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Original Research Article

The use of *GST-μ* Gene and Isoenzymes as Biomarkers to Evaluate the Mutagenicity and Hepatic Carcinogenicity in the Mouse by Carmoisine 'E122'

Mohamed S. Salama[1], Mohamed A. Ismail[2*], Mohamed A. Shahin[2] and Heba M. Yassin[2]

[1]Faculty of Science, Ain Shams University, Cairo, Egypt

[2]Department of Biology, Faculty of Education, Ain Shams University, Cairo, Egypt

***Corresponding Author E-mail: mohesmael@hotmail.com**

Abstract

In the past few years there has been a sharp rise in the use of food and beverages colourants to improve their characteristics. More worryingly is the commonly used colourant in most pharmaceutical syrups and coating of pills and capsules, particularly in children medicines. So, the present work was planned to study the effects of carmoisine as a common synthetic food-drug colorant in Egypt on the liver of the male mouse. The work plan was designed to cover four parameters: Morphological or external symptoms, protein expression and seven isozymes changes that might appear on the carmoisine-treated animals. Besides, molecular studies involving two main aspects: firstly, application of restriction endonuclease (RE) digestion (*Sma*I, *Xba*I, *Hind*III) on genomic DNA of mice liver in both treated and control groups. Secondly, PCR analysis to investigate the capability of carmoisine to induce mutations in *GST*-μ gene in treated groups comparable to control ones. According to FAO/WHO, the mice were given the Acceptable Daily Intake (ADI) dose of carmoisine (0.02mg/kg b.wt). This dose was also given in half and double fashions for 45 and 90 days for each. The results revealed that the carmoisine caused many morphological symptoms reflected mainly by agitation and a marked loss of their body weights in a significant decrease ($p<0.05$). The protein banding patterns in the liver carmoisine-treated mice exhibited distinct collapses when analyzed by native-PAGE and SDS-PAGE. The study also detected several changes in the profiles of the seven isozymes analysis for all treated groups. The RE detected liver DNA alterations. However, the *GST*-μ gene was not affected by this treatment, whereas the band at ~160 bp was found in entirely treated and control groups. Such phenomenon reflected the absence influences of carmoisine as a carcinogenic agent as dosages and periods used in this study on male mice, so further sequencing investigation is required for the evaluation of the safety of that E122.

Keywords: Carmoisine, *GST*-μ, Isozymes, Liver, Mice, PCR, Protein Expression, Restriction Endonucleases.

INTRODUCTION

Colour plays a significant role in acceptability of food stuffs, but unfortunately not all coloring agents are harmless. However, there was an increase in the use of toxic synthetic colorants over the last 30 years (Tuormaa, 1994; Sawaya et al., 2008). In Egypt, there has been a sharp increase in the use of synthetic food colorants in the past few years (Salah, 1994; Amin et al., 2010; El-Sheikh; Al-Degs, 2013). One of the most famous food

additives which are used as coloring substance is carmoisine (azorubine) (Dehghan et al., 2011). The present used food colorant in this study “carmoisine” is commonly and currently used in the manufacture of soft drinks, packet soup mix, jellies, sweet confectionery, cheese cake mixes, Swiss roll, sponge puddings, chocolate mousses, jams, milk shake mixes, brown sauce, mouthwashes, …. Etc (Wolff and Oehme, 1974; Hartman et al, 1978; Chanlona et al., 2005; Minioti et al., 2007; Gupta et al., 2009) in either domestic and export food supplies (Yamjala et al., 2016). Besides, carmoisine is a widely used in making ice-cream (Giovine and Bocca, 2003) and in drugs and cosmetics (Snehalatha et al., 2009; Amin et al., 2010). The most use of carmoisine as a reddish dye is in the meat coloration as an improvement and attractive agent for clientage by making it visually aesthetic or to reinstate the original appearance lost during the production process (Knowles et al, 1974; Yamjala et al., 2016). Furthermore, El-Ashmawy and Abdel-Aziz (1989) also notified the uses of that dyes in pharmaceuticals, such as drug capsules and pediatric drugs, as well as for cosmetics, as hand lotions. On extremely view, Tripathi et al. (2007) announced that carmoisine as a synthetic colour which was added to foods to replace natural colour lost during processing was adulteration needing to surveillance. So, Chakma and Moholkar (2016) selected and employed carmoisine as a model pollutant in their study on degradation/mineralization of pollutants. Also, in this concern, Villaño et al. (2016) compiled carmoisine with the most artificial colorants, ingested by humans, as a native in foods or added by the industries during processing as described together with European Union or United States regulations. Carmoisine is chemically classified as a mono-azo compound of artificial (synthetic) food color additives (Scotter and Castle, 2004). Azo dyes have been used for many years and the extent of such use is related to the degree of industrialization. Since intestinal cancer was more common in highly industrialized countries, a possible connection may exist between the increase in the number of cancer case and the use of azo dyes (Chung, 1983). Some experts claimed the most azo dyes were potentially carcinogenic for the most suspected groups of all food additives (Branen et al., 2002). The same authors also added that, all azo dyes were similar in structure, and any person who exhibited a reaction to one of such dyes could also develop symptoms from another.

The liver was used as in this study as a target organ for investigation as due to its essential role in the metabolism and detoxification of many toxic agents (Cohen, 1982; Klatskin and Conn, 1993 and Burkitt et al., 1996). In addition to the previous studies Sweeney et al. (1994), who explicated that the carmoisine-azo compound may reach the intestine directly after oral ingestion or through the bile after parenteral administration.

From the pharmacological point of view, Flaminio et al. (1988) and Tragni et al. (1990) concluded that the biological reduction of metabolic disposition for red azo dye " carmoisine" was into two main metabolites; 1-naphthyl- amine- 4- sulphonic acid (NA) and 2- amino- 1-naphthol- 4- sulphonic acid (ANA).

Concerning the genotoxicity of carmoisine, it was found that carmoisine induced various pictures of chromosomal mutations in human germ-line (Combes and Haveland-Smith, 1982) and in the bone marrow cells of rats (Ali et al., 1998). Whilst, the biochemical assay of the previous authors (Ali et al., 1998) exhibited statistically significant (p<0.05) decrease of DNA contents, while the RNA contents were statistically significant (p<0.05) increase at certain periods (30, 60 & 90 days) of treatments when compared with the normal tissues of brain, liver and kidney. Such findings were also a rather detected by Haveland-Smith and Combes (1980), who investigated that carmoisine caused DNA damage and mutations in bacterial assay systems of *Escherichia coli* strains and *Salmonella typhimurium*. Using cytogenetic analysis, Aguilar et al. (2009) claimed to have proven that important alterations in the morphology of somatic chromosomes occurred in *Secale cereale* (rye) in the presence of carmoisine. Mahfoz et al. (2010) observed that the azroubine (carmoisine/E122) caused a reduction in mitotic index, relative changes in the frequency of mitotic phases and induced a wide range of chromosomal abnormalities of mitotic cell division in *Allium cepa* cells. These changes were accompanied by a great variation in nucleic acids content. However, vitamin C is one of antimutagenic agents that could minimize the genotoxicity induced by carmoisine. Furthermore, in (2011), Agrawal et al. reported that the blend dye of food containing (sunset yellow and carmoisine) was found to increase the different types of chromosomal aberrations in bone marrow cells of mice. Furthermore, there are several evidences have indicated that other some food additive colours can also induce genotoxic effects in different organisms (Mpountouka et al., 2010; Soltan and Shehata, 2012; Ismail and Sakr, 2016). Furthermore, there are several evidences have indicated that other some food additive colours can also induce genotoxic effects in different organisms (Mpountouka et al., 2010; Soltan and Shehata, 2012; Ismail and Sakr, 2016).

Julin (1981) and Twaroj (1983) reported that the artificial food additives particularly, azo dyes and benzoate preservative are common causes of chronic urticaria and angioedema in both adult and children. The hyperactive children, who also frequently suffer from eczema or asthma, or may also develop learning difficulties or respiratory problems after food colorant intake were studied by Goulstone (2001). The author suggested that such behavioral problems may be due to intake of diets or foods and drinks containing synthetic colors (including carmoisine), flavors and preservatives.

The author also concluded that carmoisine was one of the E-number that is recommended to be avoided by hyperactive children.

The basis of electrophoretic analysis of isozymes (or isoenzymes) was laid dawn in 1957 when Hunter and Mohler discovered these isoenzymes (Stebbins 1989 and McMillin 1983). The concept of isoenzyme is defined as the different molecular forms in which protein may exist with the same enzymatic specificity (Buth, 1984). The isoenzymes are defined as multiple molecular forms of a single enzyme. These forms usually have similar, if not identical, enzymatic properties, but slightly different amino acid compositions due to differences in the nucleotide sequence of the DNA that codes for the protein. Often, the only difference among isoenzymes is the substitution of one to several amino acids resulting from differences in coding sequences of the genes controlling them (Micales and Bonde, 1999).

Glutathione transferases (*GST*; also known as Glutathione S- Transferases) are a group of multigene, multifunctional proteins that catalyze glutathione (GSH)-dependent reactions like conjugation, isomerization and reduction as part of the cellular detoxification mechanism of extracellular xenobiotics and biotransformation of intracellular toxicants (Neeraja et al., 2005). So, glutathione S- transferases (*GST*) protein might protect tumor target cells from accumulating additional mutations (Hayes and Pulford, 1995). Glutathione S- transferases-mu (*GST-μ*) is a gene code for enzyme putatively involved in the detoxification of chemical carcinogen in diets (Stewart, 2005). The same author reported that the polymorphism of *GST-μ* gene has been studied as a possible determinant of differential susceptibility to chemically- induced cancers. In the same aspect, Hayes et al. (2005) reported that *GST* (including *GST*-mu class) exhibited genetic polymorphism, this variation can increase susceptibility to carcinogenesis and inflammatory disease. The glutathione S-transferases (*GST*), which are part of an important family of dimeric phase II metabolic enzymes involved in the detoxification of xenobiotics, might modulate the adverse effects of exogenous toxins (Salinas and Wong, 1999; Li et al., 2015). Several accumulating evidences suggested that the important role of *GST* gene products in responsible for activating and detoxifying xenobiotics, which could influence cancers (Koh et al., 2011), and congenital anomalies (Lammer et al., 2005; Li et al., 2015). In close association with the current study, El-Wahab and Moram (2013) detected a significant decrease in the content and activity of *GST* in both blood and liver of carmoisine-administered male rats compared to control ones.

