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Toxic Effects of Oral Administration Doses of Hydroxylated C_{60} Fullerene on Liver in Swiss Albino Mice

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The toxic effect of hydroxylated C_{60} fullerene (fullerenol) in liver of swiss albino mice has been studied using 100 and 500 mg/kg of hydroxylated C_{60}. Polyhydroxylated fullerenes, $C_{60}(OH)_{29}$, was synthesized in high yield through a high-speed vibration milling method and characterized using FTIR, mass spectroscopy, and thermal gravimetric analysis to estimates the number of hydroxyl groups attached to the fullerene cage. This investigation aimed to evaluate the toxicity of fullerenol in the liver of mice. Mice were divided into 3 groups, the first group served as control group, second and third groups received oral administration doses of 100 and 500 mg/kg of fullerenol, respectively, for 8 consecutive days. The results revealed that dose of 100 mg/kg showed less toxic effects represented by relative healthy liver with ability of regeneration, minor fibrosis, apoptosis gene-expression and no oxidative stress gene-expression were seen. Whereas, dose of 500 mg/kg of fullerenol caused pathological liver changes with heavy presence of apoptosis and oxidative stress gene-expressions.

Keywords: Hydroxylated C_{60} Fullerene, Liver–Mice, Oxidative Stress, Apoptosis.

1. INTRODUCTION

Fullerenes, discovered in 1985,[1] are a family of carbon allotropes composed entirely of carbon in the form of a hollow sphere. The smallest, most stable and also the most abundant fullerene is C_{60}. Many studies show that C_{60} is composed of carbon atoms with predominantly sp^2-hybridization, and the arrangement of these atoms indicates an outline of 20 hexagons and 12 pentagons (which provide the curvature).[2–4] The strain introduced by the curvature of the surface enhanced the reactivity of C_{60}.

The non-functionalized fullerene cages are soluble only in non-polar organic solvents (solubility of 5 mg/ml in toluene), making them incompatible with biological systems. However, since the preparation of fullerenes in multigram amounts in 1990, a wide variety of chemically modified fullerenes have been synthesized.[5] From then, a large number of experimental studies have been performed on their chemical derivatization to understand the basic chemical properties and obtain new derivatives with interesting electronic, catalytic, or biological properties. One successful attempt has been performed, it increases the water-solubility of fullerenes, which functionalizes of their cages with carefully selected hydrophilic groups.[6–9] Fortunately, the exterior of the fullerene carbon cage has a rich synthetic organic chemistry, which allows a wide variety of water-soluble derivatives to be prepared. One approach to increase their water solubility is to covalently modify their surface, for example by attaching hydrophilic groups as is achieved in the hydroxylation of the fullerene cage exterior. Such hydroxylation is of great importance because it is crucial in the development of new materials with potential applications in material and medical science. Thus, several studies have focused on the hydroxylation of the fullerenes not only because of the simplicity of the synthesis, but also because of the possibility of further molecular modifications such as conjugation of the bio-reactive ligands.[10, 11]

Since fullerene discovery, it had attracted considerable interest in many fields of research including material science and biomedical applications.[12, 13] The use of C_{60} is being considered for drug delivery and recently within a number of cosmetic products, such as face creams.[14] As underivatized and derivatized fullerenes are becoming increasingly available, it is of great importance to assess their safety and environmental impact. Many studies were performed on toxicity of fullerene *in vitro* as well as

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in vivo in various model systems. Available data clearly shows that fullerene C_{60} has no toxicity in a large variety of living organisms.[4, 15] Jia et al. observed the cytotoxicity test protocol for carbon nanotubes and fullerene (C_{60}) in alveolar macrophage after a 6-h exposure *in vitro*, and the results showed that the cytotoxicity increases by increasing the dosage of SWNTs, however there is no significant toxicity was observed for C_{60} up to a dose of 226.00 μg/cm^2.[16] The present study was carried out to investigate whether moderate and high doses of fullerene C_{60} derivative can cause toxicity in liver of swiss albino mice or not and hence can be used in biomedical applications.

2. MATERIAL AND METHODS

2.1. Experimental

In this part, the synthesis and characterization of hydroxylated C_{60}-fullerene is presented, followed by biological treatment on albino mice.

