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## Amelioration of the therapeutic efficacy of 5-Fluorouracil loaded chitosan nanoparticles in experimentally induced Hepatocellular Carcinoma

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### Abstract

Hepatocellular carcinoma (HCC) is the most common type of liver cancer. The most important risk factor for the development of HCC is cirrhosis regardless of etiology. 5-Fluorouracil (5-FU) is widely used in the treatment of cancer. Drug resistance remains a significant limitation to the clinical use of 5-FU. The present study aimed to evaluate the therapeutic efficacy of 5-FU loaded chitosan nanoparticles in experimentally induced HCC. To achieve our purpose, one hundred and five male Swiss Albino mice were divided randomly into two major groups: Group A: comprised 25 mice served as normal control, Group B: comprised 80 mice received a daily oral dose of 0.06% DAB (165 mg/kg body.wt.) for 30 days after which the water was replaced with 0.05% aqueous solution of Phenobarbital (PB). Five chosen mice randomly from groups A and B at the time intervals 15, 30, 45 and 60 days were sacrificed to follow up with the development of HCC by biochemical and histopathological examination. Animals of group B were divided into 3 groups Group I: included 20 mice served as an untreated group, group II: included 20 mice injected intraperitoneally with 5-FU only (40mg/kg body.wt) every 2 days for 16 days, group III: included 20 mice injected intraperitoneally with 5-FU Cs NPs. Each group was further divided into two subgroups 10 mice each, one subgroup treated with ultrasonic waves; meanwhile the other subgroup without ultrasonic waves exposure. At the end of the experiment, animals were sacrificed, serum ALT, hepatic ALT, and hepatic MDA were estimated; HCC was histopathologically monitored in all studied groups. There was 276.5%, 145.7% and 438.5% increase in serum ALT, hepatic ALT and hepatic MDA levels respectively comparing to the corresponding control. Liver tumors that ultimately became neoplastic were produced after 45 days. US exposure triggered a significant decline in serum and hepatic ALT activity ( $P = 0.001$ ) and in hepatic MDA ( $P = 0.009$ ) within 5-FU loaded Cs NPs group. Moreover, tumor growth delay and more enhanced correction in hepatic architecture was obtained by a combination of US and 5-FU loaded Cs NPs therapy. Based on these results, we can conclude that the use of 5-FU loaded chitosan nanoparticles in combination with low-intensity ultrasound ameliorates the efficacy of 5-FU as anticancer therapy for HCC.

**Keywords:** Hepatocellular carcinoma, chitosan nanoparticles, 5-FU, Oxidative stress.

## 1 Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide (El-Serag, 2002) and the third most common cause of cancer death. Despite advances in prevention techniques, screening, and new technologies in diagnosis and treatment, incidence and mortality continue to rise. The most important risk factor for the development of HCC is cirrhosis regardless of etiology. Hepatitis B and C are independent risk factors for the development of cirrhosis. (Balogh et al., 2016). Liver Transplantation offers the benefit of removal of cancer as well as the harboring risk of de novo HCC in a cirrhotic liver (Kulik et al., 2018). The disadvantages of most anti-cancer drugs that are currently available include low bioavailability, poor selectivity because they can act on both tumor cells and healthy cells, and immunosuppression that can cause complications and even patient death (Shah et al., 1985).

Continuous oxidative stress, which results from the generation of reactive oxygen species (ROS) by environmental factors or cellular mitochondrial dysfunction, has recently been associated with hepatocarcinogenesis. In liver disease, the infiltration of activated phagocytic cells provides an additional source of ROS production that promotes oxidative stress and damage to proteins, lipids, and DNA (Kaplowitz, 2000). ROS react with polyunsaturated fatty acids to induce the release of toxic and reactive aldehyde metabolites such as malondialdehyde (MDA) (Cheeseman, 1993, Saad et al., 2014). Malondialdehyde (MDA) is a marker for oxidative stress that is formed by lipid peroxidation and that was shown to accumulate in serum of chronic hepatitis patients (Nagev et al., 2002).

5-Fluorouracil (5-FU) is widely used in the treatment of cancer. Over the past 20 years, an increased understanding of the mechanism of action of 5-FU has led to the development of strategies that increase its anticancer activity. Despite these advances, drug resistance remains a significant limitation to the clinical use of 5-FU. Modulation strategies, such as co-treatment with leucovorin and methotrexate, have been developed to increase the anticancer activity of 5-FU. (Longley et al., 2003).

Chitosan, a natural cationic polysaccharide, is prepared industrially by the hydrolysis of the aminoacetyl groups of chitin, a naturally available marine polymer. Chitosan is a non-toxic, biocompatible and biodegradable polymer and has attracted considerable interest in a wide range of biomedical and pharmaceutical applications including drug delivery, cosmetics, and tissue engineering (Giri et al., 2012). Chitosan nanoparticles are a drug carrier with wide development potential and have the advantage of slow/controlled drug release, which improves drug solubility and stability, enhances efficacy, and reduces toxicity. Because of their small size, they are capable of passing through biological barriers in vivo (such as the blood-brain barrier) and delivering drugs to the lesion site to enhance efficacy (Shi and Fan, 2002).

A new method of drug targeting to a tumor is based on the localized release of drug at the tumor site by ultrasound focusing, the application of the external ultrasound to control drug delivery and to release from nanocarriers is a relatively novel approach. In the application ultrasound triggers the release of the drug from micelles as well as decomposing the membrane, thus enhancing the cellular uptake of both

encapsulated and released drugs (Rapoport et al., 2003).

## Materials and Methods:

### 1. Chemicals:

Chitosan (Cs) was obtained from Across Organics (Newjersey-USA). 5-fluorouracil (5-FU), Sodium Tripolyphosphate (TPP) and Phenobarbital (PB) were purchased from Sigma Aldrich Chemie (GmbH-Germany), 4-dimethylaminoazobenzene (DAB) was purchased from Bio Basic (Canada Inc), Acetic acid was purchased from (ADWIC, Egypt), Alanine Transaminase (ALT) kit from Diamond Diagnostics (Egypt) and MDA estimation kit from Biodiagnostic (Egypt).

