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Original Research Article
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POTENTIAL PERFECTION EFFECTS OF SILVER NANOPARTICLES AGAINST CISPLATIN SIDE EFFECTS IN HEPATOCELLULAR CARCINOMA INDUCED IN SPRAGUE DAWLEY ALBINO RATS: HEMATOLOGICAL, BIOCHEMICAL, HISTOPATHOLOGICAL, AND IMMUNOHISTOCHEMICAL ALTERATIONS

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Abstract: Silver nanoparticles (AgNPs) have various applications such as their use in the medical field. As they have been reported to show antimicrobial and anti-tumor effects. The main purpose of the current study was to explore the anti-cancer effects of AgNPs administration alone or in a combination with cisplatin (CP) against hepatocellular carcinoma (HCC) in rats. Seventy-five rats (Sprague Dawley albino rats) were used in the present study. Rats were assigned to 5 groups. Group 1 served as normal control. Group 2 was injected intraperitoneally (IP) with a single dose (200 ml/kg) of diethylnitrosamine (DEN), then one week later carbon tetrachloride (CCl4) was injected IP (0.2 ml/kg) two times weekly for 14 successive weeks for the induction of HCC. Group 3 was treated with AgNPs (4 mg/kg) daily. Group 4 was treated with CP (6 mg/kg) once a week. Group 5 was treated with a combination of AgNPs (4 mg/kg/daily) and CP (2.5 mg/kg/weekly). Groups 3, 4, and 5 were treated for 3 successive weeks after induction of HCC. Hematological, biochemical, antioxidant activities, proinflammatory cytokines, and apoptotic genes were evaluated in the current study. Hematological results denoted normocytic normochromic anemia in all examined groups except group 2 which showed macrocytic hypochromic anemia, with thrombocytopenia and leukocytosis in all groups. Significant hypoproteinemia, hypoalbuminemia, and hypoglobulinemia were detected in all groups, with no significant changes ($P < 0.05$) in globulin in group 5. Significantly decreases in GSH and SOD were recorded in all groups. While the serum AST, ALT activities, and levels of total bilirubin, urea, creatinine, IL-6, TNF-α, AFP, VEGF, BAX, and caspase-3 were markedly elevated. The results revealed a remarkable improvement in group 5 than groups 3, and 4. The obtained results were supported by immunohistochemical and histopathological investigations of the liver tissue..

Key words: AgNPs; cisplatin; hepatocellular carcinoma; hematology; biochemical; TNFα; IL6

Introduction

Nanoparticles are an important branch of nanotechnology (1). Nanotechnology is concerned with particle structures ranging from 1-100 nm (2). Nowadays, there is a significant increase in nanoparticle usage, especially in the biomedical field. One of the most important nanoparticles is AgNPs, which are used in medicine, pharmaceutics, and dentistry. AgNPs have anti-cancer, anti-inflammatory, anti-bacterial, anti-fungal, and anti-viral activities. AgNPs are expected to present a new perspective on the detection, protection, and treatment of tumors (3). AgNPs are considered potentially ideal choices for cancer therapy due

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to their novel and unique antiproliferative and apoptosis-inducing properties (4). Hepatocellular carcinoma (HCC) is primary liver cancer. HCC is the sixth most common cancer in the world (5) and is considered a great challenge for clinicians owing to its increasing morbidity and mortality. HCC progresses from fibrosis, and cirrhosis to cancer (6, 7). Chemotherapy is considered one of the strategies of cancer treatment that may be used alone or in a combination with the other types (8, 9). Cisplatin is one of the most well-known chemotherapeutic drugs used for various types of cancer. Cisplatin interferes with DNA repair mechanisms causing DNA damage and then inducing apoptosis in cancer cells. Cisplatin causes several harmful side effects despite its anticancer activity such as nephrotoxicity (10, 11), decrease immunity, allergic reactions, gastrointestinal problems, and hemorrhage (12). Moreover combined treatment of cisplatin with other sensitizing agents is an effective method to overcome the resistance of cisplatin (13).

This study aimed at investigating the potential ameliorative effects of silver nanoparticles against cisplatin side effects in hepatocellular carcinoma induced in Sprague Dawley albino rats.

Material and methods

The experimental design was approved by the Committee of the Animal Welfare and Research Ethics, Faculty of Veterinary Medicine, Zagazig University, Egypt. (ZU-IACUC/2/F/124/2022).

Induction of Hepatocellular carcinoma:

The induction of Hepatocellular carcinoma (HCC) occurs in two steps (initiation and promotion) (14). For initiation, DEN was dissolved in corn oil and then injected intraperitoneally by a single dose (200 mg/kg bwt) (15). For promotion, CCl4 (CCl4/olive oil; 1:1; 1 ml/kg) was given intraperitoneally one week later after DEN administration by a dose of 0.2 ml/kg bwt two times weekly for 14 successive weeks (16).

Synthesis of AgNPs

Preparation of aqueous silver nitrate:

The preparation of colloidal AgNPs was according to a previous report (17).

Reduction by tri-sodium citrate

$AgNO_3$ of high purity of more than 99% (Alpha Chem. Company, India) was dissolved in 100 ml of distilled water. Heat the Silver nitrate solution near boiling (95–98°C), then Add 2% tri-sodium citrate solution (reducing and stabilizing agent) dropwise, one drop per second, and continue heating at 95–98°C (near boiling) for 15 min. The solution turned into a light yellow color which indicates the formation of AgNPs. Wait until reaching room temperature, and then store in the dark at 2–8°C (17).

Characterization of AgNPs.

Nanoparticles' size and shape were measured by transmission electron microscope (TEM) descriptions (Figure 1A, and 1B), Dynamic light scattering (DLS) determined the particle size and particle size distribution as described in (Figure 1C) and Ultraviolet (UV) visible spectrophotometry of AgNPs shows a typical absorption peak (Figure 1D).

