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Adverse effects of MSG and aspartame on the liver of Female Albino Rats and their offspring and the possible ameliorative role Opuntia ficus-indica Fruit

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ABSTRACT

Monosodium glutamate (MSG) and aspartame are the most food addaitives allover-the world. Prolonged xposure to the two additives results in several adverse effects on the differnet body organs. Recently, natural palnts and their fruits are considered as a new medical approach for alleviating of many diseases. In the curunt work we attempt to evaluate the possible ameliorative role of Opuntia ficus-indica Fruit against adverse effects of MSG and aspartame on the liver of mother's rats and their offspring. 36 pregnant rats were used in these studies, they were classified into 6 groups(six for each); control, MSG, aspartame, MSG+ Opuntia ficus-indica Fruit, Aspartame+ Opuntia ficus-indica Fruit and MSG+Aspartame + Opuntia ficus-indica Fruit. At the end of weaning (21 day postnatal), the mothers rats and and their pups were sacrificed, the blood was collected and the whole liver was removed to investigate the histopathological and biochemical changes induced in the liver. The obtained results revealed severe histopathological signs in the liver of MSG and aspartame supplemented mother's rats and their pups, these signs included dilated hepatic sinosoids, dialted central veins, pyknotic hepatocytes and presence of multiple Kupffer cells. Also, remarkable apoptosis appeared in the hepatocytes that indicated by strong expression of P53 and weak expression for Bcl-2. An obvious increase in the liver enzymes (ALT&AST) and caspase-3 but significant decrease in the levels of antioxidants (SOD&CAT) were observed in MSG and aspartame supplemented mother's rats and their pups. Suppelementaion of Opuntia ficus-indica Fruit juice was successfully ameliorated the deleterious histopathological and biochemical alterations induced by MSG and aspartame. This amelioration was mainly attributed to the vital phytochemical and antioxidant nutrients in this fruit.

Keywords: MSG, Asparatme, Gestaion, Liver, apoptosis, pups.

1. Introduction

The change in dietary habits has caught the attention of regulators and the scientific community as a whole, since the substitution of in natural by industrialized foods has contributed significantly to lowering the quality of people's diet, a situation observed due to the indiscriminate use of chemical additives in processed foods (Polonio and Peres, 2009).

The use of food additives has increased greatly in recent years and as a result, it is estimated that 75% of the contemporary diet is made up of industrialized foods. The interaction between some food additives and the general public has not been very peaceful. Despite their wide use, these are substances capable of triggering adverse reactions, just as any other drug does, including allergic reactions, behavioral changes and carcinogenicity (Cardoso et al., 2017). One of the most widely used food additives are Monosodium glutamate and aspartame.

Monosodium glutamate (MSG) is commonly used as a flavor enhancer or food additive (Alao et al, 2010). MSG is the sodium salt of the non-essential amino acid-glutamic acid (NHIC, 2008). It is known to have some adverse effects in humans and experimental animals. MSG is metabolized in liver and eliminated through the kidney. Glutamic acid is transformed into alanine in intestinal mucosa and lactate in liver (Bhattacharya et al., 2011). Chronic administration of MSG (4mg/k and above) induced oxidative stress in experimental animals.MSG causes retinal degeneration, endocrine disorder, addiction, stroke, epilepsy, brain trauma, neuropathic pain, schizophrenia, anxiety, depression, Parkinson's disease, Alzheimer's disease, Huntington's disease, and amyotrophic lateral sclerosis (Adrienne, 1999; Eweka and Adjene, 2007). Subsequently it was documented that MSG produces oxygen derived free radicals (Singh and Ahluwalia, 2003). It is reported that MSG causes changes in the liver parenchyma of mice around central vein, dilated sinusoids, inflammatory cells and nuclei were pyknotic (Bhattacharya et al, 2011). MSG is known to have some adverse effects in humans and experimental animals. Excessive consumption of MSG (above 4mg/kg) induced oxidative stress in

experimental animals.MSG causes changes in the liver parenchyma of mice around central vein, dilated sinusoids, inflammatory cells and pyknosis of nuclei (Bhattacharya et al., 2011). Tawfik & Al-Badr (2012) reported that MSG at low doses is capable of producing adverse alterations in the liver and kidney functions.

Aspartame is a low-calorie artificial sweetener widely consumed in over 100 countries worldwide. Since then consumption of aspartame has increased as a sweetener in a variety of drinks and processed foods as well as a low-calorie alternative to table sugar. Aspartame differs from other dietary sweeteners, since it is rapidly metabolized upon ingestion into 3 components, namely phenylalanine, methanol and aspartate (Humphries et al., 2008). When aspartame is absorbed by the body, aspartic acid is transformed into alanine and oxaloacetate (Stegink, 1984), phenylalanine is transformed into tyrosine and phenylethylamine and phenyl pyruvate to a lesser extent and methanol is transformed into formaldehyde and then converted to formate (Opperman et al., 1984). Formic acid from methanol is the major metabolite accountable for its harmful effects of acute intoxication in humans and animals (Butchko, 2002). Even though the metabolism of aspartame provides around 4 kcal/g of energy (Gougeon et al., 2004), it has its own side effects in adults, children and in fetal life exposure. During pregnancy, rats exposed to aspartame (14 mg/ kg b.wt) have shown significant declines in maternal fetal weights and umbilical cord length (Portela et al., 2007). Additionally, studies have shown DNA-protein cross-link formation induced by formaldehyde, formed from methanol (Trocho et al.,1998) hepato-renal toxicity (Haliem et al.,2011) and impairment of the pituitary-thyroid axis induced by aspartame exposure (Haliem & Nesreen,2013). Previous studies have shown that aspartame demonstrate its effect on oncogene manifestation and neurotoxicity mechanism (Christian et al., Gallus et al.,2007; Gombo et al.,2007). Reports state that consumption of aspartame may lead to histopathological lesion in liver and alteration in a genetic system of the mother albino rats and offspring (Azza et al., 2012).

Children and adults consume aspartame unintentionally to a larger amount in excess than the Food and Drugs Administration (FDA) approval (Oyama et al., 2002) which leads to serious health complications because of its metabolites. Upon consumption of aspartame, its metabolites increase in the blood (Stegink, 1987) and are mainly metabolized by the liver, since the liver is the chief organ in the breakdown, where xenobiotics of drugs and chemicals metabolism takes place to a large extent. The prolonged consumption of aspartame resulted in increased methanol and its metabolites which are responsible for the generation of oxidative stress (Ashok and Sheeladevi, 2012).

The medicinal plants and phytochemicals have demonstrated the efficacy of traditional medicine to prevent the onset and progression of some chronic diseases. Cactus pears are sweet edible fruits from the cactus (Opuntia spp.) that belong to the Cactaceae family (Zorgui et al., 2009). These fruits have been used in traditional medicine for the treatment of several diseases (Serra et al.,2013) and contain a wide variety of trace elements, sugars and other bioactive compounds, such asbetalains, carotenoids, ascorbic acid, flavonoids and other phenolic compounds (Díaz Medina et al.,2007). Opuntia species (spp.) exhibit a lot of beneficial properties and high biotechnological potential. Opuntia species have been used for centuries as food resources and in folk medicine for the treatment of chronic diseases like obesity, cardiovascular and inflammatory diseases, diabetes, and gastric ulcer) and many other illnesses (Young et al., 2005). Opuntia ficus-indica is most famous species that cultivated for economical and medicinal purposes in several countries. The fruits of this species are recognized as a rich source of nutritional compounds with health-promoting activities, including antioxidant (Moussa-Ayoub et al., 2011), neuroprotective, anti-inflammatory, cardio-protective, anti-diabetic (Kaur,2012), anti-clastogenic (Madrigal-Santillán et al.,2013) and anti-genotoxic actions (Brahmi et al .,2011). In addition, they have protective effects on erythrocyte membranes (Alimi et al., 2012) and on acute gastric lesions (Kim et al., 2012) and also, they improve platelet function (Wolfram et al., 2003) and cancer chemoprevention (Zou et al., 2005). Several reports have been documented the medicinal role of Opuntia ficus-indica fruit like in prevention of coronary artery diseases (CAD (Osuna-Martínez et al., 2014), antidiabetic (Hahm et al., 2011), alleviation of obesity (WHO, 2016), anticancer (Antunes-Ricardo et al., 2014) and in Wound healing (Ribeiro et al., 2015).Accordingly, the current work was mainly designed to evaluate the possible ameliorative role of Opuntia ficus indica fruits extract against (MSG) and aspartame induced hepatotoxicity in pregnant rats and their offspring.

2. Material and methods

2.1. 1. Chemicals

1.1. Monosodium glutamate (MSG) in form of white crystals was purchased from local area of Damanhur markets.

1.2. Aspartame was purchased from Amyria Pharmaceutical Company, Cairo, Egypt. It was available in the form of tablets, each tablet contained 20mg.

2.2. 2. Preparation of Opuntia ficus indica fruit juice

Mature prickly pears of O. ficus indica (purple-skinned) were purchased from local area of Damanhur markets. The whole unpeeled fruit (10Kg) was washed, ground by a Musermax double bladed mill and filtered through a colander (0.5 mm mesh size) to remove the seeds. The resulting juice was centrifuged at

3000 × g for 10 min to discard hard fibers. The clarified juice (10.5 L) was then collected and stored at −21°C until use.

3. Experimental animals.

For this study, forty-eight (36 females and 12 males) Wistar albino rats weighing 180-200 g (8-9 weeks) were obtained from the Holding Company for Biological Products and Vaccines (VACSERA, Cairo, Egypt). The animals were kept in wire-bottomed cages in a room under standard condition of illumination with a 12-hours light-dark cycle at 25 ± 1°C and 50% relative humidity. They were provided with tap water and balanced diet of libitum. After acclimatization period of two week; the animals were mated in the special matting cages (1 male: 3 females) overnight. After 3-4 days and ensuring of pregnancy via observation of vaginal plug and using vaginal smear method, pregnant females were separated from males and the day 0 of pregnancy (E0) was determined.

Mothers rats were weighed at the beginning of the experiment, at birth and at postnatal day (PND) 21 (weaning). Also, the pups were weighed at PNDs 21 days.

4. Experimental groups

The pregnant rats were randomly divided into six groups as follows, six for each group (n=6).

Group I (control): They were received daily oral dose of 0.5ml saline solution Group II (Opuntia): they were given a daily oral dose of Opuntia juice (4ml /100g b.wt). The choice of Opuntia dose was choose according to Alimi et al.(2013).

Group III (MSG): they were given a daily oral dose of MSG (400mg/Kg. body weight). The dose was in accordance to previous studies (Diniz et al., 2004 &Waer; Edress, 2006).

Group IV) (Aspartame): they were given a daily oral dose of 0.5ml of aspartame (40mg/Kg. body weight (Abd Elfatah et al., 2012).

Group V (MSG & Opuntia): the pregnant rats were given a daily oral dose of MSG 400mg /kg b. wt. simultaneously with Opuntia juice in a dose of 4ml /100g b.wt.

Group VI (Aspartame and Opuntia): the pregnant rats were given a daily oral dose of aspartame (40mg /kg b. wt.) simultaneously with Opuntia juice in a dose of 4ml /100g b.wt.

All the above groups were exposed to the appropriate dose of treatment from the 4th day of pregnancy till the end of weaning.

5. Sample collection and tissue preparation

At the end of the experimental period (21th days postnatal), the mother's rats and their offspring (at 21 days old) were sacrificed by decapitation. Blood samples were collected; serum was separated by centrifugation at 860 Xg and kept frozen at – 20oC for estimation of physiological parameters. The animals were dissected and the whole liver of mothers and their offspring was removed immediately, washed in normal saline and cut into two equal halves. One half was fixed in 10% neutral buffered formalin for histological and immunohistochemical studies and the other half was kept frozen for estimation of biochemical parameters.

6. Investigated parameters

6.1. Histological technique for haematoxylin and eosin stain.

Formalin fixed liver was dehydrated with an ascending ethanol series, cleared with xylene, and embedded in paraffin. A 5-6 μm thick sections of liver were obtained, stained with hematoxylin and eosin (Bancroft & Gamble, 2008). The obtained sections were investigated under bright field light microscope and microphotographed.

6.2. Immunohistochemical staining technique.

The Principle: The demonstration of antigens by immunohistochemistry (IHC) is a two-step process involving first, the binding of a primary antibody to the antigen of interest, and second, the detection of bound antibody by a chromogen. The primary antibody may be used in IHC using manual techniques or using BioGenex Automated Staining System. BioGenex offers a variety of Super Sensitive detection systems including Link-Label and Polymer-based technologies to detect the chromogenic signal from the stained tissues and cells.

6.2.1 Immunohistochemical labeling of BCl-2.

The liver embedded paraffin sections were blocked by goat serum for 20 minute at 37 °C to inhibit non-specific antibody binding and then incubated separately with primary antibodies (mouse-anti-human BCl-2) at 4 °C overnight. After being the sections washed 3 times in PBS (3 minutes each time), followed by incubation in biotin-labelled goat anti-mouse IgG at 37 °C for 30 minutes. The sections washed again with PBS, followed by incubation with streptavidin peroxidase complex for 30 minutes at 37 °C. Staining was visualized with 3, 3′-diaminobenzidine (DAB) for 10 minutes at room temperature. Finally, the sections were counterstained by haematoxylin solution.

6.2.2 Immunohistochemical labeling of P53

For immunohistochemical detection of P53 protein, the paraffin-embedded tissue sections of liver were incubated with primary antibodies (monoclonal antibody RM-2103-R7, 7.0 ml of antibody pre-diluted in 0.05 mol/l tris–HCl, pH 7.6 containing stabilizing protein and 0.015 mol/l sodium azide, Produced by Epitomics, Inc. Using Technology Licensed Under Patent no. 5,675,063, Thermo Fisher Scientific, Anatomical Pathology, 46360 Fremont Blvd., Fremont, CA 94538, USA) for 16 min. at 37◦C. Sections were stained with p53-

immunoperoxidase stain (Cuello, 1993). The intensity of nuclear staining for P53 was recorded as weak, moderate, or strong reaction.

The immuno-histochemical prepared slides (BCl-2 and P53) were examined, microphotographed using an Axioscop 2 plus microscope (Zeiss, Germany) with a Leica DFC 320 digital camera (Leica, Germany).

