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The Curative Effect of Berberine Nanoparticles and Cisplatin Combination Therapies Against Hepatocarcinogenesis-Induced By *N*-Nitroso-Diethylamine In Male Rat

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Abstract

This study aimed to investigate whether berberine nanoparticles (BBR-NPs) and/or cisplatin supplementation could prevent hepatocarcinogenesis-induced by N-nitroso-diethylamine (DENA) in male rats. Male *Wistar* albino rats were divided into five groups; Group 1: Control; Group 2: DENA-CCl_4; Group 3: DENA-CCl_4+Cisplatin; Group 4: DENA-CCl_4+BBR-NPs; Group 5: DENA-CCl_4+Cisplatin+BBR-NPs. DENA-CCl_4 significantly increase AST, ALT, ALP, LDH, GGT, AFP activities and total bilirubin, while, 5′ NT, total protein and albumin decreased. DENA-CCl_4 treatment caused increment in MDA levels and reduction in SOD, CAT, GPx and GSH in liver tissues. Moreover, DENA-CCl_4 increase the gene expression of ADAM17 and TNF-α however, P53 was declined. In addition, DENA-CCl_4 caused severe histopathological lesions in the liver tissue. Interestingly, administration of berberine nanoparticles alone or in combination with cisplatin improves the hepatocarcinogenesis induced by DENA-CCl_4 on the physiological, biochemical, molecular and histological levels by decreasing oxidative stress and preserving gene expression of ADAM17, TNF-α and P53. The present findings suggest that BBR-NPs with cisplatin might offer a promising strategy for the prevention of liver cancer.

Keywords: Berberine Nanoparticles, Cisplatin, N-Nitrosodiethylamine, Liver, Rats

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1. INTRODUCTION:

On top of surgery and radiotherapy, the use of chemotherapy has increased along with a supporting treatment [1] as it is highly toxic and most of the patients suffer from its adverse effects [2].

Cisplatin is one of the best-selling and potent anticancer drugs [3]. Different clinical uses of cisplatin, include sarcoma, cancers of soft tissue, bones, muscles and blood vessels [4], however, its use is limited due to its side effects on other organs of the body [5].

Various approaches have been proposed to cope with the side effects associated with chemotherapy drug treatment. Nanotechnology is an interdisciplinary research field developed with chemistry, engineering, biology, and medicine, and has various useful applications in cancer biology, such as early detection of tumors, discovery of cancer biomarkers, and development of novel treatments [6]. Its application in medicine includes drug delivery, both *in vitro* and *in vivo* diagnostics [7].

Berberine (BBR) (*Berberis vulgaris*) is an isoquinoline alkaloid, which could be found in the rhizome, root and stem bark of several plant species [8]. Berberine has antioxidant [9], anti-inflammatory, antimicrobial, antipyretic [10] and immune-regulative activities [11].

Novel berberine nanoparticles (BBR-NPs) were successfully synthesized by the ionic cross-linking method. BBR-NPs were spherical and homogeneous in shape. Moreover, they exhibit good stability and had an ideal releasing profile *in vitro* [12].

This study aimed to investigate whether berberine nanoparticles and/or cisplatin supplementation could prevent hepatocarcinogenesis-induced by N-nitroso-diethylamine (DENA) in male rats.

2. MATERIALS AND METHODS

2.1. Chemicals

N-Nitroso-Diethylamine (DENA) and Carbon Tetrachloride (CCl_4) were purchased from Sigma chemical company (St, Louis, Mo, USA). Berberine (BBR) and Chitosan (CS) were purchased from the Swanson Company and Oxford lab Chem. India, respectively. While, Cisplatin was purchased from Mylan Company.

2.2. Experimental animals

Fifty healthy, male *Wistar* albino rats (150±10g) were obtained from the animal house of Medical Research Institute, Alexandria University, Egypt. The animals were housed in metal cages (40×25×30 cm). The experimental animals were allowed to acclimate under laboratory conditions (22-25°C, 12h light/dark cycle and relative humidity) for at least two weeks prior to the experiment. They were kept on a standard balanced laboratory diet and tap water *ad libitum.* All animal procedures and the experimental protocols were carried out according to the guidelines of the National Institutes of Health (NIH).

2.3. Experimental design

The rats were divided into five groups, each of 10 rats as follows:

Group I: Control; rats were treated orally with olive oil for 60 days and considered as a control group.

Group II: DENA-CCl_4; rats were treated intraperitoneal with the DENA (200 mg/kg) as a single dose and followed by CCl_4 (1.5 ml/kg/day) for 60 days [13].

Group III: DENA-CCl_4+Cisplatin; rats were treated intraperitoneal with the DENA (200 mg/kg) as a single dose followed by carbon tetrachloride CCl_4 (1.5 ml/kg/day) for 30 days, then they were treated intraperitoneal with cisplatin (8 mg/kg/week) for 30 days [14].

Group IV: DENA-CCl_4+BBR-NPs; rats were treated intraperitoneal with the DENA (200 mg/kg) as a single dose and followed by CCl_4 (1.5 ml/kg/day) for 30 days, then they were orally treated with berberine nanoparticles (BBR-NPs) (1mg/kg/day) for 30 days [15].

Group V: DENA-CCl_4+Cisplatin+BBR-NPs; rats were treated intraperitoneal with the DENA (200 mg/kg) as a single dose followed by CCl_4 (1.5 ml/kg/day) for 30 days, then they were treated intraperitoneal with cisplatin (8 mg/kg/week) and orally treated with berberine nanoparticles (BBR-NPs) (1mg/kg/day) for 30 days.

2.4. Preparation of berberine nanoparticles (BBR-NPs)

Nanoparticles were formed suddenly upon incorporation of 6 ml of tripoly phosphate, aqueous solution (0.5 mg/ml) to 15 ml of the chitosan (CS) acidic solution 0.5 mg/ml containing a concentration of berberine 1mg/ml under magnetic stirring for 15 minutes. The zeta potential of drug chitosan-loaded berberine nanoparticles was measured by zetasizer (Malvern Zetasizer 3000HS, Germany). The zeta potential was determined by adding nanoparticle samples in electrophoretic cell where an electrical field of 15.2 V/cm was applied. The particle size was measured by dynamic light scattering (DLS). Polydispersity index (PDI), a measure of the distribution of molecular mass in a given polymer sample, was measured by dynamic light scattering (DLS). The shape of the berberine nanoparticles (BBR-NPs) is determined by using transmission electron microscope.