Experimental animals and management

A total number of 75 clinically apparent healthy adult male albino rats of 6 to 8 weeks with average body weight (200-250 g) were used in the experimental trials. These rats were obtained from the central animal house of the Faculty of Veterinary Medicine, Zagazig University. The experimental animals were assigned to five groups, each consisting of fifteen rats, and were kept under standard hygienic conditions in metal cages, fed on a balanced ration, and a good source of water and light (12 hours dark/light cycle). Daily cleaning and changing of water and food were done for experimental animals. One week before the experiment, all animals were kept under observation to ensure that they were free from bacterial infections and parasitic infestation.

Experimental design

The 75 male adult albino rats were assigned into 5 groups, 15 rats in each group. Group 1 was kept as normal control. Group 2 was injected intraperitoneally by DEN (200 mg/kg bwt) at a single dose, then one week later, CCL4 was injected intraperitoneally by (0.2 ml/kg) 2 times weekly for 14 successive weeks for the induction of HCC (16) and served as an HCC induced group. Group 3 was injected intraperitoneally by AgNPs (4 mg/kg b.wt) daily for three successive weeks (18) continuously after the induction of HCC. Group 4 was injected intraperitoneally by cisplatin (6 mg/kg b.wt) once a week for three successive weeks after induction of HCC (19). Group 5 was treated with a combination of AgNPs (4 mg/kg/IP/daily) and CP (2.5 mg/kg/IP/weekly) for three successive weeks continuously after induction of HCC.

Blood sampling

The blood samples from all individuals in all experimental groups were collected from the tail vein at the end of the experiment (20). Blood samples collected for hematological examination were collected on EDTA in clean Wassermann tubes, while that used for biochemical and antioxidant parameters were collected in a plain centrifuge test tube without anticoagulant to separate serum.

Tissue sampling

Specimens from the liver were collected within 21 days of treatments from all groups for immunohistochemical and histopathological examination.

Hematological studies

Red blood cells (RBCs), hemoglobin (Hb) concentration, packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular hemoglobin (MCH), and total leukocytic counts were determined using an automatic cell counter (ABX micros 60 hematology analyzer manufactured by HORIBA ABX SAS).

Biochemical and antioxidant assays

The biochemical tests were performed by using test kits of Diamond–Egypt, Spectrum, Spinreact, Cloud–Clone Corp (USA), (Reagents for ELIZA kit), H CUSABIO, DRG ELISA, BIO VISION, Egyptian Company for Biotechnology (S.A.E), and Bio-Med Diagnostic). Serum biochemical profiles including aspartate transaminase (AST), and alanine transaminase (ALT) were estimated (20). Total protein was measured by ELISA Kit (Cat. No MBS1600724), albumin was measured by ELISA Kit 09/17 (Catalog NO E4364-100), and Serum globulins were calculated by subtracting the obtained albumin level from the obtained total proteins level (20), while urea was measured by using a colorimetric assay kit (Catalog NO K375-100), moreover, creatinine was estimated by ELISA Kit rev 03/18 (Catalog NO E4370-100), total bilirubin was estimated by using ELISA Kit 12/19 (Catalog NO E4794-100), serum glutathione (GSH) was measured according to (21) and superoxide dismutase (SOD) was assessed (22).

Pro-inflammatory cytokines and HCC markers

Tumor necrosis factor alpha (TNFα) was estimated by ELISA Kit (Catalog Number SEA133Ra), while ELISA Kit SEA079Ra was used in the estimation of interleukin 6 (IL6), for VEGF, ELISA Kit (Catalog Number CSB-E04757r) was used, and for alpha-fetoprotein (AFP) ELISA Assay kit (SEA153Ra) was used.

Gene expression analysis by quantitative real-time PCR

Liver samples were used for evaluating the expression of apoptosis-related genes, BAX and caspase-3. Total RNA was extracted from tissue lysate by Direct-zol RNA Miniprep Plus protocol (Cat NO R2072, ZYMO RESEARCH CORP. USA), then the quantity and quality were calculated by Beckman dual spectrophotometer (USA). For reverse transcription of extracted RNA (RT-PCR), Super Script IV One-Step RT-PCR kit (Cat NO12594100, Thermo Fisher Scientific, and Waltham, MA USA) was used. After RT-PCR, the data were expressed in Cycle threshold (Ct) to calculate the Relative Quantification (RQ) (relative expression) using the 2-ΔΔCt method (23).

Histopathological examination

Liver tissue Specimens of the sacrificed rats were examined macroscopically, then fixed in neutral buffered formalin (10%). From all liver samples, the Paraffin sections (5-micron thickness) were prepared, stained by H&E (hematoxylin and eosin), and then examined microscopically (24).

Immunohistochemical analysis of Bcl2 in the hepatic tissue

This technique was performed on 3–5 μm thickness sections of liver tissue (25). then anti-Bcl-2 of the rat was diluted at 1:200 and then incubated with a liver section for 60 min (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Then the primary antibodies were diluted in TBS (Tris-buffered saline) 1% and BSA (bovine serum albumin). Then add the secondary antibody against rat immunoglobulin which was incubated for 15 min (Biotinylated Link Universal–DakoCytomation kit). Then a reddish-to-brown color appeared after adding 3-amino-9-ethyl carbazole (AEC) for 15 min at the sites of immunolocalization of the primary antibodies. Moreover, the specimens were counterstained with hematoxylin (1 min) and mounted with Aquatex fluid (Merck KGaA, Germany). All sections were incubated under the same conditions with the same concentration of antibodies and at the same time; for the immunostaining to be comparable among the different experimental groups.

Statistical analyses

The obtained data were analyzed using the One-way ANOVA procedure, by using PASW statistics 28 (SPSS Inc., USA). $P \leq 0.05$ was considered statistically significant.