6.3 Frozen tissue analysis

6.3.1 Estimation of caspase-3

Liver tissue homogenate from each group (mothers and their offspring) was centrifuged at10,000 g for10 min) and pellets were lysed in a nice-cold cell lysis buffer(50mM HEPES, pH7.4, 0.1% CHAPS, 100mM NaCl,1mM dithiothreitol (DTT), and 0.1m MEDTA).The assay was carried out in a 96-well plat with each well containing 10µl cell lysate, 80µl of assay buffer (50mM HEPES, pH7.4,0.1% CHAPS, 100mM NaCl, 10mM DTT, 0.1mM EDTA,and10% glycerol), and10µl of caspase colorimetric substrate (2mM).For measurement of caspase3 activity,caspase3 colorimetric substrate(Ac-DEVD-pNA; Cal- biochem) was used. The plate was incubated at37 °C for 1h. Cleavage of the chromophore pNA from the substrate molecule was monitored at405nm.Caspase3 activity was expressed as pico moles of pNA released per microgram of protein per minute (Zhang et al., 2012).

6.3.2 Determination of lipid peroxidation and antioxidant enzymes

A part of the liver tissue homogenate was mixed with an equal volume of 10% trichloroacetic acid (TCA) and centrifuged at 3000 rpm for 15 min at 4°C. Its supernatant was used to determine the content of malondialdehyde (MDA) (indicator of lipid peroxidation) enzyme. The remaining part of the homogenate was centrifuged at 17000 × g for 60 min at 4°C and the supernatant was used for estimation the activity of superoxide dismutase (SOD) and catalase (CAT).

Lipid peroxidation was determined by estimating the level of thiobarbituric acid reactive substances (TBARS) that was used to measure the extent of MDA formed as a result of membrane lipid peroxidation. The assay was based on the formation of a pink colored complex in a reaction between MDA and TBARS, according to the method of Ohkawa et al.(1979) using commercial kits (Biodiagnostic, Cairo, Egypt). The colorimetric absorbance was determined at 532 nm. Specific activity was presented as nmol/mg protein.

Antioxidant markers SOD and CAT were measured by a colorimetric method using commercial kits (Biodiagnostic, Cairo, Egypt) according to the manufacturer procedures. Total SOD activity was determined by assaying the autoxidation and illumination of pyrogallol at 440 nm. SOD activity in liver tissues was determined based on the ability of the enzyme to inhibit nitroblue tetrazolium (NBT) reduction by superoxide. Data were expressed as U/mg hemoglobin (Kakkar et al., 1984). CAT enzyme activity was measured according to the method described by Aebi (1984). The enzymatic activity was expressed in units (1 U decomposes 1 mmol of H_2O_2 per minute at pH 7.0.

6.3.3 Molecular detection of apoptosis by Agarose gel electrophoresis (genetic study):

The various fragments of DNA can be easily separated by using agarose gel electrophoresis by applying an electric field across the gel. DNA which is negatively charged at neutral pH, migrates towards the anode. The rate at which the fragments migrate through the gel is a function of their lengths, as small fragments moving much faster than larger fragments the relationship between the size of a DNA fragment and the distance it migrates in the gel is logarithmic. Staining of the gel with ethedium bromid dye, that bind to DNA, generates a series of bands, each corresponding to the fragments whose molecular weight can be established by calibration with DNA molecule of known weight (Southern, 1979). The gel electrophcretic results were analyzed by gel pro-computer program.

6.4 Blood analysis

6.4.1 Liver functions

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined by the method of Reitman and Frankel (1957), for colorimetric method. Serum Alkaline phosphatase (ALP) activity was determined by Belfield and Goldberg (1971) colorimetric method. Serum total bilirubin was estimated by the method described by Walter and Gerade (1970), for colorimetric method. Serum albumin test was performed by the method devised by Doumas et al. (1971), which is a colorimetric method. Serum lactate dehydrogenase (LDH) was estimated according to the method of Weishaar (1975).

Statistical analysis: Data are expressed as mean± standard error {n=5 per group} statistical analysis one way ANOVA followed by post hoc test means in the same row with different superscript (*) are significantly different when $p<0.05$ * significant at value <0.05, **significant at p- value<0.01 and ***significant p value < 0.001 in comparison with control.

Results

1. Body weight Changes

The obtained results revealed that, the mean body weights of MSG induced mother's rats were significantly higher ($P<0.001$) however, a high significant decrease ($P<0.001$) was noticed for aspartame induced mothers rats. In MSG or aspartame induced offspring the body weight showed low significant increase if compared with control. In the two protective groups of mothers and their offspring (MSG & aspartame post supplemented with *Opuntia* fruit extract) the mean body weights showed non-significant changes ($P>0.05$) in comparing with

control **(Fig. 1&2).**

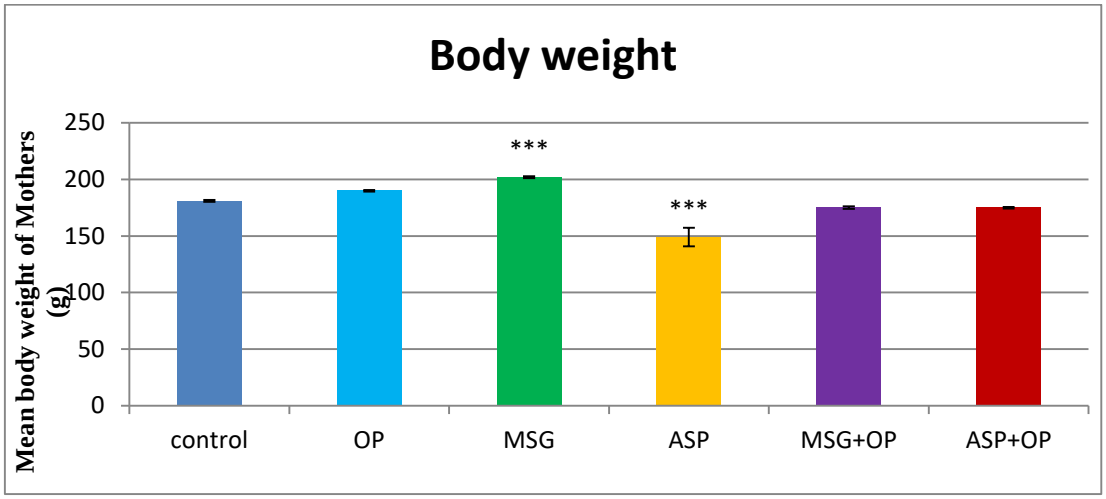


Fig 1: Showing the body weight (g) of mothers rats among different studied groups.

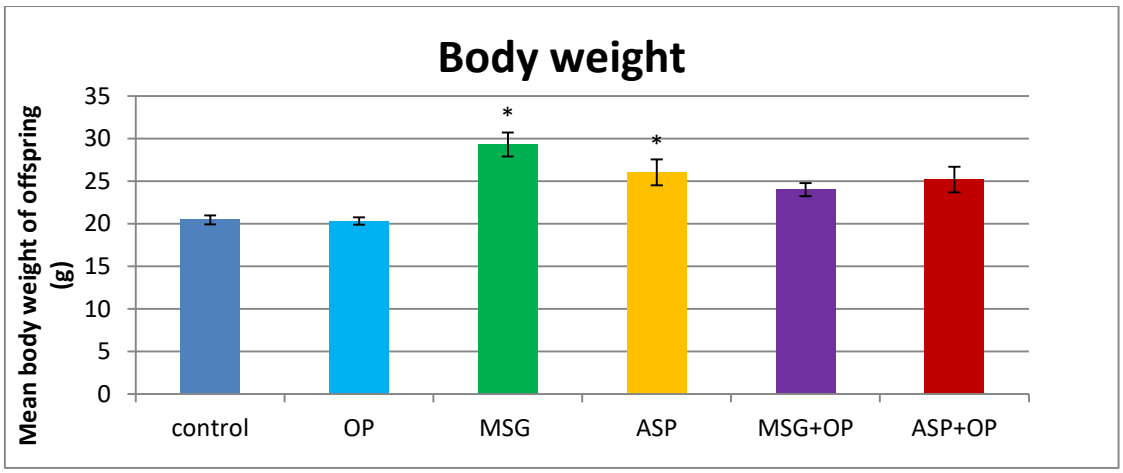


Fig 2: Showing body weight (g) 21 days among different studied groups.

2. Histological observations

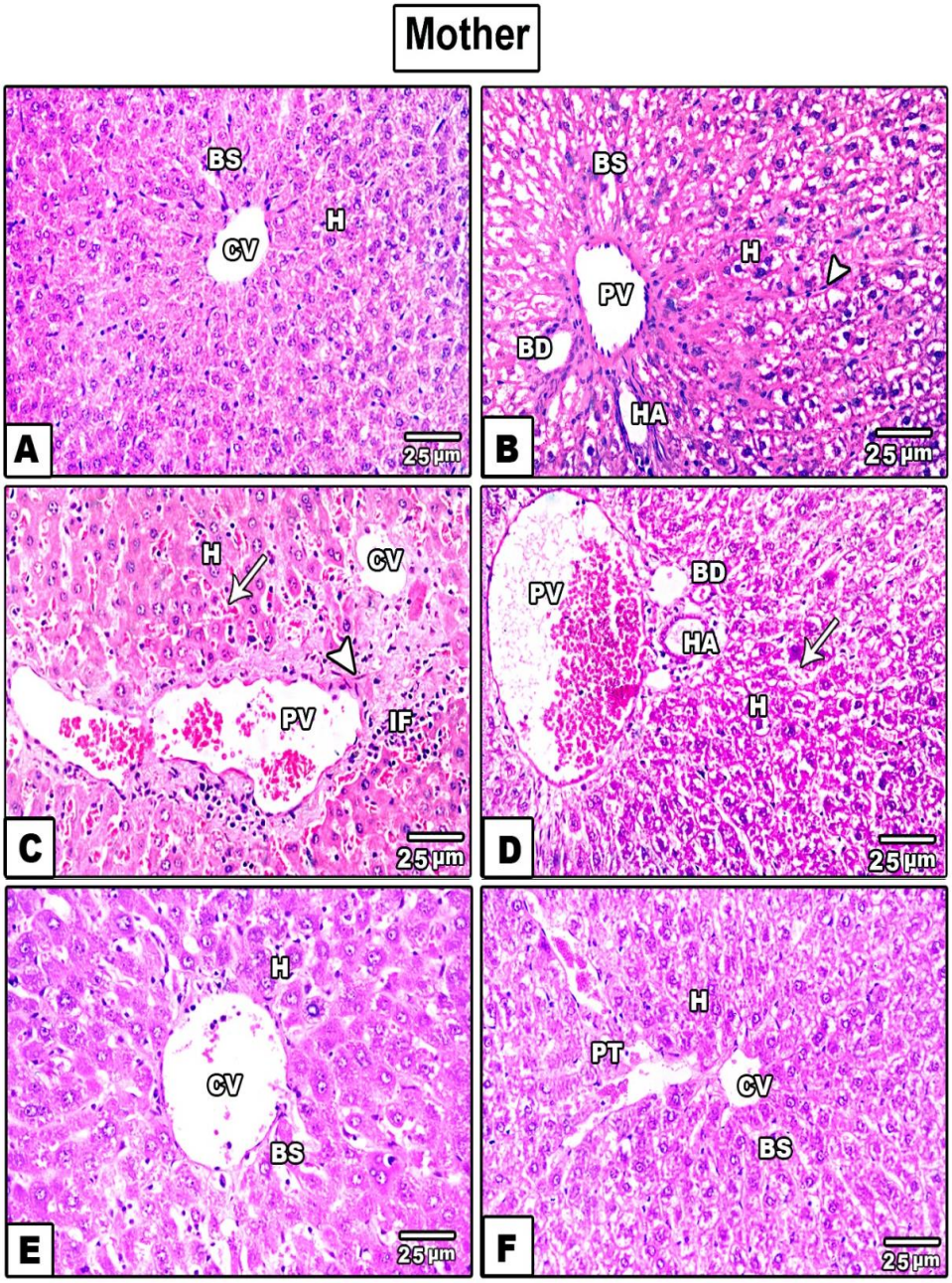
In control and *Opuntia* supplemented mother's rats **(Fig.3 A&B)** and their offspring **(Fig4 A&B**), the liver sections displayed normal histological structure. In further detail, the hepatic lobules appeared regular and separated from each other by connective tissues. Each hepatic lobule consists of hepatic strands radiating from a central vein and separated by blood sinusoids. In mothers, the blood sinusoids appeared narrower than those of their offspring. Blood sinusoids are lined with endothelial and little Kupffer cells. The hepatic lobules are interconnected by the portal areas which represented by a branch of the hepatic vein, a branch of the hepatic artery and bile ductule. The hepatocytes are polyhedral in shape with rounded centrally located nuclei.

The examined liver sections of MSG **(Fig.3 C)** and aspartame **(Fig.3 D)** exposed mother's rats, revealed damaged hepatocytes that have lost their characteristic appearance with scattered fatty degeneration spots. Moreover, dilated blood sinusoids with obvious hemorrhage, excessive Kupffer cells and hepatocyte infiltration, around the congested portal vein were also noticed. On the other side, the liver sections of 21 day's old rats induced with MSG displayed severe disruption in liver architecture, including hypertrophied hepatocytes, vacuolation and dilated blood sinusoids (Fig.4C). Moreover, in aspartame treated offspring the liver sections appeared with mild degenerative changes if compared with the disruption induced by MSG (Fig.4D).

For mothers (Fig.3E&F) and their offspring (Fig4.E&F), the two ameliorative groups (MSG, aspartame post supplemented with *Opuntia* fruit extract) the liver sections showed remarkable recovery in their histological architecture in spite of little damage of hepatic cells still noticed in some area of the liver sections for mothers rats.

3. Immunohistochemical Observation:

3. 1 Immunohistochemical localization of B-cell lymphoma (BCL-2)



The liver sections of control mother's rats (Fig.5A) and their offspring (Fig.6A) displayed a moderate to strong immune expression for BCL-2. Such expression was more confined in the cytoplasm of hepatic cells. On the other side, the liver sections of MSG (Fig5B) and aspartame (Fig.5C) induced mothers rats, displayed weak to negative BCL-2 immuno-reactivity. Moreover, the liver sections of 21 days old rats for the two induced groups with MSG and aspartame showed weak to negative BCL-2 expression (Fig.6B&C).

In MSG and aspartame treated mothers (Fig.5E&F) and their offspring (Fig.6E&F) post supplemented with *opuntia* extract, the liver sections revealed moderate to strong BCL-2 immunoreactivity.

3.2. Immunohistochemical localization of P53 in liver tissue

In control groups of mothers rats (Fig.7A) and their offspring (Fig.8A), the liver sections displayed negative or very weak expression for P53 protein. In contrast to control, the MSG and aspartame-induced mother rats (Fig.7B&C) and their offspring (Fig.8B&C), the liver sections exhibited moderate to strong positive P53 immune expression. In comparing with other induced groups, the P53 immuno-reactivity was apparently intensive in in the liver sections of aspartame- induced offspring. The P53 activity was more prominent in the nuclei and cytoplasm of hepatic cells. Moreover, a weak immune reaction for P53 protein was recorded in the liver sections of the two ameliorated groups (MSG& *opuntia* extract and aspartame &*opuntia* extract) of mothers rats (Fig.8E&F) while their offspring displayed negative immunoreactivity for P53 protein (Fig.8E&F).