2.1.1. Materials

Materials used for the hydroxylation of fullerenes are C_{60} (MER, 99+%), toluene (Aldrich, 99.9%), potassium hydroxide (Aldrich, 85+%) and sephadex G-25 (Aldrich, dry head diameter of 20–80 μm and bed volume of 4–6 mL per g of dry Sephadex).

2.1.2. Methods

2.1.2.1. Preparation of Hydroxylated C_{60}-Fullerene ($C_{60}(OH)_x$). The hydroxylation of C_{60} was performed under High-Speed Vibration Mill (HSVM) conditions in which a mixture of C_{60} and KOH was vigorously shaken under HSVM and at room temperature, and the resulting reaction mixture was readily dissolved in water, Eq. (1).[7, 8] The crude solution was filtered through a 0.5 μm PTFE membrane, and water was partially removed under reduced pressure. The concentrated crude sample was chromatographed on a Sephadex G-25 size-exclusion gel column with distilled water as the eluent to remove residual salts. The sample was collected (pH 6–7) as a dark brown solution over a short time interval to obtain final fullerenols with a narrow distribution range of hydroxyl number. The collected brown solution was concentrated and added dropwise to methanol for crystallization, and the collected brown solids of fullerenols were kept at 50 °C under vacuum for further characterization.

$$C_{60} \xrightarrow[\text{HSVM(1 hr)}]{O_2/KOH} C_{60}(O)_n^-(OH)_m \xrightarrow{H_2O} C_{60}(OH)_x \qquad (1)$$

2.2. Instrumentation and Characterization

2.2.1. Characterization of $C_{60}(OH)_x$

The resulted product of our preparation was characterized using the following techniques: FT-IR spectroscopy, Thermal Gravimetric Analysis (TGA), and Mass Spectroscopy (MS). This variety of characterization techniques was deemed essential for the elucidation of the chemical structure and approximating the number of the hydroxyl groups attached to the fullerene surface.

FT-IR. The infrared spectra of the prepared samples were recorded in the wave number range from 400–4000 cm^{-1} using the Thermo Scientific Nicolet FT-IR spectrometer (USA manufactured).

Thermal gravimetric analysis (*TGA*). The number of hydroxyl groups attached to the fullerene surface was estimated using TGA technique in which the weight changes in a well dried material are monitored as a function of temperature under a controlled atmosphere. Using a TA instrument Q5000 (USA manufactured), an average 10 mg sample was analyzed in platinum pans over the temperature range of 25–1000 °C at a heating rate of 20 °C/min. The number of hydroxyl groups attached to the fullerene cage was estimated by calculating the ratio of the weight loss at 250 °C, corresponding to the dehydroxylation of hydroxyl group, to the weight loss at a temperature >300 °C, corresponding to the decomposition of the fullerene cage.

Mass Spectroscopy (*MS*): To confirm the number of hydroxyl groups attached to the fullerene cage, mass spectra of the prepared hydroxylated C_{60}-fullerene were collected using mass spectroscopic technique (thermo scientific^{TM}, USA manufactured). In this technique, a small amount of the hydroxylated fullerenes, dissolved in water, was applied to the target plate and its mass spectra were compared to that of pure samples of C_{60} (FW of C_{60}-fullerene = 720.67 g/mol).

2.2.2. Animals

Male mice of the swiss albino strain weighing 25 ± 30 g were used for the experiment from the animal house of King Saud University. The animals were acclimated to 22 ± 1 °C and maintained under conditions of 12-h periods of light and dark, with free access to clean water and commercial mice food. The animals were housed in polypropylene cages inside a well-ventilated room. The animals were treated and killed according to the guidelines of International Society for Applied Ethology.

2.2.3. Histopathological Preparation and Analysis

The animals were randomly divided into three groups; each group consists of 10 mice in the beginning of the study. The first group served as mice of control group received clean water; however, mice of the second and third groups received daily oral administration of 100 and 500 mg/kg, respectively, of hydroxylated fullerene dissolved in water for 8 days. All animals were sacrificed at one day-post of the end of the experiment.

2.2.4. Liver Index

At the end of the experimental period, each mouse was weighed and their livers were then removed and weighed.

