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Effectiveness of Ginkgo Biloba Extract to Improve the Toxic Effects of Sodium Benzoate Administration in the Liver of Male Albino Mice.

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ABSTRACT

The use of preservatives in food may be harmful for human because of the hidden cumulative effect. Ginkgo biloba is a medicinal plant contains mixture of polyphenols with antioxidant properties that has been recently used in medicine to improve health in human. Therefore the purpose of this study is to investigate the protective effect of ginkgo biloba on liver damage induced by sodium benzoate administration. Twenty-four experimental male albino mice were divided into four groups. The first group served as control and was given disH_2O. The second group was given 120 mg/kg body weight of sodium benzoate orally, thrice weekly, for 2 weeks. The third group was exposed to sodium benzoate and supplemented with ginkgo biloba extract. The fourth group was supplemented with 150 mg/kg body weight of ginkgo biloba extract. Results revealed that sodium benzoate caused an abnormal distribution of liver proteins as well as DNA damage with histopathological changes and Bax expression in the liver of treated groups. Ginkgo biloba co-treatment with sodium benzoate appears to ameliorate the adverse effects of sodium benzoate in the hepatic tissue as it takes most of the affected parameters close to normal.

Keywords*:* Sodium benzoate, Ginkgo biloba, Protein expression, DNA damage, Bax expression.

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INTRODUCTION

Food preservatives are substances used to improve the food's taste and appearance by preserving its flavor and preventing it from souring [1]. Sodium benzoate SB (benzoate of soda) is a commonly used food preservative in acidic foods such as salad dressings, carbonated drinks, jams and fruit juices and condiments [2]. SB is considered as a bacteriostatic and fungistatic agent in acidic conditions, once it enters into cells, interacts with the anaerobic energy production pathways resulting in suppression of the development of food-spoilage microorganisms [3]. Owing to this feature, SB is broadly employed in other fields and is one of the indispensable constituents of pharmaceutical and cosmetic products [4 and 5].

Today, it is difficult to imagine foodstuffs without any food additives, nevertheless preservatives affect the health of long-term consumers even in small concentrations and may cause migraine, nausea, vomiting, diarrhea, rhinitis, bronchospasm, anaphylaxis and hyperactivity in children. Furthermore, there may also be induced damage at the molecular level, involving chromosome damage [6]. Studies on laboratory animals have implied that intake of SB has adverse effects on organs such as heart, spleen, kidney, brain, and liver and causes alterations in plasma ions, congenital malformation, vertebral column deformity [7 and 8].

Villanaueva *et al.* [9] reported that many aromatic compounds (including benzoates family) are extremely carcinogen and are metabolized by living micro-organisms, producing active compounds which react with cellular DNA and alter cellular structure. Also, Yang *et al.* [10] stated that SB can cause changes in genes expression through releasing free radicals. Since the liver is the principle organ for various metabolic and detoxification reactions, it is important to continue to study the adverse effects, that sodium benzoate may exert on this vital organ.

Ginkgo biloba (GB) extract has been investigated more recently as an alternative therapy for metabolic disorders. GB extract which is a rich mixture of polyphenolic compounds (ginkgolides, bilobalides, quercetin), is a well-known phytotherapic compound often employed as coadjuvant supplement in type 1 and 2 diabetes [11], neurodegenerative diseases [12] and obesity [13]. In addition, GB has been shown to possess hepatoprotective effect in several animal models of liver disorders, including liver fibrosis induced by different toxic substances [14 and 15]. GB is used as a therapeutic agent in modern pharmacology in many forms such as tablets, oral liquids or injectable solutions [16].

Because there is little numbers of in vivo studies in mammals that have been conducted on the genotoxic as well as histopathologic effects of SB, the aim of this study was to first assess the genomic and histologic alterations that might be induced by SB in the liver of male mice and second to test the hepatoprotective potential of ginkgo biloba.

MATERIALS AND METHODS

Experimental Animals

Twenty-four adult male mice (Mus Musculus), weighing 25-30 g (8-12 weeks), were obtained from the animal house of the National Research Center, Cairo, Egypt. Before initiating the experiment, the mice were allowed to acclimatize for one week in clean and hygienic polypropylene cages and administered food and water ad libitum. They were raised under standard environmental conditions (12 h dark/12 h light cycle; temperature 23–25 °C; relative humidity 40%–60%). The study was conducted according to regulations of the ethics committee of the National Research Centre which gave its consent in accordance with the National Regulations on Animal Welfare and Institutional Animal Ethical Committee.

Chemicals

Sodium benzoate (C_6H_5COONa) with E numberE211 is obtained from Oxford Lab Chem Company, India while Ginkgo biloba tablets are obtained from NATROL LLC Company, USA.

Animal Grouping

The experimental animals were divided into four groups (6/group) according to the following scheme:

Group I (C group): Control group received 0.5 ml of distilled water.
Group II (SB group): Received sodium benzoate at a dose of 120 mg/kg.bw [17].
Group III (GB+ SB group): Pretreated with ginkgo biloba then with sodium benzoate.
Group IV (GB group): Received ginkgo biloba at a dose of 150 mg/kg.bw [18].