2.5. Preparation of serum and tissue homogenates for biochemical studies

After 60 days of the experiment, animals were sacrificed and blood samples were allowed to clot by centrifugation at 3000 g for 5 minutes. The serum was separated and stored at -20°C until biochemical parameters assay.

The liver tissues were quickly removed, washed with saline and cut into pieces. One gram of liver was homogenized with 9 volumes of phosphate buffer (0.1M, pH 7.9) and then centrifuged at 10,000 g for 20 min and the supernatant was saved to be used for determination of oxidative stress markers and antioxidant enzyme activities.

2.6. Preparation of tissue sample for light and electron microscopical studies

The right lobe of liver from control and treated groups was excised and divided into 2 portions. The first portion allowed to fix at room temperature overnight in 10% formalin solution, then processed to be stained routinely with Haematoxylin and Eosin [16].

The second portion of the liver right lobe was fixed by immersing them immediately in F1G4-formalin/glutaraldehyde fixative (pH 7.2) at 4°C for 3 hours, washed in 0.1M phosphate buffer, post fixed for 1h in 0.1M phosphate buffer and 1% osmium tetroxide (at room temperature) then washed in buffer for several times. After fixation, the tissues were dehydrated through a graded ethanol series and then the infiltration was carried out using a series of propylene oxide and Epon mixture. Embedding was carried out in an oven adjusted at 58ºC using Araldite-Epon mixture. Semi thin sections (1µm) were cut with a glass knife on LKB Ultramicrotone and examined by light microscope after being stained with 1% Toluidine blue. The ultrathin (50 nm) sections of selected area were picked up on 200 mesh naked copper grid. After being double stained with uranyl acetate and lead citrate, the sections were examined by using Joel 100 CX transmission electron microscope at the Faculty of Science, Alexandria University.

2.7. Determination of biochemical parameters

Alanine aminotransferase (ALT: E.C. 2.6.1.2) [17] and aspartate aminotransferase (AST: E.C. 2.6.1.1) [18] were estimated. Alkaline phosphatase (ALP: E.C. 3.1.3.1) and 5-nucleotidase (5-NT) were determined according to Wan *et al.* [19] and Heppel & Hilmoe [20], respectively. While, lactate dehydrogenase (LDH: E.C. 1.1.1.27) gamma-glutamyl transpeptidase (GGT: E.C. 2.3.2.2) and total bilirubin were estimated according to Young & Friedman [21] method. Total protein (TP) concentration [22] and albumin (ALB) [17] were determined. Bates [23] method was applied for the determination of alfa-fetoprotein (AFP). Superoxide dismutase (SOD, E.C. 1.15.1.1) was assayed by the method of Marklund and Marklund [24]. Catalase (CAT, E.C. 1.11.1.6) and glutathione peroxidase (GPx, E.C. 1.11.1.9) activities were estimated by the method of Aebi [25] and Paglia & Valentine [26], respectively. Reduced glutathione (GSH) [27] and lipid peroxidation (MDA) [28] were also determined.

2.8. Total RNA isolation and PCR analysis:

Liver tissues were quickly removed, washed with saline and cut into pieces and stored at

-80ºC until used for molecular studies. The total RNA was isolated from the frozen liver of different experimental groups using the phenol/guanidine-based Isol-RNA Lysis Reagent™ (5 PRIME GmbH, D-22767, and Hamburg). The isolated RNA from control liver tissues and the other experimental groups were reverse transcribed into cDNA using reverse transcriptase. The resulting cDNA was used as templates for subsequent PCR amplification using specific primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control, ADAM metallopeptidase domain 17 (ADAM-17), tumor necrosis factor TNF and p53 (Neb New England Biolab) (Table 1). Data analysis was carried out employing the DDCt method. Results were presented as fold difference of ADAM17, TNF-α and p53 mRNA expressed in different experimental liver tissues.

Table (1): Specific gene primer sequences and corresponding PCR conditions

Gene	Primer sequence	Annealing temperature	Product length
GAPDH	Sense, 5, TACCCCACGGCAAGTTCAATGG-3, Antisense,5, AGGGGCGGAGATGATGATGACCC-3,	58.0^{0}C	255
ADAM17	Sense, 5,TAGCAGATGCTGGTCATGTG-3, Antisense,5,TTGCACAC AGGTCAAAG-3,	54.4^{0}C	157
TNF-α	Sense, 5,CAGACCCTCACACTAGATCATCTT-3, Antisense, 5,CAGAGCAATGACTCCAAAGTAGA-3,	58.6^{0}C	250
P53	Sense, 5,CAGACCCTCACACTAGATCATCTT-3, Antisense,5,CAGAGCAATGACTCCAAAGTAGA-3,	46.0^{0}C	70

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2.9. Statistical analysis

All data are expressed as Mean±SD. Statistical evaluation was conducted by one-way ANOVA. A probability level of P≤0.05 was selected as indicating statistical significance. Most of the changes between various groups were compared by Duncan.

3. RESULTS

3.1. Characterization of berberine nanoparticles (BBR-NPs)

The particle size, polydispersity index and zeta potential were 370 nm, 0.425, and 4.99 mV, respectively (Figure 1, 2 & 3). The berberine nanoparticles (BBR-NPs) showed homogeneous spherical shapes under transmission electron microscope (TEM), having more or less uniform size distribution with particle size in the range of 30–40 nm.

