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# Camel *(Camelus Dromedarius)* Milk Antibodies Ameliorated Diethylnitrosamine-Induced Hepatocellular Carcinoma in Wistar Rats

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## Abstract

Hepatocellular carcinoma (HCC) is an extremely aggressive solid tumor and relates to numerous cases of cancer-associated deaths globally. Current studies reveal that natural compounds retain various therapeutic effects. This study was aimed to investigate the beneficial antitumor effects of camel (*Camelus dromedarius*) milk antibodies (CM-IgG) on diethylnitrosamine (DENA)-induced hepatocellular carcinoma in male Wistar rats. Hepatocarcinoma was induced in rats using DENA (50 mg/kg, twice/week) for 2 weeks followed by $CCl_4$ (1 ml/kg, trice/week) for 6 weeks. At week 17th, HCC-bearing rats were orally administrated CM-IgG (100 mg/kg, orally) daily for 4 weeks. Liver enzyme activities and levels of alpha-fetoprotein (AFP) were measured in serum. Lipid peroxidation, nitric oxide, reduced glutathione (GSH) levels and superoxide dismutase (SOD) activity were determined in liver homogenate. Histological analysis using hematoxylin and eosin stain was examined in liver tissues. Hepatic mRNA gene expression of placental glutathione-s-transferase (GST-P) was determined by qRT-PCR. Treatment HCC-bearing rats with CM-IgG significantly reduced liver injury biomarkers and attenuated oxidative stress as well as enhanced antioxidant status. Moreover, CM-IgG significantly alleviated hepatocellular morphology alterations and down-regulated GST-P gene expression levels in liver. Our results revealed the potential role of total immunoglobulin IgG purified from camel milk in amelioration of DENA-induced hepatocellular dysfunction and oxidative stress in hepatocarcinoma-bearing rats.

*Keywords*: Diethylnitrosamine, Camel antibodies, HepG2 cells, Hepatocellular carcinoma, Oxidative stress, GST-P.

## 1. Introduction

Currently, many researchers are focused on studying some constituents derived from natural sources that might simply protect and improve our health. Milk, natural biological fluid, considers as a rich resource of all required nutrients for mammalian new-borns, children growth and adult nourishment (1). In addition, milk has an array of biologically active constituents like casein and whey proteins (2). Cow and camel milk are nearly similar in casein contents, whereas whey protein components are lower in cow milk; also this proportion of whey protein to casein contents in camel milk is higher than in sheep, buffalo and goat however it is lower than in human, mare and donkey milk (3).

Whey proteins composition in camel milk is unlike that of bovine milk whey, since ß-lactoglobulin is absent in camel milk as in human milk (4). Camel milk can play a vital role in our healthcare system. It contains a variety of protective proteins like casein, lactoferrin, lysozymes, lactoperoxidase, vitamin C, serum albumin, immunoglobulins (4) which in turn provide camel milk with potential health benefits. There are several reports about the medicinal effects of camel milk and its ingredients; antioxidative, antihypertensive, antimicrobial, and anticancer effects (5–8). A previous study showed that camel milk might be recommended as a nutrition protein source for children have allergy to cow milk (9). Camel milk

exerted a higher hypoglycemic activity by 49.2%, compared to buffalo and cow milk, and enhanced both liver and kidney (10) and it could stimulate wound healing (11). Camel milk and some of its components showed an activity against HCV viral infection *in vitro* and *in vivo* (12–16).Camel milk contain antibodies much more than that found in bovine or in buffalo milk, IgG is the predominant antibody in camel milk (17). Camel antibodies were undertaken into consideration in the medicinal research applications, as camels naturally possess a special unique structure of immunoglobulin G isotypes (IgG2 and IgG3); devoid of light chains, loss first constant domain CH1 hence their antigen-binding site constitutes only of a single variable domain of the heavy chain, referred to as heavy-chain antibodies (HCAbs). While IgG1, the conventional form of antibody, contain both light and heavy chains. Therefore, The HCAbs (IgG2 and IgG3) have a lower molecular weight than IgG1 (4,18). Molecular weights of the camel antibodies differ from those of sheep, cow, goat, buffalo, mare, and human (4) their sizes in camel are 10-times smaller than those in human; however their antigen affinity is similar which permits the absorption of these antibodies simply through small intestine and pass in the blood-stream and exert their actions. It had been demonstrated that naïve polyclonal camel IgG purified from CM has the ability to inhibit the infectivity of HCV *in vitro* and exhibited a potent signal against synthetic peptides of HCV (19).

The liver, centre of metabolism, regulates the physiological and biological functions in body such as growth, nutrients sourcing, detoxification of toxic agents and drugs and homeostasis (20). Chronic hepatic diseases are major worldwide health problems that trigger about 800,000 deaths per year globally. Hepatocellular carcinoma (HCC) is one of the most malignant liver tumours with high aggressive growth behaviour, elevated mortality, and increased rate of recurrence (21). HCC ranks the fifth of the most widespread malignancies worldwide and the second main cause of cancer-associated deaths (22). Its incidence in Egypt is increased rapidly due to viral hepatitis infections (HCV and HBV) that make it a major health problem (23). The most important recognized risk factors that lead to hepatocarcinoma development include toxic materials like aflatoxin B1, drinking alcohol, metabolic disorders like diabetes and non-alcoholic fatty liver disease (NAFLD), environmental pollutants, and viral hepatitis chronic infections (24). These factors contribute to chronic hepatic inflammation in which immune cells like macrophages, mast cells, lymphocytes, and kupffer cells were activated and triggered an oxidative stress through generation of reactive oxygen species (ROS)