All doses were given through oral intubation three times per week. After two weeks, animals were sacrificed under di ethyl anesthesia and liver tissues were detached for protein banding patterns and comet analysis as well as histological, histochemical and immunohistochemical examinations.

SDS-Polyacrylamide Gel Electrophoresis of Protein

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis SDS-PAGE was used to separate proteins dependent on the molecular weights of their polypeptide chains. An aliquot contains 50 µl protein solution was placed in a 2x volume of sample buffer containing 10% glycerol, 5% ß-mercaptoethanol, 4% SDS, 0.1M Tris-HCl buffer (pH 6.8) and 0.01 % bromophenol blue. Sample and the mixture tissue were heated in thermo-mixer at 90^0C for 2 minutes.

An aliquot containing approximately 100 µl of protein of such preparation was loaded onto acrylamide gel and electrophoresis was carried out until the bromophenol blue track reached three-quarters through the gel in approximately 4 hours at 40 mA & 100v on a vertical plate gel containing 12% acrylamide separating gel and 1.0 cm of 4% acrylamide stacking gel. Gel and electrode buffer (pH 8.3) was prepared [19 and 20].

Following electrophoresis, protein bands were stained with a solution of 25 % methanol, 10 % glacial acetic acid and 0.025 % Coomassie blue R. 250 at room temperature for 1 hour, and later distained by shacking in 10 % glacial acetic acid for a minimum of 30 min. Data were analyzed by an image analysis system (Gel Analyzer 3 software program) to show the differentiation in protein bands using protein marker as a stander. The mobility of the protein and of the marker dye calculated as:

$$\text{Rf (retardation factor)} = \frac{\text{migration distance of protein}}{\text{migration distance of dye}}$$

Single Cell Gel Electrophoresis (SCGE)/Comet Assay

Comet assay was carried out under alkaline conditions, as described by Singh *et al*. [21]. To obtain single cells from the liver, the sample must be finely minced using sterile scissors and the cells are harvested by centrifugation at 500 rpm for 5 min. A freshly prepared 20 µL of single liver cells in 1ml Phosphate buffer saline (PBS) was mixed with 150 µL of 1% low melting agarose LMA at 37 ºC and spread onto microscope slide pre-coated with 1% LMA and a coverslip was applied to spread the sample then the slides kept on icebox until the solidification of agarose. Slides were placed overnight in ice-cold freshly prepared lysis solution [2.5 M NaCl, 100 mM EDTA, 10 mM Tris–HCl, pH 10 with 1% Triton and 10% DMSO were added fresh] to lyse cells and allow DNA unfolding. The slides were then placed on a horizontal gel electrophoresis tank, facing the anode. The unit was filled with fresh electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 13.0) and the slides were placed in an alkaline buffer for 30 min to allow DNA unwinding prior to electrophoresis for 30 min at 25 V (300 mA). Slides were washed gently thrice with neutralization buffer (0.4 M Tris-HCl, pH 7.5) to remove excess alkali and detergents. Each slide was stained with ethidium bromide (20 µg/ml) and stored at 4°C in dark sealed boxes until being scored.

Evaluation of DNA damage: Slides were coded and examined using a fluorescence microscope equipped with appropriate filters, connected through a camera to a computer–based image analysis system (Comet Assay software) provided by a range of parameters such as comet tail length, % DNA in tail, tail moment and olive tail moment. Images of 50 randomly selected cells from each mouse were analyzed. Cells were graded by eye into five categories, based on the distance of migration and the perceived proportion of DNA within the tail, and given a value of 0, 1, 2, 3 or 4; from undamaged 0 to maximally injured 4 [22].

Histological and Histochemical Studies

The livers were immediately fixed in 10% neutral formalin and processed routinely for paraffin embedding technique. These sections of 5-7µm thick were made using a rotary microtome and stained with hematoxylin and eosin [23]. Also, Feulgen method for DNA demonstration was prepared [24].

Immunohistochemical Studies

Liver sections with 5µ thickness were deparaffinized in xylol and hydrated in decreasing series of ethanol. Endogenous peroxidase activity was blocked by incubation in methanol containing 0.3% H_2O_2 for 15 min at room temperature, followed by a rinse in 0.1 M PBS (pH 7.4) for 5 min. The sections were then treated with citrate buffer (pH 6) for 15 min at 98°C as antigen retrieval. Before the application of specific primary antibodies, nonspecific background staining was prevented by incubation with goat serum diluted 1:10 v/v in PBS for 50 min. After that the sections were incubated overnight at 4°C with primary antibodies, including the monoclonal antibody against Bax (Mouse Monoclonal anti-Bax; sc: 7480, Santa Cruz) at 1/100 diluted in PBS containing 10% normal goat serum (NGS). After washing twice with PBS the sections were incubated with secondary antibody biotinylated anti-mouse IgG (Santa Cruz ABC Peroxidase Mouse IgG Staining Kit) at 1/100 for 50 min. The sections were then incubated with peroxidase-conjugated avidin-biotin for 30 min at room temperature. Finally, after washing, the sections were incubated with diaminobenzidine (DAB) as chromogen and counterstained with hematoxylin. The control was performed by omitting anti-Bax antibody.