### 2. Methods

#### A: Preparation of nanoparticles (in vitro study)

##### I. Preparation of hollow chitosan nanoparticles

Chitosan has the ability to gel spontaneously on contact with multivalent polyanions due to the formation of inter- and intramolecular cross-linkage mediated by these polyanions. Among some polyanions investigated, tripolyphosphate (TPP) is the most commonly used because of its non-toxic property and quick gelling ability (Nagarwal et al., 2011, Aydin and Pulat, 2012)

Procedure:

Chitosan 0.2% (w/v) solution was prepared in 1% (w/v) acetic acid aqueous solution. Tripolyphosphate was dissolved in deionized water at the concentration 0.07% (w/v), then chitosan solution was flush mixed with an equal volume of TPP solution and the formation of chitosan-TPP nanoparticles started spontaneously via the TPP initiated ionic gelation mechanism. The pH of the final mixture was adjusted at 5.5 with 0.1N NaOH. The nanoparticles were formed at selected chitosan to TPP weight ratio of 2.9:1(w/w). The nanoparticles suspensions were gently stirred for 60 minutes at room temperature

using (Newtec.co magnetic stirrer MG model 2004). The mixture then was centrifuged for 60 minutes at 14,000 rpm by cooling centrifugation at 4°C (Hettich MIKRO 120 Centrifuge) and particles were separated and washed with deionized water then allowed for drying before being subjected to applications. Pellet was suspended in deionized water using ultrasonication (Branson ultrasonic cleaner B-220 50/60HZ ) for 3 min. The colloidal suspension was pre-frozen at - 80 °C for 24h. D-Trehalose 5% was added as a cryoprotectant to the colloidal suspension before the final freeze-drying. Nanoparticles were freeze-dried at -50 °C for 12h by lyophilization, and powder nanoparticles were used for further characterization (Nagarwal et al., 2011, Aydin and Pulat, 2012)

##### II. Preparation of 5-fluorouracil loaded chitosan nanoparticles

Ideally, a successful nanoparticulate system should have a high drug-loading capacity thereby reduce the number of matrix materials for administration. Drug loading can be done by two methods: Incorporating at the time of nanoparticle production (incorporation method) or absorbing the drug after the formation of nanoparticles by incubating the carrier with a concentrated drug solution (adsorption /absorption technique). Drug loading and entrapment efficiency very much depend on the solid-state drug solubility in the matrix material or polymer (solid dissolution or dispersion), which is related to the polymer composition, the molecular weight, the drug-polymer interaction and the presence of end- functional groups (ester or carboxyl). For small molecules, studies show the use of ionic interaction between the drug and matrix materials can be a very effective way to increase drug loading (Mohanraj and Chen, 2006).

Procedure:

5-FU loaded nanoparticles were obtained by the above-described procedure and the ratios of Cs and TPP remained unchanged. (0.05 w/v) of 5-FU were incorporated in the Cs solution prior to the formation of nanoparticles in order to investigate the effect of the initial 5-FU concentration on the nanoparticle characteristics and in-vitro drug release. Also, supernatant was subjected to analysis for unloaded drug concentration by UV-Visible spectrophotometry at 265 nm (Mohanraj and Chen, 2006).

### **B. Animals and induction of HCC.**

One hundred and five male Swiss Albino mice, three months old, weighing (25 – 40 g) were used. The animals housed in stainless cages at the Animal House Unit at Medical Technology Center-University of Alexandria under the following conditions; 12hrs dark/light cycle 22±2°C and 50±10% humidity. All animals received human care in compliance with the European Convention on Animal Care. Animals were handled according to the rules and regulations of the Institutional Animal Ethics Committee.

The animals were divided randomly into two major groups: Group A: 25 mice fed on a normal balanced diet and water *ad libitum* and served as normal controls. Group B: 80 mice fed the same diet as in the first group plus 0.06% DAB at a daily dose of 165 mg/kg body.wt. per mouse administered orally through a specially made fine pipette. (DAB was dissolved in paraffin oil) till 30 days after which the water was replaced with 0.05% aqueous solution of Phenobarbital till they were sacrificed (Bhattacharjee et al., 2009a, Biswas et al., 2005).

Five chosen mice randomly from groups A and B at the time intervals 15, 30, 45 and 60 days were sacrificed to follow up the development of liver tumor HCC by histopathological examination.

After confirming the developing of the liver tumor by histopathological examination, animals of group B were left two weeks to induced chronic administrated animals termed chronic group divided into 3 groups as follow: Group I: 20 mice served as an untreated group. Group II: 20 mice injected intraperitoneally with 5-FU only (as free drug). (40mg/kg body weight) (Tominaga et al., 1993) was injected intraperitoneally every 2 days for 16 days (Li et al., 2008) Group III: 20 mice injected intraperitoneally with 5-FU chitosan nanoparticles. On the same dose and schedule as group II. For group, I, intraperitoneal injections of the same volume of PBS were administered on the same schedule. Each group was further divided into two subgroups each of 10 mice, one group treated with ultrasonic waves, whereas the other subgroup received no treatment with ultrasonic waves. The mice were sacrificed on day 21<sup>th</sup>.

### **C. Blood and tissue sampling:**

Blood samples were collected via ocular puncture from etherized mice, allowed for clotting at room temperature for about 30 minutes then serum was separated under centrifugation at 4000 rpm at 4°C and assayed at the same day.

Animals were sacrificed, livers were quickly removed, washed with saline, bottled then each liver sample was weighed and divided into two parts. one part was kept in 10% formalin and processed for paraffin sections for histopathological studies. The second part was homogenized with cold 50mM phosphate buffer in (1:5) (w/v) at pH 7.4, followed by centrifugation at 4000 rpm for 15 minutes in a cooling centrifuge, the supernatant separated for MDA level and ALT activity on the same day.

### **D. Biochemical investigations**

### 1. Serum Alanine aminotransferase activity (ALT)

The enzymatic activity of ALT in serum was estimated colorimetrically according to the method of Reitman and Frankel. Briefly, 0.5 ml of the substrate (2 mM  $\alpha$ -ketoglutarate, 0.2 M L-alanine in 0.1M phosphate buffer pH 7.4) was incubated at 37° C for 5 minutes. 0.1 ml of freshly prepared serum was added to the aliquot then incubated at 37 oC for 30 minutes. At the end of incubation, 0.5 ml of 2, 4-dinitrophenyl hydrazine was added, and the aliquot left for 30 minutes at room temperature. 0.5 ml of 0.4 N NaOH was added, and the aliquot was again left for 30 minutes. Absorbance was then recorded at 505 nm against water blank (Reitman and Frankel, 1957).