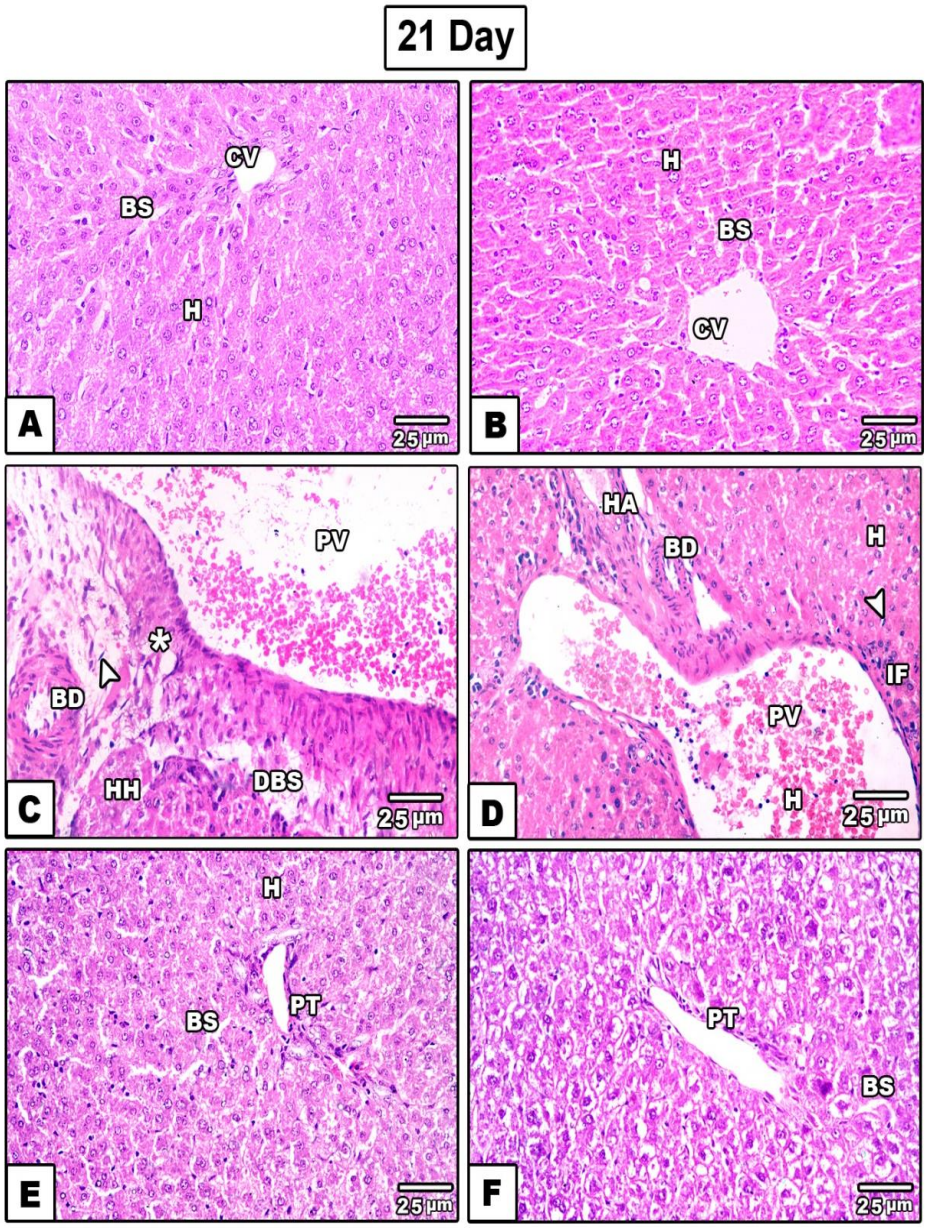
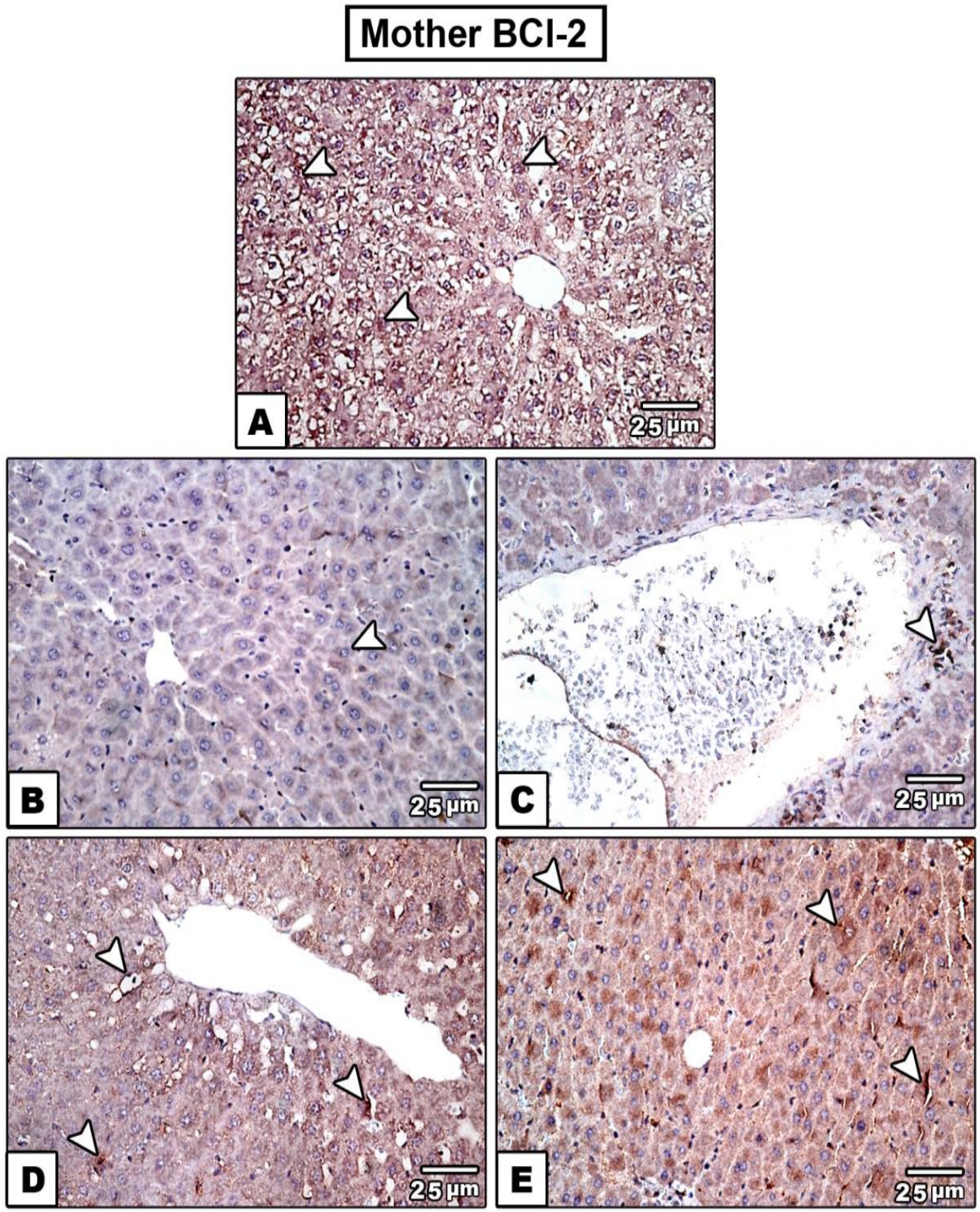


Fig.3:

Photomicrograph of histological sections through the liver of mothers rats for different studied groups. **A**:control group. **B**: *Opuntia ficus* supplemented group, **C**: MSG treated group**, D**:Aspartame treated group, **E**: MSG& *Opuntia* group and **F:**ASP& *Opuntia* group. **Note**:**In A&B** the liver sections appeard with normal histological architecture. In **C&D**: the liver sections showing degenerated hepatocytes, dilated blood sinusoids (arrow), hemorrhage, excessive Kupffer cells (arrow head) and hepatocyte infiltration around the congested portal vein were also noticed. In E&F, the liver sections showing remarkable amelioration in their histological architecture that tend to be more or less as control. (H&E X=250)

Abbreviations: Blood sinusoids (BS), bile ductule (BD), central vein (CV), hepatocytes (H), hepatic arteriole (HA), hepatocyte infiltration (IF, portal tract (PT).and portal vein (PV),

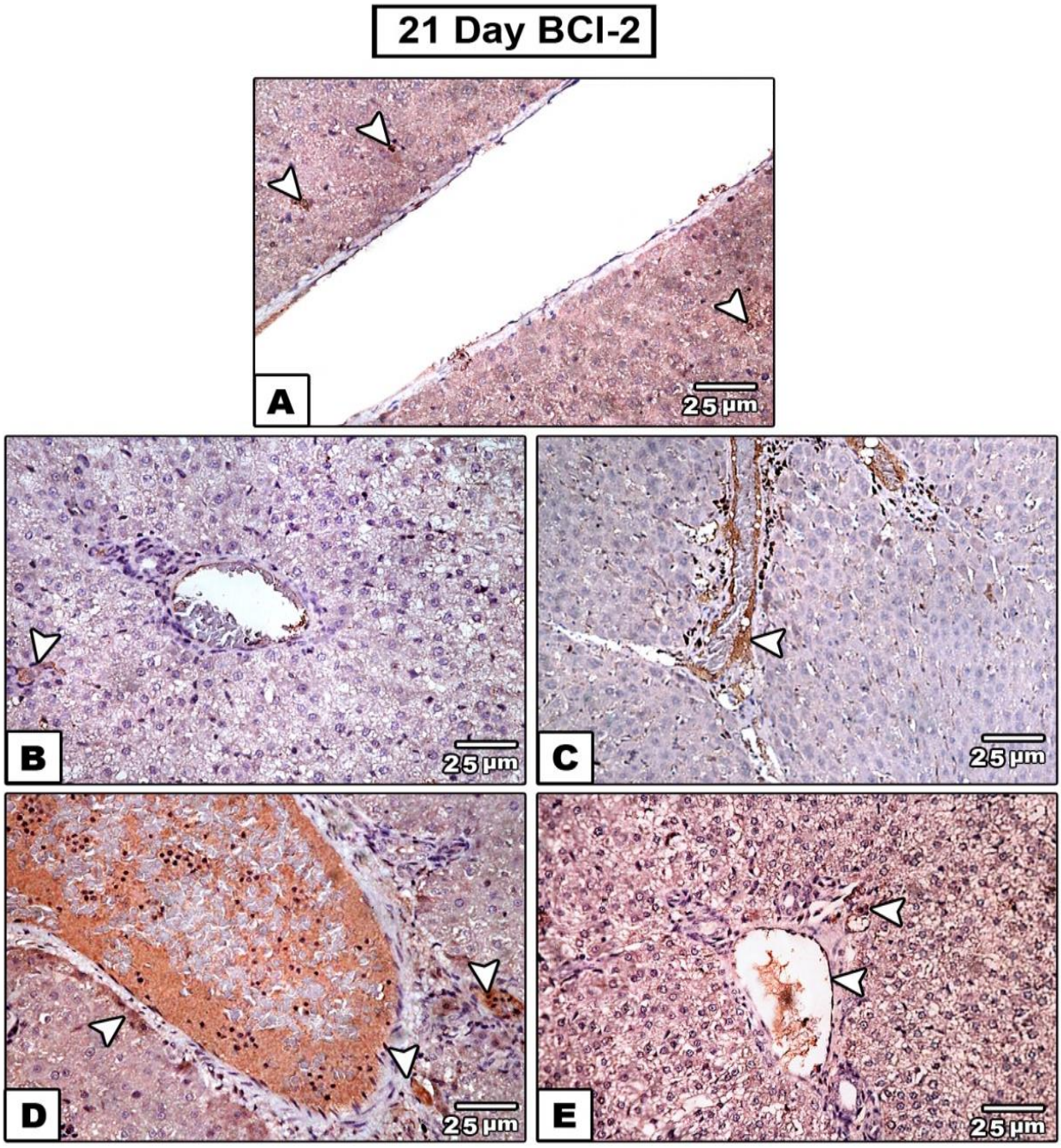
Fig.4: Photomicrograph of histological sections through the liver of 21 days old rats for different studied groups **A**:control group, **B**: *Opuntia ficus* supplemented group, **C**: MSG-treated group **D**:Aspartame-treated group, **E**: MSG& *Opuntia* group and **F:**ASP& *Opuntia* group. **Note**:**In A&B** the liver sections appeard with normal histological architecture. In **C&D**: the liver sections showing hypertrophied hepatocytes, cytoplasmic vacuolation (star), dilated blood sinusoids, hemorrhage, excessive Kupffer cells (arrow head) and hepatocyte infiltration around the congested portal vein was also noticed. In E&Fthe liver sections showing remarkable amelioration their architecture that tend to be more or less as control. (H&E X=250)



Abbreviations: Blood sinuosoids (BS), dilated blood sinusoids (DBS), bile ductule (BD), central vein (CV), hepatocytes (H), hypertrophied hepatocytes(HH), hepatic arteriole (HA), hepatocyte infiltration (IF), portal vein (PV) and portal tract (PT).