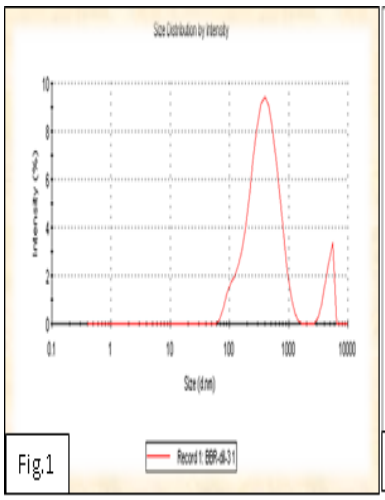

Fig.1

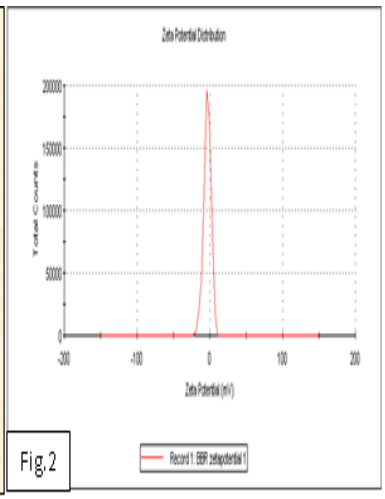

Fig.2

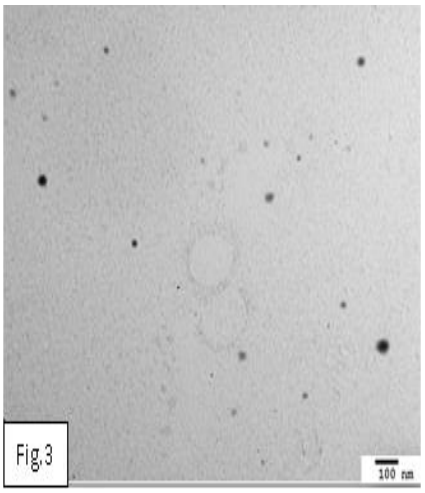

Fig.3

Fig. (1): Size distribution of berberine nanoparticles (BBR-NPs). **Fig. (2):** Zeta potential of berberine nanoparticles (BBR-NPs). **Fig. (3):** Transmission electron microscopy (TEM) of berberine nanoparticles (BBR-NPs).

3.2. Assessment of hematological parameters in the different studied groups

The derived data in table 2 showed a significant (*P≤0.05*) decrease in the RBCs, Hb, Hct and platelet counts and a significant (*P≤0.05*) increase in WBC counts in DENA-CCl_4 and cisplatin groups as compared to the control ones (Table 2). In comparison to the carcinogenic group, BBR-NPs and their combination with cisplatin showed a significant increase (*P≤0.05*) in the values of RBCs, Hb, Hct and platelet counts and a significant (*P≤0.05*) decrease in WBC counts as compared to DENA-CCl_4 group. The MCV and MCHC were insignificantly (*P≤0.05*) decreased. It was obvious that the best results in improving the hematological parameters were obtained in the combination group (DENA-CCl_4+Cisplatin+BBR-NPs).

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Table (2): Hematological parameters in the different studied groups

Parameter	Control	DENA-CCl_4	DENA-CCl_4 +Cisplatin	DENA-CCl_4 +BBR-NPs	DENA-CCl_4 +Cisplatin+BBR-NPs
RBCs ($X10^6$cell/µL)	7.079 ±0.617 [a]	4.526 ±0.516 [b]	4.564 ±0.481 [b]	6.205 ±0.678 [c]	6.120 ± 0.614 [cd]
Hb (g/dl)	15.080 ±1.964 [a]	12.570 ±0.777 [b]	12.542 ±1.471 [b]	14.250 ±2.880 [c]	14.900 ±1.751 [cd]
Ht (%)	44.578 ±4.180 [a]	34.884 ±2.767 [b]	42.360 ±4.646 [b]	42.360[c] ±2.238	43.880 ±0.943 [cd]
Platelets ($x10^3$/µL)	396.501 ±4.997 [a]	289.400 ±0.273 [b]	287.000 ±0.455 [b]	344.600 ±0.315 [c]	361.202 ±0.767 [cd]
MCV (fL)	88.950 ±2.921 [a]	85.503 ±3.109 [a]	86.875 ±4.661 [a]	87.950 ±3.7969 [a]	88.425 ±4.257 [a]
MCHC (gm/dl)	32.951 ±2.014 [a]	33.021 ±1.080 [a]	32.250 ±1.281 [a]	34.175 ±1.408 [a]	32.704 ±1.213 [a]
WBCs ($X10^3$cell/UL)	7.912 ±.228 [a]	13.202 ±1.169 [b]	12.1875 ±.311 [b]	13.725 ±1.088 [c]	9.237 ±0.723 [cd]

-Values are expressed as mean±standard deviation; (n=10).

- In the same raw, different letters indicate statistically significant differences at $p \leq 0.05$.

-The letters a, b, c, d are called Duncan letters and are used to detect the least significant difference (LSD) in ANOVA study.

3.3. Assessment of liver enzymes in the different studied groups

The injection of DENA-CCl_4 and cisplatin significantly raised ($P \leq 0.05$) liver enzyme activities (AST, ALT, ALP, LDH, GGT and 5^-NT) in respect to the control. BBR-NPs and their combination with cisplatin significantly ($P \leq 0.05$) decreased the activity of these enzymes when compared to DENA-CCl_4 group. The present results clearly demonstrated that treatment of DENA-CCl_4+Cisplatin+BBR-NPs have the best effect in reducing the elevated levels of liver enzyme.

Table (3): Serum levels of ALT, AST, ALP, LDH, GGT and 5-NT in the different studied groups.

Parameter	Control	DENA-CCl_4	DENA-CCl4 +Cisplatin	DENA-CCl_4 +BBR-NPs	DENA-CCl_4 +Cisplatin+BBR-NPs
ALT (U/L)	46.91 ±0.656 [a]	74.78 ±0.418 [b]	61.00 ±0.106 [b]	52.08 ±0.165 [c]	47.40 ±0.752 [cd]
AST (U/L)	115.00 ±0.129 [a]	217.42 ±0.255 [b]	237.31 ±0.268 [b]	144.23 ±0.300 [c]	116.20 ±0.432 [cd]
ALP (U/L)	281.25 ±0.342 [a]	868.30 ±0.244 [b]	725.00 ±0.223 [b]	383.90 ±0.291 [c]	322.60 ±0.296 [cd]
LDH (U/L)	113.11 ±0.367 [a]	337.50 ±0.661 [b]	325.25 ±0.320 [b]	201.50 ±0.166 [c]	130.50 ±0.385 [cd]
GGT (U/L)	12.83 ±0.164 [a]	16.14 ±1.908 [b]	15.25 ±3.109 [b]	13.50 ±1.893 [c]	12.75 ±1.258 [cd]
5-NT (ng/ml)	4.93 ±2.232 [a]	11.17 ±2.47 [b]	10. 50 ±4.82 [b]	9.95 ±2.654 [c]	5.44 ±2.317 [cd]

-Values are expressed as mean±standard deviation; (n=10).

- In the same raw, different letters indicate statistically significant differences at $p \leq 0.05$.

-The letters a, b, c, d are called Duncan letters and are used to detect the least significant difference (LSD) in ANOVA study.