Sasaki et al. (2002) determined the genotoxicity of 39 chemicals currently in use as food additives (involving seven azo dyes) using comet assay with 8 mouse organs. All seven dyes induced DNA damage in the gastrointestinal organs at close to doses of the acceptable daily intakes (ADIs).

The present study aims to explore possible impacts of a certain synthetic food colorant "carmoisine" as a red monoazo dye on liver of albino male mice "*Mus musculus*" CD-1 after intraperitoneal injection, to answer some questions about safety of that colorant, which is widely used in Egypt in different food stuffs. However, few studies mentioned that carmoisine may cause irreversible toxicities on experimental animals. The study apply the biomarkers of molecular studies involving two main aspects, restriction endonuclease digestion for genomic DNA and PCR analysis to investigate the capability of carmoisine to induce mutations in the glutathione S-transferase (*GST*) gene (the μ form), in treated groups comparable to control ones, besides the biochemical analysis of total protein electrophoresis and isozymes parameters.

MATERIALS AND METHODS

The Experimental Animals

The animals used throughout this study were adult male Swiss albino mice (*Mus musculus*) CD-1 and their weights ~19 g. The animals were obtained from Schistosome Biological Supply Program (SBSP) Theodor Bilharz Research Institute. Mice were housed in suitable cages for two weeks for adaptation to laboratory conditions and for maturation. All animals were fed on standard diet (hay, wheat and milk). Food 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 Examined Material

The synthetic food red colorant additive used in the present investigation is carmoisine (also named azorubine or acid red 14), taking colour index of E122 (Lodi et al., 2015). The chemical name of carmoisine is [2-(4-sulfo-1-naphthylazo)-1-naphthol-4-sulfonic acid, di-sodium salt] and according to Püssa (2013) it has the chemical formula of $C_{20}H_{12}N_2Na_2O_7S_2$.

Carmoisine is a synthetic acid of a mono-azo dye containing both -N=N- and C=C chromophore groups (pyrazolone dye) (Blumel *et al*., 2002). It is a red dye; soluble in water and used in coloring food (C.I. Food Red-3) (Chanlona et al., 2005; EFSA, 2012; Yamjala et al., 2016). The adoption for the food additive carmoisine (azorubine) was in 1987 as published in the FAO/ CODEX food and nutrition paper series (JECFA, 1999).

Dosage and Administration

The acceptable daily intake (ADI) of carmoisine for human dosing is 4 mg/ kg b.wt./ day according to European Union (EU) permission (Scotter and Castle, 2004). Such EU was allocated with numerical ADIs were studied, initially based on reports by Food and Agriculture Organization (FAO/WHO, 1974); World Health Organization (WHO, 1991); Joint Expert Committee for Food Additives (JECFA, 1999) and (Scotter and Castle, 2004).

However, in the present study a preliminary experiment was performed to determine the smallest amount of dosage of a food colorant (carmoisine) that will be applied on mice. The acceptable intake dose for mouse is 0.02 mg/ kg b. wt. daily, after the recommended table of evaluation and design of the toxicity tests by Paget and Barnes (1964) and Paget (1970). Accordingly this dose (0.02 mg/ kg b. wt. daily) of carmoisine was also given in half (0.01 mg/kg b. wt. daily) and double (0.04 mg/kg b. wt. daily) fashions to find out the possible effects of low and high doses for that food colorant.

The three estimated doses of carmoisine in the present study (0.01, 0.02 and 0.04 mg/kg b. wt.) were given for the periods 45 and 90 days. All the used experimental doses of a food colorant were diluted with saline solution (0.9% NaCl). Such doses were injected intraperitoneally at the lower left side of abdomen in the experimental mice, and were subjected to morphological aspects.

2-4-Design of Experiment

Eighty four adult male mice were allocated into seven groups (12 mice for each):
Group (C): were served as control; they were given standard diet, water and sacrificed after both 45 and 90 days.
Group (T1): were injected with the dose 0.01 mg/kg b.wt. daily for 45 days.
Group (T2): were similarly treated with the dose 0.01 mg/kg b.wt. daily for 90 days.
Group (T3): were injected with the dose 0.02 mg/kg b.wt. daily for 45 days.
Group (T4): were similarly treated with the dose 0.02 mg/kg b.wt. daily for 90 days.
Group (T5): were injected with the dose 0.04 mg/kg b.wt. daily for 45 days.
Group (T6): were similarly treated with the dose 0.04 mg/kg b.wt. daily for 90 days.

At the end of each course of drug treatment, mice were decapitated, immediately dissected and their liver were rapidly excised, and processed for biochemical and molecular investigations.

External or Morphological Symptoms

The present study was following up the external symptoms which were encountered in the present experimented mice post carmoisine application under the precise observation and were recorded in the three dose levels and periods of treatments. The means (±SD) of body weights (g) of mice for both control (C) and carmoisine-treated groups (T1-T6) are measured at the initial work before treatment, and then followed by weighing at the end of experiment.

Biochemical studies

Native PAGE protein, SDS-PAGE protein and isozymes electrophoresis

This was performed according to Laemmli (1970) and Sambrook et al. (1989) and includes 30% acryl amide mixture, 10% separating gel, 5% stacking gel, running buffer, SDS loading buffer, Coomassie blue R-250 staining and destaining solution and prestained standard protein marker (Gibco-BRL). Seven individual samples (6 control groups and their control ones) were examined electrophoretically for the native protein gel. The results of native protein gel electrophoresis were analyzed using gel documentation and analysis software gel-pro analyzer (Media Cybernetics) version 3.0.

Samples preparation (Extraction of proteins and isozymes)

Native protein, SDS electrophoresis pattern of denatured proteins, and isoenzymes were studied in the liver of both treated and non-treated animals. After sacrificing the animals, 0.5g of liver tissue was grinded with 1ml of distilled water using a mortar and pestle until liquefying the tissue then centrifuged for 10 min at 12.000 rpm at 4°C. Supernatants were transferred to new tubes and stored at -20°C until analysis was performed. The molecular weight (M.W.) of protein bands were estimated by comparing their electrophoretic mobility's with these of known standard M.W marker loading in the same gel.

Sample application and identification of isozymes

20µl from liver extracts + 20µl from sample buffer were applied into gels (one sample in each well). The gel was run at 40 V. Until the samples reach the stacking gel, then the volt was raised to 80 V. until the tracing dye reached the end of the gel which took about 4 to 5 hours. The gel was incubated in the buffer in dark at 37°C until the specific bands appear (limited time for each, as the

type of isozyme) in according to Dietz and Lubrano (1967); Hayes et al. (1987) with a simple modify. After electrophoresis, the gels were incubated in the appropriate substrate until the isoenzyme patterns were developed and the reaction was stopped by tap water and gel was photographed.

According to Dietz and Lubrano, 1967; Atzpodien et al., 1968; Cheliak and Pitel, 1985, the electrophoretic profiles of seven experimental isoenzymes were depended on the following specific chemical reactions:

α–Naphthol + fast blue BB give 2 levels of brown precipitates for showing acid phosphatase (ACP) and alkaline phosphatase (AKP) isoenzymes. In addition, α– or β-naphthol + fast blue RR by the two isozymes α -esterase (α-EST) and β-esterase (β-EST) give yellowish brown and red coloured precipitates, respectively. Whilst the aldehyde oxidase (AO) isozyme is illustrated from the reaction of (benzaldehyde +NBT +PMS) giving acid + formazan of bluish precipitate. Besides, reaction of L-lactate + NAD in the presence of lactate dehydrogenase (LDH) isozyme gives pyruvate + NADH (which enters the second reaction with NBT+PMS) to form formazan (blue precipitate). Furthermore, the reaction between D, L malic acid +NAD when gives the products of oxaloacetate + NADH (which in turn reacts with the two chemicals (NBT +PMS) in the presence of malate dehydrogenase (MDH) isozyme will formed formazan as a blue precipitate.

Molecular Studies

Isolation of Genomic DNA

The DNA was extracted from mice's liver using "BP-10-Spin column genomic DNA isolation kit for animals" (Bio Pioneer Inc, Cat No 92191). About 30 mg of liver tissue were used to isolate the genomic DNA from each control and treated groups. The main used molecular analysis probes were derived from Aquardo et al. (1992).

Before any analysis, it was important to determine the concentration and purity of isolated DNA, this was done by estimating UV absorbance at wave length of 260 and 280 nm using a spectrophotometer. A solution of isolated hepatic DNA gave a ratio of UV absorbance of >1.8 at 260 and 280 nm, indicating that DNA was sufficiently free from proteins (Glasel, 1995; Dehghan et al., 2011).

Restriction Endonuclease (RE) Analysis of DN Purified genomic DNA of liver of each mouse was digested using three restriction endonucleases six – cutters as described by Maniatis et al. (1982):

*Xba*I	*Sma*I	*Hin*dIII
5'-T/CTAGA-3'	5'-CCC/GGG-3'	5'-A/AGCTT-3'
Sticky end	Blunt end	Sticky end

The resulting cleavage fragments were subjected to electrophoresis. To perform the digestion of genomic DNA of mouse's liver, the following reagents were added in individual micro-centrifuge tubes:

-10 X enzyme reaction buffer 2.5µl
-Genomic DNA 3µl
-Enzyme 10units
-Distilled water to final volume of 20µl

The tubes were mixed by flicking repeatedly with fingers and then centrifuged for few seconds to bring the reaction mixture to the bottom of the tube. The tubes were incubated at 37°C for 2 hours. 4µl of 6X ficoll loading dye was added to 20 µl reaction mixture. This loading dye contains EDTA, which will inhibit the enzyme action by chelating Mg++, thus preventing further nuclease activity.

Polymerase Chain Reaction (PCR) For *GST* Gene Family

Three oligo-primers were synthesized at Metabolon International AG/Deutschland corresponding to sequences in the exon 4 and 5 regions of *GST*-µ gene and the same *GST* multigene family as described by (Hollstein et al., 1993) to discover *p53* mutations in hepatocellular carcinoma patients (HCC). The sequences of these primers were as following sequences:

OLF_1: 5'-CGC CAT CTT GTG CTA CAT TGC CCG -3'
OLF_2: 5'-ATC TTC TCC TCT TCT GTC TC-3'
OLF_3: 5'-TTC TGG ATT GTA GCA GAT CA-3'

Primers OLF_1 and OLF_2 anneal to both genes of *GST* multigene family, while OLF_3 anneal to only *GST*-µ gene. The three primers are used together in polymerase chain reaction (PCR) yielding a constant 160-bp fragment in all samples and a polymorphic 232-bp fragment seen only in the *GST*-µ positive genome (Hollstein et al., 1993).