and reactive nitrogen species (RNS) as well as production of large quantities of pro-inflammatory mediators like cytokines and chemokines (25). Diethylnitrosamine (DENA) is the most broadly genotoxic hepato-carcinogenic compound that can generate degenerative, proliferative and neoplastic lesions in hepatocellular carcinoma in animal model (26,27). Metabolic biotransformation of DENA is catalyzed by the hepato-cytochrom P450 (CYP) system (28) causing accumulation of free radicals and reactive oxygen species (ROS) that induces an imbalance in oxidants/antioxidants status and initiate peroxidative injury to cells (28). It has been reported that DENA could be used with carbon tetrachloride ($CCl_4$) to yield an ideal animal model of hepatocarcinogenesis that resembles to stages that occurred in human hepatic cancer starting from pre-neoplastic foci formation, nodules of neoplastic ending with HCC nodules (29,30). Although, there are a variety of HCC treatment strategies, their therapeutic outcome remains ineffectual, especially at intermediate and advanced stages (31). Therefore, developing more effective treatment strategies are of paramount importance to prevent or retard the hepatic alterations induced due to hepatocarcinogenesis. In previous studies revealed the antitumor effects of camel milk on chemically-induced hepatocellular carcinoma (7,32). Therefore, our study was designed to investigate the potential antitumor effects of a camel milk constituent (purified total immunoglobulin IgG [CM-IgG]) on DENA-induced hepatocarcinoma in rats.

## 2. Materials and Methods

### 2.1. Chemicals

Ammonium sulphate was purchased from Loba Chemie Pvt. Ltd. Company (Mumbai, India), Sephadex G-100 was supplied from Amersham Pharmacia Fine Chemicals (Uppsala, Sweden). VISKING® Diaylsis Tubing–regenerated cellulose-MWCO (12–14 kDa) was purchased from SERVA Electrophoresis Company (Germany). Diethylnitrosamine and carbon tetrachloride were purchased from Sigma Aldrich Company (USA). All other chemicals used were of high analytical grade and were purchased locally.

### 2.2. Camel Milk Samples

Raw milk samples were purchased from a camel farm Research Station existing in Marsa Matrouh, Egypt that follows Animal Reproduction Research Institute (ARRI), Egypt.

## 2.3. Purification of Camel Milk Antibodies (CM-IgG)

Camel milk whey (CMW) was prepared according to the procedure described previously (33). In brief, camel milk was defatted via centrifugation under cooling conditions then the collected skimmed milk was acidified using 1M HCl to pH 4.3 followed by centrifugation to precipitate casein. The resultant CMW was filtrated then dialyzed overnight against phosphate-buffered saline (PBS) and analyzed by SDS-PAGE electrophoresis. Total Camel IgG (CM-IgG) were purified from CMW by precipitating with 45% ammonium sulphate followed by stirring the mixture for 24 h at 4°C then centrifuged for 30 min at 4°C at 5000 rpm and the precipitant was re-suspended, dialyzed using PBS (pH 7.4) for 24 h at 4°C (34). Subsequently, the dialyzed sample was applied to gel filtration using Sephadex G-100 column. The elution profile was monitored at 280 nm and the eluted peaks were then analyzed by SDS-PAGE electrophoresis. All eluted fractions contained bands corresponding to the purified camel IgG isotypes (CM-IgG) were assembled in one container and saved at -20°C till use. Protein content of the pooled total IgG was determined by the Bradford method (35) using bovine serum albumin (BSA) as a standard. To analysis and characterize the purified total IgG purity, it was subjected to electrophoresis under non-reducing conditions with the use of a 10% SDS-PAGE gel electrophoresis. The gel was stained with 0.1% Coomassie blue R250 stain for protein detection according to previous standard protocols (36).

## 2.4. *In Vitro* Assessment of CM-IgG Cytotoxicity using Sulforhodamine B (SRB) Assay

HepG2 cell line was incubated with CM-IgG at different concentrations. The effect of CM-IgG on cell viability was evaluated by a rapid and highly accurate colorimetric approach called sulforhodamine B (SRB) cytotoxicity assay. Cell survival was investigated as represented previously (37). In brief, HepG2 cells were cultured in an exponential growth phase, washed, trypsinized and re-suspended in RPMI medium. Cells were placed on a microtitre plate and incubated for 24 h in a humidified atmosphere in order to a partial monolayer was formed. The cells were then treated with different concentrations of the purified CM-IgG in PBS (0, 5, 12.5, 25, 50, 100 and 200 µg/ml) in six-replicates using a constant volume of 20 µl while maintaining the total well volume of 200 µl. Then cells were incubated for 48 h at 37°C in a humidified 5% $CO_2$ incubator. The alterations of the cell cultures were scanned using an inverted microscope (Olympus IX2 model). 50 µl of cold 50% trichloroacetic acid was added to the 200 µl culture medium in each well. Then

microtitre plate was incubated for 30 min at 4°C and consequently washed 5 times with deionized water afterwards, it was left to dry at room temperature for at least 24 h. About 100 µl (0.4% w/v) of SRB (Sigma, USA) prepared in 1% acetic acid was added to each well and left for 20 min at room temperature. SRB was then removed and before air drying, the plate was washed 5-times with 1% acetic acid. Bound SRB was solubilized with 200 µl of 10 mM un-buffered Tris-base solution and the plate was left on a plate shaker for at least 10 min. The optical density (O.D.) was measured at 492 nm. Dose-response curve was plotted as mentioned by Papazisis (37); the estimated values expressed as percentage of the control optical density and $IC_{50}$ value of CM-IgG on HepG2 cell viability (50% inhibitory concentration) was estimated by regression analysis using GraphPad prism 7 software (CA, USA).

