine and their metabolites when administered twice, at 24 h intervals, by oral gavage *in vivo* gut micronucleus assay on mice. Moreover, Sayed et al. (2012) verified the mutagenic action of SY dye daily for 1, 2 or 3 weeks (0.325 mg/kg b.wt./day alone or with selenium and A, C and E vitamins) in mice with significant chromosomal aberration values in the liver and germinative cells, DNA fragmentation, and increase of morphological abnormalities in spermatozooids of those animals. Furthermore, the cytotoxicity of SY dye at the two doses and under the two exposure times evaluated (24 and 48 hours) in root tip cells of *Allium cepa* L. had a statistically significant increase of cellular aberrations as compared to their controls (Gomes et al., 2013).

Curcumin (CU) is a polyphenol derived from the herbal remedy and dietary spice turmeric, possessing diverse anti-inflammatory and anti-cancer properties following oral or topical administration (Sharma et al., 2005). Because curcumin is not a medication and does not require FDA approval, the preliminary studies used a curcumin dosage of 36 to 180 mg per day for human (Monson and Schoenstadt, 2008). Furthermore, Prasad et al. (2014) reported that more than 6000 articles

published within the past two decades have discussed the molecular basis for the antioxidant, anti-inflammatory, antibacterial, antiviral, antifungal, and anticancer activities assigned to this nutraceutical 'CU'. The antioxidant properties of curcumin may be due to H-atom transfer from CH₂ group at the center of the heptadione link along with that of its phenolic -OH group (Jha et al., 2015). Furthermore, according to Franceschi et al. (2015), CU is classified from bioactive nutraceuticals.

The study of intraperitoneal CU dosage (100 mg/kg) in the mouse has suggested that CU was first biotransformed to dihydrocurcumin and tetrahydrocurcumin, and these compounds were subsequently converted to monoglucuronide conjugates (Pan et al., 1999). Beside, the study by Garcea et al. (2004) detected low nM levels of the parent compound 'CU' and its glucuronide and sulphate conjugates were found in peripheral blood samples, in portal blood and trace levels of products of its metabolic reduction were detected also in liver 6–7 h after the seventh dosage of CU. In (2014), Lestari and Indrayanto reported that demethoxycurcumin and bis-demethoxycurcumin (curcuminoids) are the main secondary metabolites of curcumin (*Curcuma longa*) and other *Curcuma spp.* However, Shoji et al. (2014) investigated that CU was conjugated when absorbed through the intestine confirming curcumin glucuronide was the major metabolite of CU found in the plasma after oral administration of CU in rats. The same authors also added that CU metabolites were responsible for the CU bioactivity.

Safety reports suggested that the dietary consumption of turmeric 'CU' up to 1.5 g per person a day were not associated with adverse effects in humans (Eigner and Sholz, 1999). In extremely support, a study of high dose oral CU, Cheng et al. (2001) administered up to 8 g daily of CU for 3 months to patients with pre-invasive malignant or high risk pre-malignant conditions, stating that no toxicity was observed. Also, in preclinical studies of CU, no toxicity has been observed from 2% dietary CU (approximately 1.2 g/kg b.wt.) administered to rats for 14 days (Sharma et al., 2001) or from 0.2% dietary CU (approximately 300 mg/kg b.wt.) administered to mice for 14 weeks (Perkins et al., 2002). Furthermore, in patients with advanced colorectal cancer treated with CU, it was well tolerated at all dose levels up to 3.6 g daily for up to 4 months (Sharma et al., 2004).

In three different malignant colon cell lines, CU inhibited proliferation, induced apoptosis and caused accumulation of cells in the G2/M phase of the cell cycle (Hanif et al., 1997; Chen et al., 1999; Mori et al., 2001). Similar results of CU inhibitor have been observed in breast, kidney, hepatocellular, lymphoid, myeloid, melanoma, oral epithelial and prostate cell lines derived from malignant tumours (Han et al., 1999; Bhaumik et al., 2000; Plummer et al., 2000; Yan et al., 2005). In addition, CU has shown growth inhibitory effects *in vitro* in cancer

cell lines derived from human prostate, large intestine, bone and leukaemia (Dorai et al., 2000). The findings by Dujic et al. (2009) suggested that the combination of CU and light used as a new therapeutic concept to increase the efficacy of CU in the treatment of cancer.

Ji et al. (2012) reported that the CU, one of the most studied chemopreventive agents, whereas extensive research over the last half century revealing that CU can inhibit the proliferation of various tumour cells in culture, prevent carcinogen induced cancers in rodents and inhibited the growth of human tumours in xenotransplant or orthotransplant animal models. Moreover, Milano et al. (2013) found that nano-CU enhanced the T cell mediated immune response, and effective with a promising approach against esophageal adenocarcinoma. In (2015), Franceschi et al. stated that in Western countries, CU as a herbal medicine, bioactive nutraceutical and dietary supplement is becoming more popular for improving the quality of life of neurological patients by treating aging and memory loss.

MATERIALS AND METHODS

The Experimental Animals

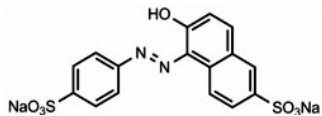
Subject selected throughout this study were 30 adult male Swiss albino mice (*Mus musculus*), morphologically healthy with weights ranged between 24 and 26 g at the beginning of experiment. The animals were obtained from Theodor Bilharz Research Institute, Giza, Egypt. Mice were housed in suitable cages for two weeks for adaptation to laboratory conditions. All animals were fed on standard diet (hay, wheat and milk). Food pellets and water were available *ad libitum*. Mice were kept under suitable laboratory conditions during the whole period of experimentation. As recommended by Ferdowsian and Beck (2011), all experimental procedures were performed taking into account the ethical and scientific considerations regarding animal testing and research.

The Applied Chemicals and Dosages

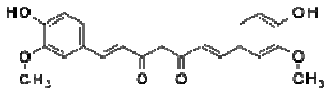
The two food additives used in the present investigation were the synthetic colorant supplement 'sunset yellow' FCF (Code No.: E110) or also known as yellow No. 6 (NTP, 1981; Hajimahmoodi et al., 2013) and the natural yellow colorant 'curcumin' (Code No.: E100) powder was derived from the turmeric rhizomes (*Curcuma longa*) (Duvoix et al., 2005).

The permitted synthetic food colourant (sunset yellow FCF; E110) is available in the form of pure powder and was obtained from El-Gomhouria Co. (Cairo, Egypt). According to Yamada et al. (2001); EFSA (2009), sunset yellow has the chemical name [disodium 6-hydroxy-5-(4-sulfonatophenylazo)-2-naphthalene-6-sulfonate], having

the molecular formula 'C₁₆H₁₀N₂Na₂O₇S₂' and the structural formula as follows:



The natural food colorant 'curcumin' (E100) was supplied from Faculty of Agriculture, Ain Shams Univ., Cairo, Egypt. Curcumin is a bis- α , β -unsaturated β -diketone (Sharma et al., 2005). According to Payton et al. (2007); Hu et al. (2010); Calaf (2014); Ghaedi and Mosallanejad (2014) curcumin has the chemical name [1, 7- bis (4-hydroxy-3-methoxyphenyl) -1, 6-heptadiene-3, 5-dione; diferuloylmethane] and its structural formula is the following:



Sunset yellow FCF (E110) is a mono-azo dye (-N=N-) authorized as a food additive in the EU and previously evaluated by both committees JECFA (1982) and SCF (1984) for establishing the ADI (acceptable daily intake) and marking of this agent. Sunset Yellow FCF is the coal-derived food and cosmetic colouring dye (Axon et al., 2012). On a worldwide basis, the control of the use of food dyes is based on the Acceptable Daily Intake (ADI), which is based on the results of international research and the recommendations of the Codex Committee on Food Additives and Contaminants (CCFAC) (Ganesan et al., 2011).

According to Reagan-Shaw et al. (2008), the formula of dose conversion of ADI of Sunset Yellow FCF from human to mouse was based on the body surface area (0.007m²) amended for mouse and calculating 30 mg/kg b.wt./day. On the same loom, curcumin has been evaluated by JECFA (2004), and then re-evaluated by EFSA (2010a) to determine the safety of curcumin (E100) as a food colorant additive and allocated an ADI of this natural additive. So, after application of the modified equation depending on the body surface area (Reagan-Shaw et al., 2008), the estimated equivalent mouse dosage of curcumin is 37 mg /kg b.wt./daily.

Both modified oral dosages of additives for mice (30 mg/kg b.wt./daily and 37 mg/kg b.wt./a day) of a synthetic colorant sunset yellow FCF (E110) and a natural coloring curcumin (E100), respectively, were freshly dissolved (in case of sunset yellow) or suspended (in case of curcumin) in water, each orally feeding with 1ml / 25 g b.wt a daily for 30 days.

Design of Experimental Animals

Thirty adult male mice were allocated into three groups,

assigning as follows:

Group "1": Ten mice were considered as control being orally feeding with the same quantity of water solvent daily under the same conditions of feeding with tube like other treated groups.

Group "2": Animals of this group (10 mice), each was treated in the same route with the modified human ADI dosage of 30 mg sunset yellow /kg b.wt./ daily for 30 days (SY-group).

Group "3": Ten mice, each was oral gavage (by feeding tube) with the equivalent human ADI dose (37mg curcumin/ kg b.wt./ daily) for 30 days (CU-group).

During the period of investigation (30 days), all experimental mice were kept under close observation to detect any marked morphological (clinical) symptoms resulting from food additives treatments.

Each group was subjected to cytological and cytogenetical testing. At the end of each course of chemical treatment, peripheral blood of tail was collected from mice, immediately the mice dissected and their livers and bone marrow cells were rapidly excised, and processed for micronucleus assay, cytological investigation and chromosomal preparation, respectively. According to Sharma et al. (2010), the percentage of body weight gain or loss was calculated as follow:

$$= \frac{\text{Mean final weight} - \text{Mean initial weight}}{\text{Mean initial body weight}} \times 100$$

Ultrastructural Preparations

For ultrastructural evaluation by transmission electron microscopy as described by Dykstra et al. (2002), freshly excised liver specimens were cut into small blocks (1X1 mm³), fixed directly in cold 4FIG (i.e., 4% formalin +1% glutaraldehyde adjusted at pH 2.2) for 24 hours, then were post fixed in 1% osmium tetroxide in 0.1M phosphate buffer (pH 7.3), dehydrated in ascending grades of ethyl alcohol, clearing in propylene oxide for two changes, 5 min each, and embedding in Epon-epoxy-resin. Semithin sections (1 μ m) were stained with toluidine blue and investigated under a bright-field light microscope. Ultrathin sections (400-500A^o) were cut with a diamond knife on an ultramicrotome and mounted on formvar-coated grids, stained with uranyl acetate and lead citrate (Reynolds, 1963). The stained grids were examined and photographed by JEOL-JEM-1400.EX-ELECTRON MICROSCOPE at the Central Laboratory of Faculty of Science, Ain Shams University. The photographs were printed on KODABROMIDE F5s GLOSSY black and white Schwarzwieb Kodak.

Chromosomal Preparation

The technique as given by Sharma and Sharma (1994)



Figure 1. External symptoms of male mice after treatment with 30mg/kg b.wt. sunset yellow (E110) for 30 days of SY-group (Figures. b - g), showing hair loss with ulcerated, itching and skin rashes (blue arrows), tail with abscessed tumour (black arrows) and little enlarged or hypertrophied descending testes. Whilst the other figure (a) represents a normal appearance of mouse after oral feeding with 37mg/kg b.wt. curcumin (E100) for 30 days (CU-group).

with a little modification was employed in the present study. Observation was made using bright field and photographs were taken with a 100 X oil objective lens. 1000 well spread metaphases from each group of animals were examined.

Mitotic index was calculated by counting the divided cells among at least 1000 metaphase spreads/ each animal group (n=5) and expressed in percentage, as conducted by Molineux et al. (1994).