Finally, the liver index was calculated by dividing the liver by the body weight and then multiplying by 100. The means and SEM were calculated by SPSS 16.0 and were represented by graphical analysis using Microsoft excel.

2.2.5. Biochemical Analysis

Blood samples for analysis were drawn into centrifuge tubes and centrifuged after half hour. Blood samples were centrifuged at 3000r/m for separation of serums and stored at −8 °C until assay. Serums were used for the estimation of Alanine aminotransferase (ALT), Aspartate Aminotransferase (AST) and alkaline phosphatase (ALP).

2.2.6. Histopathological Analysis

2.2.6.1. Histopathological Preparation. Livers were collected and cut into small pieces, fixed in 10% neutral buffered formalin. Following fixation, specimens were dehydrated, embedded in wax, and then sectioned into 5 μm thickness. Sections were stained with hematoxylin and eosin, Masson's Trichrome.

2.2.7. Histopathological Score of Liver

Liver sections stained with HE (hematoxylin) and M. Tr (Masson's Trichrome). were examined for pathological score of the following criteria: ballooning, fatty degeneration, inflammation, hyaline degeneration and fibrosis. Scoring values registered according to Table I.[27]

2.2.8. Immuno Histochemistry

Paraffin embedded liver sections were deparaffinized in xylene and rehydrated in descending grades of alcohol and finally distilled water. Sections then were heated in citrate buffer (pH 6) within microwave for 5 min. After that sections were washed with PBS buffer for 5 min and incubated in peroxidase blocking solution for 10 min. Sections were incubated overnight at 4 °C in diluted primary antibody (anti-caspase3ab13585, anti-malondialdehyde ab194225), then incubated in biotinylated goat anti-mouse (ab128976) as secondary antibody for 30 min, followed by incubation in avidin-biotin complex for 30 min, then incubated in DAB (ab64238) as chromogenic substrate for ten min. The stained sections were counter stained with Mayer's haematoxylin, and dehydrated within ascending grades of alcohol and cleared with two changes of xylene, mounted with cover slip based on DPX mountant, (all reagents from Abcam company). Liver sections were examined under microscope for brown immunoreactivity color and photos at mag. ×400. Reaction for gene-expressions scored as, −ve = no reaction, + = sight, ++ = moderate and +++ = intense.

Table I. Scoring criteria for the assessment of histopathological changes in liver architecture.

Changes in liver architecture Ballooning degeneration–fatty degeneration-necrosis	
Score 0	No cell damage
Score 1+	Mini hepatocytes damage (less than 25% of the tissue)
Score 2+	Moderate hepatocytes damage (25–50% of the tissue)
Score 3+	Extensive hepatocytes damage (>50% of the tissue)
	Inflammation
Score 0	No inflammatory foci
Score 1+	1 inflammatory foci per 100 hpf
Score 2+	2–4 inflammatory foci per 100 hpf
Score 3+	>4 inflammatory foci per 100 hpf
	Edema
Score 0	No edema
Score 1+	Presence of edema
	Fibrosis
Score 0	No fibrosis
Score 1+	Portal/sinusoidal minimal fibrosis
Score 2+	Portal/sinusoidal mild fibrosis
Score 3+	Portal/sinusoidal moderate fibrosis
Score 4+	Portal/sinusoidal extensive fibrosis

2.2.9. Statistical Analysis

The data were expressed as mean ± SEM (standard error of mean). Statistical significance of the control and experimental groups was evaluated by SPSS16.0. Comparison was made between control and experimental groups in liver, ALT, AST and ALP. $p < 0.05$ was considered to be significant. Correlation analysis was done by graphical representation analysis using Microsoft excel.

3. RESULTS AND DISCUSSION

An obvious obstacle was the complete lack of solubility of C_{60} fullerene in water, and one successful attempt have been performed, it increases the water-solubility of fullerenes, which functionalizes of their cages with carefully selected hydrophilic groups e.g., hydroxyl groups. The high electron affinity of C_{60} fullerenes, investigated by cyclic voltammetry,[17] showed that C_{60} is more susceptible to nucleophilic additions[18] than electrophilic additions, and these additions produce fullerene derivatives which have attracted a great deal of interest due to their outstanding physical and chemical properties.