3.4. Assessment of TP, ALB, AFP and bilirubin in the different studied groups

Table 4 represented the other indices of liver function included serum total proteins (TP), albumin (ALB), alpha-fetoprotein (AFP) and total bilirubin. The intraperitoneal injection of DENA-CCl_4 and cisplatin led to a significant reduction ($P \leq 0.05$) in TP, ALB and significant elevation in alpha-fetoprotein and bilirubin when compared to the control. On the other hand, BBR-NPs and their combination with cisplatin caused a significant increase in the levels of the TP and ALB while, alfa-fetoprotein and bilirubin were significantly ($P<0.05$) reduced in comparison to DENA-CCl_4 group.

Table (4): Serum levels of TP, ALB, α-fetoprotein and bilirubin in the different studied groups.

Parameter	Control	DENA-CCl_4	DENA-CCl_4 +Cisplatin	DENA-CCl_4 +BBR-NPs	DENA-CCl_4 +Cisplatin+BBR-Ps
TP (gm/dl)	6.907 ±0.230 ^{a}	3.125 ±0.632 ^{b}	4.080 ±0.325 ^{b}	5.060 ±0.655 ^{c}	6.010 ±0.455 ^{cd}
ALB (gm/dl)	3.507 ±0.225 ^{a}	2.012 ±0.376 ^{b}	2.210 ±0.332^{b}	2.860 ±0.868 ^{c}	3.550 ±0.303 ^{cd}
AFP (ng/ml)	8.535 ±4.252 ^{a}	27.310 ±4.340 ^{b}	28.417 ±5.940 ^{b}	11.765 ±4.820 ^{c}	9.905 ±2.317 ^{cd}
T. bilirubin (mg/dl)	0.187 ±0.028 ^{a}	3.582 ±0.122 ^{b}	3.800 ±0.372 ^{b}	1.840 ±0.101 ^{c}	1.000 ±0.111 ^{cd}

-Values are expressed as mean±standard deviation; (n=10).

- In the same raw, different letters indicate statistically significant differences at p≤0.05.

-The letters a, b, c, d are called Duncan letters and are used to detect the least significant difference (LSD) in ANOVA study.

3.5. Assessment of MDA,GSH, SOD, CAT and GPX levels in the different studied groups

The obtained results showed that MDA was significantly (P≤0.05) increased after exposure to DENA-CCl_4 and cisplatin, while, the level of GSH, SOD, CAT and GPX were significantly reduced in comparison to the control. Results presented in Table 5 clearly demonstrated that treatment with BBR-NPs and their combination with cisplatin, caused significant reduction in the elevated MDA and a significant enhancement in GSH, SOD, CAT and GPX (P≤0.05) with respect to DENA-CCl_4 group. It was obvious that the combined group (DENA-CCl_4+Cisplatin+BBR-NPs) had the most curative effect in reducing the elevated level of MDA and increment in the decreased levels of GSH, SOD, CAT and GPX (Table 5).

Table (5): Levels of MDA and GSH in the different studied groups

Parameter	Control	DENA-CCl4	DENA-CCl_4 +Cisplatin	DENA-CCl_4 +BBR-NPs	DENA-CCl_4 +Cisplatin+BBR-Ps
MDA (nmol/gm tissue)	12.060 ±4.252 ^{a}	35.840 ±4.340 ^{b}	30.900 ±4.820 ^{b}	18.330 ±5.940 ^{c}	12.990 ±2.317 ^{cd}
GSH (nmol/gm	11.400	6.522	6.800	9.990	11.270

tissue)	**±0.797 [a]**	**±0.697 [b]**	**±1.250[b]**	**±1.305 [c]**	**±1.095 [cd]**
SOD (U/gm protein)	**31.580 ±6.289 [a]**	**18.410 ±0.398 [b]**	**18.177 ±0.349 [b]**	**24.170 ±0.437 [c]**	**30.627 ±0..358 [cd]**
CAT (U/gm protein)	**8.955 ±0.897 [a]**	**2.900 ±0.697 [b]**	**2.675 ±1.250 [b]**	**6.312 ±1.305 [c]**	**7.615 ±1.095 [cd]**
GPX (nmol/gmtissue)	**2.272 ±0.446 [a]**	**0.980 ±0.469 [b]**	**0.827 ±0.138 [b]**	**1.652 ±0.508 [c]**	**2.177 ±0.160 [cd]**

-Values are expressed as mean±standard deviation; (n=10).

- In the same raw, different letters indicate statistically significant difference at $p \leq 0.05$.

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3.6. Assessment of gene expression for ADAM 17, TNF-α and P53 in the different experimental groups

The results illustrated in Fig. 4 and Table 6 represented that the administration of DENA-CCl_4 increased the relative expression of ADAM17 & TNF-α which associated with down regulation of p53 when compared to control relative gene expression levels. Cisplatin administration down regulated ADAM 17 & P53 expression than those of both DENA-CCl_4 and control groups. The treatment with BBR-NPs and their combination with cisplatin had the most effective one as it down regulated the inflammatory molecule expression ADAM17&TNF-α and upregulated the p53 expression when compared to DENA-CCl_4 group (Table 7) (Fig.4).

Table (7): Relative gene expression of ADAM 17, TNF-α and P53 in the different experimental groups

Gene	Control	DENA-CCl4	DENA-CCl4 +Cisplatin	DENA-CCl4 +BBR-NPs	DENA-CCl4 +Cisplatin+BBR-NPs
ADAM 17 (µg)	**1.318 ±0.102 [a]**	**3.728 ±0.201 [b]**	**0.423 ±0.003 [d]**	**1.112 ±0.091 [c]**	**0.120 ±0.007 [e]**
TNF-α (µg)	**0.841 ±0.073 [a]**	**2.219 ±0.100 [b]**	**1.904 ±0.073 [d]**	**1.700 ±0.050 [c]**	**0.441 ±0.033 [e]**
P53 (µg)	**0.463 ±0.032 [a]**	**0.130 ±0.008 [b]**	**0.141 ±0.005 [b]**	**0.521 ±0.003 [c]**	**0.300 ±0.022 [d]**

- Values are expressed as mean ± standard deviation; (n=10).