Micronucleus Assay

The frequency of normochromatic and polychromatic erythrocytes in the peripheral blood of caudal vein that contain micronuclei among a given number of mature erythrocytes can also be used as the endpoint of the assay (Zetterberg and Grawé, 1993). In the mouse, mature erythrocytes are also an acceptable cell population for micronucleus analysis when the exposure duration exceeds 4 weeks (Hayashi et al., 1994). Thus, the peripheral blood micronucleus assay was carried out according to Hayashi et al. (1994). In which a total of 2000 immature and mature erythrocytes /animal were examined for statistical analysis after treatment continuously for 30 days with the both synthetic and natural additives (SY and CU, respectively).

The bone marrow metaphases and erythrocytes of

peripheral blood smears were observed by using Olympus microscope (E330-ADU1X, Japan) and photographed by Olympus imaging Corp (Model No. E-330. DC 7.4V, Japan).

Statistical Analysis

Statistical analysis of the data of chromosomal aberrations was carried out by *t*-test, SPSS statistics 17.0. Whereas, *p*-value ($p < 0.05$) was considered as a statistically significant, whilst *p*-value ($p < 0.0001$) was represented as a highly statistically significant.

RESULTS

External Symptoms (Morphological Features)

The most detectable physical symptoms encountered in the presently experimented mice (*Mus musculus*) post-sunset yellow application (SY-group) included fur paleness, hair loss with rough, ulcerated, itching or skin rashes (Figure 1: d-g). In addition, the most animals exhibited abscessed tumours in their tails (Figure 1: e-g) and little enlarged or hypertrophied testes (Figure 1: b and c). Moreover, animals of this treatment have manifested more abstinence for food with distinct

Table 1. Means (\pm SD) of body weights and their percentages of gain (+) or loss (-) weighting (according to the equation by Sharma et al., 2010) of male mice after 30 days of oral feeding with the equivalent ADI dose of either 30 mg or 37 mg/ kg b.wt./ daily, in both SY- or CU-group respectively, and their respective controls.

Experimental Mice Groups	No. of male mice in each experimental group	Mean (\pm SD) of body weight (g) before treatment	Mean (\pm SD) of body weight (g) after treatment for 30 days (before killing)	<i>p</i> - values	% of body weight gain (+) or loss (-)
Control group	10	(24,25,27,26,23, 25,24,26,25,27) 25.2 \pm 1.3165	(35,36,36,37,35, 35,33,38,37,39) 36.1 \pm 1.7288	<i>p</i> < 0.0001**	+ 43.3
Sunset yellow-treated mice (SY-group)	10	(28,24,28,24,25, 23,26,26,25,25) 25.4 \pm 1.6465	(25,25,23,26,20, 25,24,22,21,25) 23.6 \pm 2.0110	<i>p</i> < 0.05*	- 7.1
Curcumin-treated mice (CU-group)	10	(25,23,27,28,24, 25,26,26,24,25) 25.3 \pm 1.4944	(33,35,37,36,38, 37,38,39,39,38) 37 \pm 1.8856	<i>p</i> < 0.0001**	+ 46.2

Level of significance:
 Insignificant (*p*<0.05).
 (*) Significant (*p*<0.05).
 (**) Highly significant (*p*<0.0001)

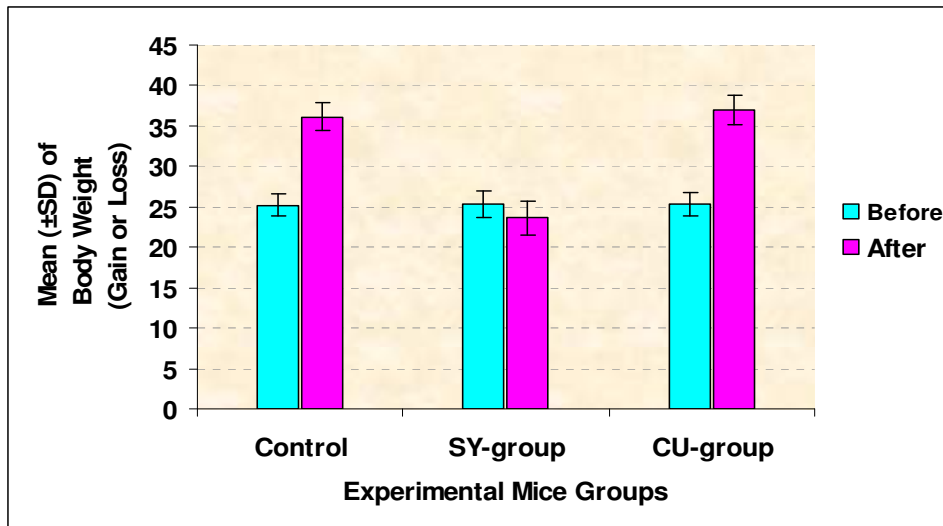


Figure 2. Histogram of means (\pm SD) of body weights (g) gain (+) or loss (-) of male mice before and after oral feeding with the equivalent ADI dose of either 30 mg or 37 mg/ kg b.wt./ daily, for 30 days in both SY- or CU-group respectively, and their respective control group, as recorded in table-1.

signs of anorexia together with progressive loss of their body weights (Table 1), and in some cases excreted red coloured-urine and -diarrhoea. Such observations became more obvious in their magnitude by the time of SY-treatment period (30 days). Furthermore, some of the experimental SY-gavage animals were inclined their activity and appetite with apathy and lethargy (Figure 1: e). In addition, most SY-administered mice were felt nervous by excessive grooming (i.e.; continuously vigorous face washing). However, in the course of oral feeding with curcumin, the previous morphological symptoms were completely absent as observed in CU-

group, reflecting a normal appearance of mice (Figure 1a).

As shown in table (1) and figures (2 and 3), the oral feeding with the equivalent ADI dose of 30 mg/ kg b.wt./ daily (SY-group) induced a loss in the percentage of body weight (g) in a significantly decrease (*p*<0.05) after 30mg/ kg b.wt./ daily of SY gavage, in comparison with respective control group. On contrast, CU-group exhibited insignificantly impact (*p*>0.05) in percentage of body weighting (g), as illustrated after CU treatment (37mg/ kg b.wt./ daily), when compared with the control mice after 30 days.

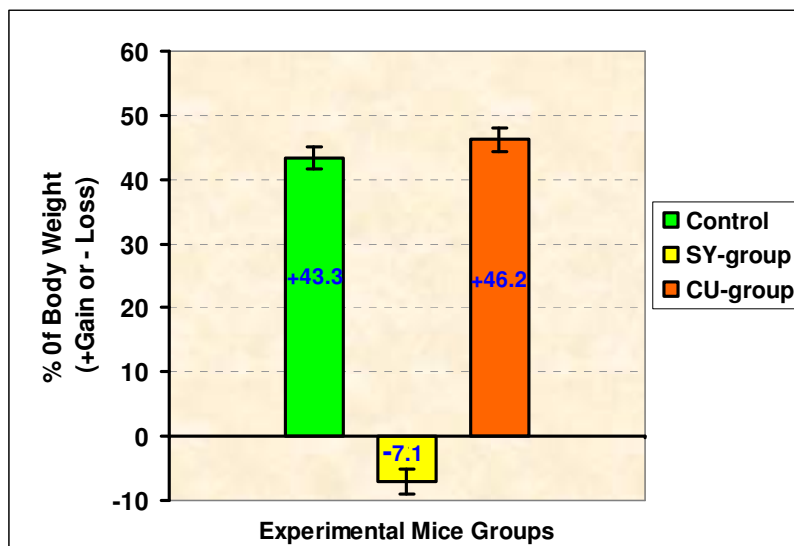


Figure 3. Histogram of means (\pm SD) of % body weights (g) gain (+) or loss (-) weighting (according to the equation by Sharma et al., 2010) of male mice before and after oral feeding with the equivalent ADI dose of either 30 mg or 37 mg/ kg b.wt./ daily, for 30 days in SY- or CU-group respectively, and their respective control group, as estimated in table-1.

Ultrastructural Observations

Group-1 (Control mice, Figures 4 and 5): Electron microscopic examination of the hepatocytes of the control mice revealed their normal ultrastructural appearance (Figure 4). The hepatocytes contain numerous mitochondria dispersed all over their cytoplasm. The mitochondria are spherical or ovoid in shape with well developed cristae (Figures 4 and 5). The rough endoplasmic reticulum consists of closely packed parallel and flattened cisternae studded with ribosomes (Figure 4).

Considerable electron-dense glycogen rosettes or granules are clearly detected in the cytoplasm of the hepatocytes (Figure 4). The nuclei of the hepatocytes are spherical, each with a distant nuclear envelope, and the nucleoplasm shows aggregations of euchromatin and heterochromatin materials (Figure 4). Hepatic sinusoids are localized between the hepatocytes and Kupffer cells with irregular in shape with their exposed surfaces are also observed (Figure 5). Numerous endocytotic vesicles and lysosomes which vary in density, shape and size are also seen in the cytoplasm of Kupffer cells (Figure 5). Kupffer cells appear differentiated with more flatter and denser nuclei and with less conspicuous cytoplasm (Figure 5). In contrast, the hepatocytes which comprise the hepatic cords have round nuclei surrounded by abundant cytoplasm (Figure 4).

Group-2 (SY-group): The mice treated with the synthetic food additive 'sunset yellow' daily with the dosage of 30 mg /kg b.wt. for 30 days (Figures 6 - 13) produced relatively drastic effects on the ultrastructure of

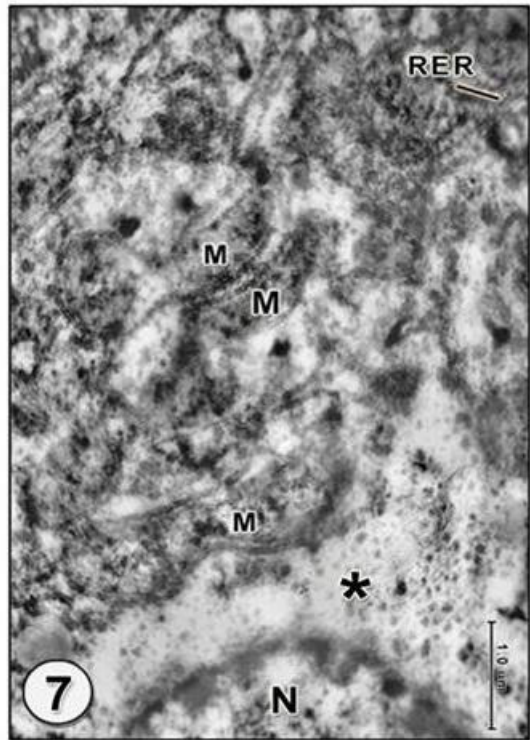
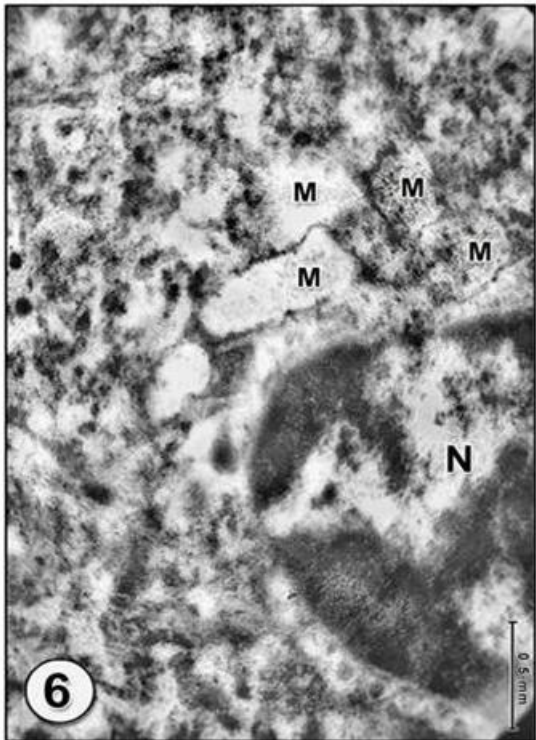
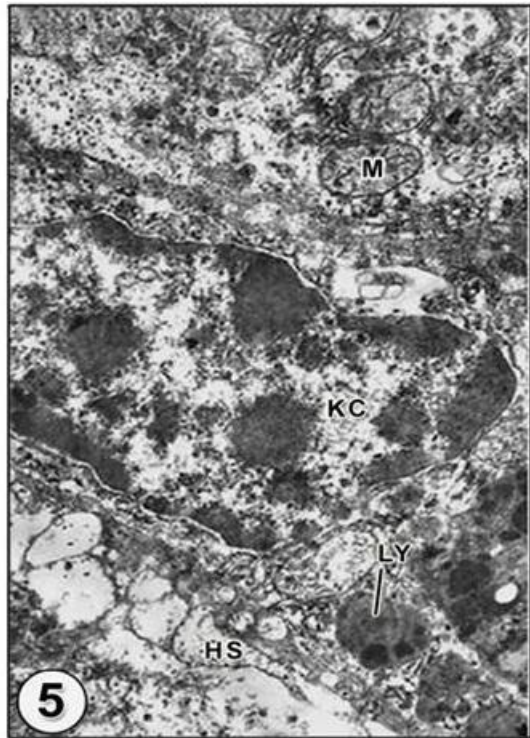
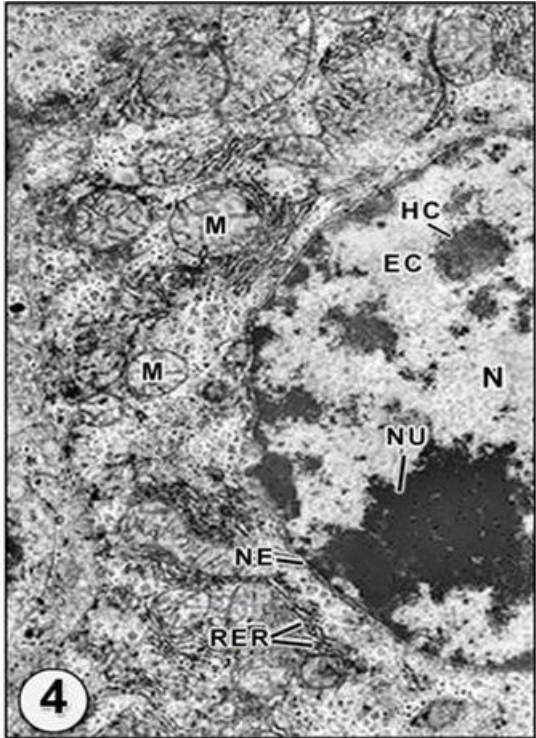
the hepatocytes as manifested by fragmentation of the rough endoplasmic reticulum into smaller stacks (Figures 7, 8 and 11) and by presence of numbers lipid droplets of variable sizes (Figures 9 and 10).