Statistical Analysis

The data of comet assay was expressed as mean ± SD and were analyzed by one-way analysis of variance (ANOVA) followed by post hoc test [25]. The difference was considered statistically significant when $p<0.05$.

RESULTS AND DISCUSSION

Influence of Sodium Benzoate on the Protein Banding Patterns of Liver

Addition of preservatives to food could prevent it from decompositions by microbial growth or undesirable chemical changes [3]. Sodium benzoate (SB) is a commonly used food and beverage preservative until now and classified as a GRAS (Generally Recognized As Safe) compound by the FDA (Food and Drug Administration) [26]. Metabolization of benzoate takes place in the liver by conjugation with glycine, where the utilization of glycine in the detoxification of benzoate results in depletion in glycine level of the body which can affect metabolic process in which glycine is involved and leads to reduction in creatinine, glutamine, urea and uric acid levels [27].

There is an increasing need to evaluate the safety of SB not only as food preservative but also in view of a rapidly expanding body of medical research, which indicates that this compound may provide inexpensive therapy for some diseases, in addition to its well-established use in the treatment of urea cycle disorders [28].

The results of the current study indicated that liver protein patterns exhibited variability in MW (24.604–104.93KD) with an RF range between 0.224 - 0.963 as noticed in Table 1 and Figure 1. Comparing the protein banding pattern from SB group with the control group, it was found that the protein bands number 4, 7, 9 and 10 were disappeared at MW 82.587, 59.158, 53.734 and 49.482 KD with RF 0.346, 0.516, 0.565 and 0.607 respectively. Furthermore, four new bands number 2, 6, 8 and 11 were appeared at MW 96.249, 62.133, 56.658 and 47.391 KD with RF 0,268, 0.491, 0.538 and 0.629, respectively. The obtained results are in agreement with those reported by Ali *et al*. [29] who investigated the different combination of Sunset yellow and SB as colorant and food additives induced a disruption of protein metabolism in liver tissues of female rats exposed to SB.

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Table 1: Results of SDS- PAGE gel scanning of sodium benzoate and gingko biloba administration for 2 weeks on liver of mice.

RF	MW	Lane1 GB+SB	Lane2 SB	Lane3 GB	Lane4 C	Frequency	Polymorphism
0.224	104.93	1	1	1	1	1.00	Monomorphic
0.268	96.249	1	1	1	0	0.75	Polymorphic
0.324	86.231	1	1	1	1	1.00	Monomorphic
0.346	82.587	1	0	0	1	0.50	Polymorphic
0.381	77.105	1	1	1	1	1.00	Monomorphic
0.491	62.133	0	1	0	0	0.25	Unique
0.516	59.158	0	0	0	1	0.25	Unique
0.538	56.658	1	1	1	0	0.75	Polymorphic
0.565	53.734	0	0	1	1	0.50	Polymorphic
0.607	49.482	0	0	1	1	0.50	Polymorphic
0.629	47.391	0	1	0	0	0.25	Unique
0.671	43.641	1	1	1	1	1.00	Monomorphic
0.779	35.306	1	1	1	1	1.00	Monomorphic
0.821	32.512	1	1	1	1	1.00	Monomorphic
0.87	29.531	1	1	1	1	1.00	Monomorphic
0.897	28.007	1	1	1	1	1.00	Monomorphic
0.926	26.458	1	1	1	1	1.00	Monomorphic
0.963	24.604	1	1	1	1	1.00	Monomorphic

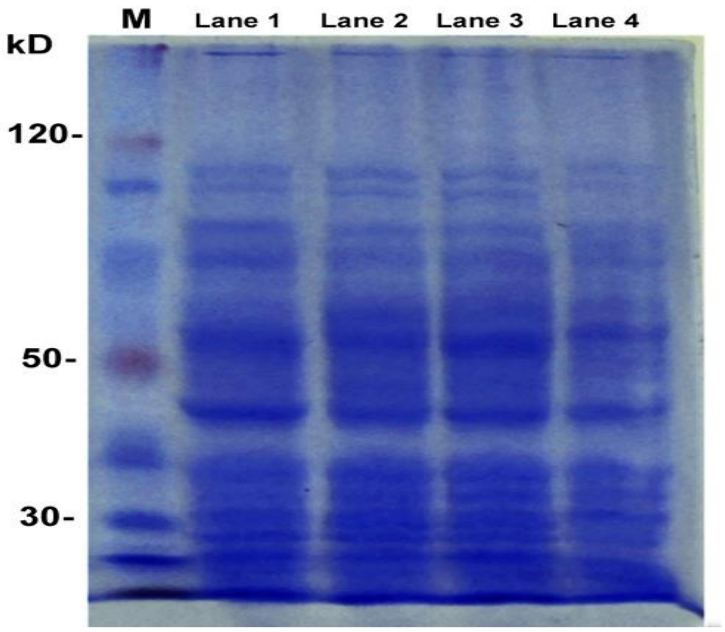

Figure 1: Separated proteins in polyacrylamide gel of male mice liver. Lane M: marker, protein standard of known molecular weights ranging from 120kDa to 30kDa, lane 1: GB plus SB group, lane 2: SB group, lane 3: GB group, lane 4: C group.