To study the *GST*-µ gene, liver DNA was used as a template. Whereas, the PCR reaction mixtures were prepared as follow:

-DNA template 2.5µl
-50 pM OLF1 4µl
-50 pM OLF2 3.3µl
-50 pM OLF3 3.3µl
-2 mM dNTPs 5µl
-10 X buffer (has it's own MgCl2) 5µl
-2 U/ µl Taq polymerase 0.2µl
- dd H2O 26.7µl

The DNA was subjected to 94°C for 4 min. for one cycle, followed by 35 cycles of denaturation (94°C for one min.), annealing (50°C for one min.) and extension (72°C for one min.). Following the last cycle, the samples were incubated for another 6 min. at 72°C to ensure that the final extension step was complete.

Gel Electrophoresis and Visualization of DNA Bands

Agarose with concentration of 2% was used for resolving the RE and PCR products at 101 volts for one hour using

1X TAE buffer. Each gel was examined on UV transilluminator filter (302 nm wavelength), to detect the ethidium bromide / DNA complex. Two types of DNA markers were used in the present study were λ DNA / *Hind*III and ΦX174 DNA/ *Hae*Ш ladders.

RESULTS

External (Morphological) Symptoms

The detectable physical symptoms encountered in the presently experimented animals post-carmoisine (azorubine/E122) application varied in their magnitude according to both dose level and period of treatment, as thereafter present. In case of high dose (0.04 mg/kg b. wt.) of carmoisine treatment, mice appeared to be unable to cling the cages, with an increase rate of respiration and a titanic chill displaying certain convulsions within 4-5 minutes subsequent to agent induction accompanied with vibrating heads and agitation state. Besides, dangling of ears and relaxing of tails were observed and both fore and hind limbs had lost their ability to movement. Then the animals exhibited staggering gait (ataxia) and dizziness, lasting few seconds, and then they occasionally fell on their sides several times, as a result of loss of their balance. By that time, the animals had lost their balance almost completely with relaxation of their limbs and resting on their abdomens; yet, the animals were unable to move. In addition, the animals appeared drowsy or lazy suffering from difficult respiration with relaxed tails about 20 minutes following the colorant agent administration, beside the testes were obviously descended. Furthermore, the ends of tail, nose and fore and hind limbs of carmoisine-treated mice were appeared marked swollen and taking reddish coloration. After that they started to restore their ability to stand on both fore and hind limbs. But nonetheless, a rather complete, recovery was achieved during a period ranging from ~30 minutes, provided that the state of dizziness, with an occasional falling on the floor of the cage, had continued for approximately an extra half an hour. Four to five hours later, they excreted large amount of red colored urine of urination. During the two periods 45 and 90 days of this treatment (0.04 mg/kg b. wt.), all animals exhibited marked increase –for about 15 days– and then decrease of their food abstinence (anorexia) followed by weakness and obviously reduced activity with a rather diarrhea. Moreover, in the case of high dose (0.04 mg/kg b. wt.) of carmoisine application, more vigorous and faster responses were encountered relative to those induced in the low dose-treated mice (0.01 mg/kg b. wt.) of carmoisine. Furthermore, marked salivation, diarrhea and frequently slight drowsiness were usually observed.

Concerning the means of body weights mice after exposure to a food colorant 'carmoisine' (T1-T6) recorded a significant decrease in a dose- and time-dependent manner in compare with respetive control (figure 1).

Biochemical Parameters

Native protein electrophoresis

In the present study polyacrylamide gel electrophoresis (PAGE) technique was used to identify the electrophoretic patterns of liver native proteins for control and treated groups. The pattern of liver proteins as shown in figure 2 was divided into 6 main zones. These zones are: albumin, fast α-globulin, transferrin (β-globulin), slow α-globulin, post transferrin and immunoglobulin (Smith, 1976). The most anodal zone is albumin, while the most cathodal one is the immunoglobulin. Seven individual samples (C and T1-T6 groups) were examined electrophoretically for the native protein gel as reflecting in figure 2.

A maximum of 14 bands were detectable out of which, 3 bands were monomorphic. These monomorphic bands had relative frequency (Rf) of 0.33, 0.51 and 0.61, and are located in the post transferrin, slow α-globulin and β-globulin zones, respectively. Eleven bands were polymorphic bands. From these polymorphic bands, one band with Rf 0.14 in the immunoglobulin zone was present in the treated samples but not found in the control one. Another band with Rf 0.48 in the slow α-globulin zone was present in control group but not found in all the treated groups. Also a band with Rf 0.65 in the β-globulin zone was found in both control and long period treated groups (T2, T4 and T6). At the same time there is one band with Rf 0.7 in the fast α-globulin zone was found in the control group and short period treated groups (T1, T3 and T5). T6 group revealed one negative unique band with Rf 0.41in the post transferrin zone, that band was present in all groups except T6 group, as demonstrating in figure 2.

The electrophoretic profile of control and treated samples will be described starting from the most anodal zone as follows:

1. Albumin zone

In the present study, albumin was resolved as a single band with Rf of 0.92. In all samples the albumin band was thin and lightly stained. The relative concentration of bands was 0.5%

2. Fast α-globulin zone

The fast α-globulin zone represents the zone that is directly cathodal to albumin. It was represented by one band with Rf of 0.7 having a relative concentration of 0.85%.

3. Transferrin (β-globulin) zone

Transferrin (β-globulin) zone had two bands, one of them with Rf 0.61 was broad and heavily stained. The dense stain indicates that the β-globulin concentration is high.

The other band located in a region very close to the

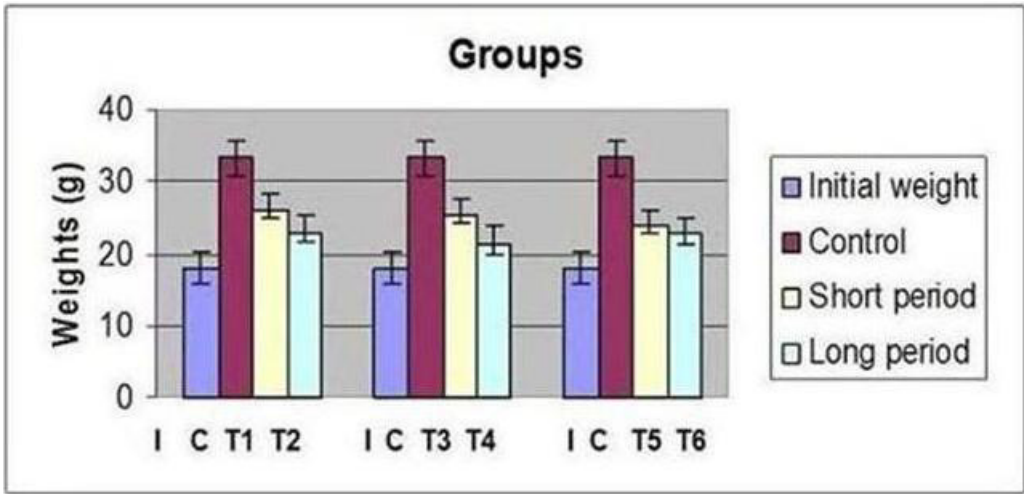


Figure 1. Revealing the body weights (g) of control and carmoisine-treated groups (T1-T6) at the end of experimental period compared with the initial weight (I) of mice before treatment. Values are represented by means (±SD) $^{n=12}$.

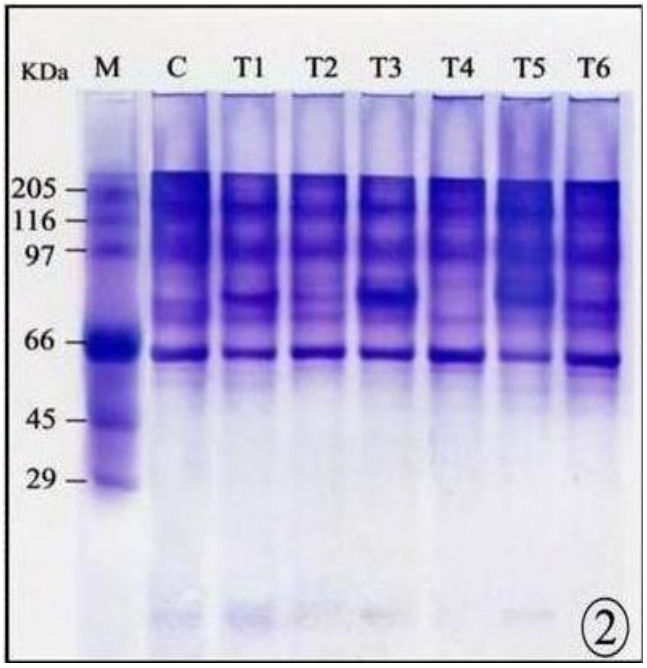


Figure 2. Electrophoretic patterns of liver native proteins for control (C) and carmoisine-treated groups (T1-T6).

previous one with Rf of 0.65.

4. Slow α-globulin zone

This zone consists of three bands closely migrating together on gel with Rf values of 0.51, 0.48 and 0.45.

5. Post transferrin zone

Post transferrin zone showed two bands with Rf values of 0.33 and 0.41. The band with Rf 0.33 stained darkly in all groups of the present investigation possessing a relative concentration equal 14.6%.

6. Immunoglobulin zone

The immunoglobulin zone had five bands with Rf of 0.14, 0.17, 0.2, 0.21, and 0.26.

SDS-PAGE

Protein banding patterns in liver of control and carmoisine-treated mice were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS –PAGE). Protein banding patterns in liver of control

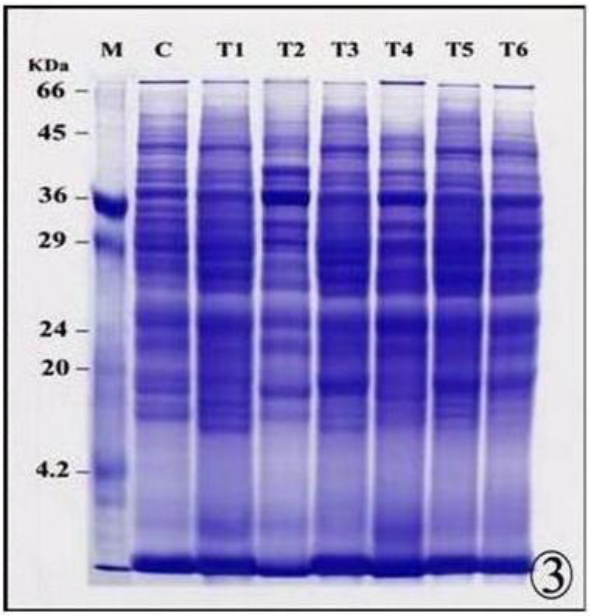


Figure 3. Electrophoretic patterns of liver SDS-PAGE of protein for control and carmoisine-treated groups.