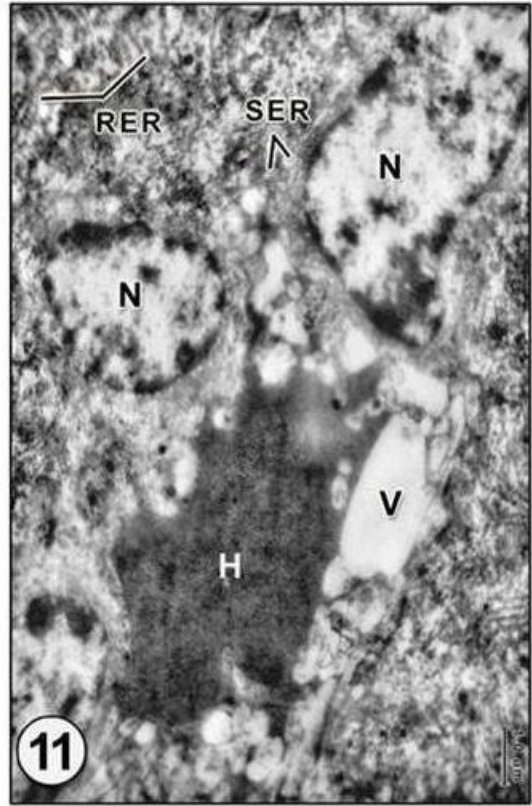
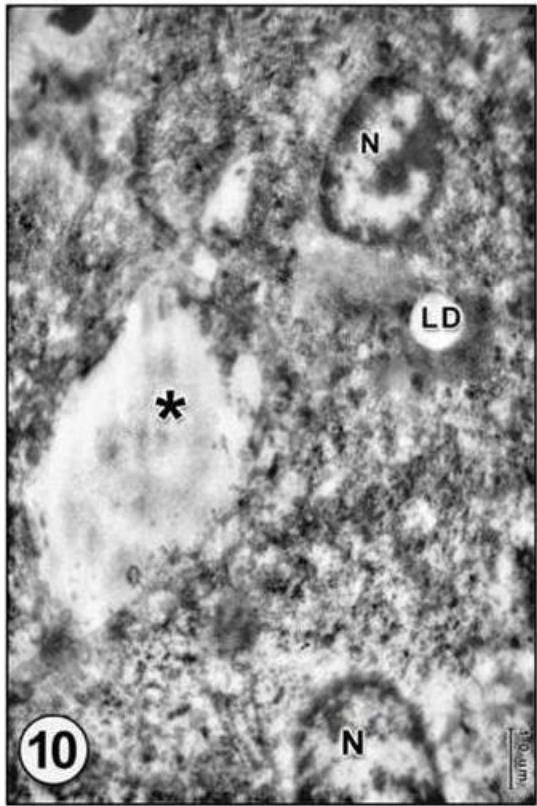
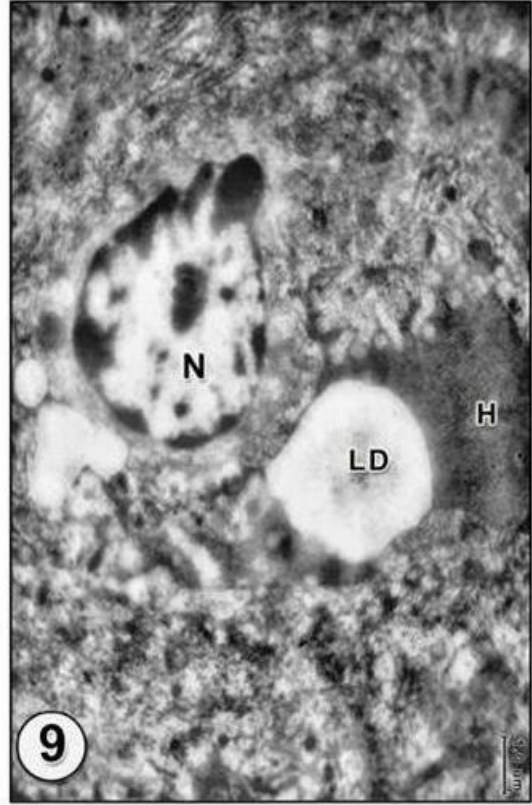
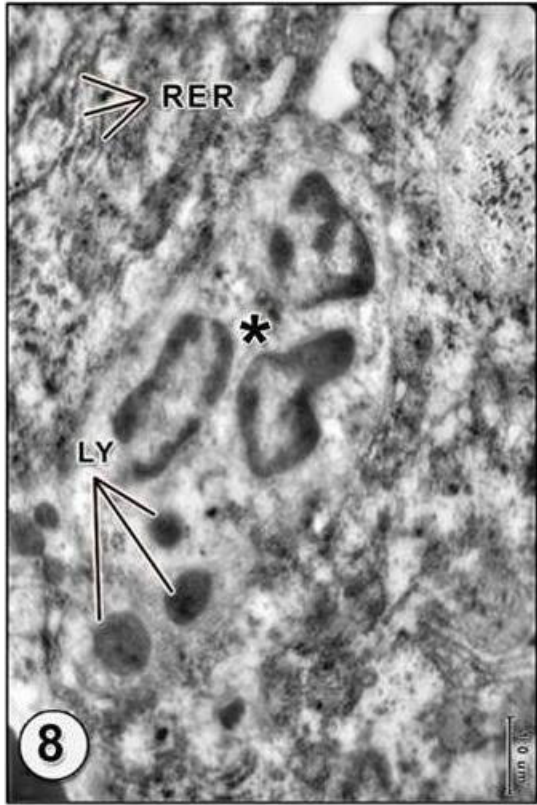
A large number of damaged mitochondria were detected in the hepatocytes of treated mice. In few hepatocytes, a considerable heterogeneity in shape and structure could be observed (Figures 6 and 7) and some of them were nearly rounded or elongated with destructed membranes with dense matrix and ill-defined cristae (Figure 7). In other hepatocytes, the mitochondria were lost their cristae and appeared as empty vacuoles or as devastated fewer bodies scattered in the cytoplasm (Figure 6).

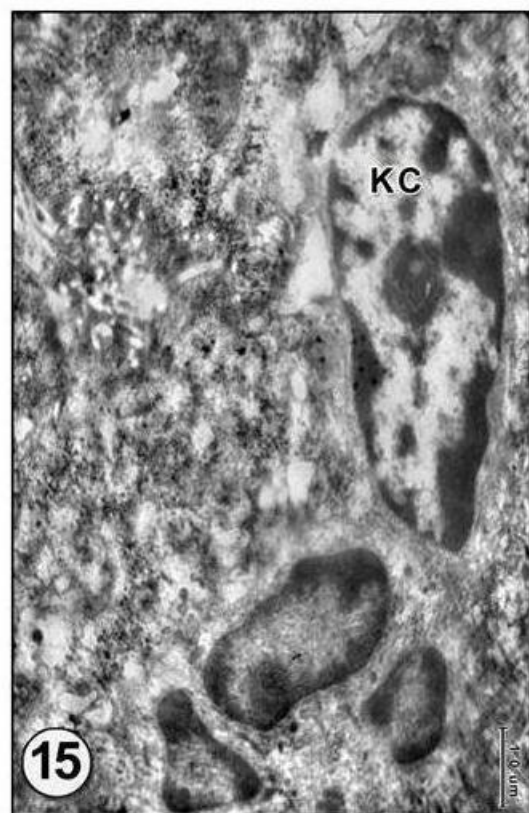
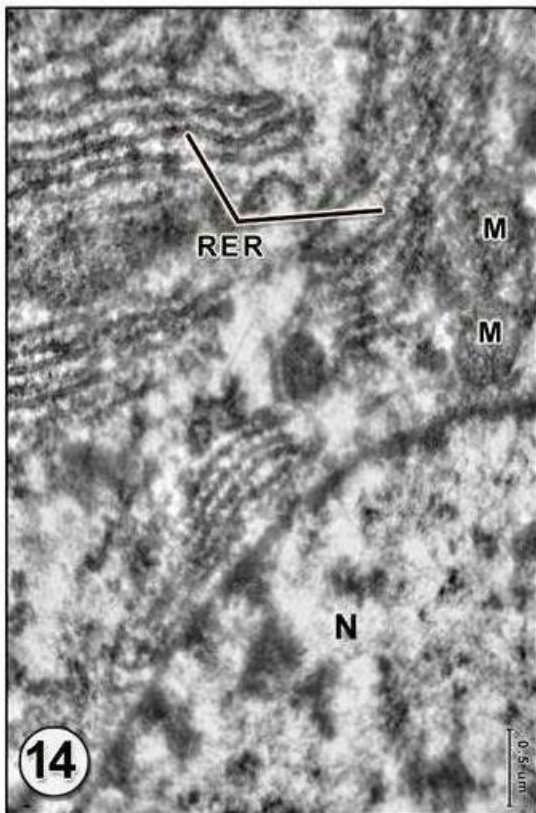
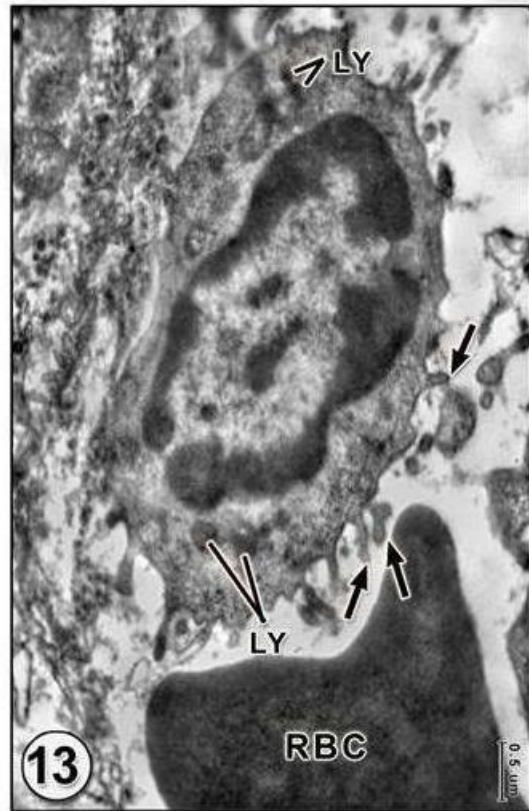
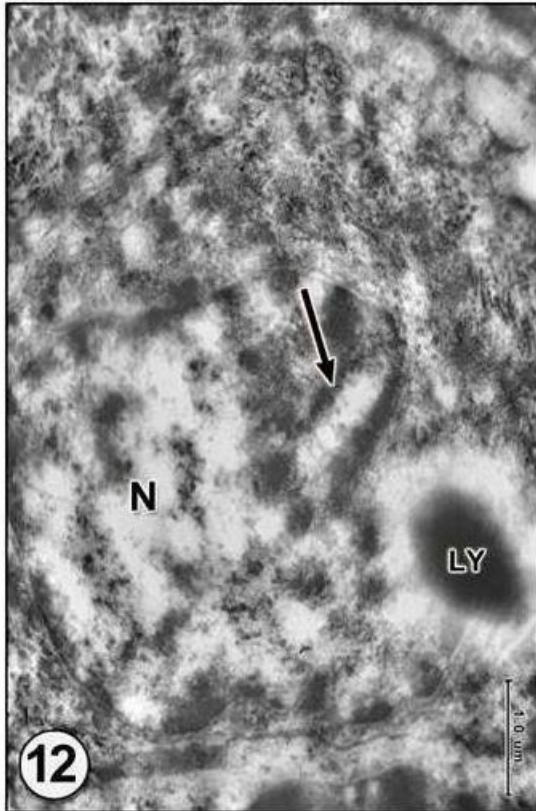
The nuclei of the hepatocytes of these treated mice have lost their spherical configuration and then acquired an irregular outline with ill-defined and partially damaged nuclear envelopes (Figures 6 and 10). The chromatin material of such cells appeared either aggregated peripherally as an apoptotic appearance taking agglomerated dense clumps (Figure 9) or distributed throughout the nuclear sap (Figure 12).