Herbal therapy has been gaining popularity among clinicians recently due to their beneficial effects with minimum toxicity [30]. The ginkgo biloba (GB) tablets is a standardized extracts of dried leaves that contains 24% ginkgo-flavone glycosides and 6% terpenoids which have antioxidant potency [31].

In our experiment, the pretreatment with GB had a beneficial effect on liver protein damage where it regulated the protein expression in the hepatic tissue of mice that had administered SB. In the GB+SB group, only 3 bands number 7, 9 and 10 have been eliminated while band number 4 reappeared again. In addition,

the two new bands number 6 & 11 does not exist in this group compared to the SB group which means that GB extract can act as protein expression regulator.

The protective effect of GB against cellular damage has been associated rather with its high antioxidant and anti-apoptotic properties. Additionally, it can upregulate the anti-apoptotic protein Bcl-2 while attenuates ROS-induced elevation of the pro-apoptotic protein like Bax. It can also stabilize the changes in mitochondrial membrane potential occurred due to excessive ROS and peroxynitrite generation [32].

DNA Damage / Comet Assay

Comet tail length is an important parameter in evaluating the DNA damage. SB induced statistically significant increase ($p<0.001$) in mean/SD comet tail length [13.41 ± 1.05 pixels] indicating DNA damage in liver cells when compared with controls [3.95 ± 0.17 pixels]. In addition, the mean/SD of percent tail DNA (10.14 ± 1.44%) was a statistically significant increase ($p<0.001$) in the animals exposed to SB compared to controls (2.24 ± 0.70%). Also, the mean/SD of % damage (18.47 ± 0.55) was a statistically significant increase ($p<0.001$) compared to controls (4.17 ± 1.26). There was also a statistically significant increase ($p=0.014$) in tail moment (0.51 ± 0.06) and olive tail moment ($p<0.001$, 0.92 ± 0.05) compared to controls (0.22 ± 0.02 and 0.34 ± 0.05 respectively) as evident in Figure 2(a). The obtained results are in agreement with those reported by Ali *et al*. [29] who investigated that SB induced an increase in the frequency of tailed nuclei (DNA damage) in liver cells of female rats.