Fig 5: Photomicrograph of paraffin embedded sections of liver of mother Albino rats stained with BCL-2 antibody. A**:** control, **B**: MSG, **C:** ASP, **D**: MSG& Opuntia, **E**: ASP& Opuntia groups. **Note:** a moderate

positive immune expression of BCL-2 in A group, weak expression in B&C and moderate to strong immune



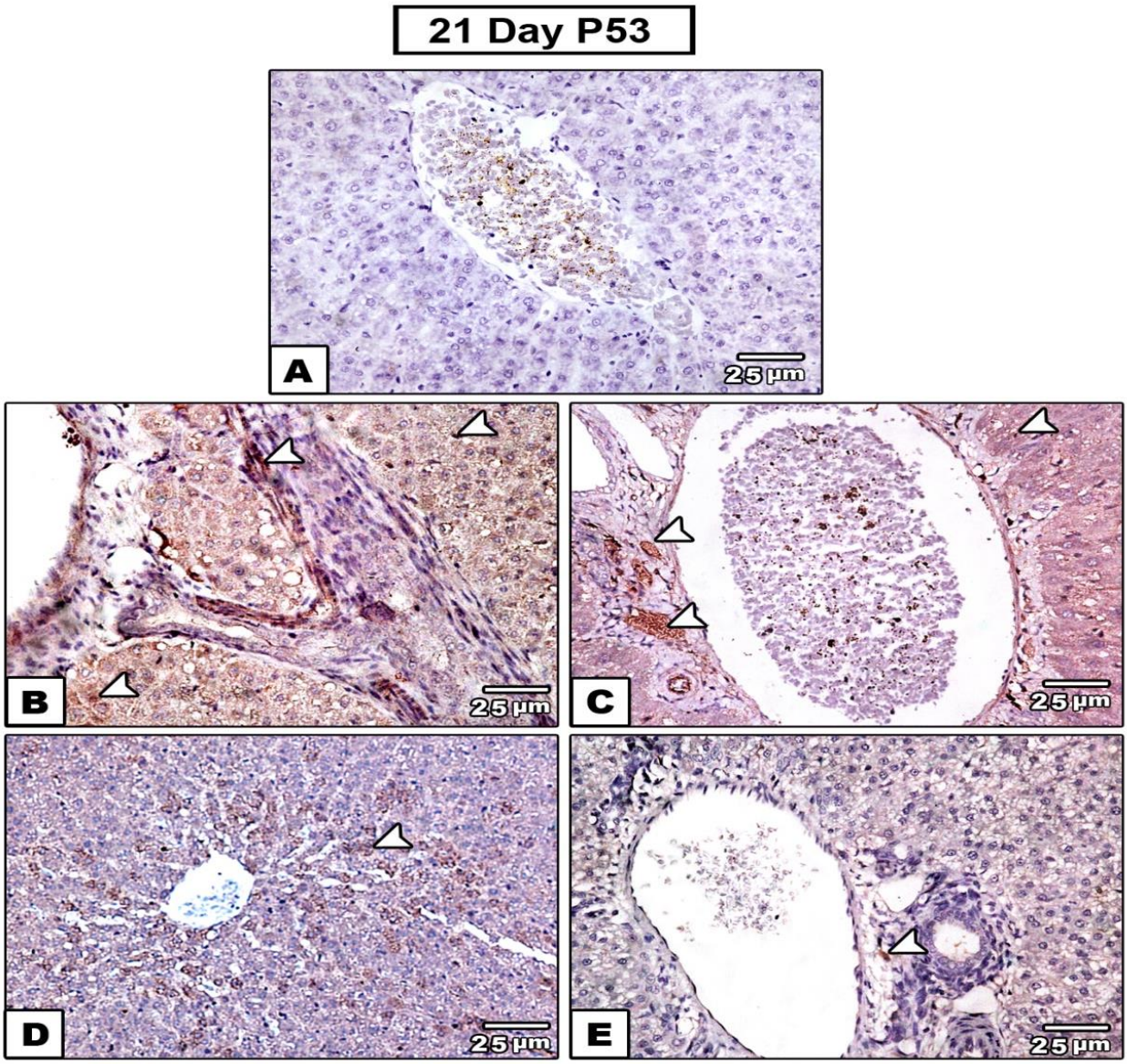
reaction in D&E groups. X=250

The arrows head referred to the degree of BCL-2 immunoreactivity reactivity

Fig.6: Photomicrograph of paraffin embedded sections of liver of 21 days old rats stained with BCL-2 antibody. **A:** control, **B**: MSG, **C:** ASP, **D**: MSG& *Opuntia*, **E**: ASP& *Opuntia* groups. **Note:** a moderate positive immune express ion of BCL-2 in A groups, weak expression in B&C and a weak to moderate immune expression of BCL-2 in D&E groups. X=250

The arrows head referred to the degree of BCL-2 immunoreactivity reactivity

Fig.7: photomicrograph of embedded formalin -fixed paraffin sections of liver of mother Albino rats stained with the P53 antibody. A**:** control, **B**: MSG, **C:** ASP, **D**: MSG& Opuntia, **E**: ASP& Opuntia groups. Note: a very weak immune expression of BCL-2 in A group, a strong expression in B&C and weak



to moderate P53 immune reaction in D&E groups. X=250

The arrows head referred to the degree of P53 *immunoreactivity reactivity*

Fig. 8: Photomicrograph of embedded formalin paraffin sections of liver of 21 days old rats stained with the P53 antibody. A**:** control, **B**: MSG, **C:** ASP, **D**: MSG& *Opuntia*, **E**: ASP& *Opuntia* groups.. **Note:** a negative to weak immune expression of BCL-2 in A group, strong expression in B&Cand weak P53 immune reaction in D&E groups. X=250

The arrows head referred to the degree of P53 immunoreactivity

4. Biochemical results in tissue homogenate:

4.1: Caspase-3

In MSG treated mother's rats and their offspring, the level of caspse-3 in liver tissue homogenate showed no significant changes with control. On the other side a highly significant increase (P<0.001) in caspase-3 activity was noticed in the liver tissue of aspartame induced mothers rats whereas, their offspring showed non-significant change with control. In the two ameliorative groups of mothers rats and their offspring, the activity of casapse-3 in liver tissue showed non-significant change with control (Figs.9&10).

4.2 Changes in tissue Antioxidants enzymes (SOD and CAT)

In MSG -treated mothers rats and their offspring , the liver SOD displayed highly significant decrease (P<0.001) if compared with control. On the other side a highly significant decrease (P<0.001) in SOD level in the liver tissue of mothers rats however their offspring displayed low significant decrease with control (Figs.11&12).

Also a highly significant decrease (P<0.001) in CAT level was noticed among MSG or aspartame treated mothers rats while their offspring showed non-significant change with control. Moreover, the level of liver tissue SOD and CAT antioxidants displayed remarkable amelioration in their levels post supplementation of *Opuntia* extract to MSG or aspartame-treated mothers rats and their offspring (Figs. 13&14).

4.3 Lipid peroxidation (Malondialdehyde =MDA)

Supplementation of MSG to pregnant rats resulted in a highly significant increased level of liver tissue malondialdehyde (MDA) either for mothers and their offspring at 21th days old (P<0.001) if compared with control. In mothers rats that supplemented with aspartame a a higly significant increased MDA was noticed (P<0.001) however their offspring declared non significant change if compared with control. On the other side, the level of liver tissue MDA showed remarkable recovery to the normal value post supplemantion of *Opuntia* extract to MSG and asparatme-treated mothers illustrating non-significant change with control(Figs.15&16).

4.4 Molecular Detection of apoptosis by agarose gel electrophoresis:

In control mother's rats **(lane1)** and their offspring **(lane 2)** the liver tissue sample did not display any detectable apoptotic bands all-over the gel. In MSG induced mother's rats (**lane3)** and their offspring **(lane4),** weak apoptotic bands in the form of a ladder-like DNA fragmentation pattern were detected while in aspartame induced mother's rats **(lane5)** and their offspring **(lane6)** a strong positive apoptotic bands were recorded. On the other hand, the two protective groups of mothers rats **(Lane 7&8**) and offspring **(lane 9&10)** the apoptotic bands were obviously disappeared**.** Accordingly aspartame supplementation was implicated in induction of remarkable liver apoptosis rather than MSG. **(Fig.17).**

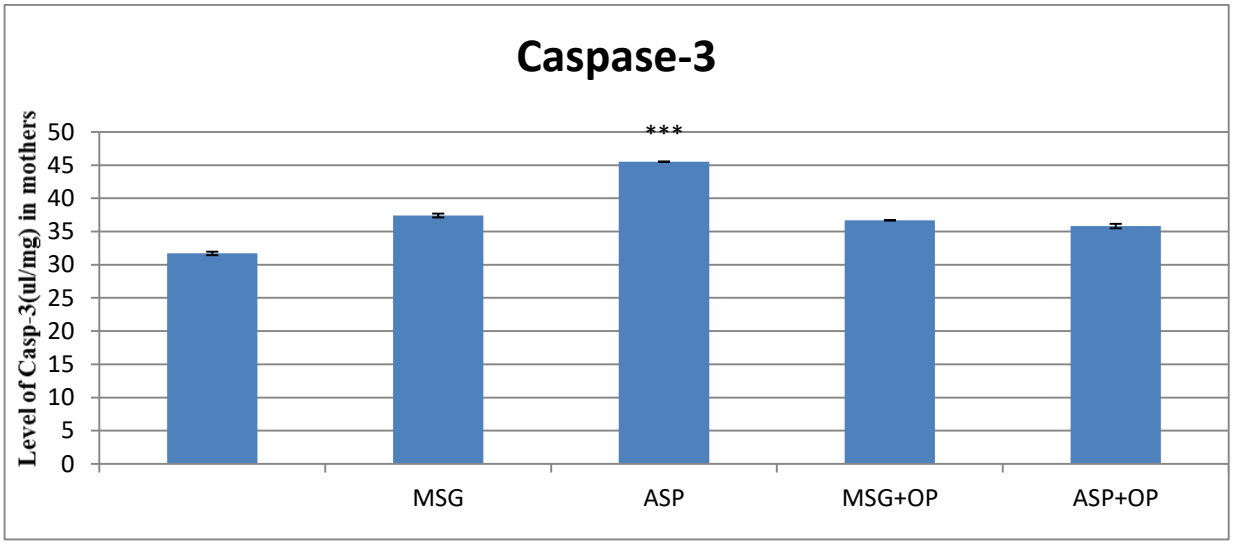


Fig.9: Showing the levels of caspase-3 in the liver tissues among different studied groups of mothers rats

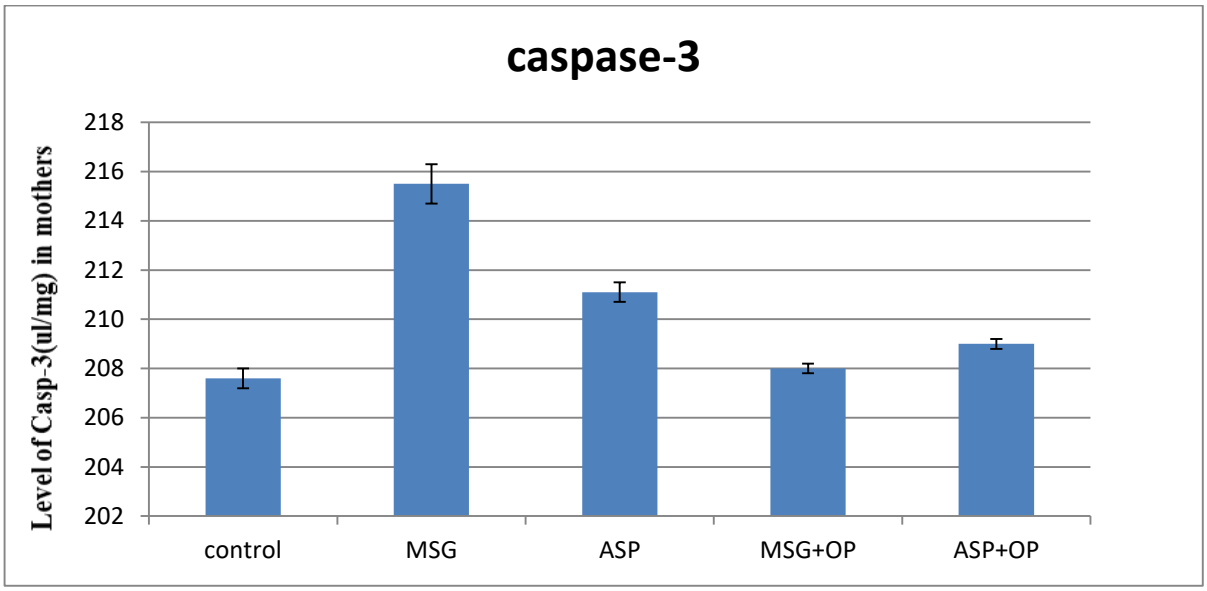


Fig.10: Showing the levels of caspase-3 in the liver tissues among different studied groups of 21 days old rats.

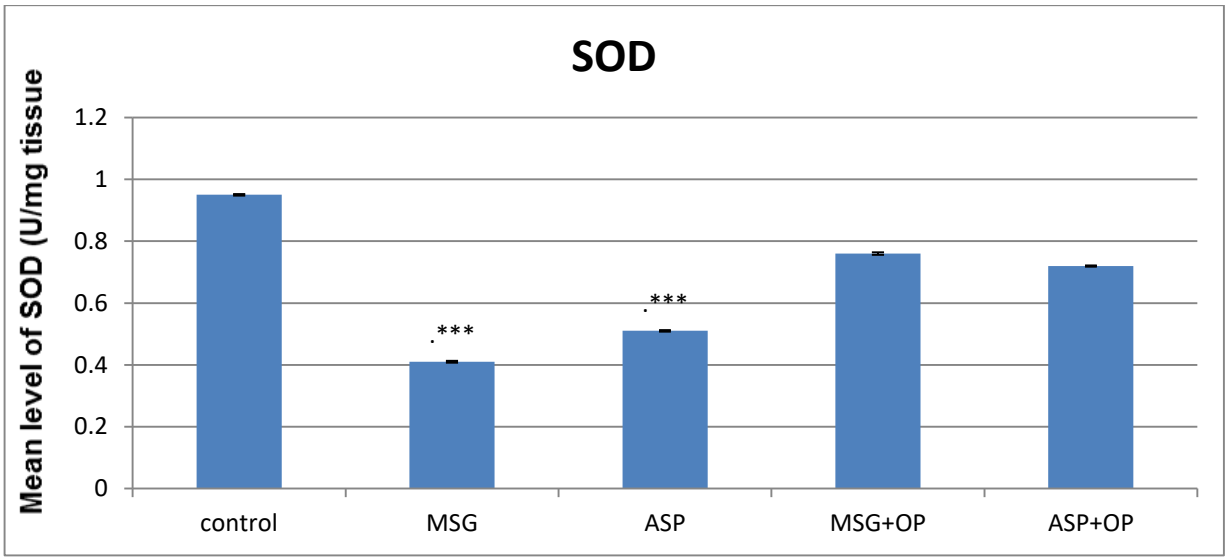


Fig.11: Showing the levels of SOD in the liver tissues among different studied groups of mother's rats.