Table 1. Acid phosphatase banding patterns of control (C) and treated groups (T1-T6).

Lanes / Rows	C	T1	T2	T3	T4	T5	T6	Rf
1	+	+	+	+	+	+	+	0.35
2	+	-	-	-	-	-	-	0.56
Total	2	1	1	1	1	1	1	

and treated mice were analyzed by SDS-polyacrylamide gel electrophoresis. The total number of bands in the profiles of all treated and control animals was 29 with approximate molecular weights ranging from 16 to 57 kilodaltons (KDa). Eight common bands at MW 42, 39, 38, 32, 29, 26.4, 24 and 17 KDa were found in the profiles of all groups as showing in figures 3. T3 group (injected daily with 0.02 mg/ kg b.w. carmoisine for 45 days) revealed the highest number of bands (25 bands), while the T4 group (injected with the same dose for 90 days) revealed the lowest number of bands (17 bands) and was deficient in two specific bands at MW 53 and 18.9 KDa, while they were detected in all other studied groups. Therefore, these two protein bands may be considered as negative specific markers for that group. On the other hand the total number of bands detected for the other groups was 23 bands for control group, 22 bands for T1 and T6 groups, 19 bands for T2 group and 20 bands for T5 group. One band of MW 16 KDa was found in short period (45 days) treated groups (T1, T3, and T5 groups). That band was absent from both control group (C) and long period (90 days) treated groups (T2, T4, and T6 groups), as showing in figures 3**.**

One band of MW 43 KDa was found in all groups except the last two groups (T5 and T6) that were injected daily with the high dose for 45 and 90 days. At the same time, time there is a band of MW 41 KDa was found in all groups except T1 and T2 groups which were injected daily with the low dose for 45 and 90 days. Also there is one band of MW 44 KDa was found in all groups except the groups that were injected daily with 0.02 mg/ kg carmoisine for 45 and 90 days (T3 and T4 groups), as illustrating in figures 3.

Isozymes Analysis

Seven isoenzyme parameters involving: acid phosphatase (ACP), malate dehydrogenase (MDH), α-β-esterases (α-β-EST), lactate dehydrogenase (LDH), alkaline phosphatase (AKP), and aldehyde oxidase (AO) were investigated in the present study to detect the possible effect of carmoisine (azorubine, E122) on the livers of mice (Figures 4-10).

Acid phosphatase (ACP)

Acid phosphatase banding patterns of treated and non-treated samples were represented in Table 1 and figure

Table 2. Alkaline phosphatase banding patterns of (C) and carmoisine-treated groups (T1-T6)

Lanes / Rows	C	T1	T2	T3	T4	T5	T6	Rf
1	+	+	+	+	+	+	+	0.07
2	+	-	+	+	+	+	+	0.23
3	-	-	+	+	+	-	-	0.34
4	+	+	+	+	+	+	+	0.9
Total	3	2	4	4	4	3	3	

Table 3. Lactate dehydrogenase banding patterns of control (C) and carmoisine-treated groups (T1-T6)

Lanes / Rows	C	T1	T2	T3	T4	T5	T6	Rf	Types
1	+	+	+	+	+	+	+	0.066	LDH5
2	+	+	+	+	+	+	+	0.21	LDH4
3	+	+	-	+	-	-	-	0.37	LDH3
4	+	+	-	-	-	-	-	0.45	LDH2
5	+	+	-	-	-	-	-	0.63	LDH1
Total	5	5	2	3	2	2	2		

Table 4. α-Esterase banding patterns of control (C) and carmoisine-treated groups (T1-T6).

Lanes / Rows	C	T1	T2	T3	T4	T5	T6	Rf
1	+	+	+	+	+	+	+	0.06
2	+	+	+	+	+	+	+	0.14
3	+	-	+	+	+	-	+	0.24
4	+	+	+	+	+	+	+	0.75
5	+	+	+	+	+	+	-	0.91
6	-	-	-	-	-	-	+	0.95
Total	5	4	5	5	5	4	5	

4. A total number of 2 bands were detected across the control samples with Rf 0.35 and 0.56. On the other hand all treated samples had only one band with Rf 0.35.

Alkaline phosphatase (AKP)

The occurrence of alkaline phosphatase bands is illustrated in Table 2 and figure 5**.** A total number of 4 bands were detected with Rf 0.07, 0.23, 0.34 and 0.9 respectively. T1 group revealed the lowest number of bands (2-bands). One negative unique band with Rf 0.23 was present in all groups except T1 group. Two common bands of Rf 0.07 and 0.9 were found in all profiles of all groups.

Lactate dehydrogenase (LDH)

The results of LDH isoenzyme for the studied groups were shown in Table 3 and figure 6. A number of 5 bands were detected and numbered as LDH1 (the fast moving ones), LDH2, LDH3, LDH4 and LDH5 (the slow moving ones).

LDH4 and LDH5 with Rf 0.21 and 0.066 respectively were detected in control as well as in all treated groups; as these two bands were monomorphic bands. The banding pattern also showed the absence of faster moving LDH1, LDH2 and LDH3 with Rf 0.63, 0.45 and 0.37 respectively from T2, T4, T5 and T6 groups at the same time these bands are present in control and T1 groups only (except LDH3 which is present in T3 group). The former groups revealed the lowest number of bands (2 bands) while the later groups revealed the maximum number of bands (5 bands). T3 group revealed only 3 bands which were LDH3, LDH4 and LDH5 while the faster moving LDH1 and LDH2 bands were absent.

α- Esterase (α- EST)

α- Esterase banding patterns of control and treated groups were represented in Table 4 and figure 7. Using α-naphthyl acetate as a substrate six bands of esterase

Table 5. β-Esterase banding patterns of control (C) and carmoisine-treated groups (T1-T6)

Lanes / Rows	C	T1	T2	T3	T4	T5	T6	Rf
1	+	+	+	+	+	+	+	0.035
2	+	-	+	+	+	+	+	0.113
3	+	+	+	+	-	-	-	0.522
4	+	+	+	+	+	+	+	0.78
5	+	+	+	+	+	+	+	0.95
Total	5	4	5	5	4	4	4	

Table 6. β-Esterase banding patterns of control (C) and carmoisine-treated groups (T1-T6).

Lanes / Rows	C	T1	T2	T3	T4	T5	T6	Rf
1	+	+	+	+	+	+	+	0.046
2	-	-	+	+	+	+	+	0.085
Total	1	1	2	2	2	2	2	

Table 7. Malate dehydrogenase banding patterns of control (C) and carmoisine-treated groups (T1-T6).

Lanes / Rows	C	T1	T2	T3	T4	T5	T6	Rf
1	+	+	+	+	+	+	+	0.085
2	-	+	-	+	-	-	+	0.116
Total	1	2	1	2	1	1	2	

activity were found in liver of mice. The bands were numbered from one to six according to the speed of migration on gel. The six bands that were detected have Rf values ranging from 0.06 to 0.95. Three bands with Rf 0.06, 0.14 and 0.75 were present in all individual samples. These bands were considered monomorphic bands. However, one band with Rf 0.91 was present in all individual samples except T6 (injected with the high dose daily for 90 days), while one positive unique band with Rf 0.95 was present in T6 group only.

β- Esterase (β- EST)

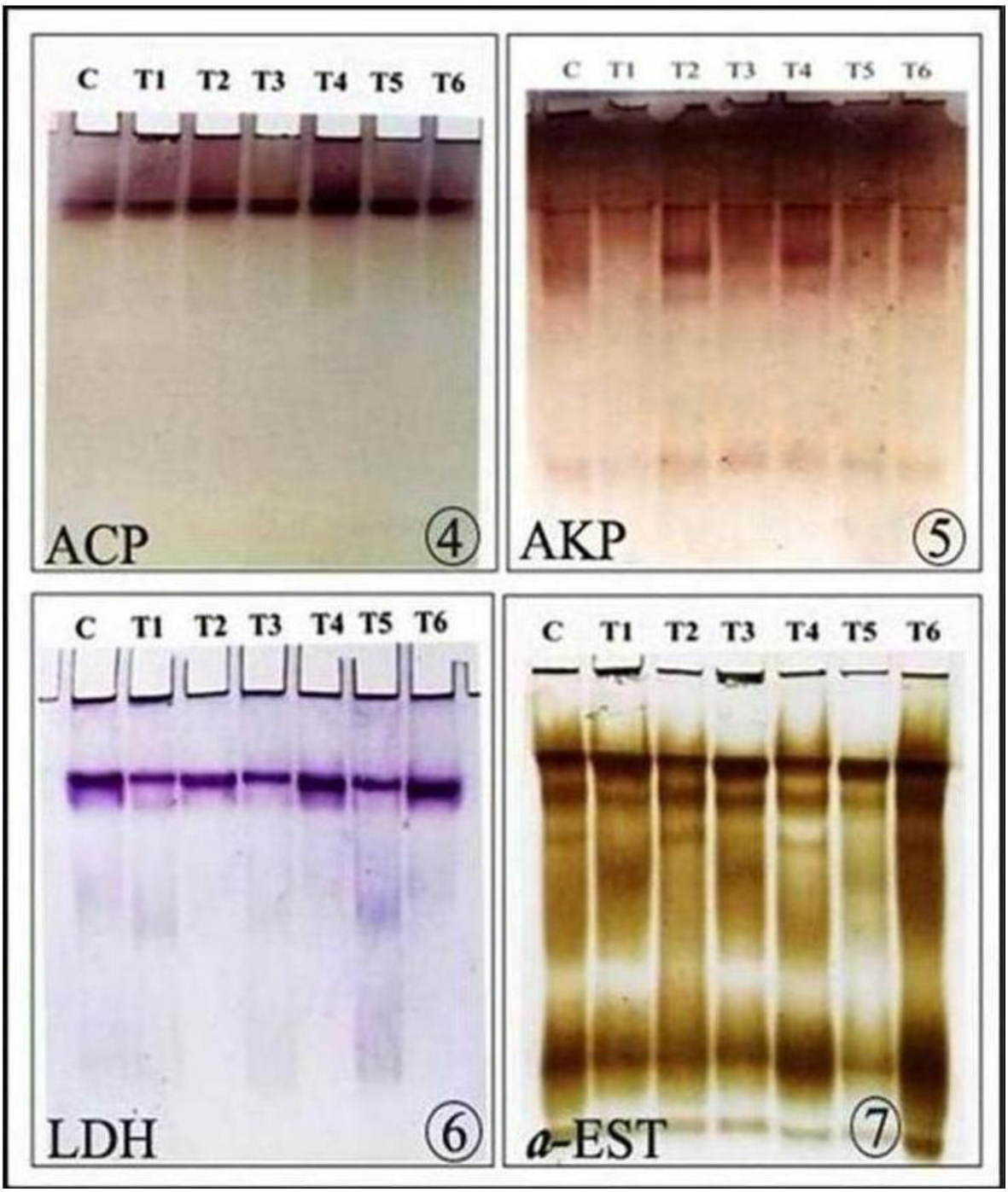
β- Esterase banding patterns of both treated and control groups were represented in Table 5 and figure 8. Using β-naphthyl acetate as a substrate, a total of five bands of esterase activity were found in liver of mice under investigation. The five bands that were detected have Rf values of 0.035, 0.11, 0.52, 0.78 and 0.95. Three bands with Rf of 0.035, 0.78 and 0.95 were present in all individual samples, these bands were considered monomorphic bands. However, one band with Rf 0.11 was negative unique band detected in all groups except in T1 group.