Results

Hematological studies:

The hematological parameters were described in table (1). Regarding the erythrogram, all groups (2, 3, 4 & 5) showed significant reduction ($P < 0.001$) in erythrocyte count, Hb level, and PCV concentration (normocytic normochromic anemia) except for group 2 which showed macrocytic hypochromic anemia in comparison with the normal control group. Moreover, all groups (2-5) showed thrombocytopenia in comparison with the normal control group. Concerning the leukogram, the total leukocyte count (TLC) revealed leukocytosis in groups 2, 3, 4 & 5 when compared with the normal control. But when compared with group 2 (HCC inducted group) it showed a significant decrease ($P < 0.001$) as declared in groups 3, 4 & 5.

Biochemical parameters and serum antioxidant activities

The changes in the biochemical parameters and serum antioxidant activities were clarified in the table (2).

Regarding liver functions, the serum total protein and albumin levels in the present study showed a significant reduction ($P < 0.001$) in all groups compared with group 1. While serum globulin level revealed a significant decrease ($P < 0.001$) in all groups except group 5.

Moreover, the serum total bilirubin, ALT, and AST activities illustrated a significant increase ($P < 0.001$) in all groups in comparison with the control. But when compared with group 2 (HCC inducted group) it showed a significant decrease as shown in groups 3, 4 & 5.

Concerning kidney function, the serum levels of creatinine and urea in groups (2-5) showed a significant increase when compared with the normal control.

Regarding antioxidant activity, the serum GSH and SOD activities showed a significant decline ($P < 0.001$) in groups 2, 3 & 4) in comparison with the normal control group, except group 5 which showed a non-significant decrease ($P > 0.05$) when compared with the normal group.

Pro-inflammatory cytokines and HCC markers

There was a significant increase ($P < 0.001$) in serum TNF-α, IL-6, AFP, and VEGF levels in all groups when compared with group 1. But it showed a significant reduction in groups 3, 4 & 5 when compared with group 2 (HCC inducted group).

Gene expression analysis by quantitative real-time PCR

The pro-apoptotic genes (BAX and caspase-3) expressed up-regulation ($P < 0.001$) in the liver of groups 3, 4 & 5, while group 2 (HCC inducted group) showed insignificant difference ($P > 0.05$) when compared with group 1 (Fig. 2 and 3).

Table 1: Hematological changes in the different experimental groups

Treatment / Parameter	Gp.1	Gp.2	Gp.3	Gp.4	Gp.5	P value
RBCs x 10^6/µl	8.60^{a} ±0.20	3.40^{d} ±0.17	7.38^{b} ±0.15	6.30^{c} ±0.19	7.44^{b} ±0.18	<0.001
Hb g/dL	16.24^{a} ±0.59	6.70^{c} ±0.25	12.92^{b} ±0.38	12.42^{b} ±0.60	13.76^{b} ±0.49	<0.001
PCV %	41.54^{a} ±0.62	22.32^{d} ±0.99	37.04^{b} ±0.62	30.66^{c} ±1.75	35.48^{b} ±1.04	<0.001
MCV fl	50.07^{b} ±0.80	65.82^{a} ±2.39	50.11^{b} ±0.93	49.37^{b} ±1.05	47.57^{b} ±0.39	<0.001
MCH pg	18.94^{a} ±0.64	19.91^{a} ±1.44	17.54^{a} ±0.66	20.03^{a} ±0.48	18.44^{a} ±0.28	0.20
MCHC %	39.06a^{b} ±1.00	30.33^{c} ±2.07	35.01^{b} ±1.13	40.68^{a} ±1.50	38.76^{ab} ±0.36	<0.001
Platelets x 10^3/µl	631.00^{a} ±7.48	194.80^{e} ±4.21	394.80^{c} ±4.21	317.60^{d} ±28.42	553.00^{b} ±35.22	<0.001
T.L.C. x 10^3/µl	10.61^{d} ±0.43	23.92^{a} ±0.61	17.14^{b} ±0.42	13.32^{c} ±0.76	12.60^{c} ±0.29	<0.001

Values within the same row carrying different superscript letters are significantly different at P < 0.01. Values represent mean ± SE.

Table 2: Some biochemical parameters, antioxidant activities, pro-inflammatory cytokines, and HCC markers

Treatment / Parameter	Gp.1	Gp.2	Gp.3	Gp.4	Gp.5	P value
ALT U/L	35.70^{e} ±0.46	159.21^{a} ±2.77	118.81^{b} ±2.92	100.86^{c} ±2.72	77.40^{d} ±1.80	<0.001
AST U/L	48.32^{e} ±0.22	188.78^{a} ±2.48	148.62^{b} ±2.93	118.14^{c} ±0.51	89.21^{d} ±2.59	<0.001
Total Bilirubin mg/dL	0.16^{e} ±0.005	1.69^{a} ±0.07	1.16^{b} ±0.01	0.90^{c} ±0.04	0.64^{d} ±0.01	<0.001
Total protein g/dL	7.43^{a} ±0.32	3.57^{d} ±0.10	4.55^{b} ±0.11	3.64^{cd} ±0.26	4.34^{bc} ±0.30	<0.001
Albumin g/dL	4.00^{a} ±0.27	1.98^{b} ±0.20	2.22^{b} ±0.20	1.76^{bc} ±0.07	1.34^{c} ±0.20	<0.001
Globulins g/dL	3.42^{a} ±0.12	1.59^{c} ±0.18	2.34^{b} ±0.20	1.88^{bc} ±0.26	3.00^{a} ±0.28	<0.001
Creatinine mg/dL	0.54^{e} ±0.006	8.62^{a} ±0.32	5.47^{b} ±0.39	4.18^{c} ±0.07	3.28^{d} ±0.07	<0.001
Urea mg/dL	6.35^{e} ±0.16	68.93^{a} ±1.00	31.03^{c} ±0.90	51.69^{b} ±3.32	25.37^{d} ±0.57	<0.001
GSH m. mol/mg	3.54^{a} ±0.16	1.26^{d} ±0.13	2.60^{b} ±0.09	2.13^{c} ±0.10	3.55^{a} ±0.13	<0.001
SOD U/ml	5.49^{a} ±0.09	0.80^{e} ±0.05	2.74^{c} ±0.06	1.97^{d} ±0.02	3.10^{b} ±0.08	<0.001
TNF (pg/ml	83.08^{e} ±0.25	420.83^{a} ±13.33	224.38^{c} ±8.65	298.09^{b} ±6.01	169.12^{d} ±6.39	<0.001
IL6 (pg/ml	62.45^{e} ±0.04	276.00^{a} ±8.78	182.56^{c} ±13.68	219.87^{b} ±2.73	120.83^{d} ±6.41	<0.001
VEGF pg/mg	8.65^{e} ±0.01	90.45^{a} ±6.21	42.60^{b} ±0.49	33.13^{c} ±2.04	20.91^{d} ±1.10	<0.001
AFP ng/mg	1.39^{e} ±0.09	17.79^{a} ±1.02	11.97^{b} ±0.26	9.30^{c} ±0.43	5.68^{d} ±0.22	<0.001