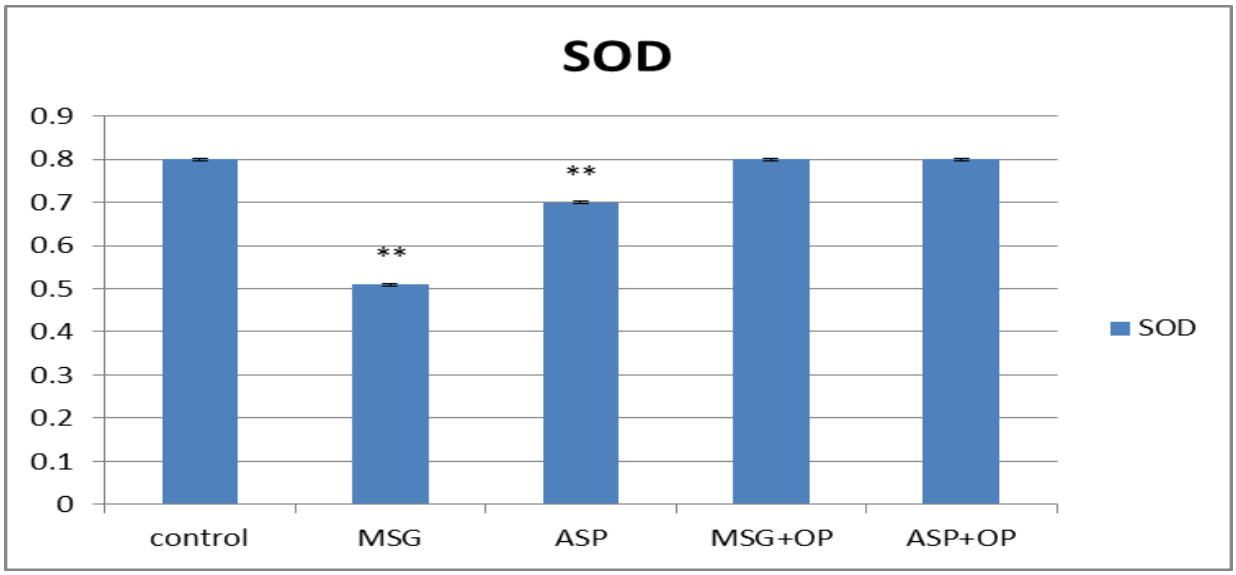


Fig.12: Showing the levels of SOD in the liver tissues among different studied groups of 21 days old rats**.**

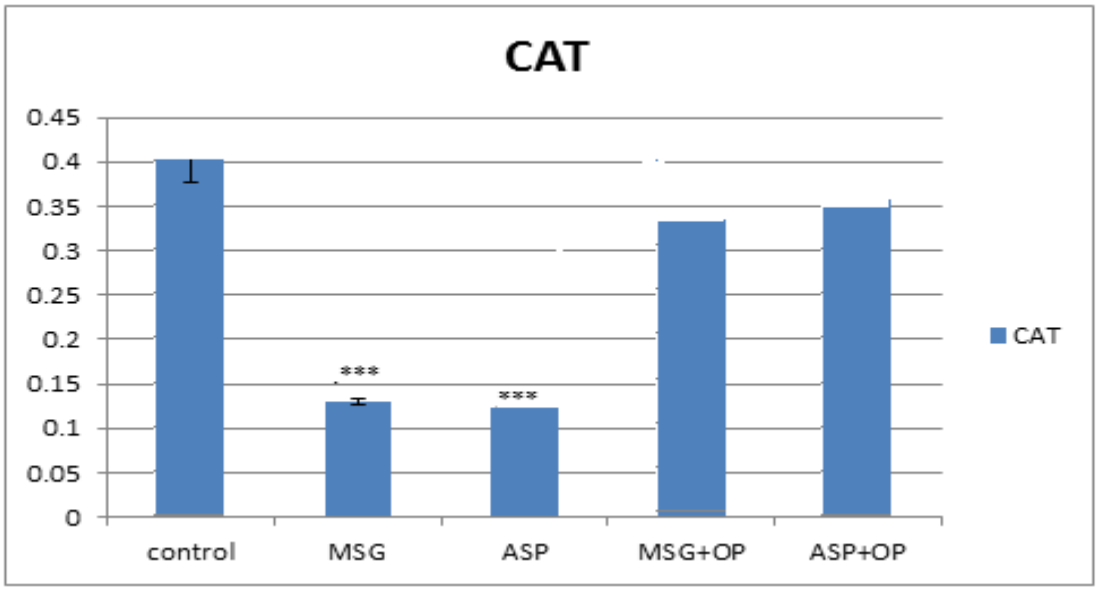


Fig.13: Showing the levels of CAT in the liver tissues among different studied groups of mother's rats.

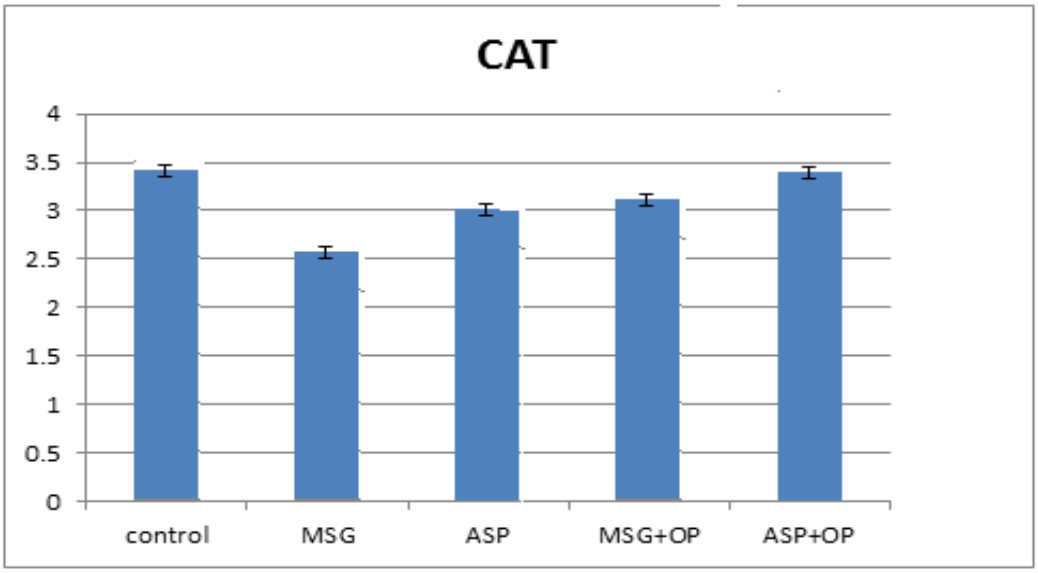


Fig.14: Showing the levels of CAT in the liver tissues among different studied groups of 21 days old rats**.**

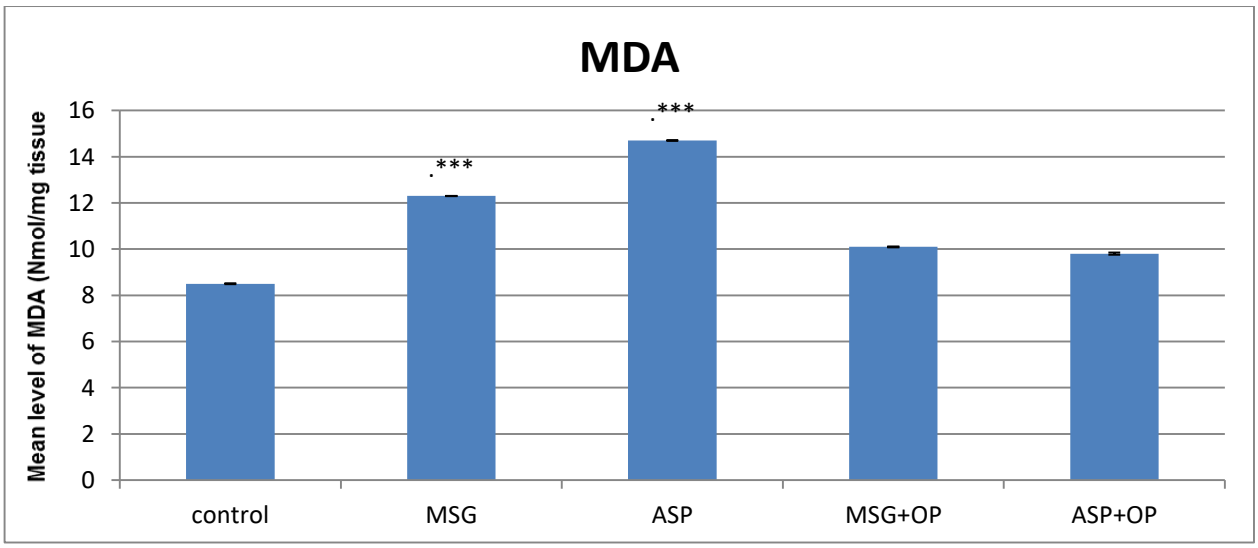


Fig.15: Showing the levels of MDA in the liver tissues among different studied groups of mother's rats.

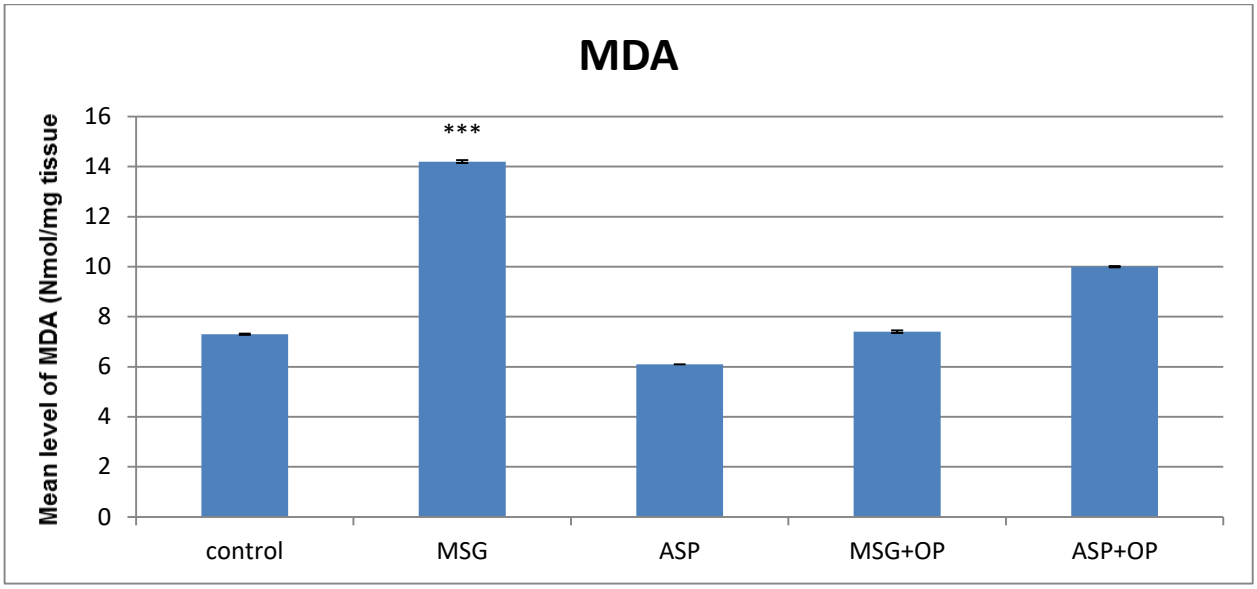


Fig.16: Showing the levels of MDA in the liver tissues among different studied groups of 21 days old rats**.**

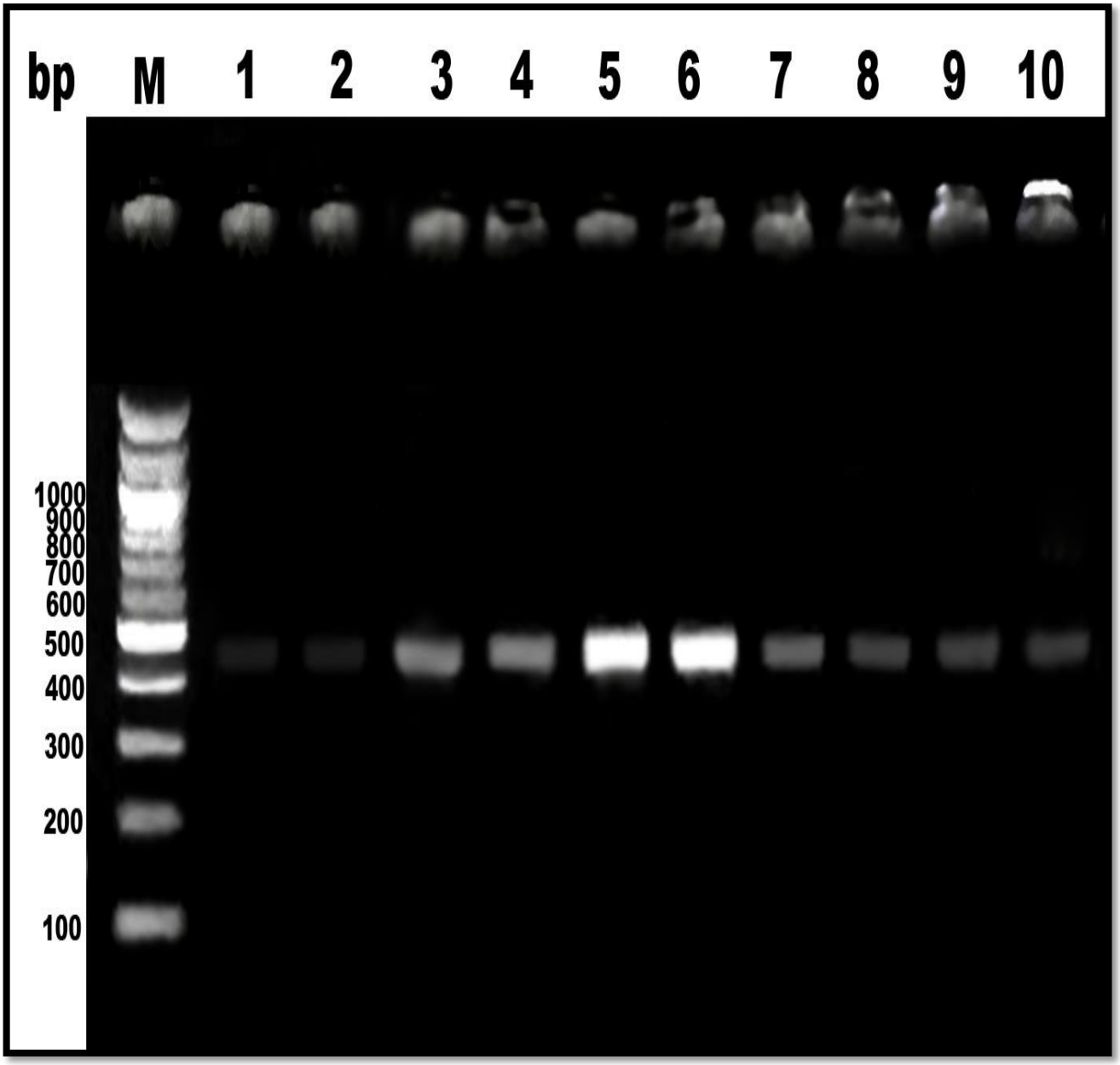


Fig.17: An electrophoretogram showing no bands of DNA fragmentation in the liver tissue of control, weak bands in MSG-treated rats, strong apoptotic bands for aspartame-treated rats and disappeared bands for the two ameliorative groups of rats (MSG or aspartame with *Opuntia* extaract).