## 2.5. Experimental animals

Healthy adult male Wistar rats (weighing 140-150 g) were obtained from the animal house of Biological Applications Department, Nuclear Research Centre, Egyptian Atomic Energy Authority, Egypt. Rats were housed in polypropylene cages at the animal facility of the radioisotopes department of Nuclear Research Centre, EAEA, Egypt for two weeks before starting the experiment. Acclimatization at a temperature of 25 ± 5°C, the humidity of 60 ± 5%, and 12/12-hour light-dark cycle. The animals were freely fed on a standard pellet diet and fresh-water ad libitum throughout the experimental period. All experimental animals were handled and treated in accordance with the ethical guidelines for care and use of laboratory animals and approved by the Institutional animal care and use committee (CU-IACUC), Cairo University (approval No. CU-I-F-55-19).

## 2.6. Hepatocarcinoma Induction

Hepatocarcinoma was induced in animals according to the method reported previously (29) with some modifications. Diethylnitrosamine (DENA) was injected intra-peritoneal (i.p.) (50 mg/kg bw) twice/week for 2 weeks to initiate hepatocarcinoma induction. After two weeks of recovery, rats were injected carbon tetrachloride ($CCl_4$) (1 ml/kg bw, dissolved in olive oil) subcutaneously (s.c.) 3-times/week for 6 constitutive weeks to promote hepatocarcinogenesis. Different treatments were initiated at the starting of 17th week.

## 2.7. Treatment Protocol

Animals were randomly divided into four groups (10 rats/group). Animals groups were as followed: **Group I**: rats received a normal diet throughout the

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experimental period, and served as Normal control (**NC**) group. **Group II**: at the beginning of 17th week, rats received CM-IgG daily (100 mg/kg bw) via a gastric gavage for 4 constitutive weeks, and served as CM-IgG-control group (**Ig**) group. **Group III**: hepatocarcinoma was induced in rats as mentioned previously, and served as Hepatic cancer control (**HCC**) group. **Group IV**: at the beginning of 17th week, HCC-bearing rats were treated daily with CM-IgG at a dose of 100 mg/kg b.wt orally for 4 constitutive weeks, and served as CM-IgG treated HCC (**HCC+Ig**) group.

The experiment was terminated at the end of 20th week; rats were fasted overnight, weighed, and then euthanized. Blood was collected from the retro-orbital vein, clotted and centrifuged for serum separation. The collected sera were saved at −80°C till used. The animals were then sacrificed under anaesthesia following Animal Ethical Guidelines. Livers of all studied groups were immediately excised, washed in ice-cold saline, blotted to dryness, weighed. A portion of liver tissue samples were collected and snap-frozen directly in liquid nitrogen and stored at -80ºC to be used, while the other part was fixed in 10% buffered formalin for histological investigations.

## 2.8. Assessment of Serum Biochemical Parameters

Serum activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma-glutamyl transferase (GGT) (38,39) were measured kinetically using the commercial assays kits purchased from Vitro Scient Co. (Cairo, Egypt). Additionally, serum levels of total protein (40) and albumin (41) were measured colorimetrically using the commercial assays kits purchased from Bio-Diagnostic Co. (Giza, Egypt) according to the manufacturer's instructions. Estimation of serum levels of globulin (G) and albumin/globulin (A/G) ratio was calculated on the basis of total protein and albumin concentrations.

## 2.9. Detection of serum alpha-fetoprotein (AFP) level with Enzyme-linked immunosorbent assay (ELISA)

Serum levels of AFP in the control and experimental groups were quantified using the available commercial Sandwich ELISA kits. A Rat AFP ELISA kit (Catalog No. MBS267612) was purchased from MyBioSource, Inc. (Canada) for AFP measurement. The absorbance of the developed colour was read at 450 nm using a 96-well plate reader Stat Fax 3200 (Fisher, Awareness Technology, Inc. USA).

## 2.10. Histopathological investigations

The liver tissue samples were fixed in 10% formal saline for 24 h, and then tissues were subjected to dehydration using ascending grades of ethyl alcohol and embedded in paraffin wax at 56°C. Subsequently, the prepared paraffin wax blocks sectioned (3-5 μm thickness) by sliding microtome. Liver sections slides were examined for routine histological alterations under the light electric microscope after staining with haematoxylin and eosin (H&E) stain.

## 2.11. Liver Tissue Homogenate Preparation

Liver tissue samples (10% wt/v) were perfused with ice-cold PBS solution, pH 7.4 containing 0.16 mg/ml heparin using a tissue homogenizer (IKA homogenizer, Germany). Homogenates were centrifuged at 4000 rpm for 15 min at 4°C (42)**.** The homogenate supernatants were used to measure the oxidant and antioxidant levels in the control and experimental groups using the commercial kits provided from Bio-Diagnostic Co. (Giza, Egypt).

## 2.12. Detection of Hepatic Antioxidants and Oxidants levels

Superoxide dismutase (SOD) activity was assessed in liver tissue homogenate (43)**.** SOD has the ability to catalyze the dismutation of superoxide anion to molecular oxygen and hydrogen peroxide. The assay depends on the ability of SOD enzyme to inhibit the phenazine methosulphate-mediated reduction of nitro-blue tetrazolium dye**.** SOD activity was expressed as U/g tissue. The hepatic glutathione reduced (GSH) levels were determined as described previously (44). The assay is based on a reduction of 5,5′ dithiobis (2-nitrobenzoic acid) "DTNB" with GSH to produce a yellow compound. The reduced chromogen directly proportional to GSH concentration and its absorbance can be measured at 405 nm. Hepatic GSH concentration was expressed as mmol/g tissue. Lipid peroxidation (LPO) was estimated in terms of malondialdehyde (MDA) production. MDA was determined according to the method mentioned by **Ohkawa et al.** in **1979** (45) through its reaction with thiobarbituric acid (TBA) forming coloured thiobarbituric acid reactive products (TBARs) that measured at 534 nm. MDA level was expressed as (nmol MDA/mg protein). Nitric oxide (NO) levels were evaluated as total nitrate+nitrite levels according the method of Montgomery and Dymock (46) and expressed as umol/L. The assay depends on the formation of nitrous acid diazotize sulphanilamide, in acid medium, and the presence of nitrite, which then coupled with N-(1-naphthyl) ethylenediamine. The resulting azo dye has a bright reddish-purple color which can be measured at 540 nm that accurately determines nitrite concentration.