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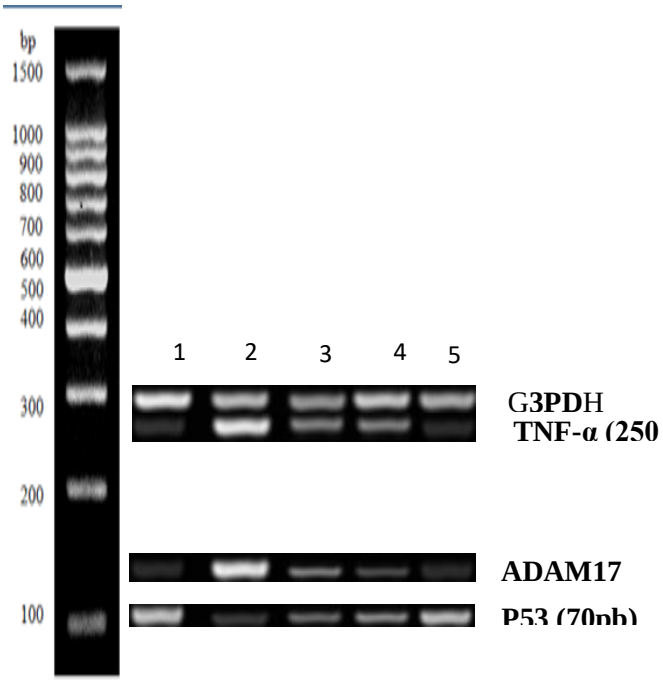

Fig. (4): Gene expression gel electrophoresis image showed gene expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a housekeeping gene, metallopeptidase domain 17(ADAM17), tumor necrosis factor α (TNF-α) and protein53 (P53), amplified by PCR among different experimental groups where lane1: control, lane2: DENA-CCl4, lane3: DENA-CCl_4+cisplatin, lane 4: DENA-CCl_4+BBR-NPs and lane 5: DENA-CCl_4+cisplatin+BBR-NPs.

3.7. Light microscopical examination

Control group (G1): Figs. 5&6 represented the normal hepatic architecture where the hepatocytes arranged in radiating cords extending to the periphery. The cords were separated from each other by blood sinusoids and contain few Kupffer cells.

DENA-CCl4 group (GII): Figs. 7&8 showed clear signs of severe hepatic injury manifested by the loss of normal hepatic architecture with congested portal veins and the proliferation of bile ductule. Most of hepatocytes with manifestations of extensive cytoplasmic vacuolization and hyperchromatic nuclei (Fig. 7). Semithin sections showed ill-defined hepatocytes with irregular nucleus, lipid droplet and fibrous capsule (Fig. 8).

DENA-CCl_4+Cisplatin group (GIII): Figs. 9&10 showed some sign of improvement in the histological structure of intact hepatic cells with some congested sinusoids (Fig. 9). The examination of semithin sections showed hepatocytes with round nuclei and granular cytoplasm, congested sinusoids with hypertrophic Kupffer cells (Fig. 10).

DENA-CCl_4+BBR-NPs group (GIV): Figs. 11&12 showed signs of improvement through hepatocytes arrangement with normal cytoplasm and some normal sinusoids but there is an affected area with collapsed sinusoid and large number of Kupffer cells (Fig. 11). Semithin revealed an affected area in contact with nearly normal one (Fig. 12).

DENA-CCl4+Cisplatin+BBR-NPs group (GV): Figs. 13&14 showed highly improved tissue where cells arranged in normal cords with no cytoplasmic vacuole. Regeneration activity is detected by the presence of a dividing stage as metaphase **(**Figs. 13**).** Semithin sections revealed the normal appearance of well identified polyhedral hepatocyte, oval or rounded nuclei separated by regular blood sinusoids (Fig. 14).

3.8. Electron microscopic examination

Control group (G1): Fig.15 displayed regular oval–shaped heterochromatic nuclei surrounded by double nuclear envelope perforated by nuclear pores. The cytoplasm contains numerous round-shaped mitochondria, parallel flattened cisterna of RER with the presence of a large number of glycogen particles and bile canaculei with long villi.

DENA-CCl_4 group (GII): Fig. 16 showed that the nucleus frequently appeared pale, many lipid droplets of different sizes, glycogen loss, clusters of polymorphic mitochondria with loss of cristae, short profile of RER, presence of peroxisomes and bile canaculei with disrupted villi.

DENA-CCl_4+Cisplatin group (GIII): Fig. 17 showed signs of improvement in certain organelles, including the nucleus where the chromatin is mainly distinguished into electron dense peripheral heterochromatin and dispersed inner euchromatin and nearly normal RER. Numerous oval or elongated mitochondria surrounded by two clear membranes, one of which separated between the mitochondria and the cytoplasm and the inner one thrown into folds project inward in a tubular cristae.

DENA-CCl_4+BBR-NPs group (GIV): The nucleus almost normal in appearance with the presence of peripheral nucleolus, the cytoplasm embodies round or elongated mitochondria, glycogen deposits were readily identifiable, RER were present in different areas of the cytoplasm or bounded to the mitochondria (Fig.18).

DENA-CCl_4+Cisplatin+BBR-NPs group (GV): Fig. 19 showed a highly improved appearance of nucleus with normal chromatin pattern, well organized cytoplasm containing a rich amount of glycogen particles**,** presence of oval shaped mitochondria. The two cell membranes of two adjacent hepatocytes are diverging from each other to form a small bile canaliculus with a number of microvilli protruded from the cell membranes into the lumen of the bile canaliculus.