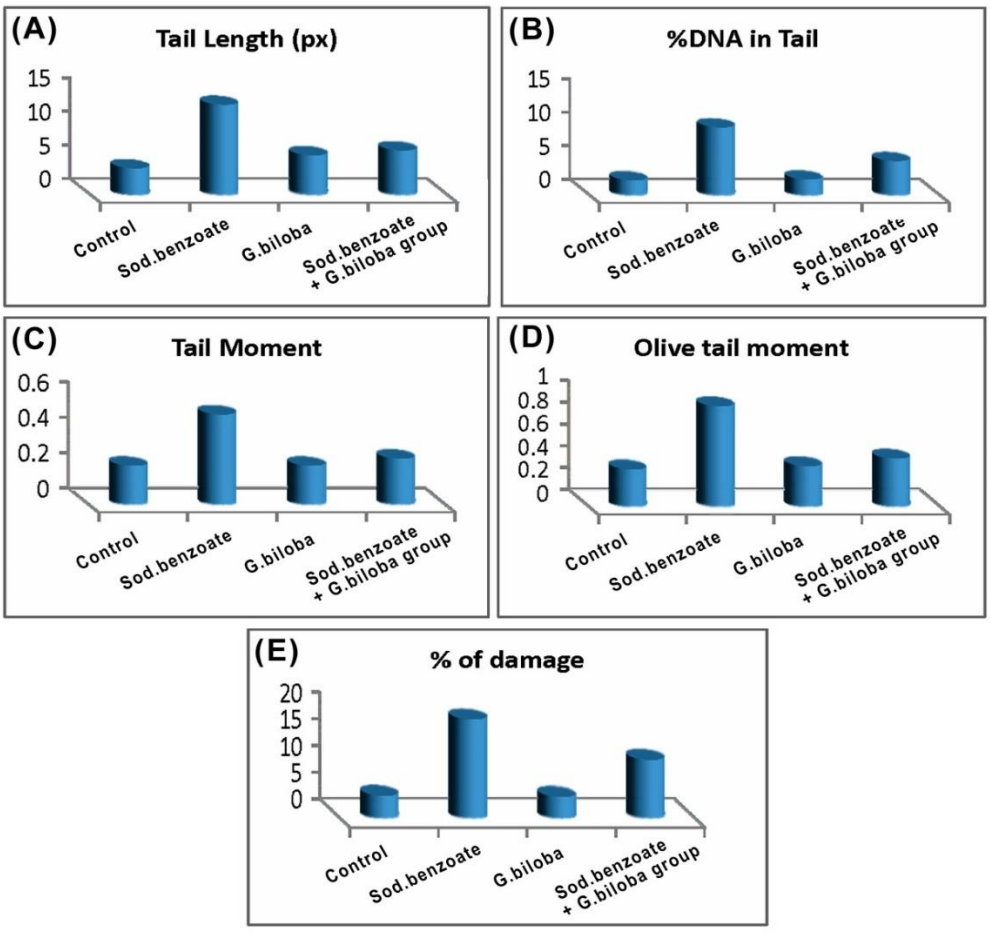

Figure 2(a): Evaluation of damaged DNA revealed statically by various comet assay parameters of male mice livers in treated and control groups. (A) Tail length (measured from the center of the head to the end of the tail). (B) Tail DNA percentage (calculated as Tail DNA Intensity/cell DNA Intensity x100). (C) Tail moment (calculated as: Tail length x % DNA in tail/100). (D) Olive tail moment (measured as: Tail DNA% x Tail Moment Length). (E) Percentage of damage (calculated as the number of damaged cells/whole cells x 100).

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Furthermore, image analysis indicated that undamaged DNA do not form a “comet” and were considered negative (stage 0), whereas cells with fragmented DNA (positive) display increased migration of the DNA from the nucleus towards the anode and had the appearance of a “comet” with brightly fluorescent head and tail formed by the DNA (stage 1- 4) as evident in Figure 2(b).