## 2.13. RNA Extraction and cDNA Synthesis

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Total RNA was isolated and purified from frozen hepatic tissues using the RNeasy Mini Kit (Qiagen, USA) according to the manufacturer's instructions. cDNA was synthesized using a high capacity cDNA reverse transcription kit (Fermentase, USA) from the isolated RNA (2 µg) according to the manufacturer's instructions.

### 2.14. Detection of GST-P mRNA by Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

qRT-PCR (Promega, USA) was used to quantify mRNA gene expression of placental glutathione-S-transferase (GST-P) using specific primers listed in **Table 1**. qPCR amplification and analysis were performed using a StepOne Real-Time PCR System (Applied Biosystems, USA) and cycling conditions (95°C 5 min, 40x at 95°C for 15 s, 60°C 60 s, and 72°C for 20 s) followed by extension at 72 °C for 5 min. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. Fold change of GST-P mRNA gene expression was calculated using the comparative threshold cycle method ($2^{-\Delta\Delta CT}$) as described previously (47).

### 2.15. Statistical Analysis

One-way ANOVA was performed using GraphPad Prism 9.0 (version 9.0.0 (121), GraphPad Software, San Diego, CA, USA) to evaluate the difference between the groups, followed by post hoc analysis using Tukey-Kramer multiple comparisons. All data were expressed as (mean ± SD) from six animals in each group. Statistical significance difference was considered at $P < 0.05$.

**Table 1: Sets of primers used for gene expression detection in rats**

| Gene | GenBank accession No. | Primer sequence | Amplified sequence (bp) |
|---|---|---|---|
| GST-P | NM_012577.2 | FW: 5`-CCTCACCCTTTACCAATCTA-3` | 462 |
| | | RV: 5`-TTCGTCCACTACTGTTTACC-3` | |
| GAPDH | NM_017008.3 | FW: 5`-ACAGCAACAGGGTGGTGGAC-3` | 252 |
| | | RV: 5`-TTTGAGGGTGCAGCGAACTT-3` | |

FW, forward; GST-P, Placental glutathione-S-transferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RV, reverse.

## 3. Results and Discussion

Hindering cancer incidence and progression by natural products may be a valuable approach in cancer chemoprevention and treatment. Nowadays, camel antibodies were applied in the medicinal research field due to their high affinity, high specificity, less immunogenicity, and recognition ability even of weakly antigenic epitopes (48) The unique configuration of camel antibodies, especially heavy-chain antibodies, attracted the attention of various investigators to be applied in numerous research and therapeutic areas, for example, applications in *in vivo* cells imaging and in cancer therapy via antibody preparations (49). Two-stage hepatocarcinogenesis induced by DENA/CCl$_4$ in rodent considered as an ideal experimental model of HCC, mimics the early stages of human HCC (50), can be used to assess effective therapeutic modalities. In the current study, we used this model to assess the potential therapeutic effects of immunoglobulin G purified from camel milk (CM-IgG) on chemically-induced hepatocellular carcinoma in Wistar rats. Our results indicated that CM-IgG treatment improved the liver injury biomarkers and histology and enhanced the antioxidant defence status as well as significantly reduced the oxidative stress in HCC-bearing animals.

### 3.1. Camel milk whey processing and IgG purification

**Figure 1** reveals the electrophoretic pattern of the camel milk whey (CMW) which was used then for CM-IgG purification. The purification was performed using a combination of ammonium sulphate precipitation followed by gel filtration chromatography. **Figure 2A** demonstrates the chromatogram of the eluted fractions from Sephadex G-100 column fractionation of the resultant 45% ammonium sulphate precipitant from CMW which were measured at 280 nm and its electrophoretic pattern analyzed by SDS-PAGE protein electrophoresis (**Figure 2B**). **Figure 3** demonstrates SDS-PAGE electrophoretic profile of the pooled eluted fractions containing CM-IgG isotypes only after being extensive dialyzed using phosphate-buffered saline (PBS) and stained with brilliant coomassie blue dye. It revealed two bands that represent IgG isotypes; band for the conventional immunoglobulin IgG1 (~190 kDa) and band for heavy-chain immunoglobulins IgG2 and IgG3 (~90 kDa). These results are paralleled with findings of previous studies (51,52)
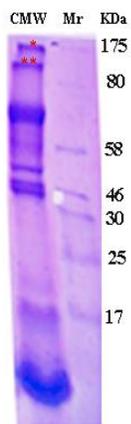
**Figure 1: Coomassie blue stained 14% SDS-PAGE electrophoretic analysis of the processed camel milk whey (CMW) proteins under non-reducing conditions.** Lane (1), CMW (*,IgG1; **, HCAbs (IgG2 and IgG3)); lane (2), protein marker (kDa).
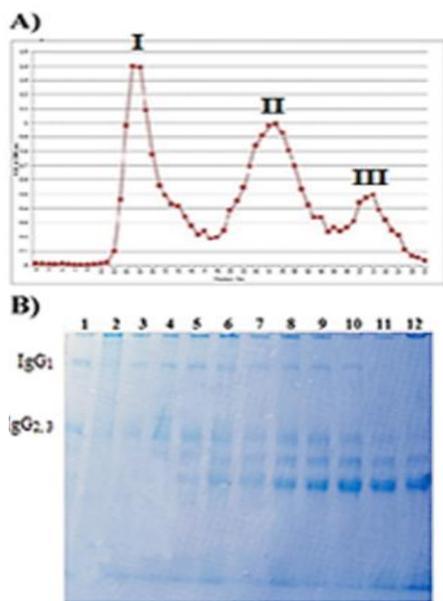


**Figure 2: Gel chromatography elution profile of 45% ammonium sulphate precipitant of CMW. (A).** Chromatogram of the eluted fractions. **(B).** Coomassie blue stained 10% SDS-PAGE electrophoretic analysis of the eluted fractions under non-reducing conditions. Lanes (1-4), peak I; lane (5-10), peak II; lane (11-12), peak III.