Lane1: Control mother. Lane2: control offspring.
Lane3: MSG-treated mother Lane4: MSG-treated offspring
Lane5: aspartame -treated mother Lane6: aspartame -treated offspring
Lane7: MSG &*Opuntia* treated mother Lane8: MSG &*Opuntia* treated offspring
Lane9: aspartame &*Opuntia* treated mother Lane10: aspartame &*Opuntia* treated offspring

5. Blood analysis

5.1. The level of ALT, AST, ALP and LDH

The obtained data of the present work revealed that the levels of serum ALT, AST and LDH for control mother's rats were localized in the normal range of standard measured liver functions of Albino rats. On the other side, the level of serum ALT was significantly higher in MSG or aspartame treated- mothers rats ($P<0.001$) while their offspring displayed low significant increase with MSG ($P<0.05$) and high significant increase ($P<0.001$) with aspartame. Moreover, the MSG treated mothers rats displayed a low significant increase ($P<0.05$) in the levels of serum AST and ALP but their offspring showed high significant increase ($P<0.001$) however their levels showed high significant increase ($P<0.001$) in aspartame treated mothers rats and their offspring if compared with control. Moreover, Post supplementation of *Opuntia* extract to MSG-induced mother's rats and their offspring the levels of ALT, AST and ALP showed non-significant change with control. On the other side, Post supplementation of *Opuntia* extract to aspartame treated mother's rats and their offspring the levels of ALT and ALP still showing low significant increase ($P<0.05$) with control however the level of AST showed non-significant change (Figs.18-23).

In MSG or aspartame treated mothers and their offspring, the level of serum LDH displayed high significant increase ($P<0.001$) if compared with control. Moreover, post supplementation of *Opuntia* extract to MSG or aspartame treated mother's rats and their offspring the levels of LDH showed non-significant change with control (Figs.24&25).

5.2 Total Bilirubin and albumin

In MSG or aspartame-treated mother's rats and their offspring, the level of serum bilirubin showed a high significant increase ($P<0.001$) than that of control however. Supplemenation of *Opuntia* extract to MSG - treated mothers rats and their offspring, the level of total bilirubin still showing low significant increase ($P<0.05$) if compared with control. On the other side, supplementation of *Opuntia* extract to aspartame treated-mothers and their offspring was succesfully improved the level of total bilirubin to the normal range as in control (Figs.26&27).

In contrast, a highly significant decrease ($P<0.001$) in serum albumin was recorded in MSG or aspartame-treated mother's rats and their offspring. In comparing with control, the level of albumin still showing low significant decrease ($P<0.05$) in MSG -treated mothers rats and their offspring that supplemented with *Opuntia* extract , however, the aspartame treated mothers and their offspring that ameliorated with *Opuntia* extract the level of albumin displayed non-significant change with control (Figs.28&29).

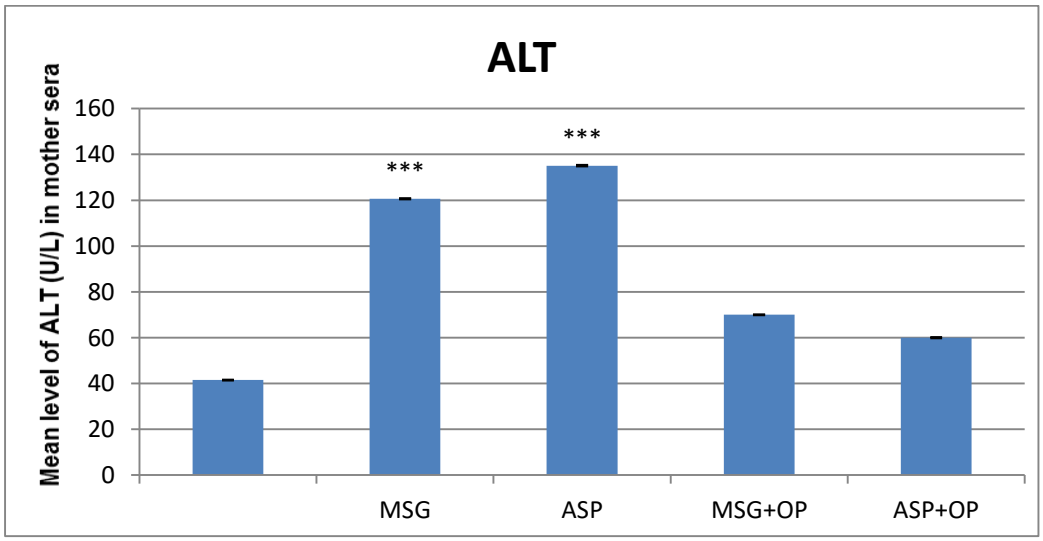


Fig.18. Illustrating the mean value of serum ALT changes among the different studied groups of mothers rats.

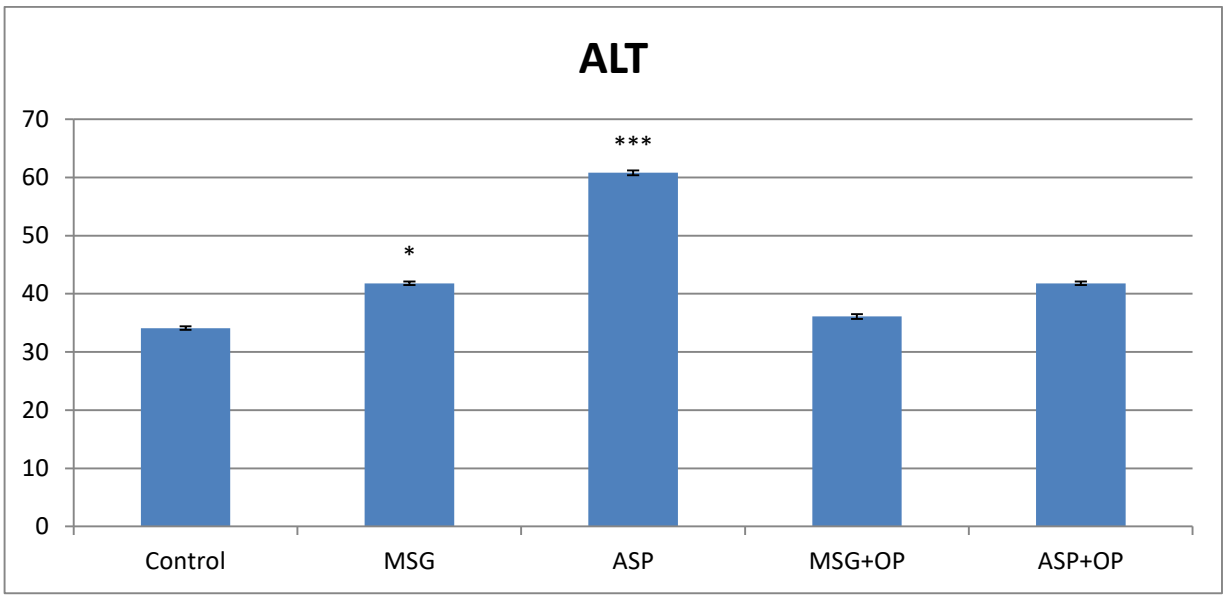


Fig.19. Illustrating the mean value of serum ALT changes among the different studied groups of 21 day offspring rats.

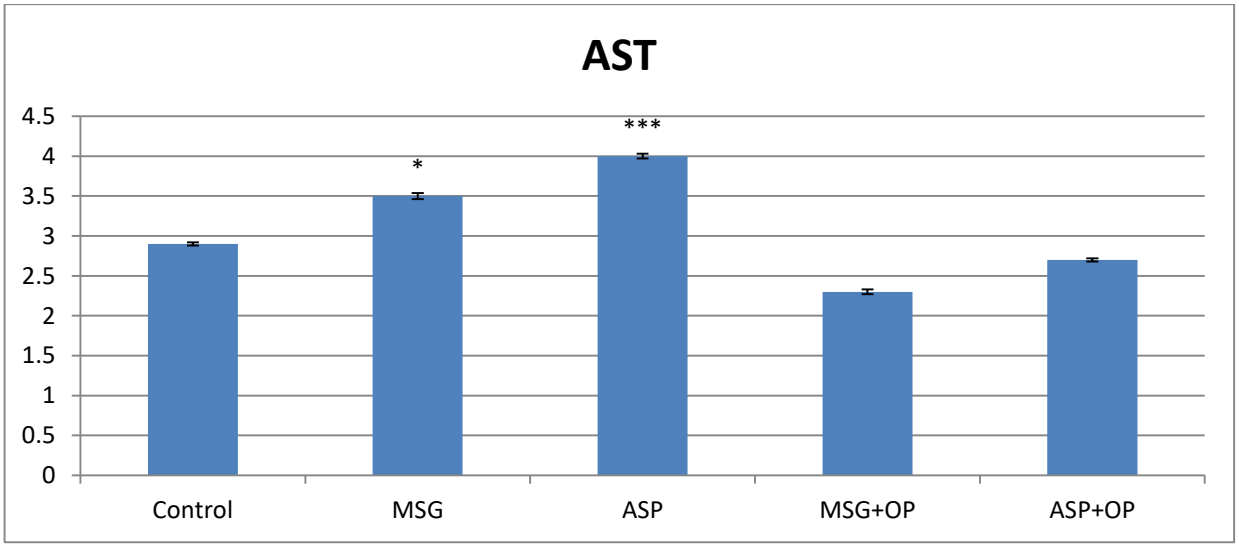


Fig.20. illustrating the mean value of serum AST among the different studied groups of mothers rats

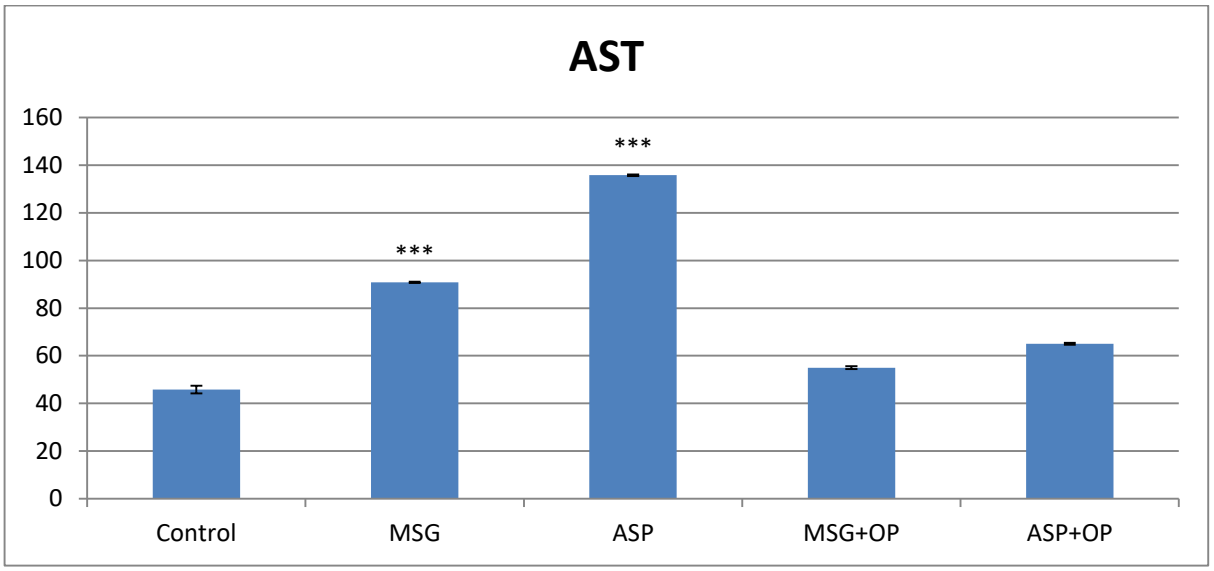


Fig.21. illustrating the mean value of serum AST among the different studied groups of 21day offspring rats.

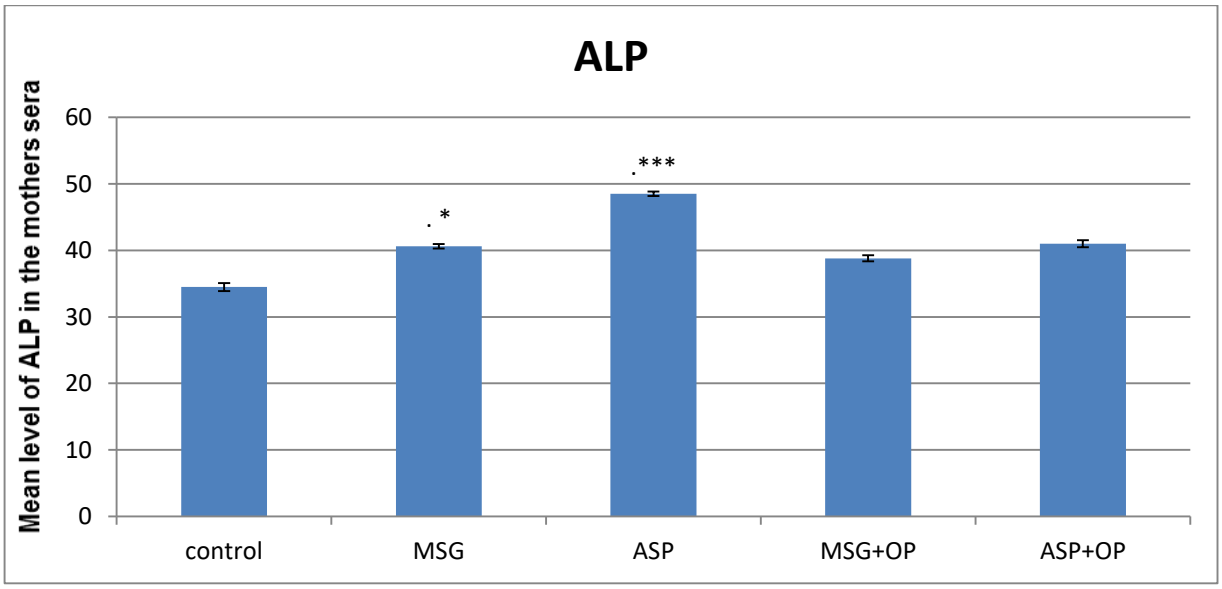


Fig.22. illustrating the mean value of serum Alkaline Phosphatase (ALP) among the different studied groups of mothers rats.