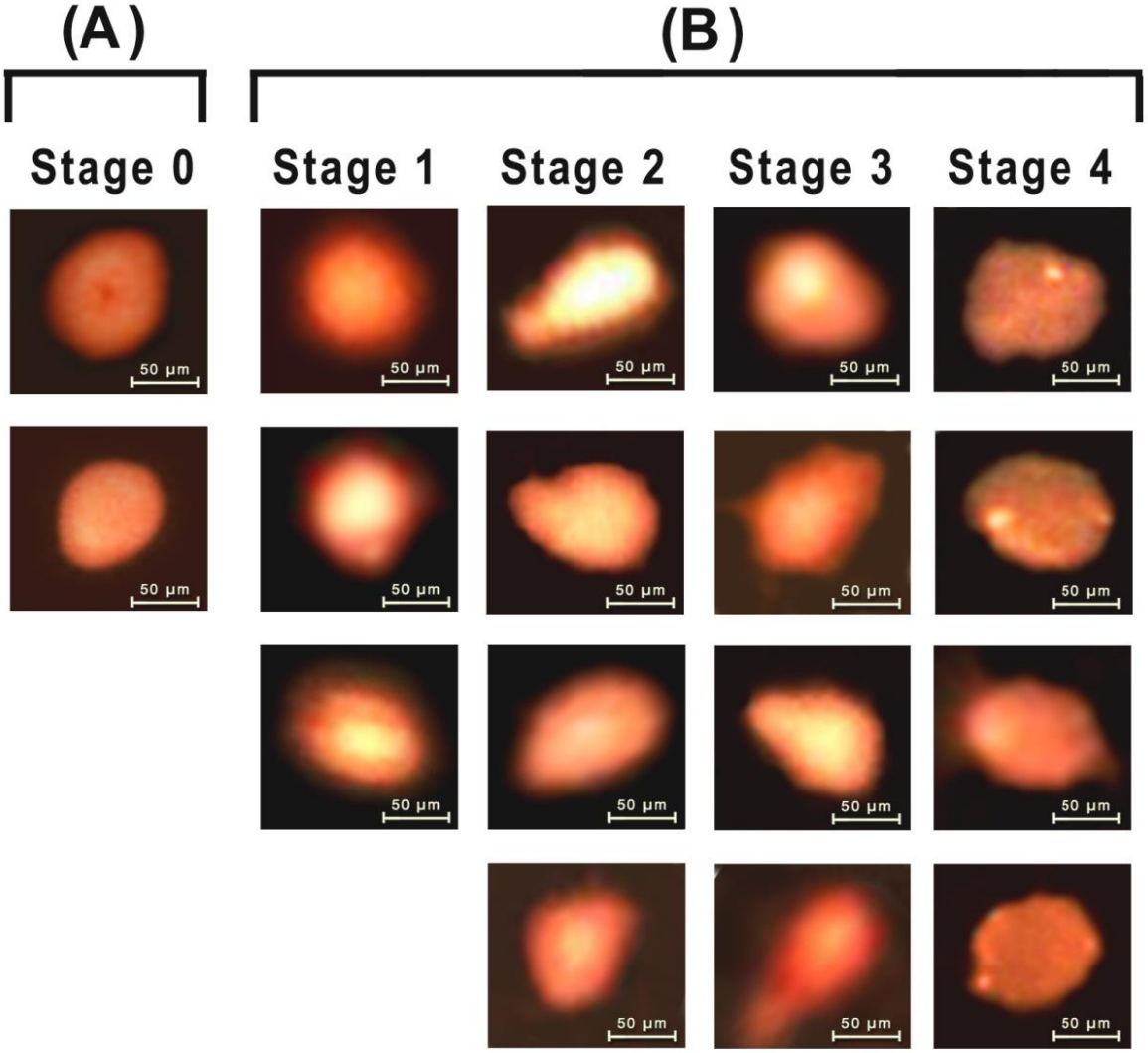

Figure 2(b): Photomicrographs of comets (from mice hepatocytes), stained with ethidium bromide ×400. They represent stages from 0 to 4 as used for visual scoring. The intensity of the comet tail relative to the head reflects the number of DNA breaks. (A) Control, the DNA is intact without a DNA tail, (B) DNA tail in damaged hepatocytes in different stages.

Studies also revealed that sodium benzoate can be metabolized in the body under irradiation to form benzene, a derivative capable of damaging mitochondrial DNA which has also been implicated in kidney and liver injury. Benzene and its hepatic metabolites would appear to cause chromosome aberrations, notably aneuploidy, as opposed to point mutations, such as base substitutions or frameshifts in the liver [28]. Benzene has also been shown to cause mutagenicity in a number of mouse tissues, including the lung and spleen; mutations at the glycophorin A locus of exposed humans; also to modulate signal transduction pathways activated by oxidative stress that are involved in cell proliferation and apoptosis [33].

On the other hand, the results revealed that mean/SD of liver DNA damage presented by comet tail length ($p<0.001$), percent tail DNA ($p=0.003$), % damage ($p<0.001$), tail moment ($p=0.032$), and olive tail moment ($p=0.002$) were statistically significantly decreased in the animals treated with GB together with SB as 6.62 ± o.77, 5.11 ± 0.91, 10.90 ± 0.79, 0.26 ± 0.13, 0.44 ± 0.13 mean/SD respectively compared to SB group. In addition, there were no significant differences ($p=1.000$) between GB plus SB group and control group in all comet parameters except in the tail length ($p=0.045$).

The hepato-protective results of GB in this study are in agreement with that of Abdul-Hamid *et al*. [15] who showed that the liver treated with GB exhibited hepato-protective mechanisms against amiodarone-induced hepatotoxicity in male albino rats. They revealed that treatment with GB improves the DNA damage of the hepatocytes by reduction in different comet assay parameters (Tail length, % DNA in tail, and tail moment). Similarly, Cavusoglu *et al*. [18] investigated the hepato-protective role of GB against genotoxic effects of glyphosate by measuring chromosomal aberrations (CAs) and micronucleus (MN) in bone marrow cells of Swiss albino mice and reported that the number of CAs and MN was always less in the GB plus glyphosate-treated groups and the effect was dose-related. In addition, GB extract has significantly possible protective effects against mitomycin C and cyclophosphamide-induced mutagenicity using the micronucleus test in mouse bone marrow and that could be related to the modulation of the cytochrome P-450 (CYP) so GB can act as an intercalating antitumor drug or by the generation of reactive oxygen species [34].

Histological and Histochemical Observations

It was found that, control and GB groups have no differences, as the liver sections showed normal hepatocytes with well-preserved cytoplasm, prominent nuclei and central vein. While SB group showed cytoplasmic vacuolization of the hepatocytes due to cell organelle deterioration specially mitochondria with consequent decrease in sodium potassium pumps which led to water accumulation. Also, SB caused focal inflammatory cells aggregation and necrotic patches that may be attributed to swelling of cytoplasm in addition to toxic effect on lysosomal membranes which in turn ruptured and liberate influential enzymes that caused disintegration and termination of many cellular components. There was also rigorous dilatation in the portal vein associated with congestion duo to toxic effect of SB causing rupture of blood vessel wall. The previous results agreed with those of Agarwal *et al*. [35]. On the other hand, GB+SB group showed restored lobular architecture of liver tissue with slight necrosis and pyknotic nuclei as seen in Figure 3.