### 2. Hepatic Alanine aminotransferase activity (ALT)

The enzymatic activity of ALT in tissue homogenate was estimated colorimetrically according to the method of Reitman and Frankel. (Reitman and Frankel, 1957).

### 3. Hepatic Malondialdehyde level (MDA)

The hepatic MDA was estimated according to the method of Ottolenghi. The formation of malonaldehyde is the basis for the well-known thiobarbituric acid (TBA) method used for evaluating the extent of lipid peroxidation. At low pH and high temperature (100°C), MDA binds TBA to form a red complex that can be measured at 534 nm (Ottolenghi, 1959).

### E. Histopathological examination of liver

Part of the liver was kept in 10% formalin and processed for paraffin sections. Paraffin sections were mounted on clean slides, placed at 37°C oven. Sections were deparaffinized in xylene, rehydrated in descending grades of alcohol to distilled water, stained with Hematoxylin, washed in tap water then stained with Eosin, dehydrated in ascending grades

of alcohol and cleared in xylene. Coverslip applied by Canada balsam and examined under a light microscope(Drury and Wallington, 1980).

### F. Statistical analysis

The statistical analysis was computed using the Mann - Whitney test. P values  $\leq 0.05$  were considered statistically significant.

### 3. Results

#### A. Biochemical results during HCC induction

##### 1. Serum ALT activity

Feeding of DAB and PB caused a significant increase in serum ALT activities through the different intervals when compared to their corresponding control animals, Figure (1).

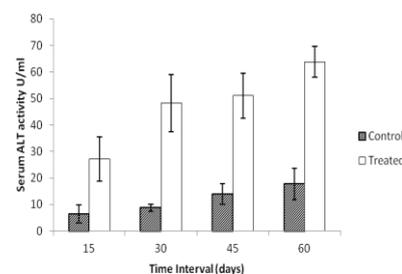


Figure (1): Serum ALT activity (U/ml)( Mean±S.D)

##### 2. Hepatic ALT activity:

The same trend as serum ALT activity, hepatic ALT levels were noticeably increased under the effect of DAB and PB feeding when compared to their corresponding control animals at all intervals Figure (2).

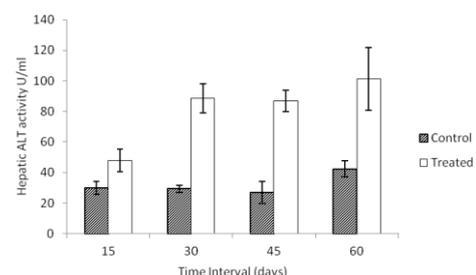


Figure (2): Hepatic ALT activity (U/ml) (Mean±S.D)

**3. Hepatic MDA level:**

Hepatic MDA level was significantly increased in DAB and PB fed mice at all intervals when compared with the corresponding control animals, Figure (3).

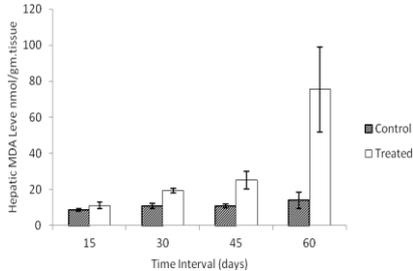


Figure (3): Hepatic MDA level (nmol/g. tissue) ( Mean±S.D)

**B. Biochemical results after the induction of HCC**

**1. Serum ALT activity**

Mice treated with free 5-FU (group II) showed no significant difference when compared to mice bearing the liver nodules in group I ( $p_1 = 0.217$ ) while treating mice with 5-FU loaded Cs NPs caused a highly significant decrease in ALT level when compared to group I ( $p_1 = 0.004^*$ ). A significant decrease in ALT level was obtained when comparing mice received 5-FU loaded Cs NPs (group III) with those received free 5-FU (group II) ( $p_1 = 0.030^*$ ). On the other hand, exposing mice to the US caused a significant decrease in ALT level when comparing both groups II and III to a group I being more significantly decreased in group III ( $p_2 = 0.014^*$ ,  $0.001^*$  and  $< 0.001^*$ ) respectively. Within the same group, the US triggered a significant decline in ALT level only in group III ( $p = 0.001$ ), Table (1)

Table (1): Serum ALT level (U/ml) in different treated groups.

	DAB + PB feeding group <b>Group I</b>		Treated Groups			
			<b>Group II</b>		<b>Group III</b>	
	Without US	With US	Without US	With US	Without US	With US
n	10	10	10	10	10	10
Range	53.0 – 75.0	48.0 – 70.0	36.0 – 72.0	38.0 – 52.0	30.0 – 60.0	13.80 – 39.10
Mean ±SD	63.80 ± 8.70	57.20 ± 8.44	54.40 ± 12.76	44.38 ± 5.85	41.80 ± 10.32	26.58 ± 9.48
Median	61.00	56.00	55.00	42.0	40.00	22.60
P		0.172		0.095		0.001*
P1			0.217			
P2				0.014*		
P1					0.004*	
P2						0.001*
P1					0.030*	
P2						< 0.001*

P : P value for Mann Whitney test between with and without ultrasound in each group  
 P1: P value for Mann Whitney test between treated and each other treated group without US  
 P2: P value for Mann Whitney test between treated and each other treated group with US  
 \*: statistically significant at  $P \leq 0.05$

**2. Hepatic ALT activity**

Mice treated with free 5-FU (group II) showed a significant decrease when compared to mice bearing the liver nodules in group I ( $p_1 = 0.035^*$ ), while treating mice with 5-FU loaded Cs NPs caused a highly significant decrease in ALT level when compared to group I ( $p_1 = 0.001^*$ ). Besides, a significant decrease in ALT level was obtained when comparing mice received 5-FU loaded Cs NPs (group III) with those received free 5-FU (group II)

( $p_1 < 0.001^*$ ). On the other hand, exposing mice to the US caused no significant decrease in ALT level when comparing group II to group I ( $p_2=0.459$ ), while US exposure significantly decreased ALT level of group III when comparing to a group I and II ( $p_2 = 0.001^*$ ,  $p_2 = <0.001^*$ ) respectively. Within the same group, the US triggered a significant decline in ALT level in group II &III ( $p = .048^*$ ,  $p = 0.001^*$ ) respectively, Table (2).