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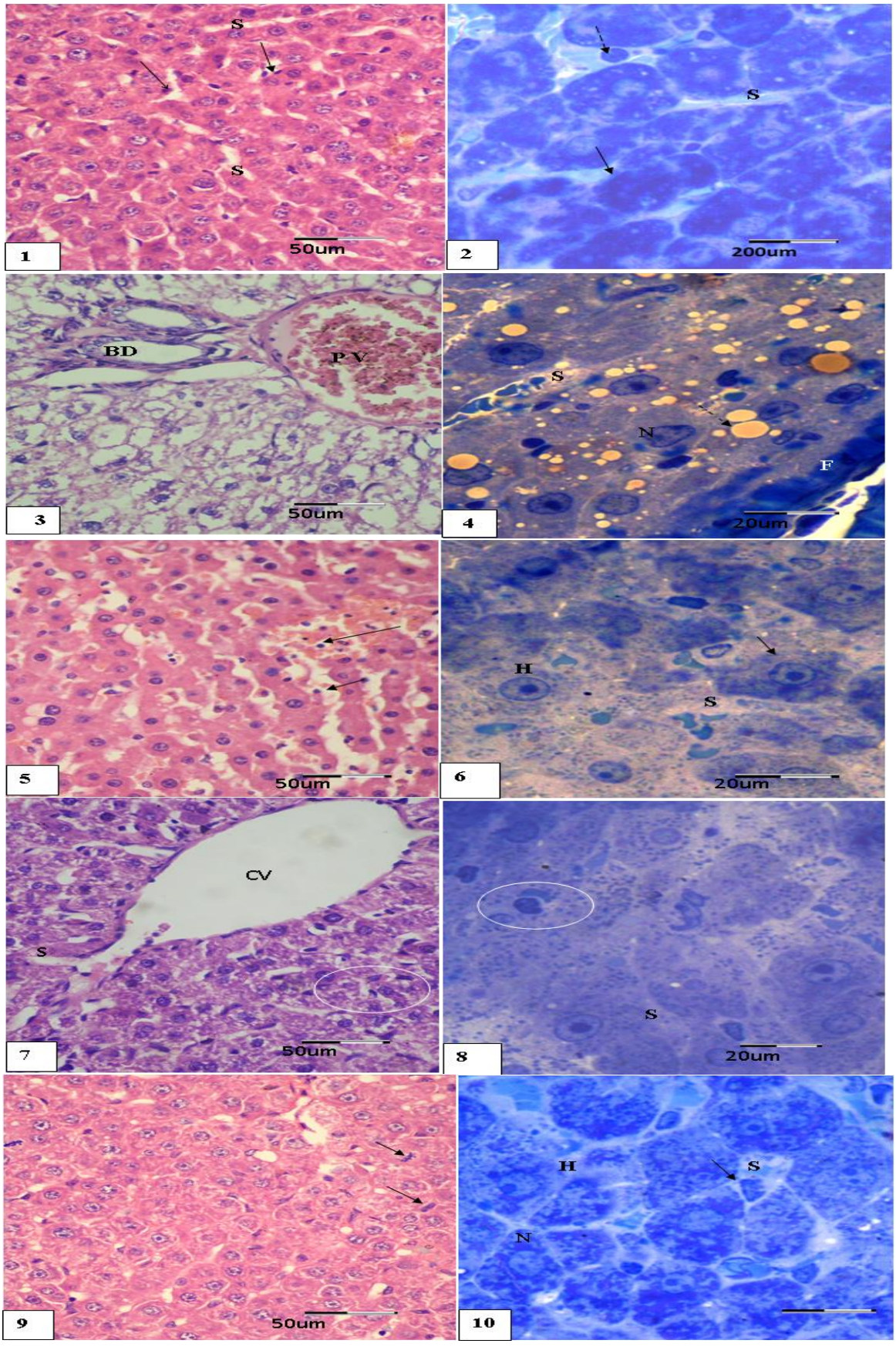

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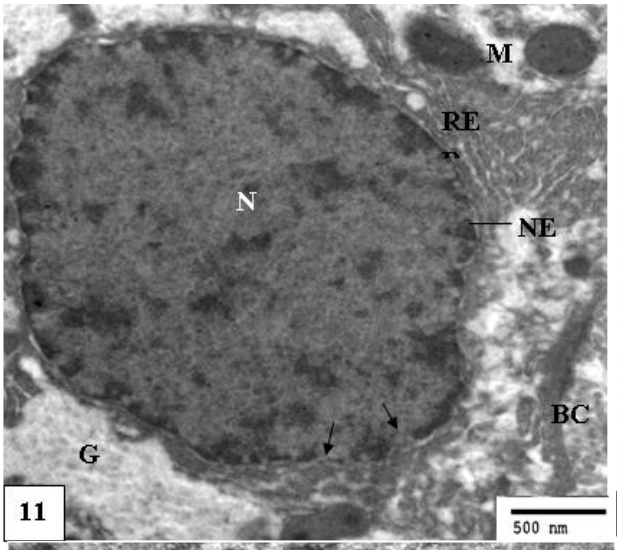

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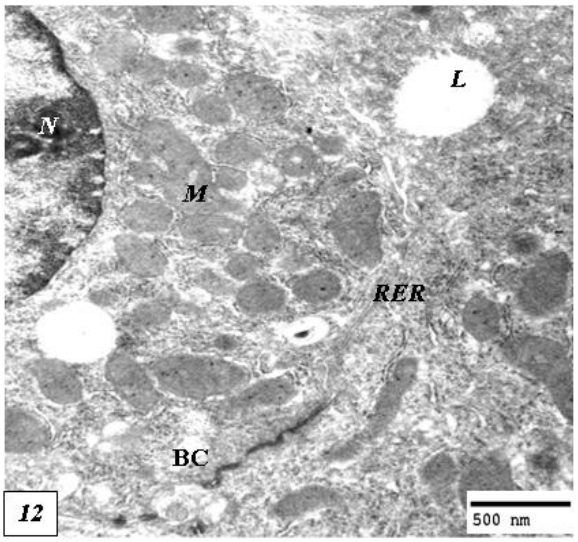

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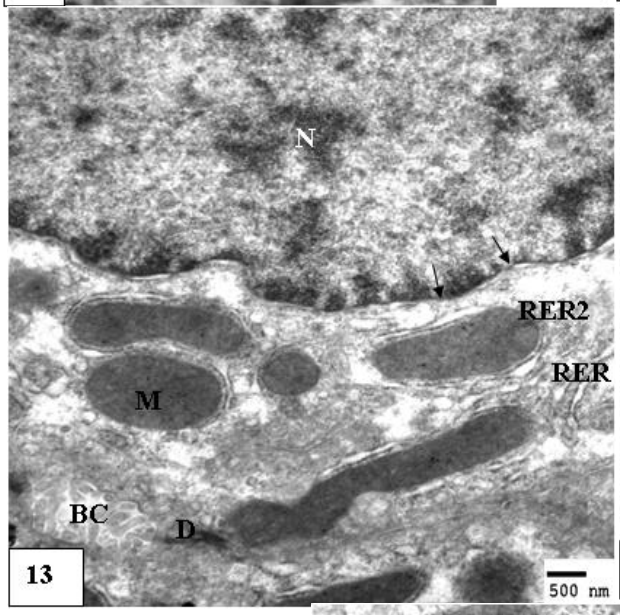