### 3.2. *In Vitro* Cytotoxicity Findings

We estimated *in vitro* cytotoxicity effects of CM-IgG on human hepatoma cell line (HepG2) and found that the purified CM-IgG exhibited an anti-proliferative activity against HepG2 cell line in a dose-dependent manner that triggered a significant reduction of cancer cell viability after 48h of incubation analyzed by sulforhodamine B (SRB) assay. **Figure 4** demonstrates a pattern of cell survival rate, where $IC_{50}$ (50% growth inhibitory concentration) of CM-IgG was estimated as ~ 120 µg/ml. These results might in consistent with a

previous study examined cytotoxicity of the crude CM on human hepatoma cell line HepG2 and they found that CM inhibited HepG2 cells survival and proliferation (53).
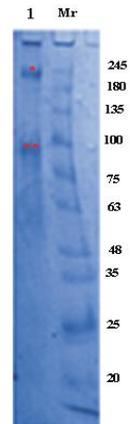


**Figure 3: 10% SDS-PAGE electrophoretic analysis of the collected pooled fractions of camel milk IgG (CM-IgG) under non-reducing conditions.** Lane (1), Two bands of CM-IgG subclasses (*conventional $IgG_1$ (~190 kDa) and **heavy-chain antibodies $IgG_2$ and $IgG_3$ (< 100 kDa)); lane (2), protein marker (kDa).
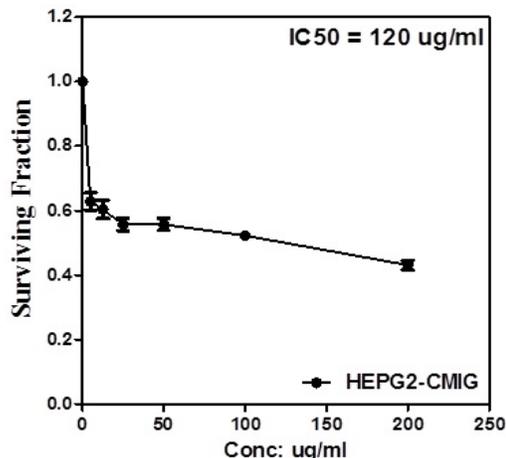


**Figure 4**: Dose-response plot of HepG2 cells with CM-IgG treatment at different concentrations (48 h incubation). Each point represents mean±standard deviation of six replicate wells.

### 3.3. Effects of Camel Milk Antibodies (CM-IgG) on Mortality Rate in the Experimental Groups in DENA-induced hepatocarcinoma in rats

The mortality rate in each group was evaluated as shown in **Table 2**. The mortality rate was higher in HCC control group (40%) and in the HCC-treated group with CM-IgG was moderate (20%). Remarkably, there was no recorded mortality upon treatment till the end of experiment in HCC-treated group (17th to 20th weeks). While in normal control (NC and Ig) groups was the least (0%).

**Table 2: Effects of CM-IgG treatment on Mortality of DENA-induced hepatocarcinoma in Wistar rats.**

| Parameters | Experimental groups | | | |
|---|---|---|---|---|
| | NC | Ig | HCC | HCC+Ig |
| Number of rats | 8 | 8 | 10 | 10 |
| Number of deaths | 0 | 0 | 4 | 2 |
| Mortality (%) | 0% | 0% | 40% | 20% |

### 3.4. CM-IgG Treatment Improve Relative Liver Index and Liver Injury Biomarkers in DENA-induced Hepatocarcinoma in Rats

Nitrosamines compounds like diethylnitrosamine (DENA) are an important category of ecological dietary carcinogens due to their ability to develop oxidative stress, inflammation and proliferation of cells in response to tissue damage that contributes to hepatocarcinoma development (26). **Table 3** shows the effects of CM-IgG treatment on relative liver index, activities of the liver injury biomarkers (ALT, AST and GGT) and AFP levels in sera obtained from both control and experimental groups. The relative liver index considered as an important factor in diagnosing the liver injury status. In the present study, the relative liver index was significantly elevated in HCC group by 67.4% when compared with NC group ($P<0.001$). These results were in agreement with a previous study (54). This elevation due to carcinogenic effects of DENA on liver tissue which induces progression of hyperplasia, hypertrophy and cirrhosis thus leads to increasing in liver weight without an obvious increase in body weight. In the present study, treatment of HCC-bearing rats with CM-IgG slightly restored to normal range compared to NC group. The hepato-specific enzymes, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma-glutamyl transferase (GGT), play an important role in assessment of liver damage and consider as a good bio-indicators for following-up hepatocellular injury. These enzymes were released to blood stream as a result of hepatocytes damage (55). Our results reavealed that ALT activity in (Ig) group almost normalized and AST activity was slightly significantly elevated when compared to NC group ($P<0.001$). In addition, GGT activity was significantly reduced in (Ig) group when compared to NC group ($P<0.001$). Rats intoxicated with DENA (HCC group) exhibited an extremely significant ($P<0.001$) elevation in serum liver enzymes activities (ALT, AST and GGT) when compared to normal control (NC) group by (45.7%, 94% and 126.3%, respectively) (**Table 3**). These results are in consistence with previous studies reported that liver carcinogenesis with DENA triggers alterations in membrane permeability and hepatocellular dysfunction that cause excessive spillage of the cytosolic enzymes (ALT and AST) into serum and liberation of the membrane-bound enzyme (GGT) into serum (56,57). Treatment with CM-IgG ameliorated the hepatic injury induced by DENA/$CCl_4$ and significantly reduced elevations in ALT, AST and GGT activities when compared with HCC group ($P<0.001$) that underlining their potential to enhance the hepatocellular membrane integrity and functions.