Values within the same row carrying different superscript letters are significantly different at P < 0.01. Values represent mean ± SE

Table 3: Effect of treatment of AgNPs and/or cisplatin and their combination on lesions scoring and quantitative assessment for the immunoexpression of the Bcl2 of rat liver

Lesions	Group 1	Group 2	Group 3	Group 4	Group 5
Thickening of the hepatic capsule	0	3	2	2	0
Macrosteatosis	0	3	2	2	0
Mononuclear cells infiltration	0	3	2	3	1
Portal congestion and haemorrhage	0	3	3	1	0
Karyomegaly of hepatocytic nucleui	0	3	3	2	0
Cytoplasmic vacuolation of hepatocyte	0	3	2	1	1
Cellular atypia	0	0	3	2	0
Cellular vaculation	0	3	2	1	1
Fibroplasia	0	1	1	3	0
Immunoreactivity of Bcl–2	***	*	**	**	**

Absent (0) mild (1) moderate (2) sever (3)
Mild expression (*) moderate expression (**) strong expression (***)

Histopathological examination

The normal control revealed normal histological structure, in which the hepatocytes orderly were arranged in normal lobular architecture with central veins and radiating hepatic cords, moreover, the portal triads showed normal histological structure (normal branches of the hepatic artery, portal vein, bile duct, and portal area) (Fig. 4A). Group 2 (HCC induced group) showing atypical mitosis with abundant accumulation of fibroplasia with inflammatory cells infiltration around hepatic lobules (Fig. 4B), with numerous mitotic figures with deep esinoohilic cytoplasm and focal aggregation of mononuclear cells infiltration (Fig. 4C) Liver from group 3 showing mononuclear cells infiltration in the portal area, cellular atypia and cellular vacuolation (Fig. 4D). While in group 4 (cisplatin group) showing abundant accumulation of fibroplasia with mononuclear cells infiltration around hepatic lobules (Fig. 4F). The Liver tissue from group 5 showing mild vacuolation of the hepatic parenchyma with few mononuclear infiltrations (Fig. 4F).

Immunohistochemical analysis of Bcl2 in hepatic tissue

Immunolabelling of Bcl-2 in the liver tissue of the negative control group had a strong expression of Bcl-2 in the hepatic parenchyma (Fig. 5A). While In group 2 (HCC induced group) showing + of Bcl-2 expression (Fig. 5B, C). The rats of group 3 had ++ of Bcl-2 expression (Fig. 5D). Group 4 possessed ++ of Bcl-2 expression (Fig. 5E). In liver sections from group 5, the reaction of Bcl-2 was ++ expression (Fig. 4F).