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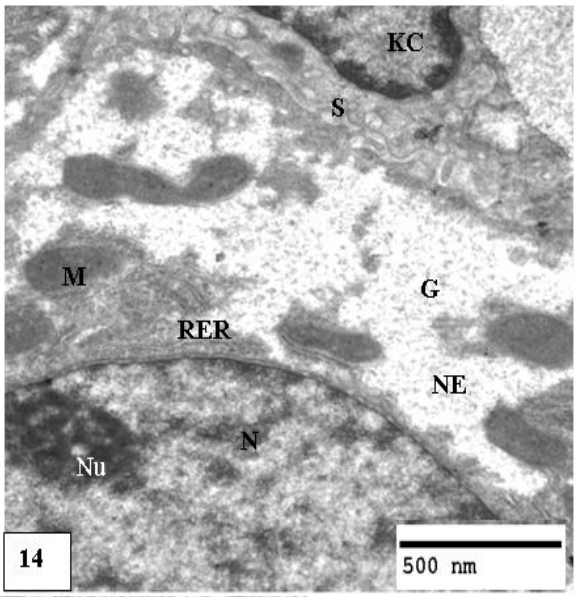

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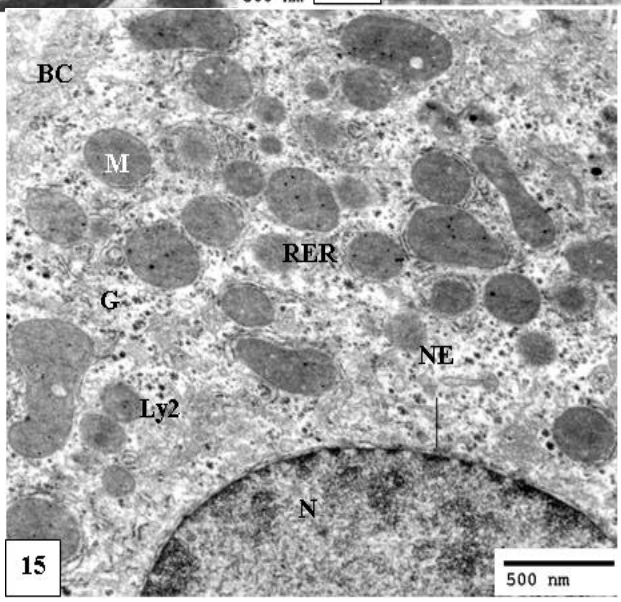

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Light micrographs:

Fig. (1): Normal control liver shoewd hepatic strands separated by blood sinusoid (S), Kupffer cell (arrows) (H&E). **Fig. (2):** Semithin section of control liver represented polyhedral cell and round nucleus with prominent nucleoli, blood sinusoids (S) lined with endothelial cell (arrow), white blood corpuscles (dashed arrow) (TB). **Fig. (3):** Portal tract of DENA-CCl_4 containing a branch of congested and dilated portal vein (PV), perforated bile duct (BD), vacuolated hepatocyte with shrinking nuclei (H&E). **Fig (4):** Semithin section of DENA-CCl_4 represented hepatocytes with an apparently irregular nucleus (N), lipid droplets of variable sizes (dashed arrow), congested sinusoid (S) and fibrous capsule (F) (TB). **Fig. (5):** represented the improved appearance of DENA-CCl_4+cisplatin hepatocytes with round nuclei separated by the congested blood sinusoid (S) and a large number of Kuffer cells (arrows) (H&E). **Fig. (6)**: Semithin section of DENA-CCl4+cisplatin showed congested sinusoid (S) surrounded by hepatocyte (H) with organized cytoplasm and a round nucleus (arrow) (TB). **Fig. (7):** Improved area of DENA-CCl_4+BBR-NPs hepatocyte with collapsed sinusoid (circle), central vein (CV**)** open into sinusoid (S) (H&E). **Fig. (8):** Semithin section of DENA-CCl_4+BBR-NPs represented affected hepatocyte with irregular nuclei (circle) and dilated sinusoid (S) (TB). **Fig. (9):** Improved hepatic tissue of DENA-CCl4+cisplatin+BBR-NPs with metaphase-stage (arrows) (H&E). **Fig. (10):** Semithin section of DENA-CCl4+cisplatin+BBR-NPs group represented polyhedral hepatocyte (H) with a round nucleus with nucleolus and sinusoid (S) contain Kupffer cell (arrow) (TB).

Electron micrographs:

Fig. (11): Control hepatocyte with round nuclei (N) and a regular chromatin pattern surrounded by the nuclear envelope (NE) perforated by nuclear pores (arrow), parralle cisterna of rough endoplasmic reticulum (RER), mitochondria (M) bile canaculei (BC) lined with well-developed microvilli. **Fig. (12):** DENA-CCl4 showed pale nucleus (N) with numerous nuclear pores, large lipid droplets (L), short profile of rough endoplasmic reticulum (RER), proliferated mitochondria (M), peroxisome (PY), deformed bile canaliculi (BC) and cell junction (arrow). **Fig. (13):** Part of DENA-CCl4 +cisplatin nucleus (N) with peripheral heterochromatin, double nuclear envelope (NE) perforated by nuclear pores (arrows), oval to elongated mitochondria (M) bounded by rough endoplasmic reticulum (RER2), (RER1) as parallel cisterna, bile canaliculi (BC) with microvilli and bounded by desmosome (D). **Fig. (14): P**art of DENA-CCl4+BBR-NPs of the improved nucleus (N) with nucleolus (Nu) surrounded by double nuclear envelope (NE) attached to rough endoplasmic reticulum (RER), small mitochondria (M) with tubular cristae, glycogen (G), sinusoid (S) with numerous microvilli contain Kupffer cell (KC). **Fig. (15):** Nearly normal appearance of DENA-CCL4+cisplatin+BBR-NPs nucleus (N) with normal chromatin pattern, numerous mitochondria (M), glycogen (G) secondary lysosome (Ly2) and healthy bile canaliculi (BC) in organized cytoplasm.

4. DISCUSSION

Nano-medicine is the medical application of nanotechnology that will hopefully lead to useful research tools and new ways to diagnose and treat cancer or repair damaged tissues and cells [29].

In the current study, transmission electron microscope (TEM) image has shown that berberine nanoparticles (BBR-NPs) were nearly spherical shape, smooth surface and size range of about 30-40 nm. The respective average diameters, measured by zetasizer, were approximately 370 nm the polydispersity index (PDI) value of BBR-NPs were 0.425 thus indicating a narrow and favorable particle size distribution. The present results were in agreement with Dounighi *et al.* [30] who used chitosan nanoparticles and stated that the nanoparticle size was about 370 nm and the zeta potential was positive. Also, they added that TEM imaging showed a smooth and spherical shape which represented the homogenous structure for nanoparticles.