Table (2): Hepatic ALT level (U/ml) in different treated groups.

	DAB + PB feeding group Group I		Treated Groups			
	Without US	With US	5-FU Group II		5-FU loaded Cs Nanoparticles Group III	
	Without US	With US	Without US	With US	Without US	With US
n	10	10	10	10	10	10
Range	84.00-111.00	69.00-102.00	76.00-93.00	72.00-90.00	35.80-71.10	35.00-52.00
Mean ±SD	100.20 ± 10.08	86.40± 14.67	85.12 ± 5.72	79.80 ± 6.55	56.80 ± 13.24	40.40 ± 6.95
Median	102.00	86.00	85.00	80.00	57.20	36.00
P		0.142		0.048*		0.001*
P1			0.035*			
P2				0.459		
P1					0.001*	
P2						0.001*
P1					< 0.001*	
P2						< 0.001*

P : P value for Mann Whitney test between with and without ultrasound in each group

P1: P value for Mann Whitney test between treated and each other treated group without US

P2 : P value for Mann Whitney test between treated and each other treated group with US

\*: statistically significant at  $P \leq 0.05$

**3.1.3. Hepatic MDA level**

Mice treated with free 5-FU (group II) showed no significant difference when compared to mice bearing the liver nodules in group I ( $p_1 = 0.064$ ), while treating mice with 5-FU loaded Cs NPs caused a significant decrease in MDA level when compared to group I ( $p_1 = 0.002^*$ ). A significant decrease in MDA level was obtained when comparing mice received 5-FU loaded Cs NPs (group III) with those received free 5-FU (group II) ( $p_1 < 0.001^*$ ).

On the other hand, exposing mice to the US caused a significant decrease in MDA level when comparing both groups II and III to a group I being more significantly decreased in group III ( $p_2 = 0.004^*$ ,  $0.002^*$  and  $< 0.001^*$ ) respectively. Within the same group, the US triggered a significant decline in the MDA level in groups II & III ( $p = 0.010^*$ ,  $0.009^*$ ) respectively, Table (3).

Table (3): Hepatic MDA level (nmol/g.tissue) in different treated groups.

	DAB + PB feeding group Group I		Treated Groups			
			5-FU Group II		5-FU loaded Cs Nanoparticles Group III	
	Without US	With US	Without US	With US	Without US	With US
n	10	10	10	10	10	10
Range	84.00-105.00	82.00-109.00	70.00-99.00	44.00-79.00	10.19-41.20	9.60-33.50
Mean ±SD	95.50 ± 9.33	95.75 ± 11.50	83.22 ± 12.40	60.40 ± 15.18	28.75 ± 9.68	19.42 ± 8.96
Median	98.50	96.00	84.00	62.00	29.69	15.60
P		0.773		0.010*		0.009*
P1			0.064*			
P2				0.004*		
P1					0.002*	
P2						0.002*
P1					< 0.001*	
P2						< 0.001*

P : P value for Mann Whitney test between with and without ultrasound in each group

P1: P value for Mann Whitney test between treated and each other treated group without US

P2 :P value for Mann Whitney test between treated and each other treated group with US

\*: statistically significant at P≤0.05

**C. Histopathological results during HCC induction**

Paraffin sections of mice liver in all animal groups were stained by H&E to study the histopathological changes under light microscopy.

**Control group:-**

Normal control (group A) liver sections architecture characterized as liver tissue with the central vein, normal hepatocytes distributed among a few numbers of eosinophilic cells and mild dilation of the portal tract (Fig.4).

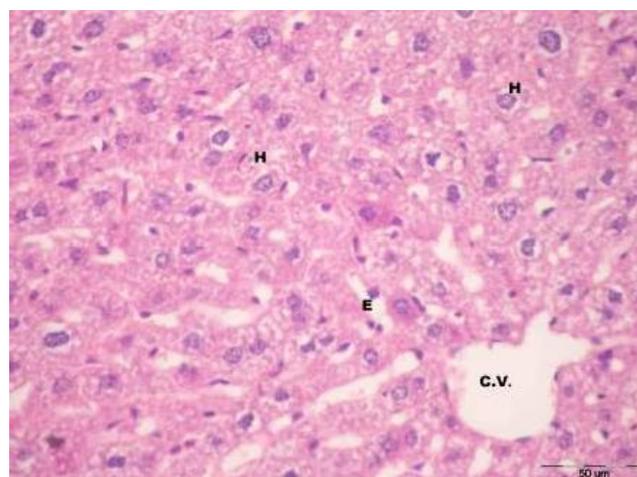


Figure (4): Paraffin section photograph of control three months old mice liver showing normal central vein (C.V.), normal hepatocytes (H) distributed among small number of eosinophilic

Liver section of the experimental control group (DAB and PB feeding group) (group B): At a different intervals of feeding DAB and PB, the liver showed mild histological changes on day 15. After 30 days, the sections had large

hepatocytes with eosinophilic cytoplasm, congested central vein and there was an increase in a number of binucleated cytoplasm (Fig.5A), follicular nodules of infiltrated lymphocytic cells with an increasing number of kupffer cells in dilated sinusoids ( Fig. 5B).

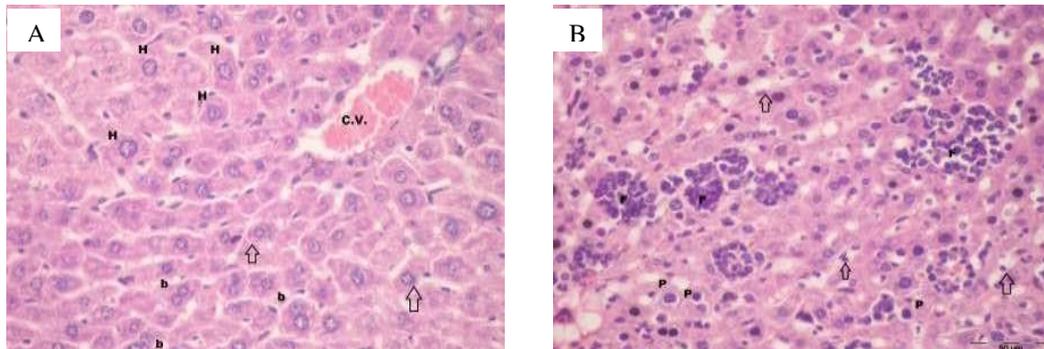


Figure (5): Paraffin section photograph of mice liver at 30 day of DAB and PB feeding. (A): showing congested central vein (C.V.), large hepatocytes (H) with eosinophilic cytoplasm, increase number of binucleated hepatocytes (b), visculated nuclei of hepatocytes with basophilic cytoplasm was noticed(↑). (B): showing follicular nodules of infiltrated lymphocytic cells (F), Small Hepatocytes with pyknotic nuclei are found (P), increasing number of kupffer cells in dilated sinusoids (↑).