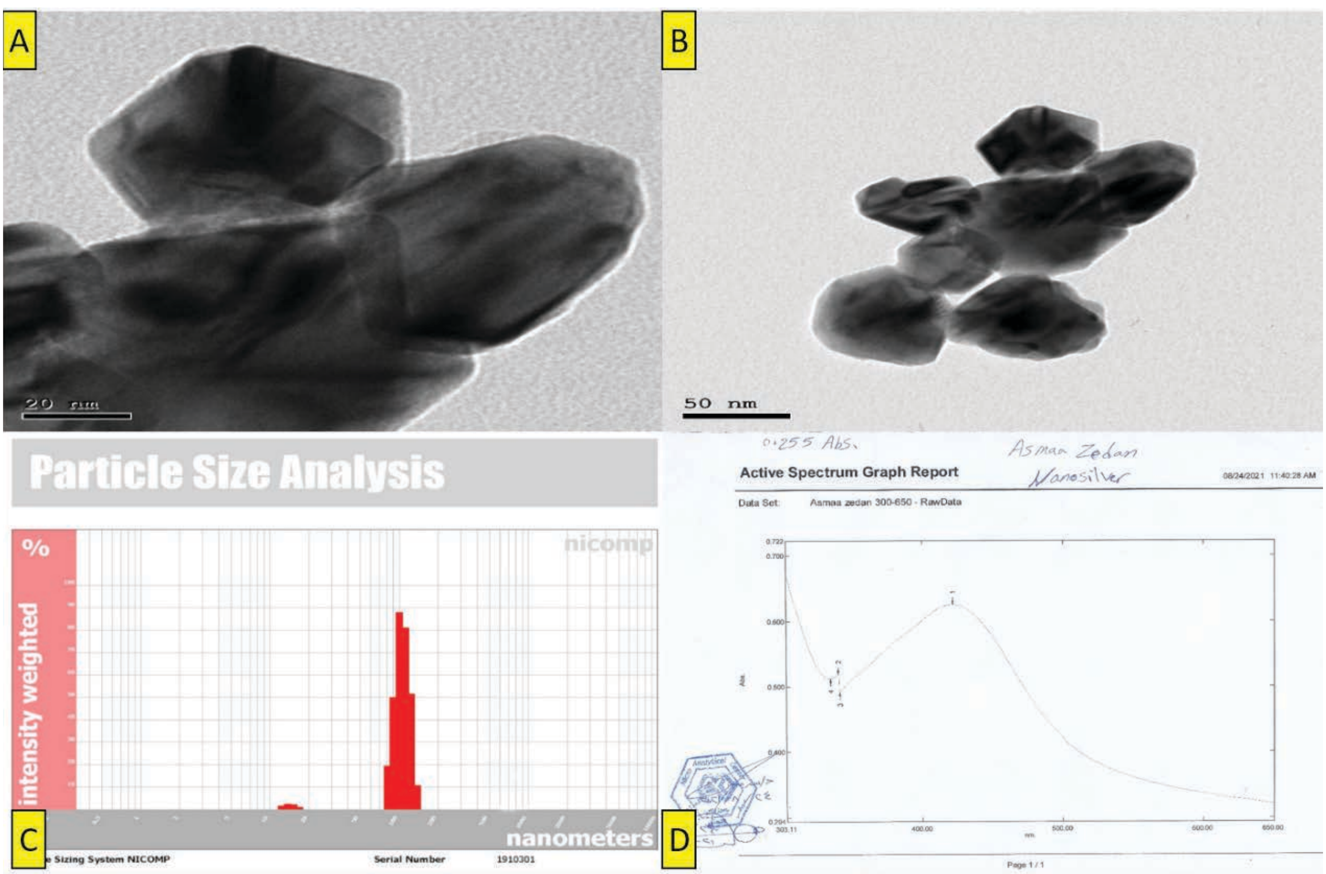

Figure 1: Characterization of the prepared silver nanoparticles; TEM image (A, B), DLS image (C), and UV (D)

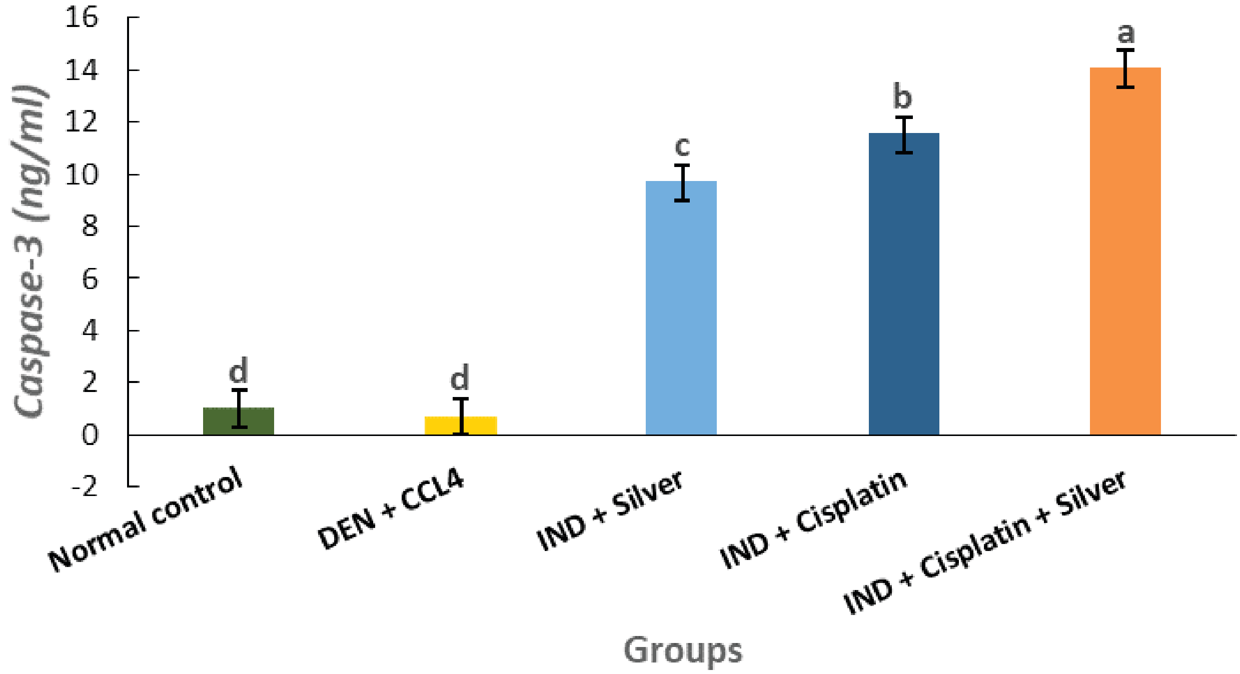

Figure 2: The changes in serum Caspase-3 level in different experimental groups. Columns carrying different letters are significantly different