In our preparation method, new and green approach for the hydroxylation of C_{60} was followed. In this approach, the hydroxylation performed without solvent and under the so-called High-Speed Vibration Mill,[7–9] HSVM, and at room temperature with a yield of approximately 83%. The obtained solid brown fullerenol is highly soluble in water.

The FT-IR spectra, shown in Figure 1, confirmed the attachment of hydroxyl groups (at 3300 cm^{-1}) to the carbon cage of C_{60}. Also, the spectra showed three characteristic bands at 1280, 1420, and 1490 cm^{-1} assigned for υC–O, δ_sC–O–H and υC=C absorption. These four broad bands are invariably reported as diagnostic absorptions of various $C_{60}(OH)_x$.[19–21]

In most previous studies, the average number of hydroxyl groups attached to the fullerene cage

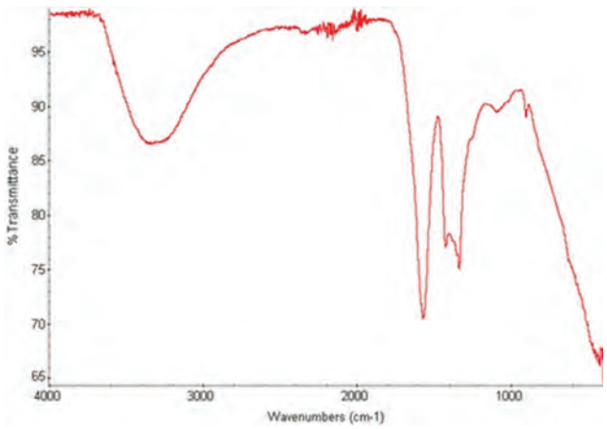

Fig. 1. The spectra shows the FTIR results of $C_{60}(OH)_x$.

was estimated using only the elemental analysis technique.[7, 19–21] However, especially for highly hydroxylated fullerenes, a substantial amount of secondary water bound to the multiple hydroxyl groups on a fullerene surface may exist.[21] Therefore, we monitored the weight loss of the prepared hydroxylated fullerenes using the thermal gravimetric analysis technique, TGA. The data obtained from TGA, Figure 2, showed that the weight loss for the hydroxylated fullerenes was observed in three temperature ranges: from room temperature to 110 °C which has been assigned to the secondary bound water from 110–300 °C which corresponds to dehydration of the attached hydroxyl, and >300 °C which has been attributed to the decomposition of the fullerene nucleus.[22] The approximate number of hydroxyl groups attached to C_{60} cage, according to the data obtained from the TGA, was 28 OH groups.

To confirm the number of hydroxyl groups attached to the fullerene cage, mass spectroscopic studies of the prepared $C_{60}(OH)_x$ were performed using mass spectroscopy whose spectra, Figure 3, showed several peaks; each one

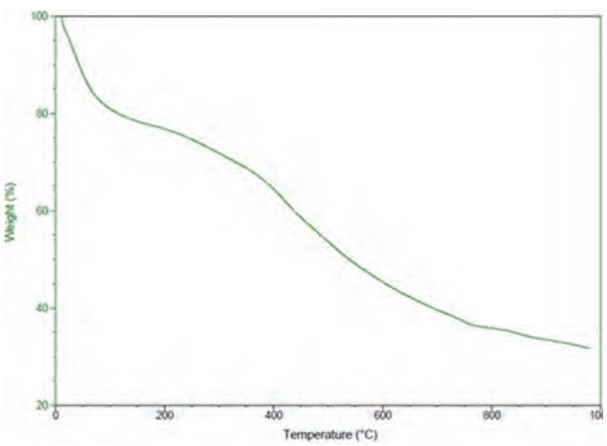

Fig. 2. Thermal gravimetric analysis (TGA) of $C_{60}(OH)_x$.