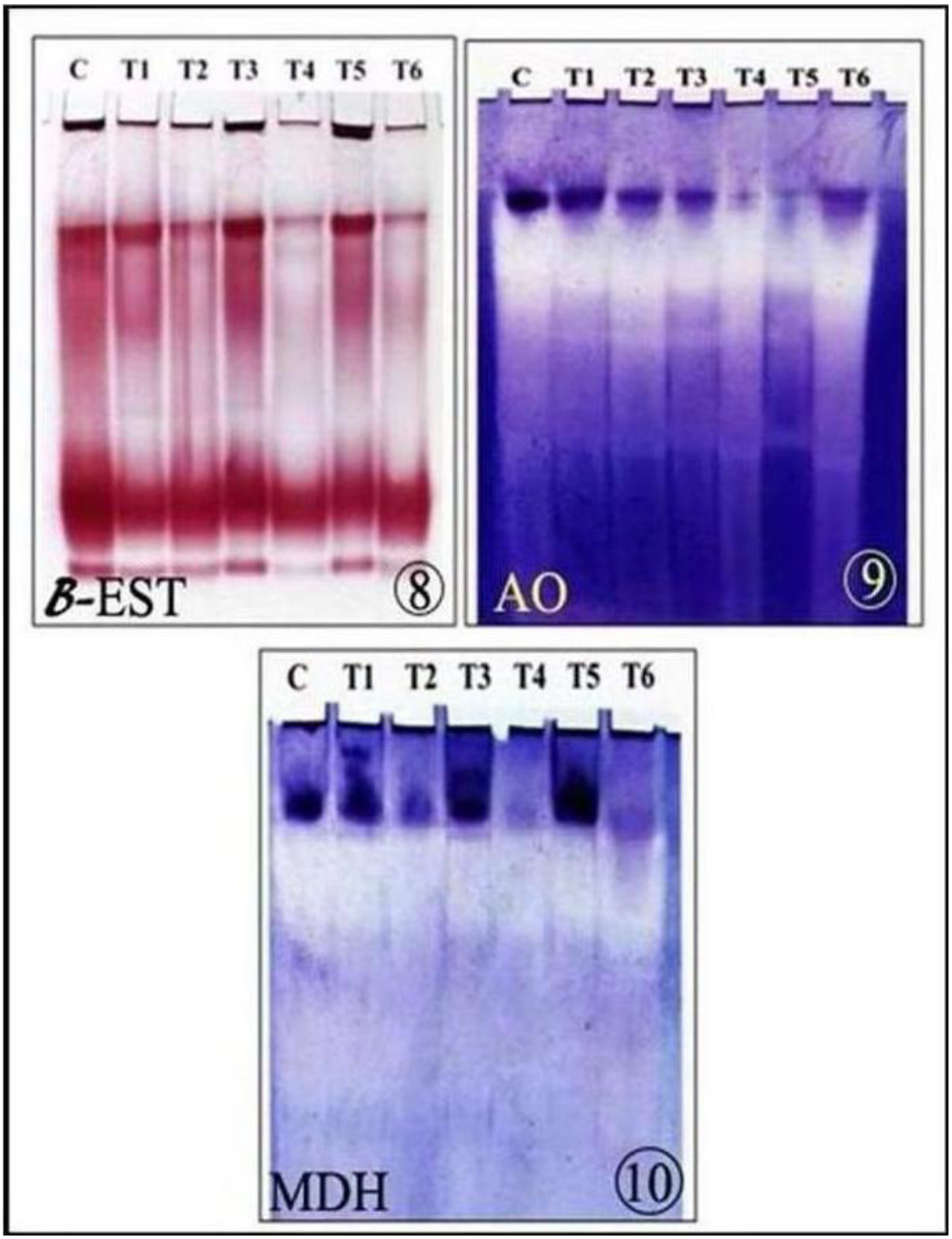
Aldehyde oxidase (AO)

Aldehyde oxidase banding patterns of both control and treated groups were represented in Table 6 and figure 9. A total number of two bands were detected across control and treated groups with Rf 0.046 and 0.085. One band, the slow moving one, was present in all samples in both treated and non-treated groups, while the second one, the fast moving one, with Rf 0.085 was present in all groups except the control and T1 groups.

Malate dehydrogenase (MDH)

Malate dehydrogenase (MDH) banding patterns of control and treated groups were represented in Table 7 and figure 10. The electrophoretic patterns of liver MDH isoenzymes exhibited mainly two bands. Only one band was monomorphic with Rf value of 0.085. The other band with Rf 0.166 was detected only in T1, T3 and T6 groups.


C T1 T2 T3 T4 T5 T6
ACP
4
C T1 T2 T3 T4 T5 T6
AKP
5
C T1 T2 T3 T4 T5 T6
LDH
6
C T1 T2 T3 T4 T5 T6
a-EST
7

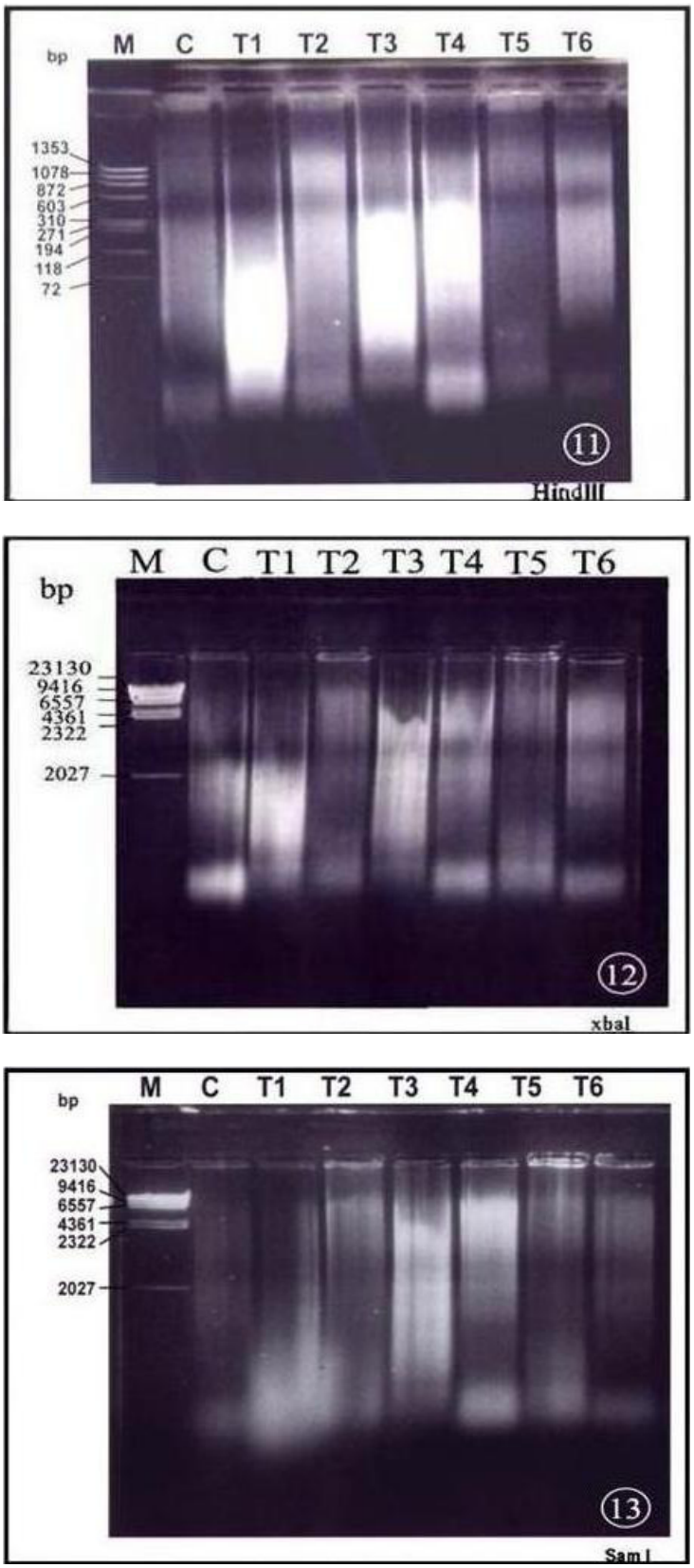


Figures 4-10. Electrophoretic patterns of seven liver isoenzymes of control (C) and carmoisine-treated groups (T1-T6), involving: acid phosphatase (ACP), alkaline phosphatase (AKP), lactate dehydrogenase (LDH), α-esterase (α-EST), β-esterase (β-EST), aldehyde oxidase (AO), malate dehydrogenase (MDH), according to arrangement figures.

Molecular Observations

Restriction Endonuclease Analysis of Genomic DNA

In the present study, the restriction patterns of treated and non treated groups were investigated using three different, 6-cutter endonucleases *Sma*I, *Hin*dIII and *Xba*I (Figures. 11-13). These enzymes cleaved genomic DNA resulting fragments had apparent low molecular sizes, some of them appeared as bands while others as smears



Figures 11-13. Genomic DNA digestion patterns of high molecular sizes DNA of control and carmoisine-treated samples (T1-T6) digested with *Hind*III, *Xba*I and *Sma*I enzymes for 2 hours at 37°C, respectively. The samples were analyzed by agarose electrophoresis.