There was a hypertrophy of phagocytic Kupffer cells which appeared distinctly activated as symptomized by the presence of many lysosomal granules in their cytoplasm and extension of filopodia from their plasma membranes (Figure 13).

Group-3 (CU-group): the mice treated with natural food coloring 'curcumin' at dosage of 37 mg /kg b.wt. a day for 30 days (Figures 14 and 15), the ultrastructural examination of their livers revealed a marked improvement of the cytoplasmic organelles. In which, the







Electron micrographs (Figures 4 and 5) of liver sections of control mice (Control-group)
Figure 4. Showing the rough endoplasmic reticulum (RER), many mitochondria (M), part of the nucleus (N) with distinct nuclear envelope (NE), well-defined nucleolus (NU) and nucleoplasm having loose euchromatin (EC) and compact heterochromatin (HC) in normal figuring. (X 16000)

Figure 5. Illustrating the normal manifested phagocytic Kupffer cell (KC) having clumps of heterochromatin (HC) and facing lumen of the hepatic sinusoid (HS). Lysosomes (LY) which vary in density, shape and size are also seen in the cytoplasm of Kupffer cell. (X 16000)

Electron micrographs (Figures 6-13) of liver sections of mice treated with 30 mg /kg b.wt. daily of sunset yellow for 30 days (SY-group)

Figure 6. Displaying necrotic nucleus (N) of a hepatocyte with dark chromatin inclusions, vacuolated mitochondria (M) with the complete deterioration of the mitochondrial cristae and in turn they appear as empty vacuolated membranous bodies. (X 13000)

Figure 7. Showing a hepatocyte with degenerated cytoplasm (*), enlarged mitochondria (M), reflecting features of mitochondrial pleomorphism and having elongated shapes with contents of dense matrix and ill-defined cristae. Fragmented rough endoplasmic reticulum (RER) and a nearly normal part of nucleus (N) are also detected in this photo. (X 15000)

Figure 8. Illustrating a multinucleated hepatocyte (*), fragmented (RER) and presence of different sizes of lysosomes (LY). (X10000)

Figure 9. Showing an apoptotic hepatocyte with a distinct depletion of its organelles. The nucleus (N) appears obviously irregular in shape and shows conspicuous clumping of its heterochromatin on the inner surface of the nuclear envelope. Notice presence of haemorrhagic area (H) and large lipid droplets (LD). (X 8000)

Figure 10. Exhibiting degenerated cytoplasmic hepatocyte (*) with necrotic nucleus (N) and lipid droplet (LD). (X 8000)

Figure 11. Exhibiting necrotic hepatocytes, haemorrhage (H) and fragmented rough (RER) and smooth (SER) endoplasmic reticula, beside vacuolated cytoplasm (V). (X 8000)

Figure 12. Displaying a severe deteriorated hepatocyte, having karyolysed nucleus (N) with a protrusion of nuclear envelop (arrow) and fused with inflating lysosome (LY). (X 15000)

Figure 13. Displaying a hypertrophy of phagocytic Kupffer cell which appear in distinctly activated state as symptomized by the presence of many lysosomes (LY) in its cytoplasm and extension of filopodia (arrows) from the plasma membrane. Note also, the blood sinusoid is dilated and inflamed with red blood corpuscle. (X 15000)

Electron micrographs (Figures 14 and 15) of liver sections of mice orally given 37 mg /kg b.wt./daily of curcumin for 30 days (CU-group):

Figure 14. Showing a hepatocyte revealing a well developed rough endoplasmic reticulum (RER) and moderate framework of mitochondria (M). Notice, the magnified part of nucleus (N) has a nearly normal chromatin distribution. (X 20000)

Figure 15. Showing a Kupffer cell (KC) with a nearly normal structure. (X 10000)

hepatic cells contained numerous mitochondria exhibiting an almost normal appearance; they were distributed throughout the cytoplasm (Figure 14). Moreover, the hepatic cells exhibited well developed rough endoplasmic reticulum in the form of parallel and flattened cisternae studded with ribosomes (Figure 14). Kupffer cells exhibited a nearly normal structure (Figure 15).

Chromosomal Aberrations in Control, Sunset Yellow–(SY-group) and Curcumin–Treated (CU-group) Mice

The keen search for chromosomal aberrations of these groups had resulted in recording certain aberrations, which were comprised two main types, namely: structural and numerical ones. The structural aberrations are represented by deletion/and or fragment, ring chromosome, break/and or gap, end to-end-association,

condensed /or sticky chromosomes and centromeric attenuation in all three experimented groups (control, SY and CU) in inconsistent records (Table 2). However, the other two structural abnormalities of the stretching chromosomes and centric fusion were not recorded in the control group, but manifested only in both SY- and CU-groups in varying values (Table 2). Moreover, the numerical aberrations are represented by different cases of polyploidy and hyperdiploidy in both SY- and CU-groups only in marked different frequencies (Table 2).

Nine varieties of chromosomal aberrations were detected in sunset yellow–treated mice (SY-group) with a total mean (34.11%) reflecting a statistical significant increase ($p < 0.05$) in comparison to only six types of abnormalities having the total mean of 2.77% in control group, as manifested by table (2) and figures (17, 20 and 23). These aberrations were constituted of both structural and numerical aberrations, which represented by

Table 2. Means (\pm SD) of chromosomal aberrations in bone–marrow cells of male mice oral fed with the equivalent ADI dose of either 30 mg/kg b.wt. of sunset yellow (SY-group) or with 37mg/kg b.wt. of curcumin (CU-group) daily for 30 days, and their respective control group.

Types of Structural and Numerical Chromosomal Aberrations	Means (\pm SD) of chromosomal aberrations in 1000 metaphases / 7 mice per each experimental group		
	Control group	SY-group	CU-group
I- Structural Aberrations:			
Deletion/ or Fragment (D/F)	(1, 2, 0, 3, 1, 2, 0) 1.2857 \pm 1.1126	(4, 4, 3, 5, 2, 6, 1) * 3.5714 \pm 1.7182	(1, 2, 0, 1, 1, 0, 1) 0.7142 \pm 0.7559
Ring chromosome (R)	(0, 1, 0, 0, 1, 0, 2) 0.5714 \pm 0.7867	(3, 5, 7, 2, 4, 3, 1) * 3.5714 \pm 1.9880	(1, 1, 0, 0, 0, 1, 1) 0.5714 \pm 0.5345
Break/ or Gap (B/G)	(1, 0, 0, 1, 2, 0, 0) 0.5714 \pm 0.7867	(3, 5, 3, 4, 2, 6, 2) * 3.5714 \pm 1.5118	(2, 1, 1, 0, 0, 0, 1) 0.7142 \pm 0.7559
End to-end-association (En)	(0, 0, 0, 1, 0, 0, 1) 0.2857 \pm 0.4879	(4, 5, 4, 2, 7, 8, 4) * 4.8571 \pm 2.0354	(1, 0, 0, 1, 1, 0, 0) 0.4285 \pm 0.5345
Stretching chromosome (St)	(0,0) 0.0 \pm 0.0 \pm	(5, 7, 6, 3, 8, 4, 9) ** 6.0 \pm 2.1602	(2, 2, 1, 1, 0, 1, 2) * 1.2857 \pm 0.7559
Condensed / or Sticky chromosomes (Co)	(0, 0, 1, 1, 0, 0, 2) 0.5713 \pm 0.7867	(3, 6, 2, 4, 7, 2, 3) ** 3.8571 \pm 1.9518	(1, 2, 1, 0, 0, 1, 0) 0.7142 \pm 0.7559
Centromeric attenuation (Ca)	(0, 0, 1, 0, 0, 1, 0) 0.2857 \pm 0.4879	(17, 10, 15, 12, 9, 11, 13) ** 12.4285 \pm 2.8199	(1, 1, 1, 0, 0, 0, 1) 0.5714 \pm 0.5345
Centric fusion (Cf)	(0,0) 0.0 \pm 0.0	(1, 0, 1, 1, 2, 0, 1) 0.8571 \pm 0.6900	(1, 1, 0, 0, 0, 1, 0) 0.4285 \pm 0.5345
II- Numerical Aberration:			
Polyploidy (Poly)	(0,0) 0.0 \pm 0.0	(5, 9, 6, 4, 5, 3, 4) ** 5.1428 \pm 1.9518	(0, 1, 1, 0, 0, 0, 0) 0.2857 \pm 0.4879
% of Total Means (\pm SD) of chromosomal aberrations/ 1000 metaphases	(9, 4, 4, 2, 0, 4, 2, 0, 0) 2.777 \pm 2.9059	(25, 25, 25, 34,42, 27, 87, 6, 36) * 34.111 \pm 22.2286	(6, 4, 5, 3, 9, 5, 4, 3, 2) 4.555 \pm 2.0682

Level of significance:

Insignificant ($p < 0.05$).

(*) Significant ($p < 0.05$).

(**) Highly significant ($p < 0.0001$).

stretching chromosomes, condensed /or sticky chromosomes, centromeric attenuation and polyploidy /or hyperdiploidy recording the means of 6.0, 3.857, 12.428 and 5.142, respectively in a statistical highly significant increase ($p < 0.0001$) (Table 2, Figures 18-21). However, the other structural abnormalities involving deletions/ or fragments, ring chromosomes, breaks/ or gaps and end to-end-association the means of 3.571, 3.5714, 3.571 and 4.857 in respective manner and a statistical significant increase ($p < 0.05$), manifesting the clastogenic potent (capable of causing breakage of chromosomes) of SY on chromosomes of mice bone marrows (Table 2, Figures 17-19 and 22). But the abnormality of centric fusion shows the lowest frequency of the entire chromosomal aberration means in SY-group, calculating 0.857 (Table 2, Figures 17 and 21).