The histopathological changes of liver tissue at 30 days of DAB and PB indicated that the carcinogen-induced tumors in a large areas of liver tissue. At day 45, the sections had angiogenesis formation, aggregation of cells with hyperchromatic nuclei and appearance of

some necrotic cells (Fig.6a). The continuous feeding of DAB and PB through 60 days caused a loss of the architecture of liver lobules with an increasing number of apoptotic cells, pyknotic hepatocytes with vacuolated cytoplasm and areas of karyolytic cells (Fig.6b).

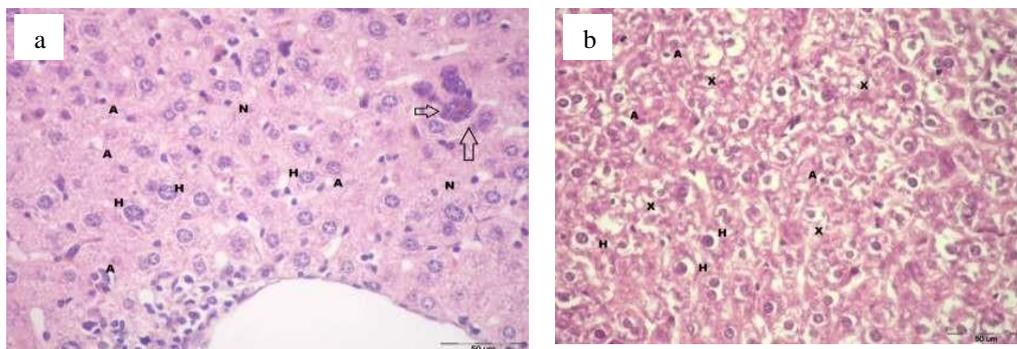


Figure (6): Paraffin section photograph of mice liver.(a): at 45 day of DAB and PB feeding showing area of multinucleated cells(↑), new angiogenesis formed (A), some necrotic cells (N) and hyperchromated hepatocytes (H), increase in kupffer cells number.(b): after 60 days of DAB and PB feeding showing pyknotic hepatocytes with vacuolated cytoplasm (H), increasing number of apoptotic cells (A), and area of karyolytic cells (X).

**D. Histopathological results due to the different therapeutic effects on HCC DAB and PB feeding group (group I)**

The liver sections of the untreated group lost its architecture with the appearance of giant cells with large nuclei and central vein congestion (Fig.4a).

While the sections exposed to the ultrasonic waves (US) showed little follicular nodules of infiltrated lymphocytic cells with angiogenesis and appearance of regenerative hepatocytes, i.e. a mild recovery caused by the effect of ultrasonic exposure (Fig.7.b).

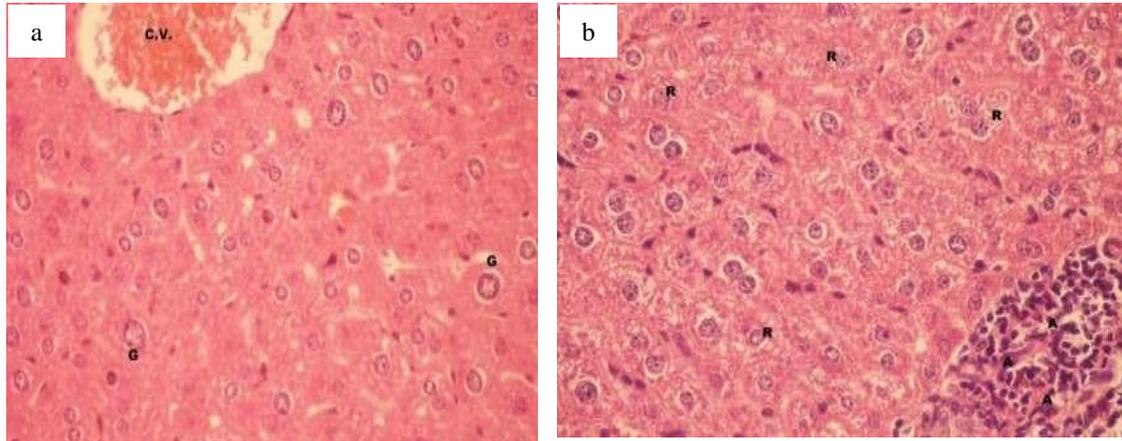


Figure (7). (a): Paraffin section photograph of mice liver of chronic DAB and PB feeding group of mice without US exposure. (b): mice exposed to US (H&E, Bar = 50 μm)

**Free 5-FU treated group (group II):**

After the administration of free 5-FU intraperitoneal injection, the sections had proliferation in the wall of the artery and bile duct, with an increasing number of proliferating lymphocytic cells and a decreased number of apoptotic cells, Figure (8.a). On the other hand,

liver sections of this group exposed to the US showed some recovery in liver tissue characterized by proliferating lymphocytes around the bile duct, dilation and congestion of the portal tract, increased number of kupffer cells and regenerative hepatocytes, Figure (8.b).