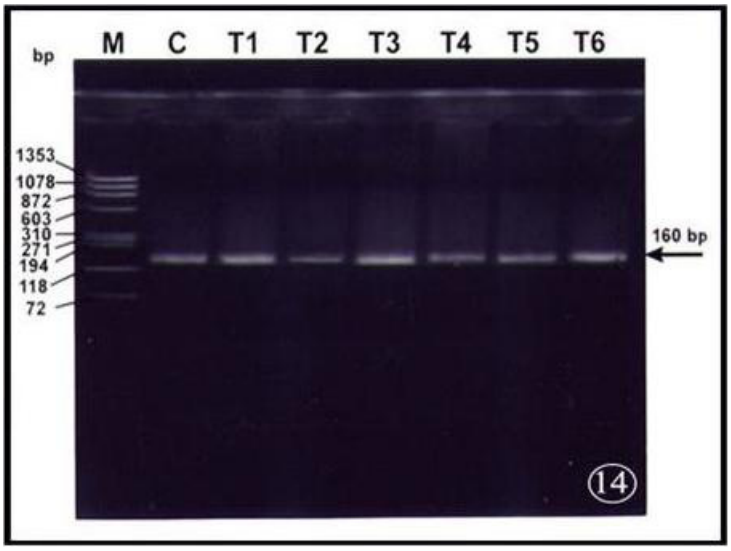


Figure 14. PCR product of *GST*-µ gene amplified with OLF primers for control and carmoisine-treated samples (T1-T6) at the band 160 bp.

of digests whatever the concentration of template DNA or restriction enzymes and the time of electrophoresis run. The resulting smears represented closely migrating DNA fragments of low molecular ratios which were not enough for any of these fragments to appear as a discrete band. The highest degree of cleavage was observed for *Sma*I which reflected a more abundant recognition sequence of that endonuclease. The lowest degree of cleavage was observed at *Hind*III**.**

***Hind*III**

The *Hind*III cleavage pattern of DNA of both control and treated groups were the same, and each of them showed a complete digestion of the major band ~23,000 bp producing 2 zones figure (11). The first zone (the slow moving one) has a molecular size in the range of ~911- ~1888 bp. The second zone (the fast moving one) has a molecular size in the range of ~ 46 - ~174 bp.

***Xba*I**

The *Xba*I cleavage of DNA of control and treated groups revealed complete digestion of the major band ~23,000 bp producing from 2 to 3 zones figure (12). The long period treated groups, i.e., T2, T4 and T6 that were cleaved with *Xba*I were the same and each of which gives 3 zones of molecular sizes in the range of ~3919- ~7500 bp, ~ 2237- ~2492 bp and ~1464-~1761 bp. Both control and T1 groups were gave only 2 zones of the same molecular sizes in the range of ~ 3596 - ~ 3800 bp and ~1082 - ~1795 bp. The last 2 remaining groups (T3 and T5), have the same number of bands (3 bands for each) of molecular sizes: ~1789bp, ~2676bp and ~3698bp for T3 group and ~1685bp, ~2356bp and ~11286bp for T5 group.

***Sma*I**

The *Sma*I cleavage pattern of DNA of control and treated groups revealed complete digestion of the major band ~23,000 bp producing bands and zones according to the group figure (13). The control and T2 groups produced 2 bands with molecular sizes ~1495 bp and ~3500 bp for control and ~325 and ~8622 for T2 group. T1 group has 3 bands two of them had the same molecular size as control group, i.e., ~1495 bp and ~3500 bp while the other band had the molecular size of ~325 bp. The remaining groups, i.e., T3, T4, T5 and T6 that were cleaved with SmaI were the same and each one gave 1 zone of molecular size in the range of ~4002-~8265 bp, and 3 bands of molecular sizes ~325 bp, ~1495 bp and ~2298 bp except for T5 where the band of 325 bp is absent.

PCR analysis

PCR product of *GST*-µ gene patterns of both treated and control groups were represented in figure (14). The figure shows the characteristic fragment ~160 bp of *GST*-µ gene in all carmoisine-treated groups (T1-T6) as well as the control one, reflecting no appearance impact of that used food colourant on *GST*-µ gene in the experimental mice.

DISCUSSION

Increasing attention has been recently paid to the toxicity of additives used in food, such as azorubine/carmoisine (Amchova et al., 2015). In connection to the presence of synthetic pigments (involving carmoisine) in food, much attention has been paid to the assessment of possible adverse human health effects, as basis for law enforcement (Diacu, 2016). So, the current study paid due attention to this topic and applied to the toxicity of the most widely used of the coloring compounds in the food, especially the most common colour of food additives (carmoisine).

The detectable external (morphological) symptoms encountered in the presently experimental animals post-carmoisine (azorubine/E122) application varied in their magnitude according to both dose level and period of treatment. During the two periods 45 and 90 days of both treatments 0.01 and 0.04 mg/ kg b. wt. of carmoisine at the present study, all animals exhibited marked decrease of their appetite followed by loss of their body weights. Such observations were confirmed by Holmes et al. (1978) in their study on rats after feeding with the dose level of 6% of carmoisine in all animals for nine weeks and by Abdel- Rahim et al. (1993) in their study also on rats for seven months at dosage of 0.2 mg /kg b. wt. From the external symptoms appeared in carmoisine-administered mice, the ends of nose and fore and hind limbs appeared swollen and marked with reddish coloration. Such observations were also detected by Galli et al. (1981 and 1982a) in male Swiss albino mice CD-1 as a very rapid distribution of that compound into the tissues after iv. administration with carmoisine (^{14}C). In agreement to the current study of reddish colouration of urine in carmoisine-treated mice, Galli *et al.* (1982b) analyzed carmoisine (^{14}C) and its metabolites in pregnant rats after 2, 16 and 64 hours by gavage dosing. They recorded that more than 90% of the radioactivity (^{14}C) was excreted in faeces and urine within 46 hours. Furthermore, Phillips *et al*. (1987) found only a trace amount of un-changed ^{14}C-labelled carmoisine in the urine of the examined rat, mouse and guinea-pig following administration of a single oral dose, beside that carmoisine was detected in the faeces of all three species. In this concern, Singh *et al*. (1997) exhibited that the aromatic amines (involving carmoisine) could be reabsorbed through the intestine to be either eliminated through urine as conjugated or retained in the target tissues to elicit toxic effects. They also detected that the caecal suspension has higher azo reductase activity than that of hepatic microsomal fraction after using the red azo dye "carmoisine" in rats. The present investigation reflected also that the ends of tail, nose and fore and hind limbs of carmoisine-administered mice were appeared marked swollen and taking reddish coloration. At which, Sweatman *et al.* (1986) observed that the case of an 8.5-year-old girl with oro-facial granulomatosis associated with clinical atopy was shown to be related to exposure to specific food additives, carmoisine, sunset yellow and monosodium glutamate. Besides, the treatment with a restricted diet resulted in considerable regression in the facial swelling which had been maintained for 6 months. Furthermore, Booth (1993) illustrated the effectiveness of dietary advice for the young boy with chronic idiopathic urticaria. An azo dye and preservative-free diet was initially advice, resulting in a total improvement in urticarial symptoms. Double- blind challenges confirmed the boy was intolerant to E122 (carmoisine). The present study also revealed that the carmoisine-treated mice appeared so lazy and suffered from difficult respiration especially after treatment with the high dose (0.04 mg/kg b. wt.). In addition to the previous external symptoms, the treated animals also exhibited staggering gait (ataxia) and agitation state as the main external observations. Such manifestations were also detected in people who are sensitive to azo dyes as due to blurred vision and constriction of their bronchi with a respiration reduction especially those suffering from asthma (Püssa, 2013). On the contrary, carmoisine had no well-documented cases of intolerance reactions in humans after oral exposure (EFSA, 2010). However, such commission attributed the absence of data on adverse clinical reactions after carmoisine treatment could be due to the lack of clinical awareness of this possibility and subsequent underreporting.

The agitation state of experimented animals accompanied with carmoisine treatment in the present study was noticed by Goulstone (2001) in children with behavioral problems. In this concern, the author recorded such problems as hyperactivity with agitation and aggressive behavior in children who use diets or drinks containing some synthetic food colours including carmoisine. These behavioral symptoms after exposure to carmoisine were also observed by Tuormaa (1994) and McCann et al. (2007) in children and Tanaka (2005) in mice administered azo dyes involving carmoisine. However, Schab and Trinh (2004) attributed such phenomenon - on a large scale - as due to accumulating neurobehavioral toxicity, which may characterize a variety of widely distributed azo artificial food colors promoted hyperactivity, non-hyperactivity and double-blind in children. Moreover, such view was supported by Lafferman and Silbergeld (1979) and Goldenring et al. (1982), who detected that the artificial food colors (or their metabolites) were strongly implicated defects in dopamine transmission. Whereas, both dopamine depletion and administration of those azo food dyes created behavioural defects in developing rats as potent neurocompetitive inhibitors of dopamine uptake by nerve endings.

On the other hand, such behavioral alterations of agitation, hyperactivity and loss of body weights of carmoisine- administered mice may be attributed to a significant increase in the rate of release of thyroid

stimulating hormone (TSH) through alteration in the pituitary-thyroid axis increasing energy consumption as a consequence of the stressing effects induced by food additives, as explained by Abdel-Rahim et al. (1993); Robert et al. (1993) and Helal (2001). In a relatively similarity of behavioral features, the current study was agreement with Püssa (2013), who published that the azo dye 'tartrazine' in alone or in combination with an another food additive 'benzoic acid may evoked migraine, blurred vision, itching, and superactivity and neurobehavioral (excitability, restlessness and concentration difficulties) in children.

In the present investigation, the biochemical study includes three main parameters which are SDS-PAGE, native protein electrophoresis and isoenzyme. The protein banding patterns in the liver of control and carmoisine- treated mice were analyzed by SDS-polyacrylamide gel electrophoresis (SDS- PAGE). Following gel analysis a number of eight common bands at MW 42, 39, 38, 32, 29, 26.4, 24 and 17 KDa were found in the profiles of all groups. T3 group (injected with 0.02 mg/ kg body weight daily for 45 days) revealed the highest number of bands (25 bands), while the T4 group (injected with the same dose for 90 days) revealed the lowest number of bands (17 bands) and was deficient in two specific bands at MW 53 and 18.9 KDa, which were detected in all the other studied groups. Therefore, these two protein bands may be considered as negative specific markers for that group. On the other hand the total number of bands detected for the other groups was 23 bands for control group, 22 bands for T1 and T6 groups, 19 bands for T2 group and 20 bands for T5 group. In close relationship to the previous results, Montaser (1998) observed that after scanning the SDS-PAGE technique of sera proteins for carmoisine- treated rats a significant increase ($p \leq 0.05$) in the number of bands (after the limited dosage of 0.11 mg/ kg b.w.) and a highly significant increase ($p \leq 0.01$) in mean percent area in both albumin and transferrin bands (after the high dosage of 0.22 mg/ kg b.w.), in comparison with the controls. In the present work, such alterations detected in T1 to T6 groups after intraperitoneal injection with carmoisine in mice may be due to the interference of food colorant with the gene expression (as thereafter discuss) and disturbs immune protein constituents (as detected by polymorphic bands in both globulin and immunoglobulin zones) to mediate the clearance of foreign particles, representing carmoisine substance.