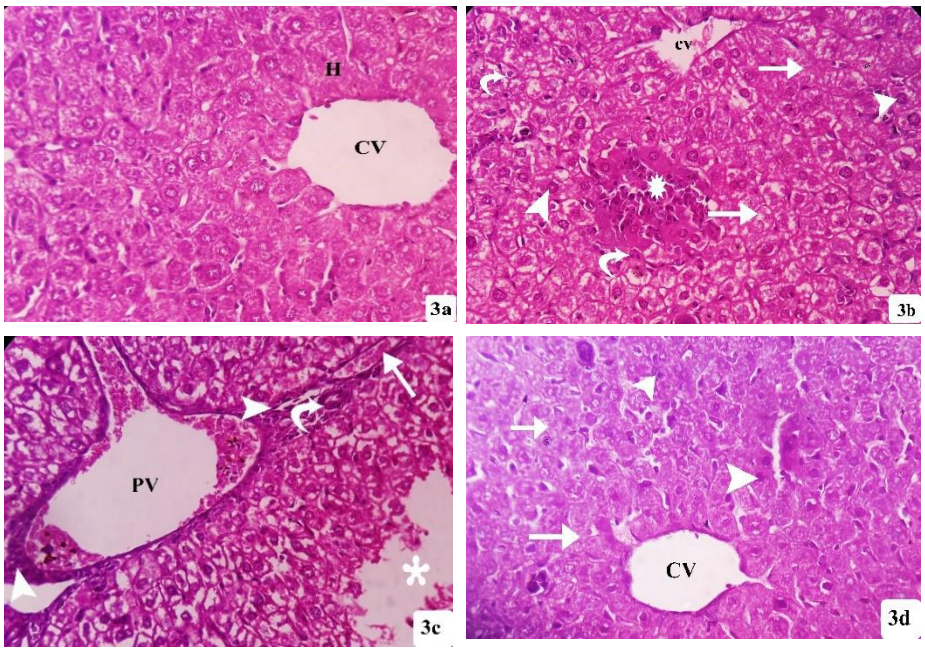

Figure 3: Histological sections from mice liver stained with H&E (400X) showing: (a) normal hepatic architecture with normal hepatocytes (H) surrounds central vein (CV). (b): SB- treated group showing necrotic hepatocytes (thin arrows), pyknotic nuclei (curved arrows), karyorrhexis nuclei of hepatocytes (arrow heads), karyolysed nuclei (thick arrow) and inflammatory infiltration (*). (c): SB- treated group showing necrosis (*), congested and dilated portal vein (PV), inflammation focii (arrow heads), congested portal artery (arrow) and thickened bile duct (curved arrow). (d): Gb+ SB- treated group showing regeneration of the hepatic archiecture with few pyknotic nuclei (arrow heads) and necrosis (arrows) around central vein (CV).

Furthermore, DNA-containing particles were seen in the nuclei as small purple particles that could be distributed in the nucleoplasm or peripherally in controls (Figure 4a). While SB group exhibited a diffuse

stainability as many hepatocytes' nuclei became faint shrunk and karyolysed (Figure 4b). That may be attributed to oxidative stress and free radical formation which led to damage of macromolecules in the cell such as lipids, proteins and DNA. This result agreed with Pongsavee [3] who examined SB effects on a human lymphocyte cell line and found that the micronuclei formation increased proportionally to the applied SB concentration and the treatment duration in vitro. He also observed chromosome breakage to occur at a dose of 2.0 mg/mL of SB at 24- and 48-hour incubation time. Based on this evidence that SB triggers micronucleus formation and induces chromosome breaks that caused damage to DNA, so he suggested that SB as food additive agent has mutagenic and cytotoxic effects in lymphocytes. On the other hand, GB administration with SB exposed a moderate normal hepatic tissue while there were few cells showing decrease in DNA reactivity (Figure 4c).