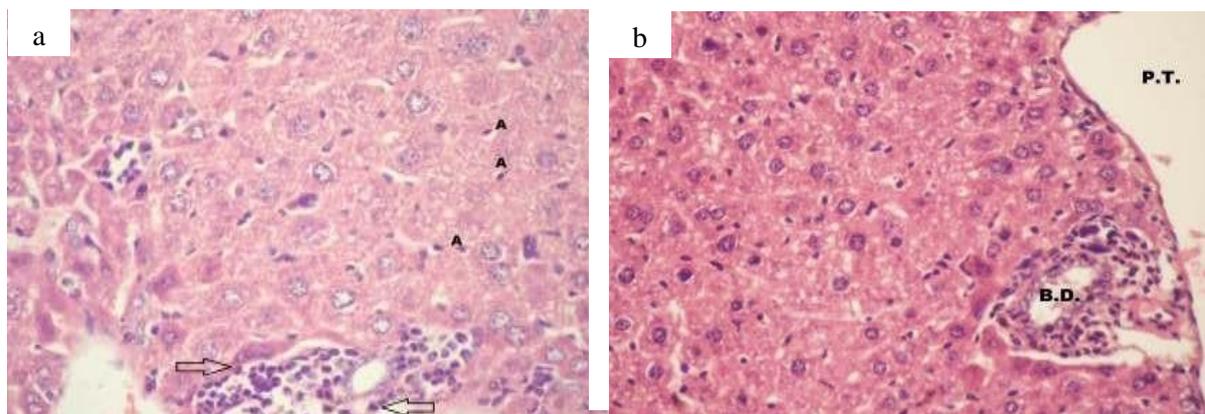
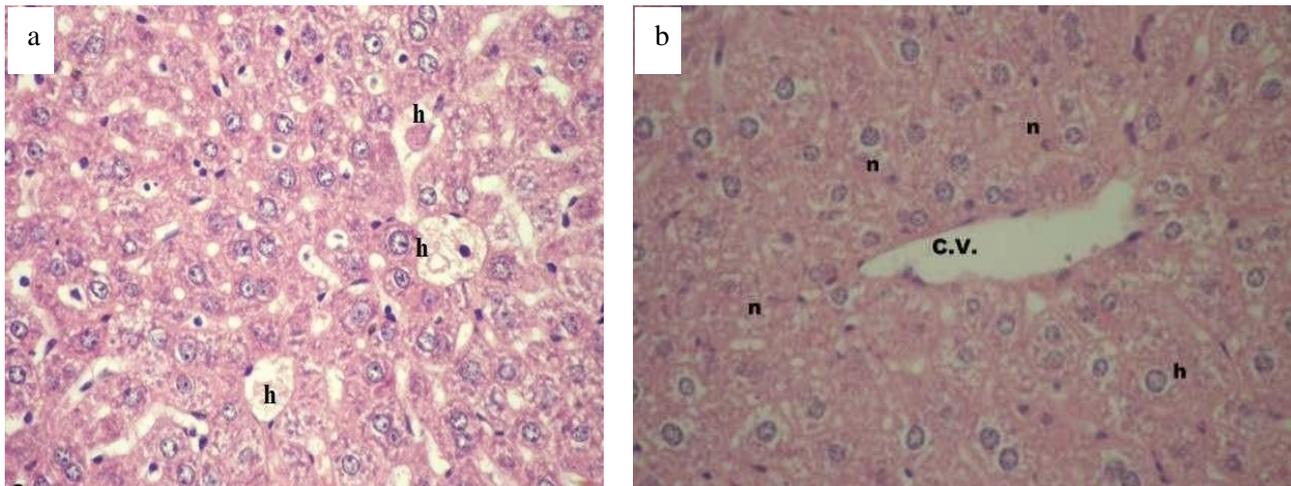


Figure (8): (a).Paraffin section photograph of mice liver of group received 5-FU injection showing increase in number of proliferating lymphocytic cells in sinusoids and histocytes ( ↑ ), decreased number of apoptotic cells (A) and proliferation in the wall of the artery and bile duct.(b). mice liver of group received 5-FU + US exposure showing proliferating lymphocytes around dilated bile duct (B.D.) and congested portal tract (P.T.), increased number of hepatocytes with visculated nuclei and eosinophilic cytoplasm, increase in number of kupffer cells, some regenerative hepatocytes. (H&E, Bar = 50 μm)

### 5-FU loaded Cs nanoparticles treated group (group III) :

The liver sections of this group of mice received 5-FU loaded Cs nanoparticles injection showed partial restoring of the architecture of hepatic lobules with the appearance of hyaline in dilated sinusoids matrix (as a results of the presence of glycosyle debris induced due to the degradation of the

polysaccharide matrix in sinusoids during digestion), Figure (9.a) with increasing number of regenerative hepatocytes and kupffer cells Figure. Also, this group exposed to the US after the 5-FU loaded Cs nanoparticle injections showed newly formed hepatocyte and other hepatocytes with large vesculated nucleus, slightly dilated central vein and few apoptotic cells, Figure (9.b).



Fig(9) : (a). Paraffin section photograph of mice liver of group received 5-FU loaded Cs nanoparticles showing dilation of sinusoids with hyaline matrix (h), the ratio of nucleus to cytoplasm is 1:2, increased number of kupffer cells.(b). mice liver of group received 5-FU loaded Cs nanoparticles and US exposure showing newly formed hepatocytes (n) and others with large vesculated nuclei (h). (H&E, Bar = 50  $\mu$ m)

## 4. Discussion

Carcinogenesis typically involves multiple steps. An initiating step that mutates DNA of the cell, the promotional stage that may involve evasion of apoptosis and uninhibited cell growth in the presence of endogenous or exogenous growth factors. As the neoplasm proceeds, the cells acquire self-sufficiency in growth and overcome inhibitory signals and immune surveillance, followed by angiogenesis and invasion of host tissue (De Minicis et al., 2013, Filler et al., 2007, Hanahan and Weinberg, 2000, Basu, 2018).

Owing to the physiologic and genetic similarities between rodents and humans, the laboratory mouse is

one of the best experimental systems in defining the pathogenesis of HCC due to the availability of gene targeting methods (Xin et al., 2017).

Chronic use of the azo dye DAB is known to act as an initiator of liver cancer. Phenobarbital (PB), is known to have a carcinogenic effect on humans, mice, and rats when administered repeatedly. When PB is used in combination with the azo-dye, their effects are more pronounced which unfailingly produce liver tumors that ultimately become neoplastic (Bhattacharjee et al., 2009a). Therefore this induced liver carcinoma serves as a good model for the study of events during carcinogenesis as well as to evaluate the anti-cancerous effect of a drug

(Bakiri and Wagner, 2013). Based on this knowledge, in the present study, we experimentally induced HCC in male adult mice using 0.06% DAB solution (initiator) at a daily dose of 165 mg/kg body wt. per mouse, administered orally till 30 days after which the water was replaced with 0.05% aqueous solution of phenobarbital (promoter) till they were sacrificed.