The magnitudes and types of curcumin–induced chromosomal aberrations (CU-group) are generally presented in tables (2) and it's nearly normal appearance illustrated in figure (16). Limited minute values of chromosomal distortions were designated in this group, recording the total percentage of 4.55%, thus exhibiting a

statistically non-significant ($p > 0.05$) effect relative to the control group, which recorded the total mean of 2.77% (Figure 23). The entire set of abnormalities have been restricted in this gavage with curcumin are being regarded to represent a statistically insignificant ($p > 0.05$) as encountered to their control (Table 2, Figures 21 and 22). However, an exception to these statistics was spotted (stretching chromosomes), counting a statistical significant increase ($p < 0.05$) in comparison to its respective control (Table 2, Figure 21).

Micronucleus Assay

The means (\pm SD) of micronucleated peripheral immature and mature erythrocytes of male mice oral fed with the equivalent ADI dose of either 30 mg/kg b.wt. of sunset yellow daily for 30 days (SY-group) or with 37mg/kg b.wt. of curcumin daily for 30 days (CU-group), and their respective control group were demonstrated in table (3) and figures (24-27). Animals treated daily with 30 mg/kg SY for 30 consecutive days induced a total mean (\pm SD)

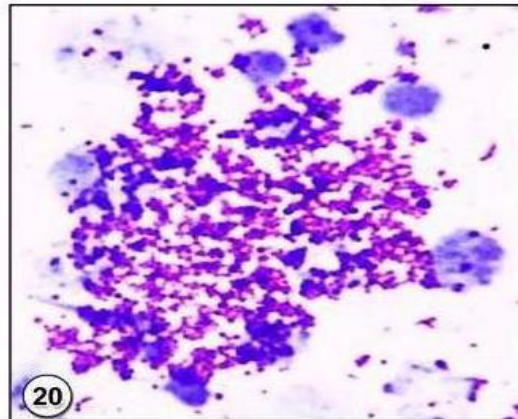
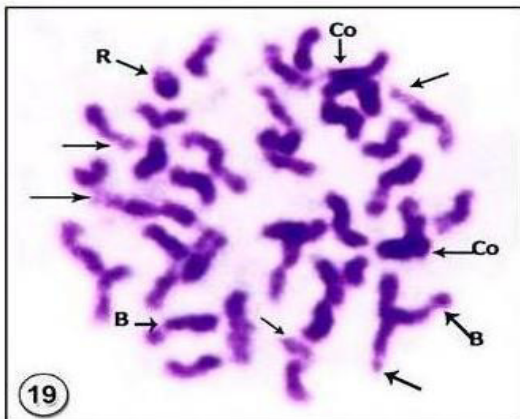
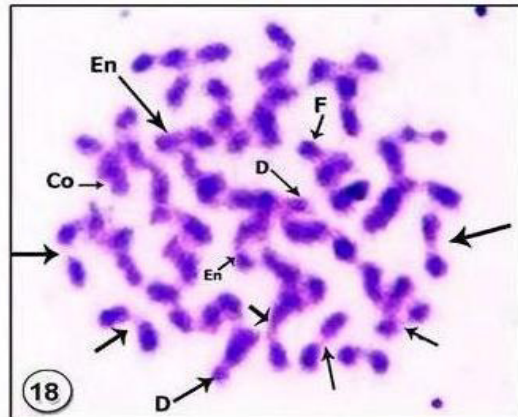
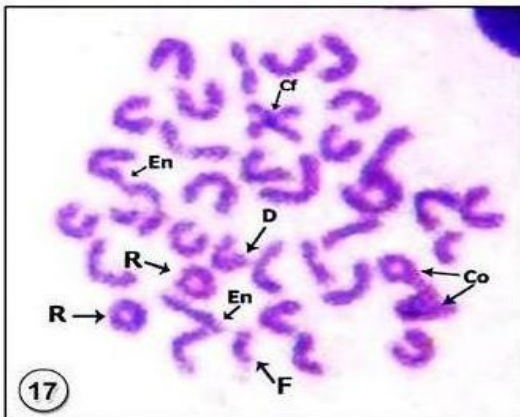
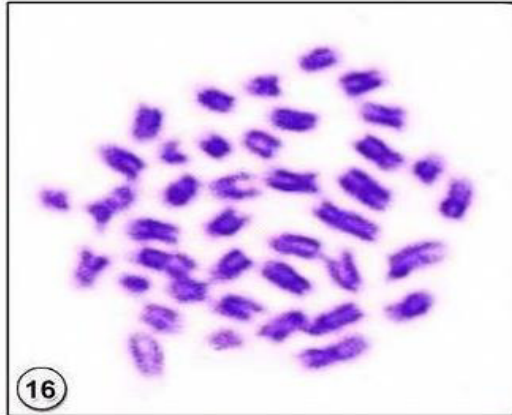


Figure 16. Normal appearance of metaphase spread obtained from bone marrow of male mice (*Mus musculus*) after oral feeding daily with the equivalent ADI dose (37mg/kg b.wt.) of curcumin for 30 days (CU-group).

All the above figures of sunset yellow-treated mice (Figures 17-20) were received the equivalent ADI dose (30 mg/kg b.wt. daily) for 30 days (SY-group).

Figure 17. A metaphase photograph of SY-group demonstrating multiple chromosomal aberrations, including, deletions (D), centric fragments (F) ring chromosomes (R), end-to-end chromatid association (En) and condensed chromosomes in sticky appearance (Co).

Figure 18. A metaphase spread of SY-group exhibiting several chromosomal abnormalities involving deletions (D), fragments (F), end-to-end chromatid association (En), condensed chromosomes (Co) and variety shapes of centromeric attenuation at stretching forms (arrows).

Figure 19. Chromatid breaks (B), ring chromosomes (R), condensed chromosomes (Co), and dispersed and stretching chromosomal ends (arrows) are obtained from metaphase spread of SY-group.

Figure 20. Multiploid metaphase (polyploidy) of chromosomes in minute and pulverized appearance, as detecting in SY-group.

(All photomicrographs are taken at X 1000).

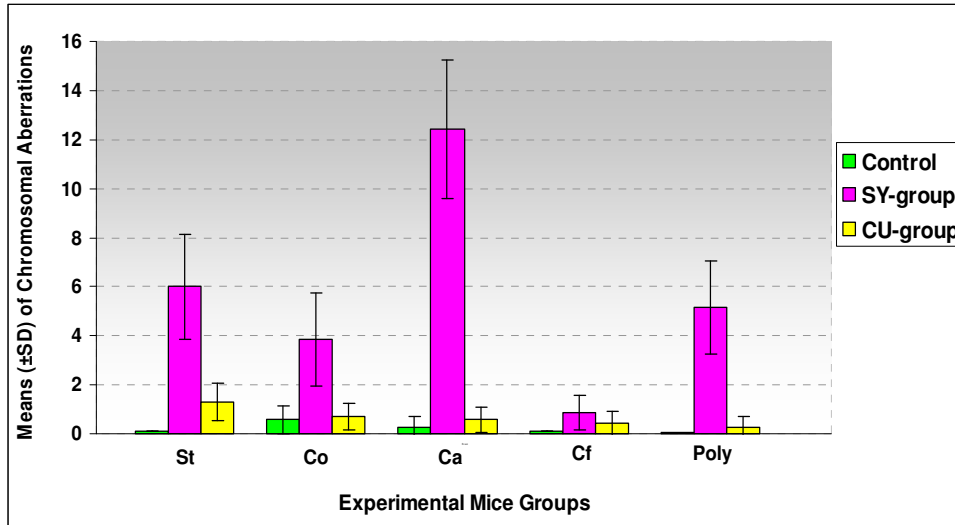


Figure 21. Histogram of means (\pm SD) of chromosomal aberrations in bone-marrow cells of male mice fed orally with the equivalent ADI dose of either 30 mg/kg b.wt. of sunset yellow (SY-group) or with 37mg/kg b.wt. of curcumin (CU-group) daily for 30 days, and their respective control group, as recorded in table-2. This figure showing structural aberrations represented with stretching chromosome (St), condensed/ or sticky chromosomes (Co), centromeric attenuation (Ca), centric fusion (Cf) and numerical aberration of different cases of polyploidy (Poly)

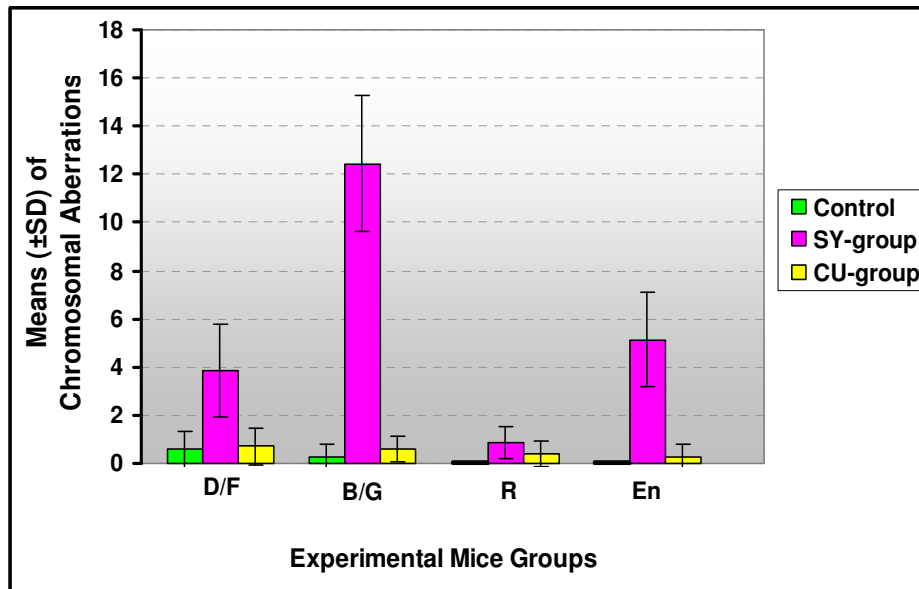


Figure 22. Histogram of means (\pm SD) of chromosomal aberrations in bone-marrow cells of male mice oral fed with the equivalent ADI dose of either 30 mg/kg b.wt. of sunset yellow (SY-group) or with 37mg/kg b.wt. of curcumin (CU-group) daily for 30 days, and their respective control group, as recorded in table-2. This figure showing deletion/ or fragment (D/F), ring chromosome (R), break or gap (B/G) and end to-end-association (En).

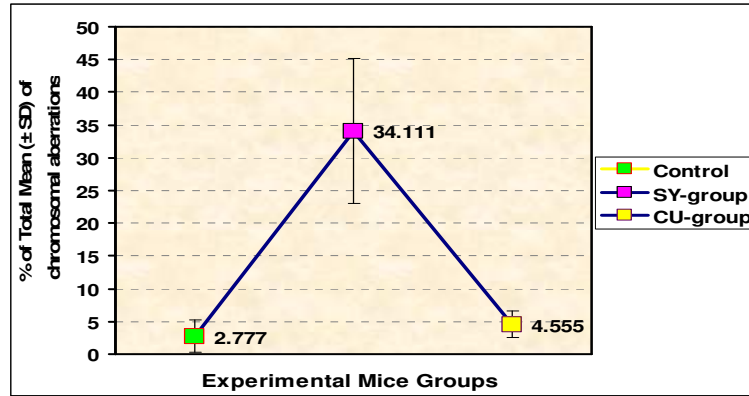


Figure 23. Graph of means (\pm SD) of chromosomal aberrations in bone-marrow cells of male mice oral fed with the equivalent ADI dose of either 30 mg/kg b.wt. of sunset yellow (SY-group) or with 37mg/kg b.wt. of curcumin (CU-group) daily for 30 days, and their respective control group, as estimated in table-2.

Table 3. Means (\pm SD) of micronucleated immature and mature peripheral erythrocytes of male mice oral fed with the equivalent ADI dose of either 30 mg/kg b.wt. of sunset yellow (SY-group)ⁿ⁼¹⁰ or with 37mg/kg b.wt. of curcumin (CU-group)ⁿ⁼¹⁰ daily for 30 days, and their corresponding control groupⁿ⁼¹⁰.