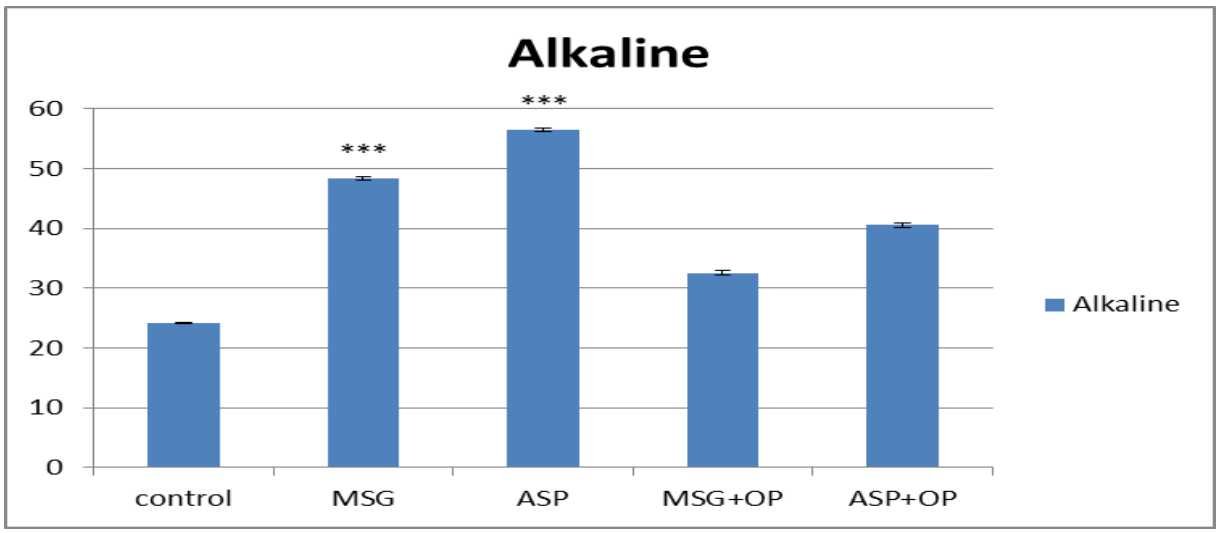


Fig.23. illustrating the mean value of serum Alkaline Phosphatase (U/l) among the different studied groups of 21day offspring.

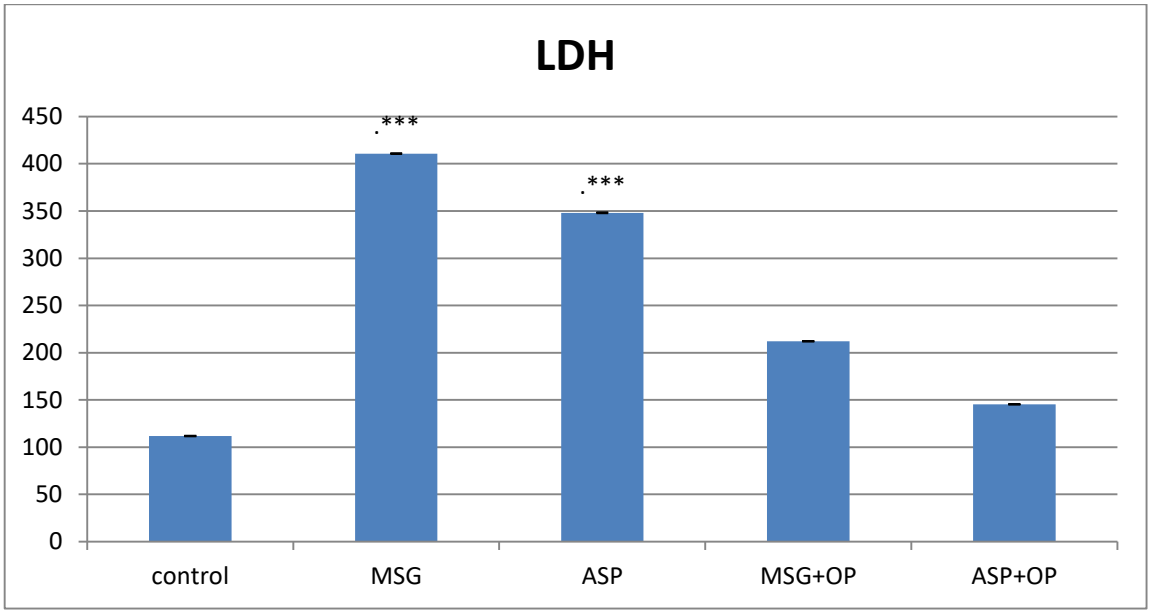


Fig.24. illustrating the mean value of serum LDH (U/l) among the different studied groups of mothers rats

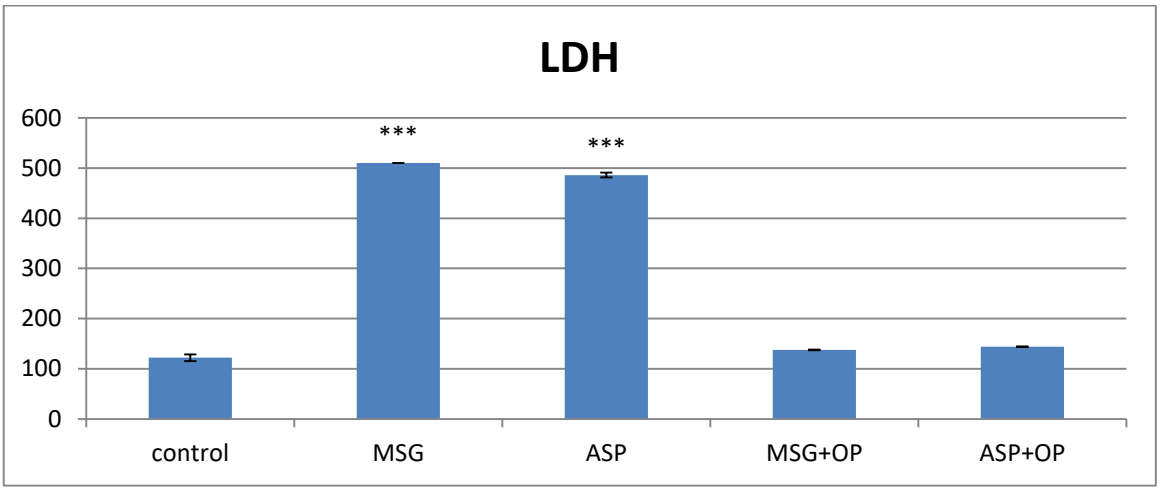


Fig.25. illustrating the mean value of serum LDH (U/l) among the different studied groups of 21day offspring.

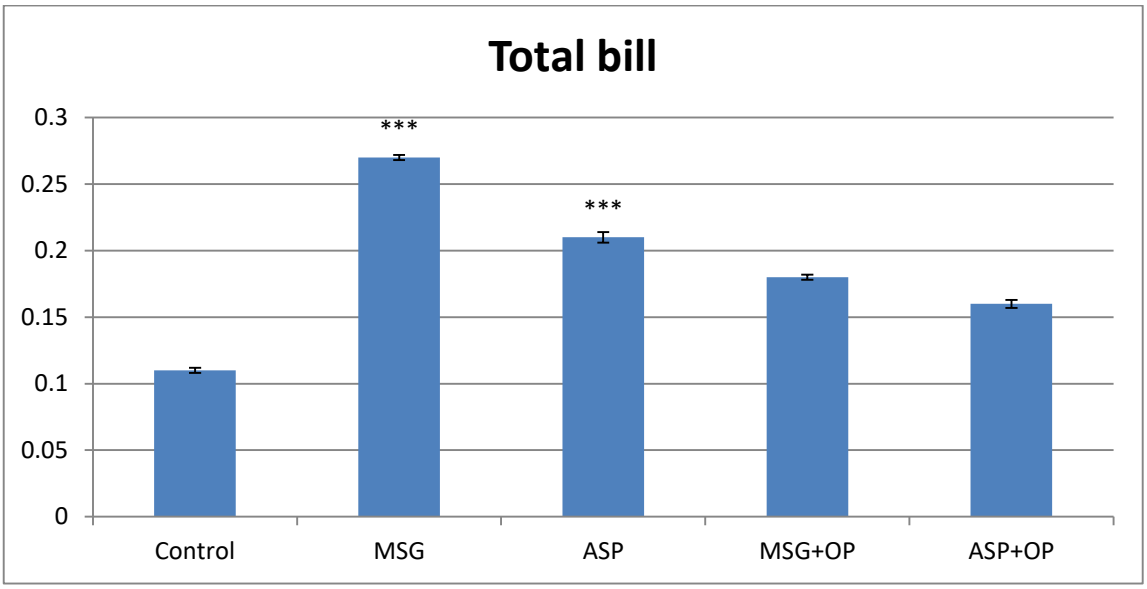


Fig.26. illustrating the mean value total bilirubin (mg/dl) among the different studied groups of mothers rats

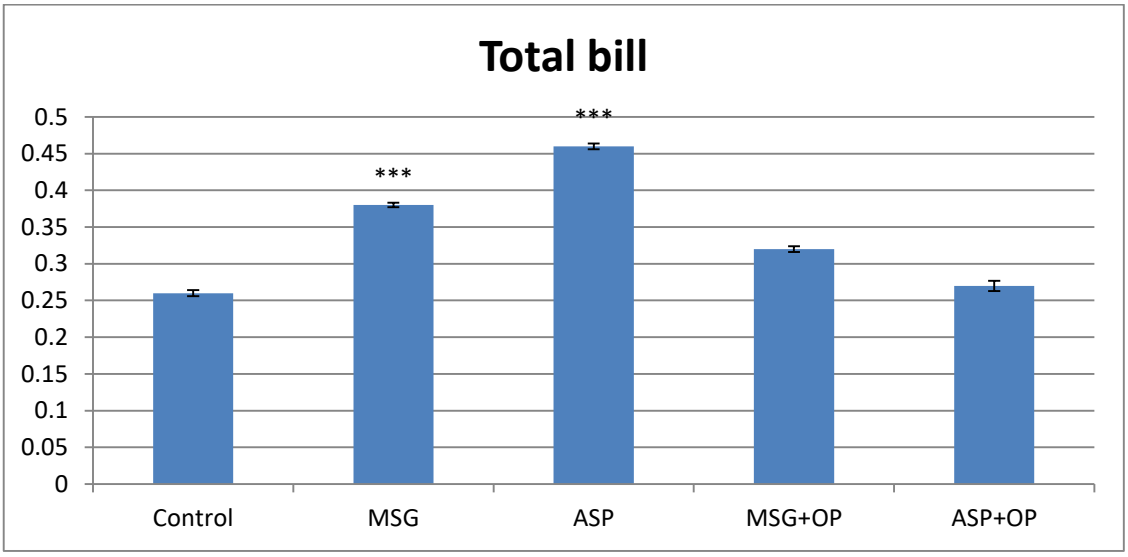


Fig.27. illustrating the mean total bilirubin (mg/dl) among the different studied groups of 21day offspring.

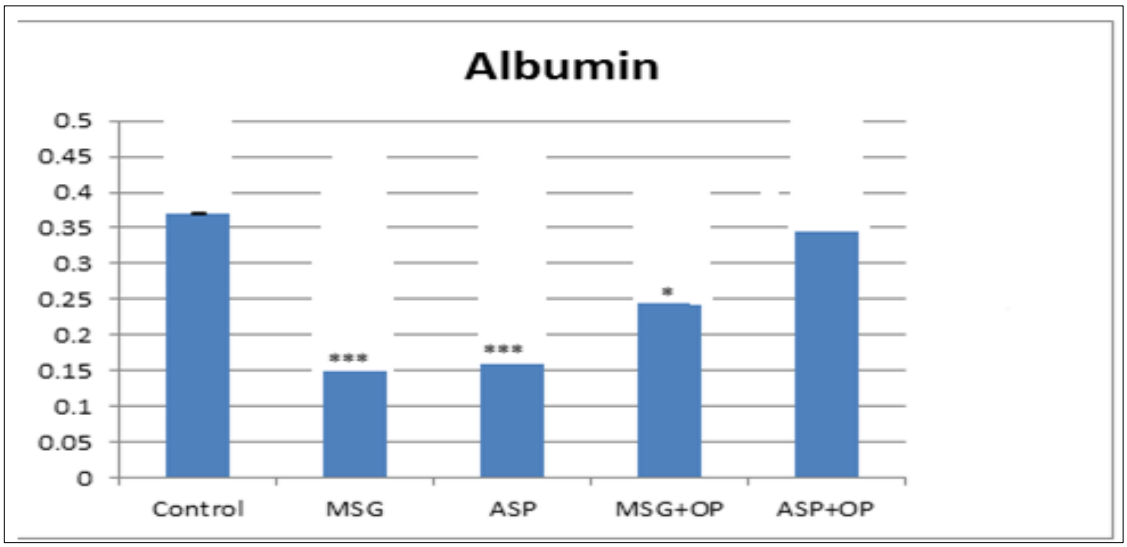


Fig.28. illustrating the mean albumin (g/dl) among the different studied groups of mothers rats.

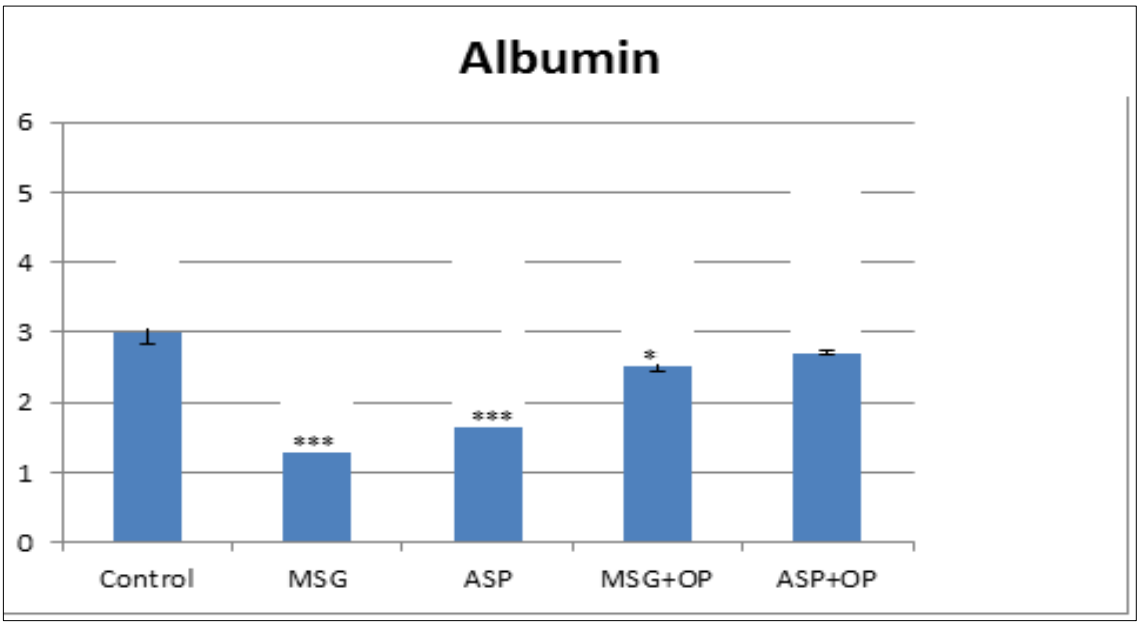


Fig.29. illustrating the mean albumin (g/dl) among the different studied groups of 21day offspring.