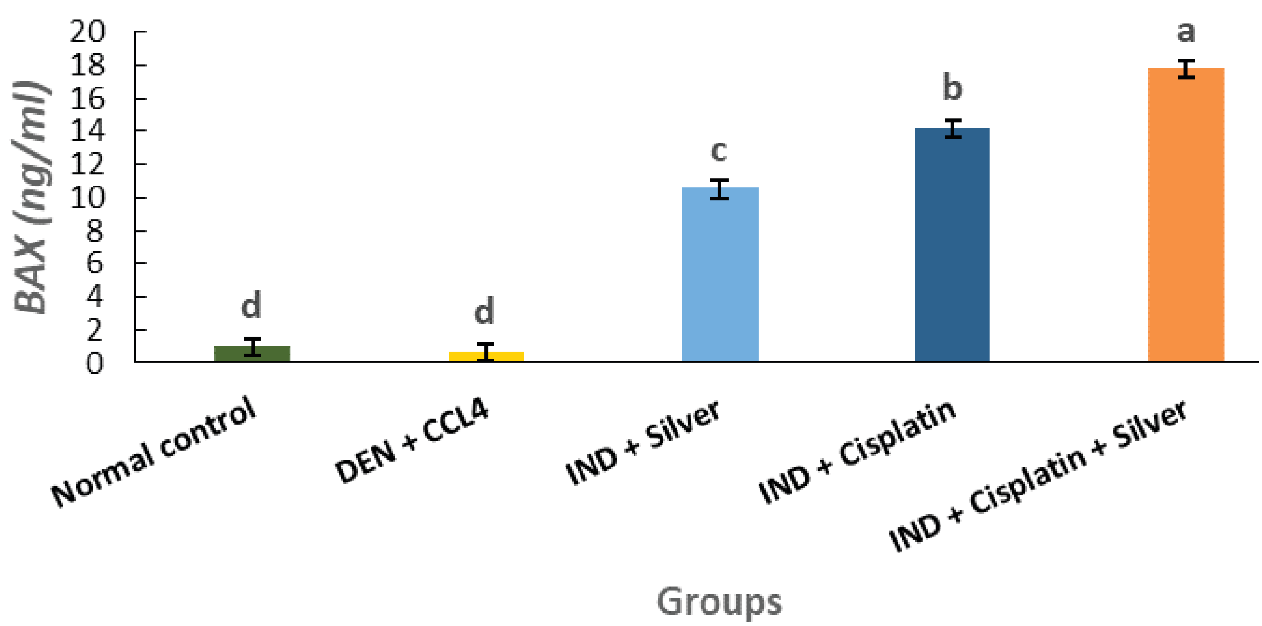

Figure 3: The changes in serum BAX level in different experimental groups. Columns carrying different letters are significantly different

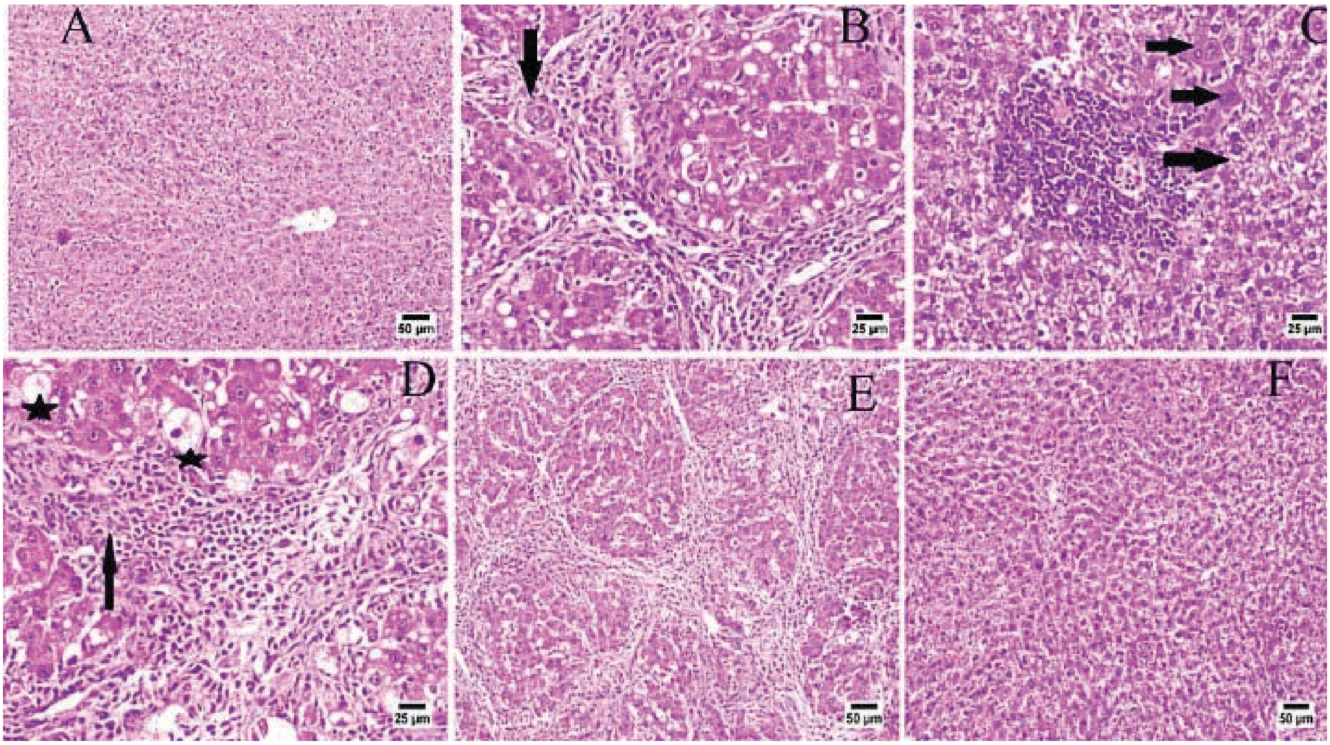

Figure 4: Photomicrographs of liver tissue. (A) normal histological structure, in which the hepatocytes are orderly arranged in normal lobular architecture with central veins and radiating hepatic cords, moreover, the portal triads showed normal histological structure (normal branches of the hepatic artery, portal vein, bile duct, and portal area) in the normal negative control. (B) atypical mitosis (arrow) with abundant accumulation of fibroplasia with inflammatory cells infiltration around hepatic lobules, (C) with numerous mitotic figures with deep esinoohilic cytoplasm (arrow) and focal aggregation of mononuclear cells infiltration of hepatocyte (C) Portal congestion and hemorrhage (arrow) and karyomegaly of hepatocytic nuclei (star) in addition to cytoplasmic vacuolation of hepatocyte (zigzag arrow) in group 2. (D) Mononuclear cells infiltration in the portal area (arrow), cellular atypia (zigzag arrow), and cellular vacuolation in group 3. (E) Abundant accumulation of fibroplasia with mononuclear cells infiltration around hepatic lobules in group 4. (F) Mild vacuolation of the hepatic parenchyma with few mononuclear infiltrations in group 5