Means (\pm SD) of micronucleated peripheral erythrocytes / 2000 RBCs / 10 mice for each experimental group		
Control group	SY-group	CU-group
(1, 0, 2, 0, 0, 1, 1, 2, 0, 1) 0.8 \pm 0.7888	(9, 7, 8, 5, 9, 10, 4, 6, 6, 7) ** 7.1 \pm 1.9119	(2, 0, 1, 1, 2, 1, 3, 1, 0, 1) 1.2 \pm 0.9189

Level of significance:

Insignificant ($p < 0.05$).

(*) Significant ($p < 0.05$).

(**) Highly significant ($p < 0.0001$).

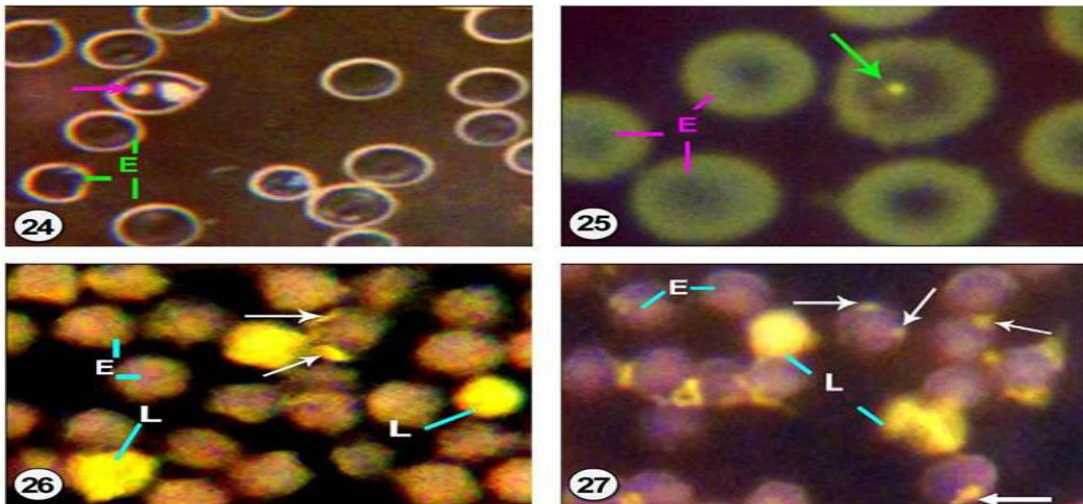


Figure 24-27. Peripheral blood smears from caudal vein of mice oral fed with 30 mg sunset yellow /kg b.wt. /daily for 30 days (SY-group), displaying micronucleated normochromatic and polychromatic erythrocytes stained with modified Giemsa stain and photographed by using the inverted image and microscopic filters for more clearness of the micronuclei. The micronuclei in erythrocytes are visualized as small rounded spots with variable degrees of yellowish appearance (arrows). The mature erythrocytes (E) appear as rounded bodies. The nuclei of leukocytes (L) take large brightly-yellow coloured irregular masses. (All photomicrographs were taken at 1000 X).

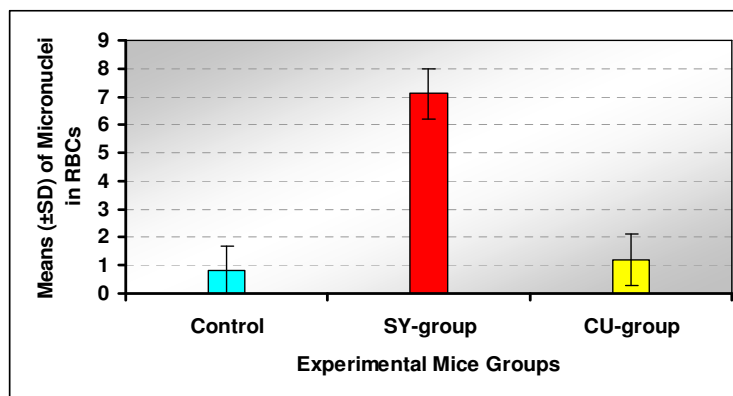


Figure 28. Histograms of means (\pm SD) and their percentages of micronucleated peripheral erythrocytes of male mice oral fed with the equivalent ADI dose of either 30 mg/kg b.wt. of sunset yellow (SY-group) $n=10$ or with 37mg/kg b.wt. of curcumin (CU-group) $n=10$ daily for 30 days, and their respective control group $n=10$, as recorded in table-3.

Table 4. Mitotic index (MI) scored in 1000 bone marrow cells per each experimental group and its percentage after treatment with the equivalent ADI dose of either 30 mg/kg b.wt. of sunset yellow (SY-group) or 37mg/kg b.wt. of curcumin (CU-group) daily for 30 days, and their comparative control group.

Animal groups	Score of divided cells / 5 mice/ total cells (1000) & Mean (\pm SD)	% of MI
Control-group	(66, 71, 69, 72, 64) = 342 68.4 \pm 3.361	34.2 %
SY-group	(89, 90, 101, 91, 96) = 467 ** 93.4 \pm 5.029	46.7 %
CU-group	(51, 63, 57, 65, 49) = 285 * 57 \pm 7.07	28.5 %

Level of significance:
 Insignificant ($p < 0.05$).
 (*) Significant ($p < 0.05$).
 (**) Highly significant ($p < 0.0001$).

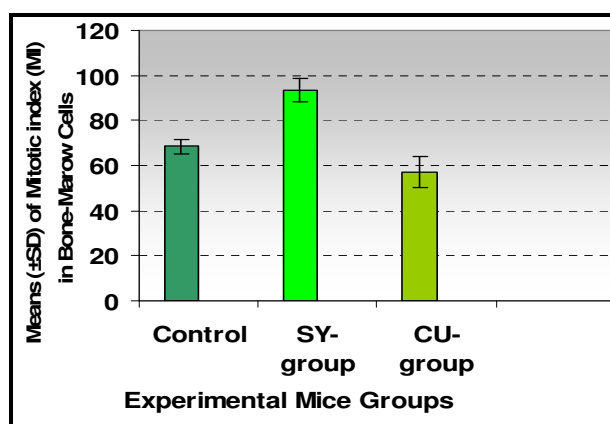


Figure 29. Histogram of means (\pm SD) of mitotic indices in bone-marrow cells of male mice treated with the equivalent ADI dose of either 30 mg/kg b.wt. of sunset yellow (SY-group) or with 37mg/kg b.wt. of curcumin (CU-group) daily for 30 days, and their respective control group, as recorded in table-4.

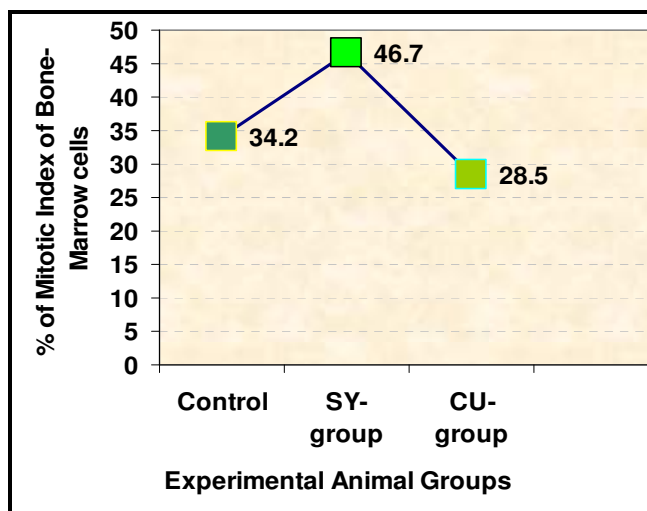


Figure 30. Graph of percentages of mitotic indices (MI) in SY- and CU- groups and their corresponding control group, as calculated in table 4.

of 7.1 ± 1.9119 in a highly significant elevation ($p < 0.0001$) in the frequency of micronucleus than the respective control level (0.8 ± 0.7888), as shown in table 3 and figure 28.

However, the averages of a total mean (\pm SD) of micronucleated erythrocytes after gavage with 37mg/kg b.wt of CU a daily for 30 days did not induce a significant increase ($p > 0.05$), recording 1.2 ± 0.9189 , as illustrated in table 3 and figure 28.

Mitotic Index (MI)

The mitotic index was based on the number of cells and mitotic figures (involving prophase and metaphase) were scored per counts of at least 1000 cells for each experimental group. Data cited in table (4) and figures (29 and 30) show the mean (\pm SD) of mitotic index (MI) in bone marrow cells of SY-gavage mice is 93.4 ± 5.029 recording 46.7 % and revealing highly significant increase ($p < 0.0001$) in comparison with controls (34.2 %). On contrary, CU-oral feeding induces a significant decrease ($p < 0.05$) in mitotic index (28.5 %) of bone marrow cells, whereas the frequency of its mean (\pm SD) scored 57 ± 7.07 when analyzed with the mitotic ratio (MI) of its respective control (68.4 ± 3.361), as illustrated in table (4) and figures (29 and 30).

DISCUSSION

During the past several decades, the technology of food processing has changed dramatically and the growth in the use of synthetic food colours as an individual dye or in the form of blends has increased enormously (Sharma

et al., 2008). The azo group dyes (involving sunset yellow) present a naphthalene ring connected to a second benzene ring by an azo bond ($-N=N-$), as formulated by Yamada et al. (2001). Those rings can contain one sulfonic group, globally representing the class of the most commonly used synthetic dyes by the food industry, mainly sunset yellow (Morrison, et al., 2012). Moreover, in the past decade, sunset yellow (SY) dye has caused controversy as to its toxic effects, and its use was forbidden in some countries such as the United States and Japan, where from the results of bioassays with mammals, it was shown to be mutagenic and even carcinogenic (Feng et al., 2012). Determination of the synthetic SY 'sunset yellow' is not restricted only as a foodstuff colorant but it is also detected in many human drugs just for colouring, such as in effervescent multi-vitamin tablets and powders (Ottó et al., 2005). Beside, the dye SY is also found in 15% of studied pharmaceutical excipients and the information on drug labels of analgesic/antipyretic, antimicrobial, mucoregulatory, cough and cold, decongestant, antihistamine, bronchodilator, corticosteroid anti-inflammatory and diabetic medications, with omission and inaccuracy causing adverse reactions (Balbani, 2006). Hence, from all the above studies, it was selected that the synthetic azo food additive 'SY' in the present study, further it was approved as a most common food dye used in different foodstuff (especially for infants and children), several cosmetic and pharmaceutical industries.

Concerning to curcumin 'CU' (diferuloyl methane), the yellow-colored dietary pigment from the rhizomes of turmeric, has been recognized as a chemopreventive agent because of its antitumor, antioxidant and antiproliferative effects (Ramachandran et al., 2005). Furthermore, the findings by Motaghinejad et al. (2015)

have demonstrated that curcumin can act as an antioxidant and antiapoptotic agent against damage induced by morphine dependence in rat isolated hippocampus. Such observations were investigated by the various dosages of curcumin caused significantly lowering lipid peroxidation, GSSG level, Bax concentration, caspase-3 and caspase-9 activities, while increasing superoxide dismutase and glutathione peroxidase activity, mitochondrial GSH level and Bcl-2 concentration. Hence, the natural CU was selected to the present study as an alternative food dye to the synthetic SY; further both food additives have nearly the same colouring and texture when dissolved. However, in this concern, Chuah et al. (2014) enhanced bioavailability and bioefficacy of an amorphous solid dispersion of CU to provide it as a more potent, efficacious formulation and help in masking the colour, taste and smell which currently limit its application as a functional food ingredient.

In the present work, the oral feeding with the designed dosage of SY (30 mg/ kg b.wt./ daily) induced a loss in the percentage of the body weight (-7.1%) of its mean \pm SD (25.4 \pm 1.646) after 30 days in a significantly ($p < 0.05$) decrease efficacy of male mice. These results were in agreement with Ershoff (1977), who obtained a marked retardation in growth with an unthrifty appearance of the fur of rats within an experimental period of 14 days after feeding with both SY and tartrazine at 5% level in their diet. But, the evaluated data by Sharma et al. (2010) revealed a highly significant increase in the body weights of female Swiss albino mice after ingestion of a food colourant ' Kesari ' powder (a blend of sunset yellow and tartrazine).