The elevation value of serum ALT has been widely used as a sensitive parameter for the assessment of liver injury degree (Kwo et al., 2017). However, its value does not correctly reflect the degree of hepatic cell necrosis. Although injured cells have an increase in its membrane permeability with an elevation in serum ALT, its elevation is not parallel to the degree of damage. Many factors as etiology of the liver disease or the severity of the liver cell necrosis can affect the mechanism of elevation (Woreta and Alqahtani, 2014)

In the present study, the results showed a decrease in body weight in DAB and PB fed group compared to the control one. These results were in agreement with Bhattacharjee et al, who found weight loss in disturbed liver proliferation activity (Bhattacharjee and Khuda-Bukhsh, 2015). DAB and PB feeding significantly increased serum and hepatic ALT activities through the different intervals when compared to their corresponding control animals. At 60 days of DAB and PB feeding, there was 276.5% & 145.7% increase in serum and hepatic ALT activity respectively comparing to the corresponding control. This result was in agreement with Pathak S et al, who manifested that DAB and PB intoxication significantly raised serum AST & ALT levels (Pathak et al., 2009).

Several carcinogens are known to be pro-oxidant that contribute to the induction of oxidative stress and hence carcinogenesis in experimental animal models (Saha et al., 2017). Oxidative stress can affect DNA via oxidation of purine and pyrimidine bases, strand breaks and microsatellite instability. Its effect on protein lead to alteration in its function while on lipids it enhances lipid peroxidative events producing MDA (Forouzandeh et al., 2017).

MDA, the end-product of lipid peroxidation, previously has been reported chronic hepatitis C (CHC) patients have increased serum and hepatic MDA levels (Khan and Ali, 2018). Li et al, reported that the MDA level in CHC patients was statistically significantly higher when compared to the healthy control group (Li et al., 2015). In the present study, the MDA level was significantly increased in the DAB and PB feeding group of mice at all intervals when compared with the corresponding control animals. At 60 days of DAB and PB feeding, there was a 438.5% increase in hepatic MDA level comparing to the corresponding control. These results were in agreement with (Bhattacharjee et al., 2009b), they found pronounced elevation of MDA in the DAB treated group of mice accompanied with lowering levels of antioxidant enzymes (Pathak et al., 2018)

In addition, chronic intake of DAB and PB is known to induce a plethora of cytotoxic and genotoxic alterations (Biswas et al., 2008). It was demonstrated that feeding of carcinogenic azo dyes produced liver damage followed by regeneration of parenchymal cells, a proliferation of bile ducts and connective tissue, and at later stages tumors developed from liver parenchyma that ended up with neoplastic characteristics (Bhattacharjee et al., 2009b). In the present study, at day 15 after DAB and PB feeding,

mice liver showed follicular nodules of infiltrated lymphocytic cells with an increasing number of kupffer cells in dilated sinusoids. Moreover, at 30 and 45 days aggressive histological changes such as angiogenesis formation were observed, aggregation of cells with hyperchromatic nuclei and appearance of some necrotic cells under the cumulative effect of DAB and PB feeding. In addition, pyknotic hepatocytes with vacuolated cytoplasm and areas of karyolytic cells were shown after 60 days. The current result was in agreement with the result of Biswas SJ et al (Biswas and Khuda-Bukhsh, 2002), who demonstrated that dietary PB with the azo dye had positive carcinogenic effect, but neither of these two when fed alone showed positive hepatocarcinogenesis in both mice and rat (Bhattacharjee and Khuda-Bukhsh, 2012). In our study, liver tissue injury began after 15 days and the continuous feeding of DAB and PB caused tumor lesion to appear after 30 days. Therefore, the cumulative feeding of DAB and PB promotes the carcinogenic event, as a toxic effect of DAB was introduced through the 60 days feeding and the addition of PB promoted the cytotoxic effect of DAB and caused weight loss in mice with disturbed liver proliferation activity and liver tumor. The feeding DAB combined with PB produces liver tumors that ultimately became neoplastic after 45 days.

A relatively novel strategy for gene and drug delivery enhancement is the application of nanoparticles in combination with relatively low-intensity ultrasound (US). This method (referred to as "sonoporation") can induce cavitations of or near cellular membranes to enhance the delivery of drugs *in vitro* and *in vivo*. In general, beneficial and reversible cellular effects can be induced by the low-intensity US, in contrast to high US intensities, which are more likely to induce

cellular death. Sonoporation is an emerging and promising physical method for drug and gene delivery enhancement *in vitro* and *in vivo* (Larina et al., 2005, Zolochovska et al., 2011).

Ultrasound can be used in combination with chemotherapy agents for several reasons. It has been shown to enhance the transport of drugs and other chemicals into cells and tissues. The cytotoxic efficiency of chemotherapeutic agents has been shown to increase under the action of ultrasound. Since ultrasound increases the local temperature of the exposed tissues, hyperthermia can be used as an additional ultrasonic advantage (Figueiredo and Esenaliev, 2012)

The mechanism of sonoporation involves the motion and disruption of nanoparticles induced by low-intensity US sonication. US increase the permeability of cell membranes and the endothelium, thus enhancing therapeutic uptake, and can locally increase drug transport. Sonoporation mediates delivery of drugs that have been incorporated into or on the surface of nanoparticles via covalent or electrostatic interactions, which allow these complexes to circulate in the blood and retain their cargo until activation by the US. US application results in localized and spatially controlled particle disruption that enhances drug delivery. Sonoporation does not appear to negatively impact cellular viability of insonated tumor cells or normal surrounding tissues after treatment with either chemotherapeutic drugs or plasmid DNA *in vitro* or *in vivo* (Horsley et al., 2019, Larina et al., 2005)

Specific targeting of tumor cells to achieve higher drug levels in tumor tissue and to overcome the side effects is the major goal in cancer therapy. Therefore, in the present study, we investigated the therapeutic efficacy of the formulated 5-FU Cs NPs in the

treatment of the experimentally induced HCC in mice as compared to the impact of the conventional 5-FU free drug injection under the absence/presence of ultrasonic exposure.