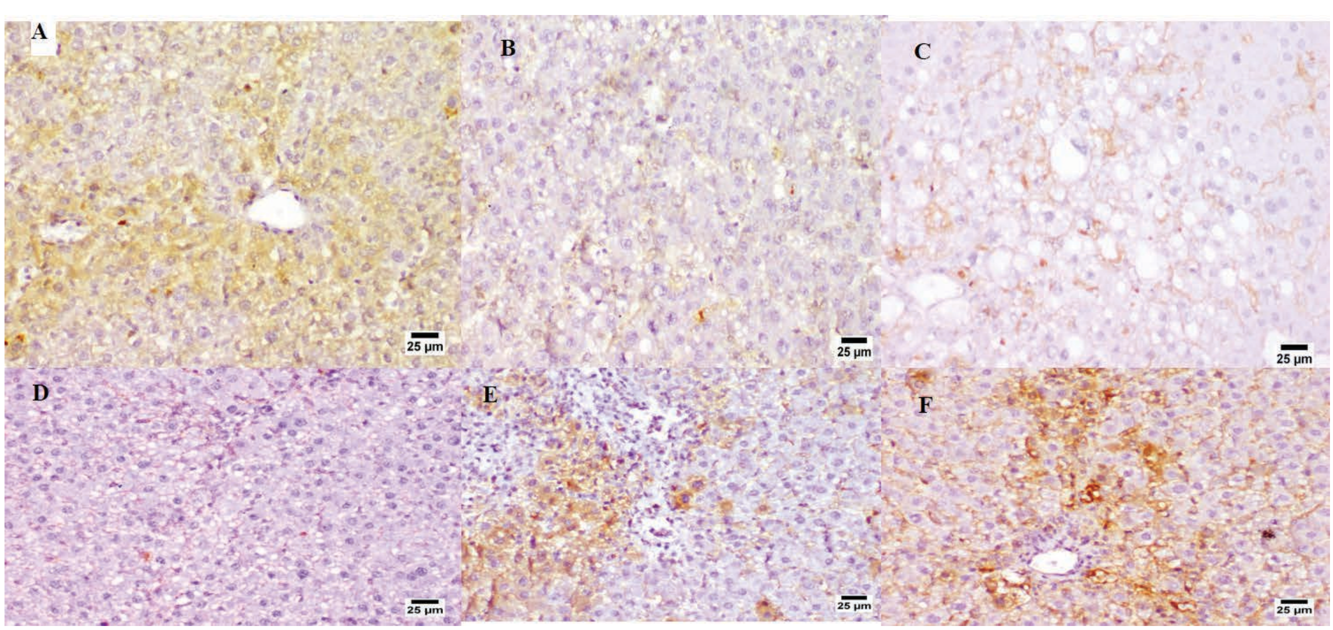

Figure 5: Photomicrograph of liver showing the immunoreactivity of Bcl–2, (A) Immunolabelling of Bcl-2 in liver tissue of negative control group showing a strong expression of Bcl-2 in the hepatic parenchyma. (B &C) group 2 (HCC induced group) showing + of Bcl-2 expression. (D)The rats of group 3 revealed + + of Bcl-2 expression. (E) The liver of rats treated with cisplatin (group 4) demonstrated a moderate positive cytoplasmic labeling of Bcl-2. (F) In liver sections from group 5, the reaction of Bcl–2 was ++ expression

Discussion

Regarding the hematological parameters changes in group 2 which exhibited a significant decline in erythrocyte count, Hb level, PCV concentration (macrocytic hypochromic anemia), and the thrombocyte count (thrombocytopenia) with leukocytosis and these results might be attributed to hemolysis and hemoglobinopathy (26). In the AgNPs-treated group, the hematological studies showed a significant decrease in red blood cell count, Hb level, PCV concentration (normocytic normochromic anemia), and thrombocyte count (thrombocytopenia). Moreover, leukocytosis had been observed, these alterations in the blood picture may be attributed to the effect of NPS on hemoglobin syntheses (during red blood cells maturation in bone marrow) (27) or suppression of circulating hormones such as erythropoietin hormone (28) resulting in normochromic normocytic anemia (29). Leukocytosis might be due to a toxic or allergic reaction against drugs or chemicals, as white blood cells represent the first line of defense, as well as it has an important role in the immunity of the body (30). The erythrogram and leukogram in rats of group 4 (cisplatin-treated group) revealed a significant decrease in the RBCs count, Hb level, and PCV concentration with the development of normocytic normochromic anemia when compared with normal control this may be attributed to the nephrotoxicity occurred by cisplatin resulting in a negative effect on erythropoietin hormone, which leads to reduction of erythropoiesis (31). The reduction in the platelet count is possibly owing to inhibiting bone marrow activity by cisplatin or due to decreased production or increased consumption of platelets (32). Group 5 (AgNps + cisplatin-treated group) revealed a significant decrease in erythrocyte count, Hb level, PCV concentration (normocytic normochromic anemia), and thrombocyte count. This might be attributed to the effect of nanoparticles on hemoglobin syntheses (27) and the suppression effect of cisplatin on hematopoietic tissues leads to impaired erythropoiesis by inhibition of the production of renal erythropoietin, which may be attributed to the same causes previously mentioned in both in groups 3 and 4.

Regarding the biochemical results, the liver function revealed a significant decline in total protein, albumin, and globulin levels with a significant increase in total bilirubin level, ALT, and AST activities in the HCC-induced group. Such results were attributed to the effect of DEN/CCl4 which induces liver dysfunction (19), moreover, DEN produced hepatic injury which is related to the disturbance in hepatocytes membrane instability and metabolism resulting in alterations in liver function (33). Such results were supported by the histopathological finding which revealed atypical mitosis, abundant accumulation of fibroplasia, inflammatory cells infiltration around hepatic lobules, numerous mitotic figures, deep esinoohilic cytoplasm and focal aggregation of mononuclear cells infiltration. n group 3, there was a significant decrease in total protein, albumin, and globulin levels. By contrast, the total bilirubin level, ALT, and AST activities are elevated, these alterations may denote stress on the liver imposed by the nanoparticles (34). But these results showed a significant improvement when compared with the HCC-induced group. Such results were supported by the histopathological findings which revealed mononuclear cell infiltration in the portal area, cellular atypia, and cellular vacuolation. In the cisplatin-treated group, the biochemical results revealed a significant decrease in total protein, albumin, and globulin levels with a significant increase in total bilirubin level, ALT, and AST activities. This result may reflect the metabolism alteration that resulted in liver malfunction (19). However the previous liver function showed significant improvement when compared with the HCC group and this is attributed to the significant reduction of HCC by cisplatin (35), this is evidenced by the enhancement of the histopathological finding which showed abundant accumulation of fibroplasia with mononuclear cells infiltration around hepatic lobules. The rats in group 5 showed significant improvement in the biochemical parameters (total protein, albumin, globulin levels, total bilirubin level, ALT, and AST activities) when compared with the HCC group (group 2), but did not reach the normal levels. Such enhancement is attributed to the effect of AgNPs and cisplatin in the treatment of HCC and this is supported by the histopathological findings which showed mild vacuolation of the hepatic parenchyma with few mononuclear infiltrations.