Discussion

The liver is the largest gland in the body that play a major role in regulation of several physiological processes, like protein synthesis, secretion of some enzymes, maintenance of homeostasis and protection of the body against the toxic agents(Williamson et al., 1996) .

Several studies revealed that food additives including MSG and aspartame have been implicated in induction of harmful effects on the different body organs (Moore, 2003; Waer and Edress, 2006; El-Beltagy, 2016; AbdElwahab et al., 2017). Also, Banderali et al. (2000) added that utilization of food additives during pregnancy and breastfeeding has dangerous effects on both mothers and their offspring.

It has also been documented that, the antioxidants properties of phytochemicals ingredients in specific natural plants play a major role in amelioration of body organs functions (Liu, 2003). Opuntia ficus indica (cactus cladode) or Opuntai is one of these plants which contain several vital components such as vitamins C and E and phenolics which play a crucial role in antioxidant activity and protective capacities (Bouhrim et al., 2018). Other studies declared that the fruit extract of Opuntia ficus indica contains specific anti-inflammatory compounds like ascorbic acid, betalains, betacyanins, and a flavonoid fraction (Kuti , 2004; Fernandez-Lopez et al., 2010).

Accordingly, the current work was mainly designed to evaluate the protective and ameliorative role of Opuntia ficus indica fruit extract against MSG and aspartame induced hepatotoxicity in pregnant mother's rats and their offspring.

The data of the present work revealed that the body weight of MSG induced mother's rats were significantly higher however, a high significant decrease was noticed among the aspartame treated mothers rats. On the other side, the body weight appeared with low significant increase among the offspring of MSG or aspartame groups. In the two ameliorative groups of mothers and their offspring (MSG or aspartame post supplemented with Opuntia fruit extract) the mean body weights showed non-significant changes in comparing with control. The data concerning the increased body weight under the influence of MSG go parallel with the findings of Bhattacharya et al. (2011) in mice, Inuwa et al. (2011) in rats and Tawfik & Al Bader (2012) in female rats. Bergen et al. (1998) postulated that, MSG could increase energy intake which consequently lead to obesity (Mozes et al., 2004) or change the metabolism of lipids, carbohydrates and proteins (Diniz et al, 2004). Other studies revealed that, the induced inflammation of liver tissue by MSG may be implicated in increased body weight (Park et al, 2000; Onyema et al., 2006).

Recent studies declared that, the decreased body weight in aspartame treated mother's rats may be due to direct inhibitory effect of aspartame on the appetite center (Anton et al., 2010; AbdElwahab et al., 2017; Otman & Bin-Jumah, 2019). The obtained data suggests that low calorie sweeteners of aspartame can accelerate both lipids and carbohydrates metabolism leading to decrease in body weight. In contrast to our results, Feijo Fde et al. (2013) reported increased body weight gain in adult rats treated with aspartame. Such conflict of result may be due to the variation in the surrounded environmental conditions or to the difference in feeding style during experiment. In the current work, the slight increase in the body weight of MSG or aspartame treated

offspring may be due to the leakage of the two food additives through the placenta or during breast feeding period.

Kazeem et al. (2011) declared that Opuntia fruit extract has a powerful role in maintaining the body weight through quick and easy assimilation of nutrients found in the blood stream and also through regulation of liver and renal functions. Such finding is accordance with the obtained result.

In the current work severe deleterious histological alterations were recorded in the liver sections of MSG or aspartame induced mothers' rats and their offspring. In mother's rats, such alterations were represented by damaged hepatocytes that have lost their characteristic architecture; scattered fatty degeneration spots, dilated blood sinusoids and excessive Kupffer cells as well as hepatocyte infiltration, around the congested portal vein. In 21 day's old rats, the histopathological signs were represented by disruption in liver architecture, including hypertrophied hepatocytes, vacuolation and dilated blood sinusoids. Similar observations were recorded in the liver sections of aspartame treated mother's mice and their offspring (Otman & Bin-Jumah, 2019) and on rats (AbdElwahab et al., 2017) and also, in MSG treated mothers rats and their offspring (Ortiz et al., 2006; Waer and Edress, 2006; El-Beltagy, 2016). Cheville (2009) reported that damaged hepatocytes in MSG treated rats is mainly due to they have various surface receptors for toxins and little oxygen. Abdel Hameed, (2004) added that the vacuolation of hepatocytes is considered as a type of cellular defensive mechanism against toxic agents, whereas, these vacuoles can collect the toxic elements and preventing them from disruption of the functional activities of these cells (Cheville, 2009). Diniz et al. (2004) found that, supplementation of MSG to adult rats was associated with oxidative stress on the liver tissue leading to hepatocellular damage.

Several studies reported that, methanol is a major byproduct of aspartame which can induce several histopatholigical signs in the liver tissue whereas; it can alter oxidant/antioxidant status of hepatocytes leading to cell injury (Giannini et al., 2005; Parthasarathy et al., 2006; Ebraheim &Metwally, 2016). Butchko et al. (2002) added that methanol and aspartic acid could liberate free radicals which result in cytoplasmic vacuoles through the reaction with the proteins and lipids of cell organelles.

In the current work a remarkable amelioration was noticed in the histological architecture of MSG or aspartame group post supplemented with Opuntia fruit extract. It has been reported that Opuntia extract is rich with phenolic compounds like catechol, cinnamic acid, phenylpropionic acid, psoralen, syringic acid, sinapaldehyde, 3′-Omethylcatechin, bisdemethoxycurcumin, epicatechin 3′-O-glucuronide and viscutin (Koubaa et al., 2017). These compounds have a considerable antioxidant activity through elimination of free radicals resulting from MSG or aspartame which are implicated in the generation of liver damage (Khan et al., 2000; Ghazi et al., 2015). Gandia-Herrero et al. (2016) declared that, the fruit extract of Opuntia contain high amount of betalains which are considered as natural antioxidants with an exceptionally high free radical scavenging power against liver damage.

Generally, cell apoptosis is accompanied by a series of physiological and morphological alterations like decreased cell size or loss, accumulation of chromatin, cytoplasmic vacuoles, and DNA damage (Chipuk et al., 2008). In the current work, supplementation of MSG or aspartame to mother rats and their offspring for consecutive 40 days was induced moderate to strong positive immune expression for P53 while a weak to negative expression for BCl-2 proteins in their liver sections if compared with control. In comparing with other induced groups, the P53 immuno-reactivity was apparently intensive in in the liver sections of aspartame-induced offspring. Moreover, the data obtained from analysis of liver tissue homogenate revealed marked significant increase in the pro-apoptotic marker; caspase3 in aspartame treated mothers only while the other groups showed non-significant change with control.

BCL-2 is an anti-apoptotic membrane bound protein which maintains the mitochondrial apoptotic pathway under various pathophysiological conditions (Cory, 1995; Antonsson and Martinou 2000; Cheng et al., 2008). Pavlovic et al. (2007) found that, the level of Bcl-2 protein is markedly reduced in the thymocytes of rats exposed to high amount of MSG, indicating that Bcl-2 protein plays a crucial role in initiation of apoptosis. Moreover, Peter et al. (1997) declared that, glutamate compounds can decreases Bcl-2 protein level in various cell type. Other studies revealed that, excess glutamate can accelerate Ca2+ influx and disturbance of the mitochondrial inner transmembrane potential, which leads to increased opening of the mitochondrial pores and consequently cell death (Khodorov et al. 2002; Kanki et al. 2004).

Iyaswamy et al. (2017) found that, the Bcl2 is down-regulated while caspase3 activity is up-regulated in the liver tissue of aspartame treated rats. Also, similar observations were recorded in the brain tissues of rabbit offspring supplemented with aspartame (Puica et al., 2008). Oyama et al. (2002) suggested that, the apoptotic effect of aspartame is mainly attributed to increased production of formaldehyde metabolites that produced by methanol oxidation. These metabolites can increase liberation of free radicals which consequently stimulate protein oxidative damages that play a significant role acceleration of apoptosis through activation of caspase3 (Ashok & Sheeladevi, 2014). Moreover, formaldehyde metabolite of aspartame is considered as toxic chemical that activate protein kinases leading to accumulation of P53 in liver cells.

The data concerning overexpression of P53 protein in the liver tissue of mother's rats under the influence of MSG go parallel with the previous studies (Ortiz et al., 2006; Oscar et al., 2006; Albrahim and Binobead 2018). Also similar observations were recorded on the brain tissues of MSG of rats (Liu et al., 1999). Narayaman et

al. (2010) explained that, the apoptotic effect of MSG was mediated by induction of oxidative stress through stimulation of NMDA (N- malondialdehyde) receptors which consequently led to increased intracellular calcium concentration and activation of different apoptotic enzymes.

In the present work the liver sections of the two ameliorated groups with cactus cladode fruit extract was exhibited a pronounced decrease in the markers P53 protein and an increase of anti-apoptotic markers BCl-2. Previous studies declared that antioxidants of Opuntia fruit extract play a crucial role in scavenging of free radicals, suggesting the capacity of this extract to induce antigenotoxicity and anti-apoptotic effects (Brahmi et al., 2011; Brahmi et al., 2012). Lee et al. (2012) added that, ascorbic acid, vitamin E, carotenoids and flavonoids in Opuntia fruits extract are considered as strong antioxidants against oxidative stress and cellular death.

The data obtained from the liver tissue homogenate revealed that, in MSG treated mother's rats and their offspring as well as the aspartame treated mothers the levels of SOD and CAT antioxidants were significantly lower while the levels of MDA were significantly higher than control. However, a non significant cahnge in the levels of SOD, CAT and MDA was recorded the offspring of aspartame treated mothers rats. Moreover, the level of liver tissue SOD, CAT and MDA displayed remarkable amelioration in their levels post supplementation of Opuntia fruit extract to MSG or aspartame-treated mothers rats and their offspring. The obtained results of this work go parallel with those of Onyema et al. (2006), who recorded remarkable reduction in the levels of CAT& SOD, and obvious increase in MDA levels in MSG-treated rats. Previous researches found that prolonged exposure to MSG can induce oxidative stress and liberation of excess free radicals in various body cells which led to elevation of lipid perioxidation; MDA that implicated in hepatic tissue damage and decreased production of antioxidants (Hazar et al., 2008; Ranawat et al., 2010)

The data concerning the decreased hepatic SOD and CAT as well as increased MDA levels under the influence of aspartame were recorded by Ashok & Sheeladevi (2015) in rats and Ebraheim & Metwally (2016) in rabbits. Previous studies suggested that, aspartame metabolites induce oxidative stress in the liver through liberation of excess free radicals and production of peroxides which are implicated in degradation of antioxidants (Mourad &Noor, 2011; Ashok & Sheeladevi, 2014). Other studies explained that, the reduction in the levels of liver antioxidant enzymes might be attributed to the cytotoxicity induced by formaldehyde resulted from methanol oxidation (Reiter, 2000; Prabhakar et al., 2005).

Castellar et al. (2003) reported that fruit extract of Opuntia ficus indica contain relative concentrations of betanin with specific phenolic and cyclic amine groups which play an essential role in scavenging of free radicals. Other study added that, Opuntia extract is considered as a good supplement for many bioactive constituents with strong antioxidant activities which attenuate the cellular oxidative damage (Livrea et al., 2013; onzález-Ponce et al., 2016). Such finding is consistent with our obtained results.

In this study, significantly increased liver enzymes (AST, ALT, LDH, and ALP) in mother's rats and their offspring induced with MSG are considered as indicative markers of liver damage that caused by the MSG-induced oxidative stress. The obtained results are consistent with the Oscar et al. (2006), who found liver cell damage based on MSG-caused remarkable increase in the levels of serum ALT and AST. Also, Onyema et al. (2006) added that administration of MSG to rats can induce hepatic oxidative stress that manifested by elevation of serum ALT level. Moreover, several studies explained that, increased liver enzymes are mainly attributed to hepatic cell injury, changes in the permeability of cell membrane or elevated aminotransferase or inhibited catabolism (El-Shenawy, 2010; Kalender et al., 2010; Albrahim & Binobead, 2018).

As previously mentioned above, methanol (byproduct of aspartame) is recognized as a toxic agent that induce liver cells damage where it is further oxidized to formaldehyde and other formate products (Trocho et al.,1998). It is well known that, damaged liver cells can liberate a variety of enzymes from the cytoplasm into the blood stream. Accordingly, their elevated concentration in the serum is a good marker for hepatocellular damage (Mitra et al., 1998; Iyaswamy et al., 2017). In the current work long-term consumption of aspartame for mother's rats and their offspring showed increased serum levels of AST, ALT, LDH, and ALP which are indicative of liver damage, together with obvious histopathological alterations (Abhilash et al., 2011). Thus, the increased activities of such enzymes in the current work might be attributed to those aspartame metabolites - induced oxidative stress. Moreover, lipid peroxidation is accompanied with increased permeability of cell membranes leading to release of membrane-bind enzymes like 5' NT into the blood stream (Subhani et al., 2016). Such results are consistent with our obtained data.

In the current work the increased levels of serum bilirubin among MSG or aspartame treated rats levels could be considered as a compensatory mechanism against cellular peroxidative alteration as reported by Pratibha et al. (2004). Marilena (1997) added that bilirubin functions in-vivo as a strong anti-oxidant, and anti-complement against increased serum MDA.

Post-supplementation of Opuntia fruit extract to MSG or aspartame treated mothers rats were significantly restored the increased hepatic enzymes. Similar observation was recorded in the liver functions of rats exposed to nickel (Hfaiedh et al. 2008), chlorpyrifos (Ncibi et al. 2008) and lithium (Saad et al., 2017). Alimi et al. (2013) explained that bioactive constituents of Opuntia extract can stabilize the cell membrane permeability and decrease the leakage of these enzymes into the blood stream. Moreover, it had been confirmed that, the

phenolic constituents found in cactus cladode extract exert their heptoprotective effects through free radical-scavenging activities (Brahmi et al., 2011).

Disclosure

The author reports no conflicts of interest in this work.

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