**Table 3: Effects of CM-IgG treatment on relative liver index, serum activities of ALT, AST and GGT and serum level of AFP in the experimental groups.**

| Parameters | Experimental groups | | | |
|---|---|---|---|---|
| | NC | Ig | HCC | HCC+Ig |
| **Relative liver index** (g liver/100 g body weight) | $2.3 \pm 0.074^a$ | $2.31 \pm 0.021^a$ | $3.85 \pm 0.3^c$ | $2.79 \pm 0.077^b$ |
| **ALT (U/L)** | $67 \pm 2.75^a$ | $66.83 \pm 3.31^a$ | $97.66 \pm 3.01^b$ | $66.71 \pm 8.05^a$ |
| **AST (U/L)** | $132.16 \pm 3.76^a$ | $140.66 \pm 5.46^b$ | $256.33 \pm 5.50^d$ | $213.50 \pm 3.44^c$ |
| **GGT (U/L)** | $3.90 \pm 0.20^b$ | $2.16 \pm 0.26^a$ | $8.82 \pm 0.70^d$ | $5.60 \pm 0.86^c$ |
| **AFP (ng/ml)** | $0.44 \pm 0.037^a$ | $0.68 \pm 0.069^{ab}$ | $5.25 \pm 0.750^c$ | $1.13 \pm 0.134^b$ |

Data were presented as mean $\pm$ SD (n=6); one-way ANOVA test followed by post hoc Tukey's HSD multiple comparisons; Different letters correspond to statistical difference (a, b, c... etc.) as significance at $P < 0.05$.

AFP, a biomarker of HCC, is a glycoprotein that expressed in hepatoblast stem cells, hepatocarcinoma stem cells and hepatoma cells. AFP can be used as a surveillance indicator for cancer progression after HCC patients are treated and a prognostic marker in tumor diagnosis and staging (58). **Table 3** demonstrates AFP level in (Ig) group which was non-significantly increased slightly when compared to NC group by (54.6%). AFP level was highly significantly elevated in HCC group ($P<0.001$) when compared to NC group by (1093.2%), confirming the incidence of HCC. These results are in agreement with previous studies (59,60) which demonstrated that rats intoxicated with carcinogens show over-production of serum AFP. In our study, on treatment with CM-IgG, AFP level was highly significantly reduced by (364.6%) as compared to HCC group ($P<0.001$), suggesting the potential anticancer effects of CM-IgG

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on DENA-induced HCC. Additionally, **Table 4** shows the effects of CM-IgG treatment on levels of total protein, albumin, globulin and albumin/globulin (A/G) ratio in sera obtained from both control and experimental animals in rats of all groups. Carcinogenesis with DENA triggers acute hepatotoxicity causing poly-ribosomes destruction and/or separation from endoplasmic reticulum (ER) and consequently obstructs protein synthesis rate (61). We observed significant decrease in serum levels of total protein, albumin and globulin in HCC-bearing rats ($P<0.001$) when compared to NC group. This reduction confirmed the impaired liver function due to alterations in hepatic protein synthesis and infiltration with tumour (62,63). Upon treatment with CM-IgG, these levels were significantly increased as compared to HCC group ($P<0.001$), suggesting the beneficial effects of CM-IgG on DENA-induced HCC on hepatocytes activity.

**Table 4: Effects of CM-IgG treatment on serum levels of total protein, albumin, globulin and albumin/globulin (A/G) ratio in the control and experimental groups.**

| Parameters | Experimental groups | | | |
|---|---|---|---|---|
| | **NC** | **Ig** | **HCC** | **HCC+Ig** |
| **Total protein (g/dL)** | $8.084 \pm 0.16^c$ | $8.084 \pm 0.17^c$ | $5.34 \pm 0.32^a$ | $7.58 \pm 0.37^b$ |
| **Albumin (g/dL)** | $4.037 \pm 0.18^c$ | $4.057 \pm 0.09^c$ | $2.37 \pm 0.16^a$ | $3.03 \pm 0.30^b$ |
| **Globulin (g/dL)** | $4.047 \pm 0.13^b$ | $4.027 \pm 0.09^b$ | $2.97 \pm 0.43^a$ | $4.55 \pm 0.30^c$ |
| **A/G ratio** | $0.99 \pm 0.07^b$ | $1.01 \pm 0.02^b$ | $0.82 \pm 0.18^a$ | $0.67 \pm 0.1^a$ |

Data were presented as mean ± SD (n=6); one-way ANOVA test followed by post hoc Tukey's HSD multiple comparisons; Different letters correspond to statistical difference (a, b, c... etc.) as significance at *P < 0.05*.