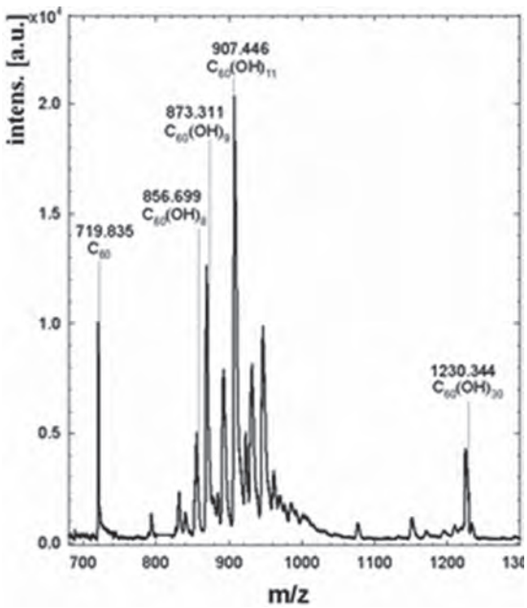

Fig. 3. Mass spectra of $C_{60}(OH)_x$.

corresponds to an adduct. This is because in mass spectrometer, the sample undergoes several decompositions by the action of energy of laser pulse applied. The peak at m/z 720 corresponds to C_{60} fullerene, and the peak at m/z 1230 corresponds to the water-soluble adducts $C_{60}(OH)_{30}$, according to the following calculation: $(1230-720)/17$ (molar mass of OH) = 30 hydroxyl groups.

From the data obtained from TGA technique combined with those obtained from MS, the hydroxylation had been successfully performed with a 29 hydroxyl groups around the C_{60} fullerene surface.

3.1. Liver Index and Biochemical Analysis

Liver index of mice group received oral administration of 100, 500 mg/kg of fullerenol respectively showed insignificant decrease comparing with control group (Table II).

Biochemical analysis of blood sera collected from the three experimental groups showed significant increase

Table II. Showed liver index and enzymes ALT, AST and ALP score in control, fullerene 100 mg/kg and 500 mg/kg groups.

Items	Control	Fullerene 100 mg/kg	Fullerene 500 mg/kg
Liver index	7.25 ± 1	6.2 ± 0.9	6.1 ± 0.8
ALT	46 ± 0.5	$54 \pm 1.7^*$	$34 \pm 1.5^*$
AST	176 ± 0.5	$268 \pm 24^*$	$143 \pm 52^*$
ALP	65 ± 0.6	69 ± 5.5	$75 \pm 0^*$

Notes: Data are mean ± SEM, *significant difference. Liver index: showed insignificant difference among groups. ALT: showed significant increase $p < 0.05$ in group received 100 mg/kg of fullerene versus control and significant decrease in group received 500 mg/kg versus control. AST: showed significant increase $p < 0.05$ in group received 100 mg/kg of fullerene versus control and significant decrease in group received 500 mg/kg versus control. ALP: showed insignificant increase in group received 100 mg/kg of fullerene versus control and significant increase $p < 0.05$ in group received 500 mg/kg versus control.

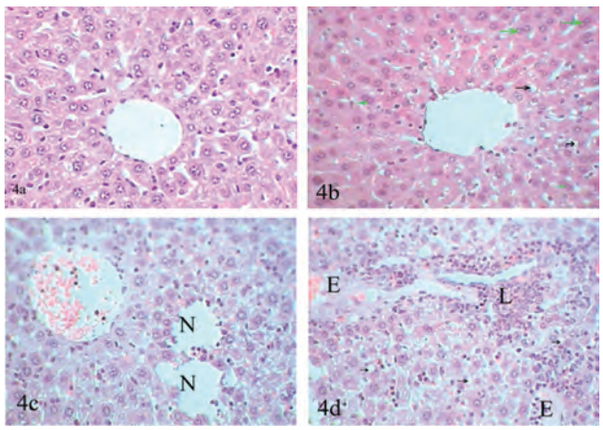

Fig. 4. (a) control liver showed normal liver, (b) liver of mice received 100 mg/kg showed relative healthy liver with binucleated cells (green arrows) and mice microvesicular fatty degeneration (black arrows), (c) liver of mice received 500 mg/kg showed necrotic foci (N), degenerated cells, (d) showed edema (E), lymphocytic aggragations (L), micro vesicular fatty degeneration (arrows). (Hx and E-mag.×400).