Regarding the changes in kidney function tests (serum urea and creatinine levels) in group 2. It showed a significant increase in the normal group, this may be attributed to alterations in the renal tissue induced by DEN (36).

In rats of group 2, AgNPs significantly reduced the levels of urea and creatinine than the HCC group but are still higher than normal and this improvement may be due to the protective action of AgNPs against alterations in the renal tissue induced by DEN (36).

The cisplatin-treated group showed a significant increase in urea and creatinine levels, these elevations might be due to the nephrotoxicity induced by cisplatin through induction of oxidative stress resulting in elevation of ROS, lipid peroxidation, and reduction of the antioxidant defense system (37, 38). Rats in group 5 revealed a significant improvement in kidney function (serum urea and creatinine level) when compared with group 2, this improvement owing to the protective effect of synthesized AgNPs against the side effect of cisplatin on renal tissue during HCC treatment, as cisplatin has a nephrotoxic effect (39). But it was still elevated than normal.

Regarding antioxidant activities, group 2 showed a significant decrease in GSH and SOD levels, this may be attributed to the oxidative toxic effect of DEN/CCl_4 which increases MDA level (40). GSH and SOD are critically needed for the scavenging of MDA (19). Also, hepatic dysfunction induced disturbances in the antioxidant defense systems and increases the reactive oxygen species (ROS) (41) which leads to increased oxidation of thiol groups of GSH to overcome the increased level of ROS (42). In group 3 there was a significant increase in both GSH and SOD levels in the HCC group (group 2), but still less than the normal control level, this improvement in GSH and SOD levels was explained by (43) who reported an enhancement in GSH and SOD levels, which probably protect the cells from functional damage (44). Moreover, in group 4, the improvement in anti-oxidant activity might be attributed to the anti-cancer and potential inhibitory effect of cisplatin on DEN and CCL4 which induced intracellular oxidative stress. AgNPs and cisplatin in group 5 had a repair effect in remodeling the oxidative stress induced by DEN and CCL4 (45, 46). In addition to the raised levels of GSH, and SOD, AgNPs could induce apoptosis in different types of cancers (47).

TNF-α and IL-6 (proinflammatory cytokines) revealed a significant increase ($P<0.001$) in group 2 owing to the effect of DEN and CCL4, which induced the expression of pro-inflammatory markers under the direct transcriptional directive of NF-κB. (48). Group 3 revealed a significant improvement compared to group 2 in the levels of TNF-α and IL-6 but did not reach the normal level, AgNPs reduced the activity of the TNFR1/NF-KB transcriptional pathway, resulting in a reduction in the TNFα- and suppress IL-6 (49). In group 4 (cisplatin-treated) there was a significant elevation in TNF-α, and IL-6 levels, cisplatin causes cellular damage and forms a platinum-based DNA adduct (Pt-DNA), which activates the p38 mitogen-activated protein kinase (MAPK) pathway and the inflammatory pathway (50) however it had lesser effects when compared with HCC group, in our opinion, this is attributed to the anticancer effect of cisplatin. Group 5 (AgNPs and cisplatin-treated) revealed a significant improvement compared to groups 2, 3 & 4 in the levels of TNF-α and IL-6, but did not reach the normal level, such improvement is resulting from the anti-inflammatory effects of Ag-NPs which had a strong inhibitory effect on Th1cells production which secrete TNF- α, IL-1β and INF-γ that involved in chronic inflammatory disorders and cellular immunity (51).

Regarding HCC markers, both AFP and VEGF showed significant elevations in the HCC group such elevation is owing to the hepatic damage and development of HCC (19, 52) and the carcinogenic effect of DEN and CCL4 (53). The previous parameter levels were reduced in groups (3, 4 & 5) than HCC inducted group but still raised than the normal control, this improvement in results may be owing to the apoptotic effect of AgNPs (54) and cisplatin (55). The apoptotic effect of AgNPs was clarified through the activation of caspase-3 and the ability of AgNPs to inhibit VEGF (56) as shown in group 3. Moreover, the downregulation of VEGF level in group 4 may be attributed to the suppressing effect of cisplatin on cell proliferation and inhibition of the angiogenesis-associated proteins (57), this is supported by our result (increasing Casp-3 and BAX levels), so cisplatin induces apoptosis and down-regulated anti-apoptotic BCL-2 gene (40), in the same trend combined group AgNPs and cisplatin-treated group revealed much improvement in the AFP and VEGF levels and also increased both Casp-3 and BAX. Such results were supported by the immunohistochemical analysis of Bcl2 in hepatic tissue, which showed strong expression of Bcl-2 in the hepatic parenchyma. While HCC induced group showed a slight expression of Bcl-2. The rats of groups 3 and 4, and 5 illustrated moderate Bcl-2 expressions.

Conclusion

AgNPs have anti-inflammatory, anti-angiogenic, and apoptotic properties so they are more recommended as a cytotoxic therapy for HCC. Despite cisplatin being the most commonly used chemotherapy now, it has side effects on hematology and biochemical assay, as well as, apoptotic and antioxidant status in HCC animals. To overcome the side effect of cisplatin, a combination of AgNPs and cisplatin (at a lower dose) enhanced the therapeutic effect on HCC with improved hematology and biochemistry, as well as, apoptotic and antioxidant status in HCC animals.

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