### 3.5. CM-IgG Treatment Alleviated DENA-Induced Hepatic Histo-morphological Alterations

The biochemical alterations caused by DENA were confirmed via histological analysis of liver tissues. Animals' liver tissues were examined macroscopically for any gross abnormalities and microscopically with H&E staining for evaluation of the cellular alterations occurred due to DENA/CCl₄ administration and treatment effects in all studied groups. As depicted in **Figure 5,** it revealed normal shiny smoothly reddish appearance of livers in NC group as well as its liver sections stained with H&E displayed a normal appearance of hepatic cells with granulated cytoplasm and uniform small nuclei, as well as normal sinusoids and normal architecture of hepatocytes. In (Ig) group, plates of normal polyhedral hepatocytes with acidophilic cytoplasm and central vesicular basophilic nuclei with occasional binucleation (arrow) were seen. These hepatocytes plates are seen radiating from the central vein (CV) and separated by endothelial-lined blood sinusoids (S) (**Figure 6**).

Oppositely, HCC group showed diffused white nodules on the liver with a pale appearance and liver photomicrograph stained with H&E showed loss of normal architecture of hepatocytes, neoplastic hepatocytes with pleomorphism, increased nuclear to cytoplasmic ratio (N/C), nuclear atypia and hyperchromasia (black arrows). Areas of hemorrhagic necrosis (H) invaded by mononuclear cellular infiltration (K; kupffer cells) are also observed (**Figure 7**), this was in accordance with previous studies (22,64,65). **Figure 8** showed no gross lesions on the liver surface of HCC-bearing rats treated with CM-Ig and its photomicrograph stained with H&E showed diffuse vacuolization of hepatocytes. Treatment HCC-bearing rats with CM-IgG reduced the DENA-induced deleterious effect in hepatocellular morphology.
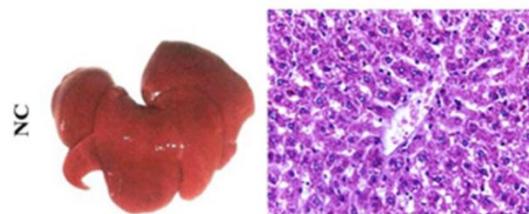


**Figure 5:** Macroscopic gross pathology of liver revealing normal shiny smoothly reddish appearance and histopathological photomicrographs stained with H&E stain of (NC) group showing regular hepatic architecture and normal hepatocytes(scale bar 50µm).
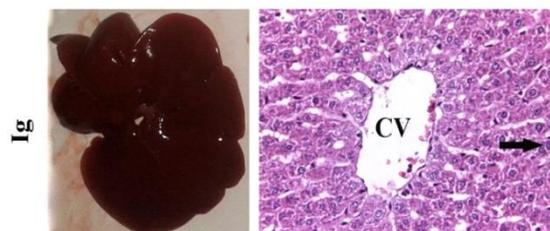


**Figure 6:** Macroscopic gross pathology of liver revealing normal smoothly reddish appearance and histopathological photomicrographs stained with H&E stain of (Ig) group showing plates of normal polyhedral hepatocytes, radiating from the central vein (CV) and separated by endothelial-lined blood sinusoids (S), with acidophilic cytoplasm and

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central vesicular basophilic nuclei with occasional binucleation (black arrow) (scale bar 50μm).
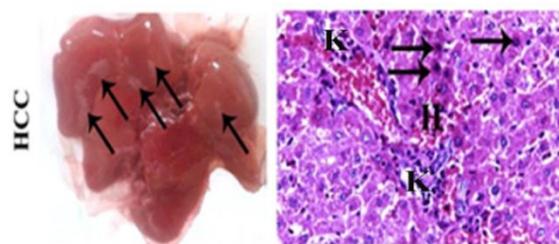


**Figure 7:** Macroscopic gross pathology of liver pale hepatic appearance with diffused white nodules were observed on their liver (arrows) and histopathological photomicrographs stained with H&E stain of (HCC) group showing neoplastic hepatocytes with pleomorphism, increased nuclear to cytoplasmic ratio (N/C), nuclear atypia and hyperchromasia (black arrows). Areas of hemorrhagic necrosis (H) invaded by mononuclear cellular infiltration (K; kupffer cells) are also observed (scale bar 50μm).
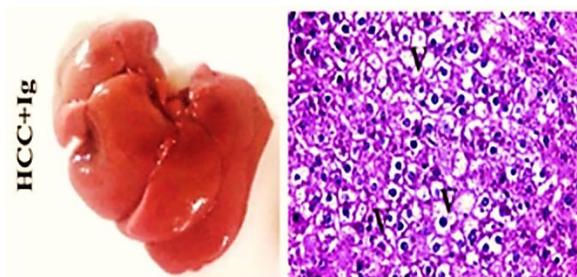


**Figure 8:** Macroscopic gross pathology of liver revealing normal reddish appearance and histopathological photomicrographs stained with H&E stain of (HCC+Ig) group showing diffuse vacuolization (V) of the hepatocytes (scale bar 50μm).