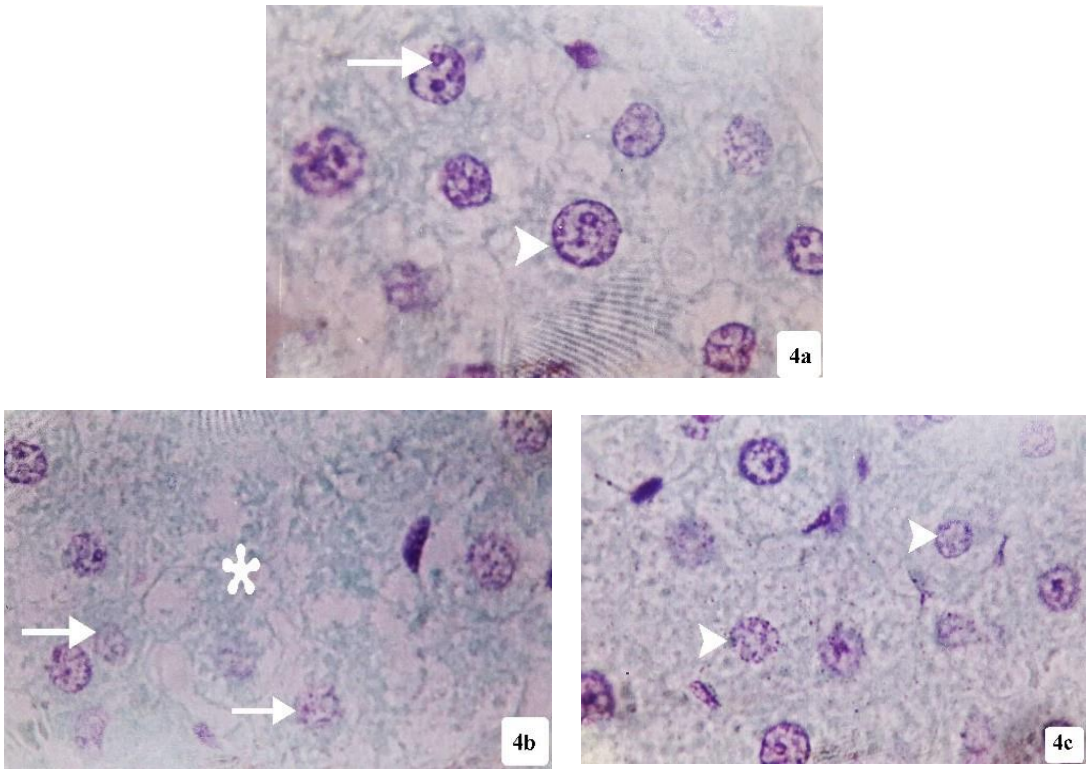

Figure 4: Photomicrographs of liver sections stained with Feulgen technique (1000X): (a) Controls showing DNA- containg particles, nucleus (head arrow) and nucleolus (arrow). (b): SB group showing decreased the amount of DNA. The nuclei became pale, shrunk and karyolysed (arrows). Also, DNA was absent in the necrotic area (*) Irregular boundary of some nuclei could be seen (c): GB+ SB group showing slight decrease in DNA reactivity in karyorrhexis nuclei of the hepatocytes (arrow heads).

Immunohistochemical Observations

The controls showed negative immunoreactivity toward Bax in the hepatocytes (Figure 5a) but Bax expression was strongly detected in the cytoplasm and nuclei of hepatocytes of SB group (Figure 5b). Bax is a typical pro-apoptotic protein in the cytosol, which may translocate to the mitochondria to induce apoptosis and its activation stimulates caspase-3 that consequently, induced apoptotic response, eventually leading to cell death [36]. Meanwhile, a slight expression of Bax was observed in the hepatocytes of the GB+ SB group (Figure 5c).

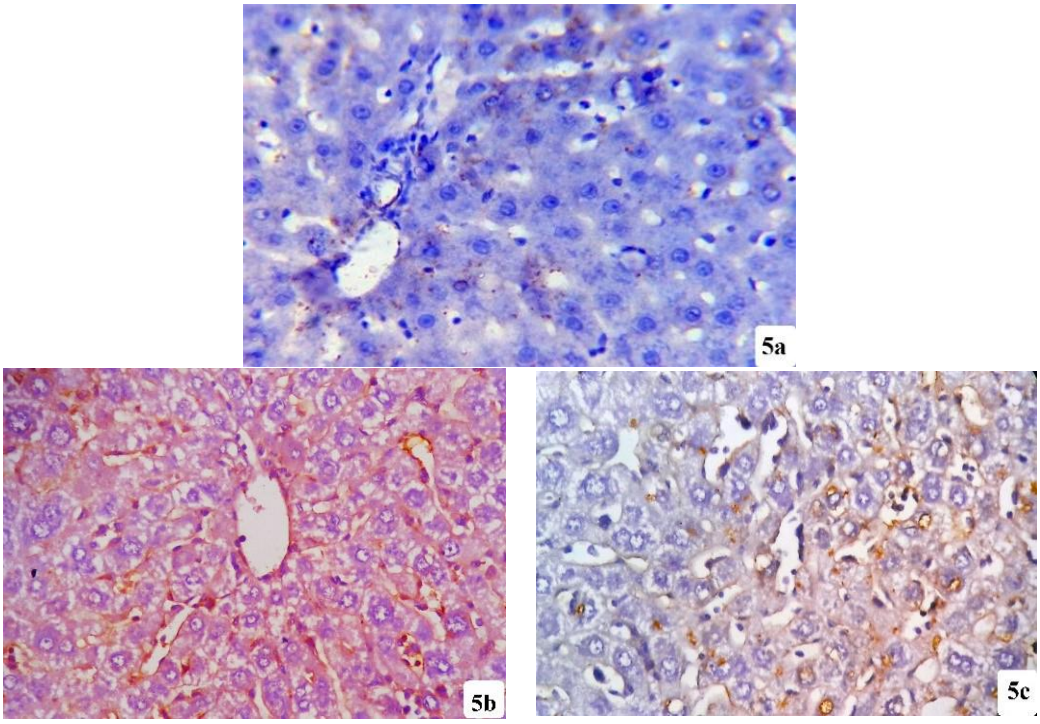

Figure 5: Photomicrographs of liver sections stained with immunohistochemical stain of Bax (400X): (a) Controls showing negative immuoreaction in the cytoplasm and nuclei of hepatocytes. (b): SB group showing increase of Bax immunoreactivity in the cytoplasm of hepatocytes. (c): GB+ SB group showing slight increase immunoreactivity of Bax expression.

In the present study, the hepato-protective effect of GB might be through increased GSH levels that scavenged the free radicals and also due to combined role of antioxidant enzyme system comprising SOD and catalase that efficiently scavenged reactive oxygen species. It also appeared that GB might have enhanced gene expression of the antioxidant enzymes [37].

CONCLUSION

These findings corroborate the genotoxicity as well as histopathological effect of sodium benzoate preservative on mice hepatic tissue and thus may have hazard effects on human and should be limited in use. The present study also revealed that administration of ginkgo biloba exerted potent protective effects against sodium benzoate induced liver damage in mice.

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