In this view, Arcadiochonn and Cullis (1995), reported that the 50-55 kDa protein was the murine equivalent of human β-2-glycoprotein-I which plays a primary role in mediating the clearance of liposomes and, by extension, senescent cells and foreign particles. Generally, Luzio and Thompson (1990) and Dutta *et al*. (1992) declared that the changes in the electrophoretic protein profile were considered a good monitor of environmental pollution. In addition, Tsai and Yang (1975) showed that the protein electrophoresis exhibited specific pattern for each response. Furthermore, by Western blotting Yamashiki *et al*. (2002) recorded that a new 54 KDa band in alveolar epithelial cells of bovine lung, was a target of the atmospheric pollutant, phenol UPD- glucuronosyl-transferase (UGT).

After using polyacrylamide gel electrophoresis (PAGE) technique, the pattern of liver native proteins in the present investigation recorded distinct six main zones in both control and carmoisine- treated mice. These zones were included on: albumin, fast α-globulin, transferrin (β-globulin), slow α-globulin, post transferrin and immunoglobulin. Moreover, the most anodal zone was the albumin zone, whilst the most cathodal one was the immunoglobulin zone. Such electrophoretic pattern of the six marked zones in mouse native protein was recorded in human (Smith, 1976), in some rabbit breeds (Abdel-Salam, 2004) and in Egyptian sheep (Gouda, 2004).

Furthermore, the results of native protein gel electrophoresis in both control and carmoisine-administered groups were analyzed using software gel analyzer to identify the similarity and dissimilarity within the experimented profiles. The analysis detected specific polymorphic bands between the experimented animals. In which one band with Rf 0.14 in the immunoglobulin zone was present in the treated samples but not found in the control one. Besides, another band with Rf 0.48 in the slow α-globulin zone was present in control group but not found in the carmoisine- treated groups. Also there was a band with Rf 0.65 in the β-globulin zone found in both control and long period treated groups (T2, T4 and T6). These new synthesis or missing of protein bands comparable to those of control may be due to the influence of a synthetic food colorant used in this study. Such explanation was based on the opinion of Salah (1994) in her study on the effects of synthetic food colorants "erythrosine, indigo carmine and brilliant blue" on male and female mice. The presence of one band with Rf 0.14 in immunoglobulin zone in carmoisine- treated samples, and its abscence in the control one reflected the induction of immune response towards the food colorant. Such phenomenon was documented by Montaser (1998) in his study on the effects of food colorants "carmoisine and fast green" on the electrophoretic profiles of serum proteins in white rats. Moreover, the same author added that both albumin and transferrin reacted differently from each other toward carmoisine and fast green stress. The present study found also one band with Rf 0.7 in the fast α-globulin zone in both control group and short period treated groups (T1, T3 &T5). In this concern, the data by Montaser (1998) indicated that the number of serum protein bands was decreased or increased especially at the globulin region, which is the immune region, as a result of exposure to carmoisine and fast green food colorants. However, the albumin/ globulin ratio may be inverted in many diseases (Robert *et al*., 1993). The present work revealed also that the protein band with Rf

0.41 in the post transferrin zone was present in all groups except T6 group (injected with 0.04 mg/ kg b.w. for 90 days). In this respect, it was reported that the increase in the percentage of transferrin indicated a hypotonic anaemia (Tietz, 1986 & Montaser, 1998). The appearance of new protein band may be illustrated by Cholod (1974), who explained that the pre- albumin region is the low molecular weight, and under the influence was defined as the post- albumin region as due to its migration distance. Also according to Dutta *et al*. (1992) regarded that the new bands formed in the protein electrophoresis were due to the breakdown of high molecular weight bands.

In the present study, seven isoenzyme systems were investigated in the liver to detect the possible effects of carmoisine (azorubine, E122) on mice. These isoenzymes included: acid phosphatase (ACP), alkaline phosphatase (AKP), lactate dehydrogenase (LDH), α-esterase (α-EST), β-esterase (β-EST), aldehyde oxidase (AO), malate dehydrogenase (MDH),

The results showed that acid phosphatase (ACP) were represented by two bands in control group, while the treated groups had only one band, which was the slow moving band. In the same aspect, Khalil (2004) found that acid phosphatase revealed only one zone of activity in sera of progeny of CD- 1 mice, while in the parents that were treated with stevia (natural sweetener) for a year this zone was more diffused. The first few reports of acid phosphatase activity indicated the presence of only single zone of enzyme activity after electrophoresis of sera in normal human individuals (Latner, 1986). However, later studies found four zones of acid phosphatase in the sera of patients with prostate carcinoma. Further observations have demonstrated the occurrence of four or five zones of acid phosphatase in cases of myeloma and carcinoma (Finlayson and Bouchier, 1995 and Tamari, 2003).

The present study used the liver as a mouse target organ to study the effects of carmoisine because the major isoenzyme of alkaline phosphatase in mature animals was isolated mainly from the liver tissues as stated by Thakore *et al*. (1981). Alkaline phosphatase (AKP) in this study was represented by four bands, two of them were monomorphic, one band was negative unique band for T1 group and the fourth band was found in long period treated groups only (T2, T4 and T6). Such observations may be attributed to that the long period of carmoisine treatment may stimulate the production of new alkaline phosphatase isoenzyme. In the same manner, Khalil (2004) found that after treatment of CD- 1 male and female mice with stivea (natural sweetener) for a year, the liver alkaline phosphatase revealed only two bands one of them was represented in almost all treated and non-treated individuals while the other band was present in two individuals only one treated and the other was non- treated. The author attributed these findings to the differences between groups and normal variations rather than the treatment effect.

The present results of lactate dehydrogenase isoenzyme (LDH) showed the presence of five bands taking numbers from LDH1, the fast moving one, to LDH2, the slow moving one. These findings were in agreement with Pasteur and Pasteur (1988) who stated that the individuals are heterozygous for lactate dehydrogenase and have four polypeptide chains showing five regularly spaced bands. They also added that lactate dehydrogenase from mouse heart shows five possible combinations of two subunits of tetrameric enzyme. Wilkinson (1970) reported that LDH1 carrying the highest negative charge and migrating fastest towards the anode during electrophoresis. He also observed that the principle isoenzymes of lactate dehydrogenase in the liver and skeletal muscle are LDH4 and LDH5. The present study revealed no changes of the two previous isoenzymes (LDH4 and LDH5) in control and all treated groups as all groups possessed two bands. Furthermore, LDH1 and LDH2 in this investigation were presented in control and T1 groups only while those bands were absent from the other groups. In the same aspect, Thakore *et al*. (1981) stated that lactate dehydrogenase isoenzyme patterns showed a gradual decrease of the faster moving LDH1 and LDH2 bands, whereas they disappeared completely when hepatic tumors found. Besides, LDH1 and LDH2 are characteristic of heart muscle, the excess of them revealed a case of infective hepatitis (Wilkinson, 1970). They also suggested that LDH5 is adapted for anaerobic glycolysis, while LDH1 is associated with oxidative metabolism, this is very important in glycolytic processes in cancer tissue.

After using α-naphthyl acetate as a substrate, in the present study, a number of six bands of α-esterase (α-EST) activity were assigned. This resolution could be attributed to differences in molecular weights and in the net charge of each isoenzyme form (Wilkinson, 1970). Three bands out of six were monomorphic bands. Another band was found in all groups except T1 and T5. Also one band was positive unique band for T6 group and the last band was negative unique for T6 group only. In agreement with the present investigation, Gouda (2004) found six bands of esterase activity in the plasma of sheep after using α-naphthyl acetate as a substrate for α-esterase isoenzyme. In the same aspect, Abdel-Salam (2004) found a total number of ten bands of esterase activity in the plasma of rabbit breeds after using α-naphthyl acetate as a substrate, some of them were present in all individuals, while some were lacking in other. She attributed these findings to five structural genes. In more depth, there is more than one genetic locus responsible for the biosynthesis of esterases. Van Zutphen *et al*. (1987) reported that results of a test performed on rabbits indicated that esterase-6 was detected in many organs, particularly the liver and small intestine, but not in erythrocytes and blood serum. Concerning the use of β-naphthyl acetate as a substrate

a number of five bands of β- esterase (β- EST) activity were found. Three bands of them were monomorphic; one band was negative unique band for T1 group and the last band was found in C, T1, T2 and T3 groups. In the same aspect, Gouda (2004) found six bands of esterase activity in the plasma of sheep after using β-naphthyl acetate as a substrate for β -esterase isoenzyme.

In this investigation, the electrophoretic pattern of liver malate dehydrogenase (MDH) exhibited mainly two bands one of them was monomorphic while the other was detected only in T1, T3, and T6 groups (0.01, 0.02 & 0.04 mg/ kg b.w. respectively) in comparison with those of control. These findings were in agreement with Khalil (2004) who observed that the electrophoretic pattern of liver malate dehydrogenase isoenzyme exhibited mainly two bands in male and female mice. Also, Abdel-Salam (2004) found two distinct bands of malate dehydrogenase isoenzyme in the sera of rabbit breeds. She attributed that to the activity of two loci, one for each band. Pasteur and Pasteur (1988) stated that in most mammals, the study of malate dehydrogenase isoenzyme revealed at least two isoenzymes in each individual. Two electrophoretically separable forms of NADP-dependent malate dehydrogenase isoenzyme had been detected in mouse and pig tissue (Wilkinson, 1970). The same author added that liver malate dehydrogenase isoenzymes differ in their electrophoretic mobility in certain strains of mice. A series of human tissues was examined by Wilkinson (1970), who found identical patterns with malate dehydrogenase concentrated in two bands. These observations were unable to detect any specific changes in the sera of patients with elevated serum malate dehydrogenase activities and concluded that the determination of isozyme pattern for this enzyme had a little value in the diagnosis. Furthermore, Wilkinson (1970) determined that the malate dehydrogenase of rat liver consists of about 62% of the cytoplasmic form and 38% of mitochondrial form. In the same aspect, Bleile *et al.* (1975) reported that the mobility of (m-MDH) was behind the origin, i.e., towards the cathode, while the mobility of the (s-MDH) was toward the anode. This was usually seen when samples were in the middle of the gel, far enough from the cathodal end at alkaline pH values. In more depth, Pasteur and Pasteur (1988) stated that the two fractions of malate dehydrogenase isozymes (cytoplasmic and mitochondrial forms) were elevated in serum of patients with hepatitis during acute phase of illness and also during recovery the mitochondrial fraction remained in abnormal state for a much longer period. Such phenomenon was attributed to the relatively longer half-life time of mitochondrial malate dehydrogenase. However, mutations of malate dehydrogenase isoenzyme do not affect the active center or produce abnormal conformations, which led to distortion of the active center when the enzymes became associated with the mitochondrial membrane (Pasteur and Pasteur, 1988).