### 3.6. Influence of CM-IgG Treatment on Hepatic Antioxidants and Oxidants Levels in HCC-Bearing Rats

As represented in **Table 5**, MDA and nitric oxide (NO) levels were insignificantly increased in (Ig) group when compared to NC group. DENA administration for HCC group caused a highly significant ($P<0.001$) elevation in levels of MDA (458.4%) and NO (737.8%) when compared to NC group. Hepatic carcinogenesis was induced in rats received DENA as a result of its reactive metabolites products and the unregulated production of free radicals that triggered an oxidative stress, interrupted the antioxidant status (56). Malondialdehyde (MDA, lipid peroxidation marker) is the best helpful guide of oxidative stress, which was elevated in HCC-induced group compared to NC group as represented in our study that also correlated with previous studies (56,57,66) that indicated DENA impacts on animal liver and how it resulted in severe injury to it. One of the leading inflammatory mediators is nitric oxide (NO), previous studies reported the association of increased cancer incidence with increased in NO production (67). In the recent study, hepatic nitric oxide (NO) was highly increased in HCC group compared to NC group. This result is in consistent with a previous study (60). This elevation may be due to the carcinogenesis effects on host cells especially macrophages and monocytes which produce and liberate excessive amount of NO by the induction of iNOS causing hepatotoxicity and DNA damage that contribute to tumour progression and rapid growth (67). In the current study, treatment HCC-bearing rats with CM-IgG triggered a significant ($P<0.001$) decline in MDA and NO levels compared to HCC group. In addition, **Table 5** demonstrates SOD activity in (Ig) group was near normal range (i.e., non-significant), while GSH level was significantly decreased (13.1% "$P<0.01$") when compared to NC group. Owing to carcinogenesis, HCC group showed an extremely significant ($P < 0.001$) reduction in both SOD activity (80%) and GSH level (63.76%) when compared to NC group. These results of the present study are in line with earlier studies (56,66) reported that this reduction may be due to the over-use of antioxidants to overcome the increment in lipid peroxidation products. Treatment HCC-bearing rats with CM-IgG triggered a significant ($P<0.001$) increase in SOD activity GSH level and compared to HCC group.

**Table 5: Effects of CM-IgG treatment on levels of malondialdehyde (MDA), nitric oxide (NO) and reduced glutathione (GSH) and superoxide dismutase (SOD) activity and in liver tissues of the control and experimental groups**

| Parameters | Experimental groups | | | |
|---|---|---|---|---|
| | **NC** | **Ig** | **HCC** | **HCC+Ig** |
| **MDA (nmol MDA/mg protein)** | $9.76 \pm 0.61^a$ | $14.83 \pm 1.64^{ab}$ | $54.50 \pm 8.08^c$ | $27.31 \pm 5.87^b$ |
| **NO (μmol/L)** | $6.43 \pm 1.18^a$ | $10.17 \pm 0.56^a$ | $53.87 \pm 8.04^c$ | $21.72 \pm 2.51^b$ |
| **GSH (mmol/g tissue)** | $65.12 \pm 3.30^d$ | $56.57 \pm 2.24^c$ | $23.60 \pm 4.01^a$ | $42.30 \pm 3.39^b$ |
| **SOD (U/g tissue)** | $5.01 \pm 0.22^d$ | $4.54 \pm 0.23^c$ | $1.00 \pm 0.07^a$ | $2.25 \pm 0.18^b$ |

Data were presented as mean ± SD (n=6); one-way ANOVA test followed by post hoc Tukey's HSD multiple comparisons; Different letters correspond to statistical difference (a, b, c... etc.) as significance at $P < 0.05$.

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## 3.7. Impact of CM-IgG Treatment on Hepatic GST-P mRNA Gene Expression in DENA-induced Hepatocarcinoma in Rats

**Figure 9A** displays the analysis of mRNA gene expression of GST-P in liver tissues by qRT-PCR on agarose gel. **Figure 9B** demonstrates the relative gene expression of GST-P, which was significantly over-expressed in HCC group versus NC group. Hepatocarcinogenesis, a multistep process, involves alterations in expression of genes which in turn leads to mutations formation. The rat placental glutathione-S-trasferase (GST-P), a detoxifying enzyme of a phase II set, is only specifically and extremely high expressed during early hepatocarcinogenesis and in hepatoma cells (68) and can be used as a dependable tumour marker for rodent hepatocarcinogenesis (69). Our results agree with these observations, which show an elevation in GST-P gene expression level in hepatic tissues of DENA-induced HCC group. While, treatment with CM-IgG down-regulated the over-expression of GST-P mRNA gene expression occurred due to DENA injection in HCC-bearing rats.
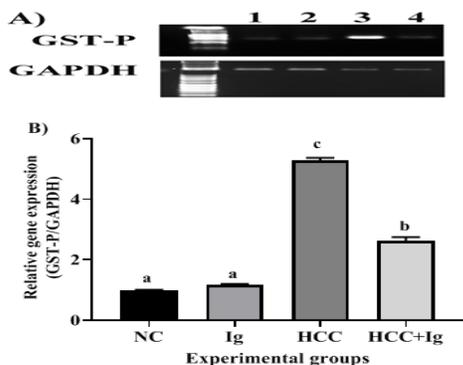
**Figure 9: Effects of CM-IgG treatment on mRNA expression of GST-P in livers of the experimental groups.** (**A**) GST-P mRNA gene expression on agarose gel. Various groups are as follow: 1, NC; 2, Ig; 3, HCC; 4, HCC+Ig; (**B**) Relative mRNA gene expression of GST-P. GAPDH used as an internal control. (Data were shown as mean ± SD, n=3). One-way ANOVA test followed by post hoc Tukey's HSD multiple comparisons; Different letters correspond to statistical difference (a, b, c... etc.) as significance at *P < 0.05*.

## 4. Conclusion

Our results suggested that the purified total IgG from camel milk (CM-IgG) can ameliorate DENA-induced hepatocellular alterations and oxidative stress in the experimental animals. CM-IgG can exert potential role on chemically-induced hepatocellular carcinoma in rats.

## 5. Abbreviations

**CM,** Camel milk; **CMW,** Camel milk whey; **CM-IgG,** Camel milk immunoglobulin G; **HCC,** Hepatocellular carcinoma; **DENA,** Diethylnitrosamine; **CCl₄,** Carbon tetrachloride; **HCAbs,** heavy-chain antibodies; **PBS,** Phosphate-buffered saline; **H&E,** Haematoxylin and eosin; **GST-P,** placental glutathione-S-trasferase; **GAPDH**; Glyceraldehyde 3-phosphate dehydrogenase.

## 6. Conflict of interests

The authors declare that there is no conflict of interests.

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