$p < 0.05$ in ALT and AST of mice group received 100 mg/kg of fullerene comparing with control group due to hepatocytes injury, whereas, mice group received 500 mg/kg of fullerenol showed significant decrease in ALT and AST levels comparing with control group may be due to high dose of fullerenol caused decreased in ALT and AST as high doses of many drugs. Moreover, ALP levels showed insignificant increase in mice group received 100 mg/kg of fullerene and significant increase $p < 0.05$ in mice group received 500 mg/kg of fullerene comparing with control group due to destruction of bile ducts (Table II). The present findings revealed that moderate and high doses caused significant changes in liver enzymes, however the low doses of fullerene caused no change in liver enzymes.[23]

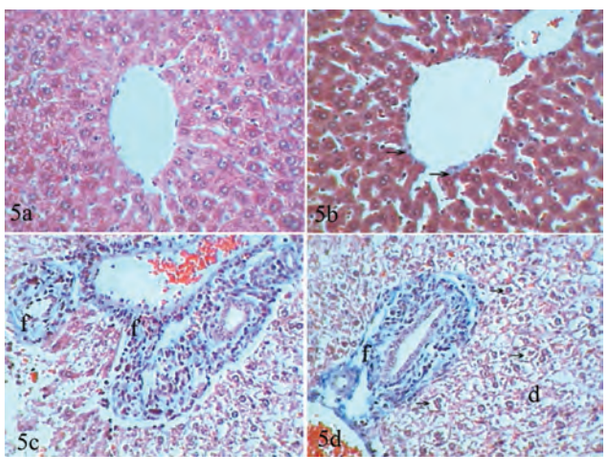

Fig. 5. (a) control liver showed normal liver without collagenous fibers depo sitions, (b) liver of mice received 100 mg/kg showed small layer of collagenous fibers (black arrows) deposited around portal vein, (c) liver of mice received 500 mg/kg showed concentric layers of collagenous fibers (f), severe inflammation, (d) showed collagenous fibers (f), ballooning cells (arrows), degeneration (d). (M.Tr.-mag.×400).

Table III. Assessment of liver pathological score in control, fullerene 100 mg/kg and 500 mg/kg groups.

Items	Control	Fullerenol 100 mg/kg	Fullerenol 500 mg/kg
Ballooning	0±0	1±0.09*	3±0.08*
Fatty degeneration	0±0	1±0.1*	2±0.1*
Necrosis	0±0	1±0.08*	3±0.1*
Inflammation	0±0	1±0.09*	2±0.1*
Fibrosis	0±0	2±0*	4±0*

Note: Data are mean±SEM, *significant difference.

3.2. Histopathological Analysis and Pathological Liver Score

Liver of control mice group showed normal liver with central vein and a network of healthy hepatocytes with abundant nuclei, some hepatocytes showed two nuclei due regeneration of cells, strands of hepatocytes were separated from each other by blood sinusoids with abundant kupffer cells that scored (0) no cell damage in pathological score Figure 4(a), without collagenous depositions Figure 5(a). Whereas mice liver received 100 mg/kg of fullerenol showed relative normal liver section with increasing number in binucleated cells comparing with control group. They also showed a few number of inflammatory cells were scattered in the tissue, that scored mini ballooning, fatty degeneration (microvesicular), necrosis in tissue and mild portal fibrosis Figures 4(b) and 5(b) (Table III). Gharbi et al.[24] revealed that after oral administration of fullerenol for 2–4 days, it accumulated in liver and spleen without no acute toxicity. Moreover, Livers

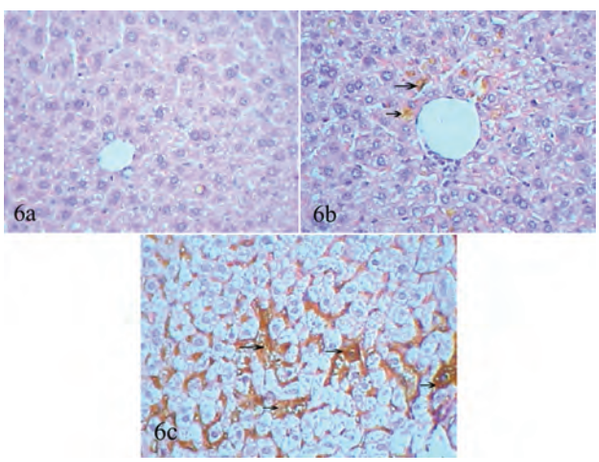

Fig. 6. (a) control liver showed no reaction for caspase 3 gene-expression, (b) liver of mice received 100 mg/kg showed slight brownish immunoprecipitate of caspase 3 gene-expression reaction, (c) liver of mice received 500 mg/kg showed intense brownish immune precipitate of caspase 3 gene-expression reaction (ABC method mag.×400).