In the present experimental study, a number of three restriction endonucleases (*Hind*III, *Xba*I and *Sma*I) were examined comparable with those of control mice, to investigate the deteriorated effects of the food colorant carmoisine on mice genome.

Such basic view of this investigation was carried out by Wolff *et al*. (1986). They used the cleavage patterns yielding by the restriction enzymes (*Bgl*I, *Eco*RI, *Hind*III and *Xba*I) to reveal the changes in Herpes Simplex Virus DNA after latency in mouse ganglia when compared with the original virus. Restriction enzymes are still widely used as an excellent tool for studying changes or damage occurred in DNA sequences of different living organisms under the effect of several biotic and abiotic stress factors (Duim *et al*., 1999; Hyytia *et al*., 1999; Thayer *et al*., 1999; Raux *et al*., 2000 and Steen, 2000). The results of the present investigation indicated that the *Sma*I cleaved the genomic DNA in both control and treated groups. The results showed that the cleavage pattern of 0.02 and 0.04 mg/ kg body weight doses for both periods were almost the same, while the cleavage pattern of control and the two periods of low doses treated groups were also the same. Such findings may be attributed to that the low dose had a limited effect on the recognition sites of *Sma*I on the genomic DNA when compared with 0.02 and 0.04 mg/ kg body weight doses. Concerning the digestion effect of *Hind*III the present investigation indicated that the restriction enzyme cleaved both control and treated groups in the same manner. These findings may be attributed to that the recognition sites of *Hind*III restriction enzyme throughout the genomic DNA of mouse CD-1 were not affected. Also, the present study revealed that the *Xba*I restriction enzyme cleaved genomic DNA of the long period treated groups (T2, T4 & T6) in the same manner beside, the cleavage pattern of control and T1 groups were the same. This may be attributed to that the period of treatment had the big action on the recognition sites of *Xba*I restriction enzyme. The appearance of smears as revealed by the figures of genomic DNA digestion patterns could be explained according to Brown (2002). The author mentioned that the starting DNA is long so it gives rise to many fragments after digestion with a restriction enzyme, then the gel may simply showed a smear of DNA because there were fragments of every possible length that all merge together, as usual result when genomic DNA was restricted.

Glutathione- S- transferases (*GSTs*) are considered biomarkers of cancer susceptibility, playing a pivotal role in protecting cells from carcinogenic and cytotoxic damage, as they are the most abundant and ubiquitous detoxification enzyme families (Clapper *et al*., 1998). Glutathione- S- transferases mu (*GST*-μ) is a gene code for enzymes involved in detoxification of chemical carcinogens in diet (Stewart, 2005). In this respect Clapper *et al.* (1998) reported that *GST*s genes especially μ- class has important role in cellular protection in combination with their large inherent

variability in expression among individuals. The present study has selected a class of *GST*s gene for in-depth investigation for several reasons reported by Clapper *et al.* (1998). First, large variability in the expression of *GST* isozyme indicates that individuals differ greatly in their ability to protect themselves from carcinogen exposure. Second, the important role of the *GST* isozymes in cancer prevention is indicated by their specificity for detoxifying highly reactive carcinogenic and mutagenic epoxides, such as those present in cigarette smoke and other chemical compounds. Finally, epidemiological studies continue to document an association between the *GST*-µ null genotype and increased risk for a variety of human cancers, including lung, bladder, and larynx cancer. The results of the present investigation for polymorphism of *GST*-µ gene after using PCR amplified segments on agarose gel were negative as the characteristic fragment of ~160-bp of the gene was found in all carmoisine-treated groups as well as control group, while the polymorphic band of ~232-bp was absent from all previous groups. Such observation confirms that of Board *et al.,* (1990) and Clapper *et al*., (1998) who mentioned that 40-60% of healthy human individuals do not express the isozyme of ~232-bp band. The glutathione S- transferases are a critical factor in determining the sensitivity to chemical carcinogenesis (Hollstein *et al*., 1993). The µ form of *GST* gene may be polymorphic due to gene deletion (Board *et al.,* 1990 and Clapper *et al*., 1998). The polymorphism of that gene was detectable on agarose gel as a positive band at ~232-bp in some individuals while the ~160-bp band is constant in all individuals (Hollstein *et al*., 1993). The findings of the present investigation for carcinogenicity and genotoxicity of carmoisine on *GST*-µ gene did not give a clear result, whereas all the carmoisine administered mice beside the control have ~160bp band reflecting the absence of marked effect on *GST*-µ gene. The observations are in agreement with Chung (1983) who classified carmoisine as a non- mutagenic azo dye with or without microsomal activation after testing the mutagenicity of carmoisine on five tester strains of *Salmonella typhimurium.* In spite of the previous view of Chung (1983), the degree of toxicity of carmoisine and of its metabolites may undergo initial reduction followed by a series of reactions leading to the formation of toxic compound(s) after interaction with other substances as postulated by Marathe *et al*. (1993).

Glutathione transferases (*GST*; also known as Glutathione-S Transferases) are a group of multigene, multifunctional proteins that catalyze glutathione (GSH)-dependent reactions like conjugation, isomerization and reduction as part of the cellular detoxification mechanism of extracellular xenobiotics and biotransformation of intracellular toxicants (Neeraja et al., 2005). The glutathione S-transferases (alpha, mu, and pi), a family of Phase II detoxification enzymes, play a critical role in protecting the colon mucosa by catalyzing the conjugation of dietary carcinogens with glutathione (Szarka et al., 1995 and Sheehan et al., 2001). Moreover, Szarka et al. (1995) used in the glutathione S-transferase (*GST*) activity of blood lymphocytes and *GST*-mu expression as biomarkers of risk for colorectal cancer. Furthermore, Shearn et al. (2016) defined the role of *GST*A4-4 in buffering hepatic oxidative stress associated with chronic alcohol consumption and that this *GST* isoform plays an important role in protecting against carbonylation of mitochondrial proteins. Whereas, such protein carbonylation contributes to the mitochondrial dysfunction associated with obesity and insulin resistance, as speculated by Doyle and Fitzpatrick (2010). As the living organisms are continuously exposed to non- natural foreign chemicals that may interact deleteriously with the organism affecting the DNA integrity (Ames et al., 1990). Such accumulated DNA damage, added to spontaneous replication errors and not corrected by the repair system, could cause irreversible mutations which in turn could lead to the development of tumors and/or progression of cancer. (Doll and Peto, 1981). So, glutathione-S transferases (*GST*) might protect tumor target cells from accumulating additional mutations (Hayes and Pulford, 1995). Furthermore, from the reasons to select *GST-µ* gene in the present study was also indicated by Zou et al. (2015), who found that the various regulation of eight *GST* gene expressions (including *GST*-µ) and its embedding in the detoxification process against chemical agents, reflecting that *GST*s isoenzymes might play divergent physiological roles in the detoxification of toxins . Dehghan et al. (2011) and Arvin et al. (2012) concluded that carmoisine (azorubine) could interact with native calf thymus DNA *via* a groove or an outside binding mode with electrochemical interaction as monitored by hyperchromic effect of absorption spectra of DNA, induced certain spectral changes, and relatively small changes in viscosity of DNA. Such interaction could led to conformational changes (as a contraction in the helix axis of DNA) in the calf thymus DNA and, therefore, putative changes in the gene expression/regulation activity (Dehghan et al., 2011). To some extent, in the present study may be due not to the effectiveness and impact of carmoisine (E122) on the *GST* multigene as to the decline dosages used (0.04, 0.02 and 0.01 mg/kg b. wt.). Whereas, the study by EFSA (2012) emphasized that the efficacy of carmoisine was safe in foodstuffs for cats and dogs at up to about 50 mg/kg dry matter. Significantly increased concentrations of serum total protein, serum albumin, serum alkaline phosphatase and hepatic malondialdehyde and significantly lowered levels of superoxide dismutase, reduced glutathione and catalase in the liver tissue of azo food colours- treated Swiss albino rat were assessed when compared with control animals (Saxena and Sharma, 2015). However, Li et al. (2015) explored how polymorphisms in sequencing of the glutathione S-transferase (*GST*) genes affect the association between cigarette smoke exposure and congenital heart defects.

Shearn et al. (2016) hypothesized that increased hepatocellular damage in ethanol-fed *GST*A4 (/) mice was due to enhanced mitochondrial protein modification by reactive aldehydes. In which the deletion of *GST*A4-4 results in increased mitochondrial post-translational modification of proteins by reactive aldehydes following chronic ethanol consumption in mice.

However, many countries in the world have banned the use of most of the azo dyes in food and their usage is highly regulated by domestic and export food supplies (Yamjala et al., 2016), so we hope that this trend prevails in all countries.

CONCLUSION

This investigation addressed the potential of food azo-carmoisine to induce mutagenicity and hepatic carcinogenicity in male mice *via* protein expression profiles in native and SDS-electrophoresis, and seven isozymes analysis, besides the molecular parameters. We concluded that carmoisine affect adversely and alters biochemical markers in the liver not only at higher dose but also at low doses. The three restriction endonucleases were used under this study, *Hin*dIII, *Sma*I and *Xba*I, reflecting damaging effects of carmoisine on liver DNA. The absence of the effect of carmoisine in mice *GST*-µ gene does not necessarily mean that the case will be the same in human beings and not implicated in adverse impacts to liver. Besides, further sequencing investigation is required for the evaluation of the safety of this food colorant. Whereas, the dangerous effects of carmoisine may be also due to the exceeding dosages which intake by children and adults. So, we need further follow-up studies to provide insight into potential adverse health effects associated with the high intakes of these artificial color additive 'carmoisine' on the test population and to limit the commercial fraud.

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