Nevertheless, the present investigation scored both curcumin (CU) and control groups achieved gaining in their percentages (+46.2% and +43.3%) of the body weight means \pm SD (25.3 \pm 1.494 and 25.2 \pm 1.316), respectively after 30 days in significantly ($p < 0.0001$) increase manners. Generally, this increasing of body weight appeared having normal convergent results between these two groups (control and CU), reflecting that the impact of CU on the body weight of mice was a slightly non-tangible in comparable with those of control ones. In this regard, CU alone or combined with piperine (a pungent alkaloid of pepper berries) in food restricted diet to obese mice had no additional effects on total body weight and fat loss (Noble et al., 2013). On the same avenue, high dosages of oral curcumin were greatly ameliorated as an adjuvant treatment for diabetes complications in several mouse models of obesity-associated diabetes (Weisberg et al., 2008).

In addition, the present morphological features revealed enlarged testes of SY-fed mice. Such phenomenon was also observed at autopsy of acute (for rat and mouse) and short-term (for rat) toxicity studies on testes at the 3% SY level of intraperitoneal LD₅₀ (Gaunt et al., 1967).

Furthermore, the present investigation showed some SY-treated animals have decreased their activity and appetite with apathy and sluggishness. Such evoked symptoms in SY-treated mice may be attributed to the effect of a synthetic azo dye "SY" on both true and pseudo-cholinesterases in rats, causing a significant decrease in these enzyme activities (Osman et al., 2004). This relationship between those enzymes (cholinesterases) and the behavioural symptom (apathy) were also indicated by Mega et al. (2005), who published that the improvement in apathy was following administration of galantamine - a cholinesterase adjustment.

The present clinical features observed that some SY-oral fed mice excreted red coloured-urine and -diarrhoea. Such symptoms became more obvious in their magnitude by the time of SY-treatment period (30 days). These findings are in agreement with those obtained by Yamada et al. (2001), who found that after oral administration of SY to rats, a major metabolite-I (6-hydroxy-5-(4-sulfooxyphenylazo)-2-naphthalenesulfonic acid), a minor metabolite-II (6-hydroxy-5-(4-hydroxyphenylazo)-2-naphthalenesulfonic acid) and unaltered SY were detected as red colored metabolites in the rat urine. Beside, they measured approximately 56% of SY was excreted into the urine and the rest was probably excreted into faeces. From another view, increased numbers of *Salmonella* strain TA100 were obtained in faecal extracts of SY-oral application on rodent species, when an exogenous metabolic activation was performed (Wever et al., 1989), causing diarrhoea.

From the most detectable morphological symptoms encountered in the presently SY-gavage mice were fur paleness, hair loss with rough ulcerated or skin rashes. These dermal results were also detected as a severe skin reaction due to excipients of an oral iron-deficiency-anaemia therapy with tablets containing SY (Rogkakou et al., 2007). At which, the patient developed a severe facial erythema with itching and skin oedema after the application of drug. In this respect, Mizutani (2009) suggested that superoxide anions, originating on SY dye by light irradiation, must attack drug-metabolizing enzymes, which was possible that dye to react with proteins on skin under lighting and may lead to rough skin. Moreover, the panel of EFSA (2010b) concluded that oral consumption of the food azo-colours (involving SY) caused intolerance and/or allergic reactions in humans after oral exposure. These reactions included urticaria, angioedema, wheezing, and leukoclastic vasculitis as well as higher hyperactivity scores in children. Furthermore, Leysat et al. (2003) found the clinical dermal effects of allergy in patients ingested antihistamine polaronil tablets containing a metabolite of SY (4-aminobenzene sulfonic acid or sulfanilic acid). Moreover, this dye can cause anaphylactic shock, angioedema, vasculitis, and thromboxane synthesis inhibition in people sensitive to its

composition; it can enhance aggressive behaviour in children, and trigger serious allergic reactions at certain people (Sardi et al., 2010). Hence, such observations are taken in consideration by EFSA (2012), who advised that in the absence of any information, the substance should be considered to be potentially harmful as a result of skin, eye, or inhalation exposure by users of the food additives.

Most SY-administered mice in the present investigation were felt more nervously by excessive grooming (i.e.; continuously vigorous their face washing).

Such behavioural actions from the beginning to the end of the grooming sequence are result of a series of muscle contractions and depend upon the mechanic outputs from motor control center in the central nervous system as a result of interaction between genetic and environmental factors during development (Broom, 1981 and Berridge, 1990). Depending on the explanation of previous authors, the excessive repeating of grooming in SY-gavage mice may be due to the effect of SY on motor control center in the central nervous system affecting on the power of stereotype allometric function on the animal face. Furthermore, in general, Ceyhan et al. (2013) interpreted the behavioural impacts indicating that exposure to artificial food colours and additives (involving SY) during the foetal period may lead to alterations in expressions of N-methyl-D-aspartate receptors, which are related to psychomotor agitation in adulthood.

From the most marks which noted in this study were some skin scratches arising from competition and struggle between male mice during compilation in their cages. Such observations were more prominent in the cages contained SY-treated mice than the corresponding CU-gavage group. These findings may be attributed to CU enhanced the angiogenesis (neovasculogenesis) and accelerated the wound healing by increased expressions of various factors, as observed in diabetic rats by Kant et al. (2015). These factors included well-formed granulation tissue dominated by fibroblast proliferation, collagen deposition, and complete early regenerated epithelial layer. Beside, CU catalyzed the well-formed blood vessels with increased microvessel density and hypoxia-inducible growth factor-1 α , stromal cell-derived growth factor-1 α , and heme oxygenase-1 (Kant et al., 2015).

The present assay investigated that the most mice exhibited abscessed tumour in their tails and hepatic foci. In this regard, livers of the azo dye-fed rats exhibited histological changes that had been classically attributed to the course and development of cancer, and nearly all rats had developed hepatomas, which were accompanied by qualitative changes in chromosomal protein antigens (Schmidt et al., 1982). These authors established that define chronological correlation between the azo dye induction of cancer and sequential changes in the immunological specificity of nonhistone protein antigens.

Liver is a specialized organ in terms of its metabolic, synthetic and detoxifying function, whereas Kupffer cell-mediated detoxification of endotoxins (Rao et al., 2004). Concerning the food azo dyes, Gomes et al. (2013) announced that the studies on the adverse health effects caused by artificial dyes, mainly those of the azo group (-N=N-), are insufficient and quite contradictory, and as such, additional studies on various system-tests such as mammalian liver should be conducted to assess the true effect of these food additives on a cellular level.

A large number of deteriorated mitochondria were detected in the hepatocytes of currently SY-treated mice.

In which some mitochondria destructed membranes with dense matrix and ill-defined cristae and others were lost their cristae and appeared as empty vacuolated bodies. In this matter, Reyes et al. (1996) tested SY to determine its effects on mitochondrial respiration in mitochondria isolated from rat liver and kidney. The authors found that both rat liver and kidney mitochondria showed similar patterns of inhibition, whereas this effect was SY-dose concentration related giving 50 % inhibition. Moreover, Effect of feeding amino azo dyes on mitochondrial swelling and contraction, as a kinetic evidence for deletion of membrane regulatory sites (Arcos et al., 1969).

Cytoplasmic and mitochondrial vacuolization were observed in SY-treated liver cells of the present study as a prominent symptom, may be reinforced the previous purpose. In which, the cytoplasmic vacuolation may attributed to disturbance of ionic milieu of the cell with consequent retention of water and sodium leading to cellular swelling (Jaarsma et al., 2001 and Wiedemann et al., 2002), thereupon the accumulation of ions and fluids in cytosol would rapidly pass through leaky membranes of cell vacuolated organelles and finally lead to cell lysis (Gores et al., 1990, Sarhan and Al-Sahhaf, 2011). Straus (1964) interpreted such vacuolation as pathological changes of permeability, whereas phagosomes and lysosomes lost their normal location and fused to form large vacuoles or spheres in kidney and liver of peroxidase-treated rats in an azo dye medium. Furthermore, the clear cytoplasmic vacuoles contain predominantly electron-lucent material consistent with phospholipid in hepatic phospholipidosis of rats (Obert et al., 2007). In this concern, lysosomes are the primary site for phospholipid accumulation (Kacew, 1987), which results from an inability of the cell to catabolize this substrate (Hostetler, 1984). So, the mitochondrial vacuolation and swelling represent an accelerated form of mitochondrial damage caused by high level of mutant superoxide dismutase accumulation (Wong et al., 1995; Higgins et al., 2002). Whereas, superoxide dismutase protects oxygen-metabolizing cells against harmful effects of superoxide free-radicals, by catalyzing the destruction of the O²⁻ free radical (Petkau et al., 1975). However, such vacuolation may be due expansion of the mitochondrial intermembrane space and extension of the

outer mitochondrial membrane (Higgins et al., 2002) consistent with ultrastructural changes observed in SY-treated liver cells of the present study. Generally in this concern, the histopathological study by Sharma et al. (2008) revealed severe degenerative changes in the liver, kidney and testes as a toxicity of another popular azo food dye blend 'tomato red' on male albino mice.

The monoazo food dye 'SY' tested in the present investigation induced ultrastructural nuclear alterations (such as agglomerated dense clumps and/or multinucleate) in liver cells of mice. Besides, the current study detected hyperdiploidy and polyploidy (as numerical chromosomal aberrations) recording mean (\pm SD) of 5.142 (\pm 1.951) and manifesting a highly significant ($p < 0.0001$) increase. In this respect, the isolated rat liver nuclei treated with azo food dyes amaranth or ponceau 3R showed dissociation of heterochromatin and the nucleoli lost their fine-granular structure acquiring a coarse-granular configuration (Yoshimoto et al., 1984). Besides, the dense structure of heterochromatin in liver nuclei treated with ponceau 3R was completely dissociated and appeared as a lace-like configuration. These results suggested that both ponceau 3R and amaranth stimulate *in vitro* RNA synthesis by causing the dissociation of heterochromatin in isolated rat-liver nuclei and were examined by electron microscopy. In this perspective, results by Ramachandani et al. (1992) suggested that permitted food colours metanil yellow and orange II caused hepatic nuclear, mitochondrial and microsomal membrane damage with increase in NADPH-dependent enzymatic lipid peroxidation in nuclear ultrastructural components (62%) of rat liver and that the effect may be more severe with the use of blend.

Moreover, Matsuoka et al. (2001) found that azo dyes and their intermediates induced micronucleus and polynuclear cells, including binucleate cells and cells with a multilobed nucleus in two Chinese hamster cell lines, and condensed the chromatin in 100% of the cells with endoreduplication and chromosomal effects. The authors also reported that some of the azo chemicals induced a characteristic karyomorphology that might reflect abnormal cell division. In this concern, the study by Srinivasan and Bhargava (2004) suggested that the interaction of azo dye metabolites with nuclear macromolecules necessitate further prior processing which actually may occur in the nucleus, whereas hepatic binding proteins translocating azo dye carcinogen metabolites from cytoplasm into nucleus in liver of rats.