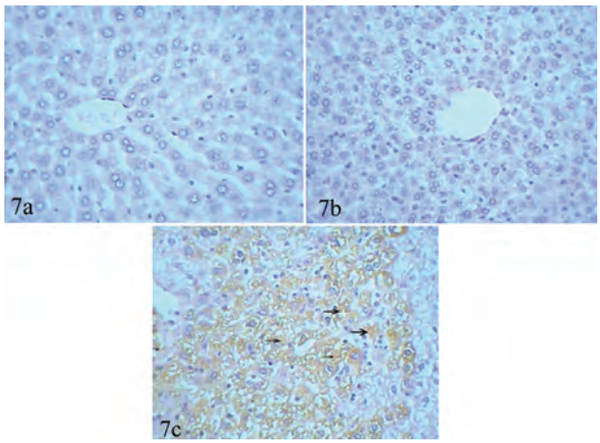

Fig. 7. (a) control liver showed no reaction for MDA gene-expression, (b) liver of mice received 100 mg/kg showed no reaction for MDA gene-expression, (c) liver of mice received 500 mg/kg showed intense brownish immunoprecipitate of MDA gene-expression reaction (ABC method mag.×400).

from mice that received 500 mg/kg of the fullerenol derivative showed histopathological changes in the liver manifested by congested portal vein, extensive ballooning and necrosis, moderate fatty degeneration, presence of edema Figures 4(c) and 5(d), severe inflammation with giant macrophages and extensive portal fibrosis Figures 5(c and d) (Table III). These findings run in full agreement with Gharbi[24] and Folkmann et al.[25] proved that C_{60} and its derivatives induced hypertrophy and hyperplasia of hepatic stellate cells that play a role in the production of extracellular matrix both in normal and fibrotic liver, then these cells transformed into fibroblast–like cells that leads to fibrosis.

3.3. Gene-Expression Localization

Control liver showed no immunoreactions for caspase3 and MDA gene-expressions (Figs. 6(a) and 7(a)). Whereas, mice received 100 mg/kg of fullerenol revealed slight brownish immunopreceipitate for caspase 3 gene-expression (Fig. 6(b)) and no reaction for MDA an evidence that moderate dose of fullerene induce apopotsis but not oxidative stress, Figure 7(b). These results agreed with Isakovic et al.[26] that fullerene considered less toxic compound but caused apoptosis at high concentrations Moreover mice received 500 mg/kg of fullerenol showed intense immune precipitate for caspase 3 (Fig. 6(c)) and MDA gene-expression, Figure 7(c), an evidence that high dose of fullerene caused apoptosis and oxidative stress in liver (Table IV). These results agreed with Folkmann et al.[25] that oral exposure to fullerenol elevated oxidative stress in liver

Table IV. Assessment of gene-expressions in control, fullerene 100, 500 mg/kg groups.

Gene-expression	Control	Fullerene 100 mg/kg	Fullerene 500 mg/kg
Caspase 3	−ve	+ve	+++ve
Maliondialdehyde (MDA)	−ve	−ve	+++ve

4. CONCLUSION

It has been considered for a long time that "the dose makes poison," thus moderate doses of fullerene C_{60} derivative considered safe doses without oxidative stress and increase regeneration capability of hepatocytes. Whereas, high doses of hydroxylated C_{60} fullerene considered a toxic material that induced hepatotoxicity, oxidative stress and apoptosis. For the promising medicinal applications of fullerene, high doses of hydroxylated fullerene should be avoided due to its toxic effects, whereas, moderate doses sufficient data should be accumulated, the suggested dose used for human should be less than 50 mg/kg .

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