The biochemical results showed mild recovery of the liver tissue in groups treated with the free form of 5-FU with no significant difference in serum ALT activity (0.1% decrease) when compared to mice bearing the liver nodules, slight decrease in hepatic ALT activity (0.16% decrease) ( $P = 0.035^*$ ) and no significant difference in hepatic MDA level when compared to mice bearing the liver nodules. These results were in accordance with Abdel-Hamid et al. who showed that transaminase activities were reduced after 5-FU treatment (Abdel-Hamid et al., 2011). The persistent increase in the MDA level may be due to resistance to the 5-FU treatment as a cellular adaptive response to ROS is a mechanism of drug resistance to 5-FU. While acute oxidative stress triggers cell apoptosis or necrosis, persistent oxidative stress induces genomic instability and has been implicated in tumor progression and drug resistance. It was shown that tumor cells that adapt to oxidative stress by increasing manganese superoxide dismutase (MnSOD), Prx I and Bcl-2, show drug resistance to 5-FU (Hwang et al., 2007).

The improvement in the histological features of the liver after treatment with 5-FU injection was supported by the same result of Abdel Hamid et al, who found that 5-FU corrected the histological changes after carcinogen administration except that some necrotic and cytotoxic effects of 5-FU were still shown (Abdel-Hamid and Morsy, 2010). Animals treated with free 5-FU with ultrasonic exposure showed moderate histological changes, increase in the number of kupffer cells with the appearance of proliferating lymphocytes, these

results described the bioeffect of ultrasound which initiate a resistant immune response toward the conventional 5-FU treatment

The therapy of 5-FU can be improved and its toxicity diminished by facilitating the specific accumulation of this anticancer agent in the tumor under US exposure. In addition, the association of anticancer drugs to delivery systems has been an interesting approach to selectively delivering these active agents and reducing their toxicity. In the present work, animals treated with 5-FU loaded chitosan nanoparticles showed improvement of liver tissues as restoring of the architecture of hepatic lobules with the appearance of a hyaline matrix in dilated sinusoids, increasing number of regenerative hepatocytes and kupffer cells. These findings were confirmed by the biochemical results of ALT activity and MDA level, which demonstrated a significant decrease in serum and hepatic ALT activity in the group that received 5-FU loaded chitosan nanoparticles when compared with the group received free 5-FU injection ( $p = 0.030^*$ ) and ( $p < 0.001^*$ ) respectively. This is in agreement with Cheng et al. who found that the levels of AST and ALT in 5-FU loaded glycosylated chitosan NPs group were lower compared with 5-FU group (Cheng et al., 2012), Also, high significant decrease in hepatic MDA levels was obtained when compared that of mice received 5-FU loaded Cs NPs with those received free 5-FU ( $P < 0.001^*$ ).

These present findings introduced the role of the nanoparticles for carrying drugs, adding a new concept for the drug delivery in carrying the chemotherapy as it is available and easy. This is consistent with previous reports where good antitumor activity of 5-FU- loaded N-succinyl chitosan nanoparticles was observed against Sarcoma

180 solid tumor with mild toxicity (Yan et al., 2006), conjugation of glycosylated chitosan to 5-FU improved the tumor-suppressive effect and the apoptotic effect of 5-FU in hepatic cancer cells, the mechanism of 5-FU loaded chitosan nanoparticles action in tumor inhibition resulted from induction of G0–G1 arrest and apoptosis mediated by the p53 pathway (Abdel-Hamid and Morsy, 2010). These studies confirmed that the colloidal nanoparticles incorporating anticancer agents can improve drug action, distribution, biotransformation and clearance of anticancer agent, increasing the selectivity of drugs toward cancer cells and reducing their toxicity toward normal cell (Serpe, 2006, Villela-Martinez et al., 2017).

In the present study, the exposure to the ultrasonic waves (US) after treatment with 5-FU loaded Cs NPs, showed newly formed hepatocytes and other hepatocytes with large vesiculated nucleus, mild dilated central vein and few apoptotic cells around the central vein. This illustrated the effectiveness of ultrasonic exposure with loaded nanocarriers by increasing vascular permeability to accumulate the nanoparticles in the target organs and improve the injury caused by a toxic substances. Further study, in mice inoculated with a human colon cancer cell line to deliver fluorouracil encapsulated in stabilized micelles, showed a significant reduction in the tumor volume compared to the group that not received the US. The result explained that the US can improve the tumor blood supply and the drug content in tumor tissue increased remarkably (Grumezescu, 2016, Myhr and Moan, 2006)

The role of US was further evaluated by the result obtained from the biochemical analysis of ALT and MDA level, where exposing mice to the US caused a highly significant decrease in serum and hepatic ALT

activity of group received 5-FU loaded Cs NPs when compared with free 5-FU group received US ( $P < 0.001^*$ ). Also, US exposure triggered a significant decline in serum and hepatic ALT activity within 5-FU loaded Cs NPs group of ( $P = 0.001^*$ ). Exposing mice to the US caused a high significant decrease in hepatic MDA level of the group received 5-FU loaded Cs NPs when compared with free 5-FU group received US ( $P < 0.001^*$ ). Also, US exposure triggered a significant decline in the hepatic MDA level within 5-FU loaded Cs NPs group ( $P = 0.009^*$ ).

The herein finding was supported by other researchers who stated that enhanced antitumor effect of liposomal doxorubicin was increased when combined with low-frequency ultrasound, observed significant tumor growth delay indicated that the US can produce bioeffects, raise the tumor hemoperfusion, increase vascular permeability and increase drug content in tumor tissues as well as improve the accumulation of the nanoparticles in the tumor (Golombek et al., 2018, Wei et al., 2013). More enhanced correction in hepatic architecture was obtained after US exposure. The US can increase drug distribution in nucleic acid, as well as decrease the genotoxicity of the azo dye. The present work illustrated that the free form of 5-FU chemotherapy is less valuable than the one loaded on nanoparticles. The role of the ultrasound exposure was much more pronounced in the group received 5-FU loaded chitosan nanoparticles than the group received the free form of 5-FU. The present findings illustrated the achieved role of drug-loaded nanoparticles in enhancing drugs delivery when associated with Ultrasonic exposure.

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