In the current SY-fed group, the injury of the hepatocytes was manifested as dilatation and fragmentation of endoplasmic reticulum (ER) and the nuclear chromatin material of such cells appeared aggregated peripherally as apoptotic clumps. In which, the apoptosis is characterized by early nuclear collapse and massive condensation of chromatin (Wylli et al., 1980). Although such changes were reported as an early signs of cell death (Sasaki et al., 2004), in which the

livers are characterized by the presence of two different morphological types of developmental cell death. Type I corresponds to cytoplasmic type degeneration and non-apoptotic death and type II to nuclear type cell death or apoptotic death (Sasaki et al., 2004). These ultra-cytological changes were also induced in rat liver cells by the azo dye (2-Me-DAB), in which the profiles of the granular endoplasmic reticulum in hepatic cells of rats became disorganized and the dispersed cisternae often appear fragmented and irregular, and induced an increase in the number of mitochondria per cell (Lafontaine and Allard, 1964). Such dramatic ER dilation, followed by DNA fragmentation, could be provoked by apoptotic agents perturbing ER functions (H-Cki, et al., 2000). Nevertheless, Liedtke and Friedman (2003) stated that the cytoplasmic type I degeneration with dilatation and fragmentation of ER, it could result from the effect of abnormal osmotic stress, whereas osmotic homeostasis is one of the most aggressively defended physiological parameters in human and rodent cells. In more recently, in the liver of mice, deletion of AQP11 resulted in disrupted the rough endoplasmic reticulum (RER) homeostasis and increased sensitivity to RER injury, showing rapid vacuolization upon metabolic challenge with amino acids feeding (Rojek et al., 2013). In apoptotic assay by Bafana et al. (2009) on direct red 28, which is an azo dye widely used in several countries, showed an initial increase in bacterial toxicity upon biodegradation due to release of mutagenic products, like benzidine and 4-aminobiphenyl, from the dye. These intermediates caused a significant DNA damage and induced apoptosis in HL-60 cells. In another cytotoxic mechanism of azo food colouring agent (such as p-anilinoaniline; a metabolite of metanil yellow) induced transient G1 and S cell cycle phase arrests; whereas cytostatic concentrations induced protracted arrests and caused DNA damage, as monitored by comet assay and morphological changes consistent with cells undergoing apoptosis and/or autophagy (Elliott and Reiners, 2008).

In addition, SY-severe deteriorated hepatocytes and phagocytic Kupffer cells obtained in the present research are symptomized by inflating lysosomes. Such results may be due to endocytosis and filling with SY fluid as suggested by Espada et al. (1997), who reported that occurrence of azo dye 'fast red' in lysosomes resulted from fluid-phase endocytosis in living and pre-fixed cells.

The current magnitudes and types of food dyes of both synthetic SY- and natural CU-induced chromosomal aberrations, recording the total mean percentages of 34.11% (as nine varieties) and 4.55% (in limited minute values), thus exhibiting a statistical significant increase ($p < 0.05$) and a statistically insignificant ($p > 0.05$) effect, in respective manner, relative to the control group (2.77%). In this perspective, Agarwal et al. (1993) studied chromosomal aberrations induced by ponceau 4R (an azo food dye) and beta-carotene (a natural food colour) were studied on bone

marrow cells of mice *in vivo*. The results indicated that ponceau 4R was more clastogenic than beta-carotene, even at a minimum effective dose inducing a significant number of chromosome aberrations.

The genotoxicity of SY interpreted as the three major structural classes of synthetic dyes (containing azo colourings) exhibited activity to genotoxicity (involving chromosome assays), which may due to the presence of certain functional groups, notably nitro- and amino-substituents which are metabolized (such as azo-reduction) to ultimate electrophiles that may be stabilized by electronic interaction with aryl rings causing activating or detoxifying of azo agents (Combes and Haveland-Smith, 1982). On another avenue, Chequer et al. (2012) suggested that the micronuclei as a result of erythrosine (US-FDA approved azo food dye) mutagenicity in HepG2 cells were arisen from clastogenic and aneugenic processes, whereas aneugenic was responsible for the DNA single strand breaks. Several studies have demonstrated the frequencies of genotoxic effects of various artificial azo dyes on the DNA damage using the micronucleus assay, such as Chequer et al. (2009) in human lymphocytes and in liver HepG2 cells caused by red-1 and orange-1.

Ring chromosomes are from the most structural aberrations detected in SY-bone marrow cells of mice, recording the mean (\pm SD) of 3.571 ± 1.988 and carrying out significant ($p < 0.05$) increase. Chromosome ends are complex structures, consisting of repetitive DNA sequence terminating in the ssDNA overhang with many associated proteins. Because alteration of the regulation of these ends is a hallmark of cancer, telomeres and telomere maintenance have been prime drug targets by inhibiting of Pot1 binding to telomeric DNA (Altschuler et al., 2012). According to that statement, SY may be affect on the regulation of DNA telomeric ends as inhibiting target of Pot1 binding to telomeric DNA and could be resulted in formation of ring chromosomes (Altschuler et al., 2012). On molecular avenue, Kashanian et al. (2012) conducted spectroscopic methods to analyze the interaction of native calf thymus DNA (CT-DNA) with sunset yellow (SY), suggesting that SY interacted with CT-DNA *via* groove binding mode, indicating that *van der Waals* interactions and hydrogen bonding were the main running forces in the binding of SY with DNA, which are evidences to show that the reaction is exothermic and enthalpy favoured. Moreover, the molecular docking experiments by Guo et al. (2011) revealed that the azo food dye 'acid red 73' was capable of interacting with the minor groove of the DNA on the basis of its curved shape, which fit well with the topology of dsDNA. Furthermore, azo food dyes amaranth, allura red and new coccine-induced colon DNA damage in rats and mice (Shimada et al., 2010).

The current data concluded that the clastogenic potent of SY on chromosomes of mice bone marrows was recorded statistical significant increase ($P < 0.05$),

involving structural abnormalities of deletions/or fragments, ring chromosomes, breaks/ or gaps and end to-end-association. Such records were reinforced by Ishidate et al. (1984) and Giri (1991), who found that SY was a positive in clastogenesis test *in vitro* using Chinese hamster fibroblasts. On the same manner, the most previous genotoxic effects of SY were also detected by another azo food colour 'carmoisine' in the rat's bone-marrow cells (Ali et al., 1998), which induced various pictures of chromosomal aberrations. These pictures were represented in stickiness, centromeric attenuation, centric fusion, end to end association and hyperploidy and deletion which were statistically significant increase ($p < 0.05$) from control group at certain periods of treatment (30 and 60 days).

Concerning the current induced ring chromosomes in CU-group were recorded the mean (\pm SD) of $0.5714 (\pm 0.5345)$ in a non-significant ($p > 0.05$) effect in a contest with control ones (3.5714 ± 1.9880). The study proposed that declining in the aberration of ring chromosomes after CU treatment may be attributed to the inhibition of telomerase, a reverse transcriptase that maintains telomere length, and induction of apoptosis by curcumin in bone marrow cells of mice, as investigated by Chakraborty et al. (2006) in K-562 cells. Thereupon, the shortening of telomeres and the kinetics of telomeric DNA loss, which were accompanied by an increase in the incidence of chromosome rearrangements and could potentially derive from telomere association and fusion, were formed ring and dicentric chromosomes (Counter et al., 1992).

The magnitudes and types of curcumin-induced chromosomal aberrations in CU-group are generally exhibiting a statistically non-significant ($p > 0.05$) effect relative to the control group. Such records are in agreement with the study on diets of mice containing 0.5% turmeric or 0.015% curcumin did not show significant differences in the incidence of structural and numerical chromosomal aberrations in their bone marrow (Vijayalaxmi, 1980).

In the mouse, the analysis of immature erythrocytes in either bone marrow or peripheral blood is equally acceptable for those in mature erythrocyte cell population for micronucleus analysis when the exposure duration exceeds 4 weeks (Hayashi et al., 1994). Moreover, the micronucleus assays have emerged as one of the preferred methods for assessing chromosomal level DNA damage because they enable both chromosome loss and chromosome breakage to be measured reliably using also simple morphological criteria (Fenech, 2008) and in identification of genetic disorders (AlFaisal et al. 2012). Hence, the present study depended on the previous purposes, showing that the animals fed daily with 30 mg/kg SY for 30 consecutive days induced a highly significant elevation ($P < 0.0001$) in the frequency of micronucleus than their respective controls. Intimately related to view on the current study, at which the food

dye sunset yellow and its metabolites were administered twice, at 24 h intervals, by oral gavage to mice and assessed *in vivo* gut micronucleus test for genotoxic effects (frequency of micronucleated cells) and toxicity of apoptotic and mitotic cells, recording moderate influences (Poul et al., 2009). The present data of micronucleus frequencies are in agreement with another azo dye 'direct violet' treatments increasing the number of micronucleated polychromatic erythrocytes with statistically significant ($p < 0.0001$) in male rats with respect to the controls (El-Rahim et al., 2008).

In the present study, the means (\pm SD) of micronucleated peripheral immature and mature erythrocytes (1.2 ± 0.9189) of male mice oral fed with the equivalent ADI dose (37mg/kg b.wt. of curcumin) daily for 30 days (CU-group), and their respective control group were did not induce a significant increase ($p > 0.05$). This assay was endorsed by Vijayalaxmi (1980), who investigated that the incorporation of either turmeric (0.5%) or curcumin (0.015%) into diets of mice did not show significant effect on the incidence of micronucleated polychromatic erythrocytes.

CU-oral feeding induced a significant decrease ($p < 0.05$) in mitotic index (28.5 %) of bone marrow cells, whereas the frequency of its mean (\pm SD) scoring 57 ± 7.07 when analyzed with the mitotic ratio (MI) of its respective control (68.4 ± 3.361). Such inclination of MI after treatment with CU may be due to its inhibitory effect *via* it's interfere with the growth factor signaling pathways through apoptotic process, as exemplified this phenomenon in preventive therapy for prostate cancer in human by progressing to its hormone-refractory state, besides it is used in food and medicine in India for centuries (Dorai et al., 2000). Furthermore, the level of mitotic index (MI) in bone marrow cells of mice under CU was relatively stable may due to the mechanism of curcumin (as an antioxidant) in controlling *in vitro* experimental breast cancer model (Alpha model) as well as in isolated cells derived from breast specimens as reported by Calaf (2014). Who found that CU interfered with multiple genes that promote carcinogenesis, therefore affecting genomic instability and preventing alteration of protein expressions involved in key signaling pathways.

Moreover, CU-oral feeding induced a significant decrease ($p < 0.05$) in mitotic index (28.5 %) of bone marrow cells, whereas the frequency of its mean (\pm SD) scored 57 ± 7.07 when analyzed with the mitotic ratio (MI) of its respective control (68.4 ± 3.361). Such results are in agreement with Holy (2002), who found that many cell types are arrested in the G2/M-phase of the cell cycle after curcumin treatment in human MCF-7 breast cancer cells exhibiting monopolar spindles, and chromosomes do not undergo normal anaphase movements. Then after 48 h, most cells eventually leave M-phase, and many formed multiple micronuclei instead of individual daughter nuclei. The author interpreted that

the production of cells with micronucleation after curcumin treatment suggested that at least some of the cytostatic effects of this phytochemical were due to its ability to disrupt normal mitosis, and raised the possibility that curcumin may promote genetic instability under some circumstances. Also, there is another support on the ability of CU to disrupt normal mitosis causing a significant decrease ($p < 0.05$) in mitotic index of bone marrow cells in mice, as resulted in the present work. This support depended on that CU (diferuloylmethane) and its derivative (monoacetylcurcumin) were selective inhibitors of eukaryotic DNA polymerase λ *in vitro* (Mizushina et al., 2003 and 2005).

CONCLUSION

However, in spite of these morphologic criteria, cytotoxic and genotoxic alterations of a synthetic SY in the present study and other investigations, it is prohibited in the world and is found as a prevalent food colour in the most food categories, beverages, cosmetics and drugs. Such suppose was also indicated by Ha et al. (2013), who recorded that among 704 foods sampled, 471 contained approved synthetic colours (especially, SY) in Korea and intake limit to a maximum of 47.8% in Indian children (Dixit et al., 2013). On conclusion, the present study tries to find alternative biosafe food additive for the synthetic sunset yellow from cytotoxicity and genotoxic aspects by supporting to use the natural curcumin as a nutraceutical, after assessment both dyes. From this perspective, this study is indicating that the use of this synthetic food additive 'sunset yellow' by the population requires greater scrutiny. Therefore, further studies are necessary varying the doses, exposure times, and system-tests to accurately evaluate the potential risks of the mutagenic agents present in the composition of this food colorant 'SY'. These results also are important to advise the committees responsible for the ADIs, such as FAO/WHO, JECFA, and ANVISA, on the establishment of appropriate tolerable limits for the use of these food additives. The implications of the present findings for the health and safety of occupationally exposed peoples are necessitated to further investigations.

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