The anemic effect of DENA-CCl_4 may be attributed to destruction of erythrocytes or the results of adverse effect of DENA on erythropoietic tissue, namely the bone marrow [31]. The catabolism and degradation of the Hb may be anther cause of DENA-CCl_4 anemic effect [32]. Also, the CCl_4 induced oxidative stress leading to

production of reactive oxygen species (ROS). Accumulation of ROS often resulted in shortened RBCs life span, hemolysis and depletion in the erytherpiotein synthesis [33]. Induction of DENA-CCl_4 caused an increase in the white blood cell counts (WBCs) which may be due to the immune response toward DENA [34].

In the current study, the anemic effect of cisplatin was a result of either suppresses the activity of hematopoietic tissues, impaired erythropoiesis and accelerated RBCs destruction because as a result of RBCs membrane permeability alterations, increased RBCs mechanical fragility and/or defective Fe metabolism [35]. Cisplatin may induce injury to renal tubular epithelial cells and subsequent renal failure that affect on the differentiation and proliferation of erythroid progenitor cell through bone marrow suppression [4]. Also, cisplatin accumulation in the renal tubular cells, leading to free radical production and lipid peroxidation, which is the main factor causing anemia by diminishing synthesis of erythropoietin due to a reduction of functional renal mass [36]. Moreover, the decrease in platelet counts might be due to cisplatin inhibiting bone marrow activity, decreased production or increased consumption of platelets and/or due to the increased platelet aggregation.

Treatment with BBR-NPs alone or in combination with cisplatin decreased the anemic effect of DENA-CCl_4 as documented by increasing the RBC counts, Hb content, Hct value and platelet counts which may be due to the antioxidant properties of berberine (BBR). BBR decreased the oxidative stress via reducing reactive oxygen species and decrease lipid peroxidation that led to membrane stabilizing activity [37]. Furthermore, it was reported that BBR suppressed oxidative stress through induction of the nuclear factor erythroid-2-related factor-2 (Nrf2) pathway [38] which is necessary for erythropoiesis and platelet development [39]. In the present study, BRB-NPs decreased the elevation in WBC counts, this may be attributed to the role of BBR in regulation of immune response [40]. These results came in agreements with Dkhil *et al.* [41] who showed that BBR was able to improve the induced alteration in both of erythrocytes count and hemoglobin content. Moreover, berberine lowers the increased number of leucocytes on *Plasmodium chabaudi*-induced hepatic tissue injury in mice that mediated by both acquired and innate immune responses initiated by the BBR.

The bioactivation of DENA by cytochrome P450 and its capability of alkylating DNA structure contributing to the carcinogenic capacity of DENA and induce hepatocellular carcinoma (HCC) as well as the proliferation of liver tumor cells [42]. These effects may be due to the DENA induced hepatocyte membrane damage and subsequent leakage of enzymes into the blood stream [43]. Also, Hemieda *et al.* [44] and Mohamed *et al.* [45] showed that induction of DENA-CCl_4 increased enzyme activities of liver, suggesting hepatocellular damage and impairment of liver function as a result of oxidative stress production causing damage in cell membrane integrity. The current result came accordance with Al-Rejaie *et al.* [46] who stated that induction of DENA increased serum indices of liver function, including ALT, GGT, ALP and total bilirubin.

Moreover, levels of LDH have been reported in hemolytic anemia, hepatocellular necrosis and hepatocellular carcinoma. The elevated levels of LDH and GGT may be due to hepatic necrosis or premalignant hepatocellular lesions induced by DENA [47].

5′-nucleotidase is an accurate marker of early hepatic primary or secondary tumors [48]. A recent study showed its elevation in liver diseases, including liver cirrhosis, chronic alcoholism, benign biliary disease and neoplasm of the liver and bile ducts [49]. Herein, the high activity of 5^{-}NT in the DENA-CCl_4 intoxicated rats are revealing of intrahepatic obstruction of bile canaliculi as a result of liver cell injury and due to increase in the fluidity of the cell membrane as confirmed by Ghaffar [50]. Moreover, Vedarethinam *et al.* [51] reported that the elevated levels of the 5^{-}NT marker enzyme in rats injected by DENA are correlated with the malignancy development of DENA action.

Cisplatin has been shown to achieve significant hepatic disturbances as indicated by an increment in the liver enzyme activities and decreased TP and ALB levels. Cisplatin distress hepatic injury through the activation of inflammatory and oxidative stress pathways causing apoptosis and anomalies in liver structure and function [52]. Palipoch *et al.* [53] showed that treatment with cisplatin causing increase enzyme leakage of LDH and GGT in the blood stream as a result of its oxidative stress mechanism.

The hepatoprotective effect of BBR-NPs refers to the antioxidant and anti-inflammatory properties of berberine phenolic compounds and membrane stabilizing efficacy [54]. The present results were in agreement with the Hu *et al.* [55] who showed that berberine decreases the level of AST, while, albumin, ALT, alkaline phosphatase, and total bilirubin levels were not changed.

AFP measurement may be useful as a sensitive marker system for the early detection of recurring HCC even before the clinical symptoms are evident [56]. Injection of the DENA-CCl_4 increased in alfa-fetoprotein (AFP) level, which may be attributed to the inflammatory response of DENA-CCl_4 [57].

The present results were in the same line of Tawfek *et al.* [58] who stated that induction of DENA-CCl_4 caused an elevation in the AFP level, suggesting the occurrence of premalignant liver changes. Similarly, Vedarethinam *et al.* [51] stated that induction of DENA induced liver damage as documented by an increase in AFP.

On the other hand, treatment with cisplatin injection after the induction of DENA-CCl_4 decreased the elevation in serum AFP which may be attributed to the strong antitumor activity of cisplatin [59].

Treatment with BBR-NPs alone or in combination with cisplatin decreased the level of AFP which may be attributed to the antitumor and anti-inflammatory action of berberine. Cameron *et al.* [60] reported that berberine induced cell growth arrest in human liver cancer cell line (HepG2) and led to inhibit the secretion of alpha-fetoprotein. Tan *et al.* [61] demonstrated that using BBR-NPs in combination with cisplatin caused enhancement of cisplatin uptake by liver cells, which could play a beneficial role in preventing cisplatin hepatotoxicity via its anti-oxidative role. Also, they stated that BBR enhances chemosensitivity, implying its potential as an adjuvant in cancer therapy when combined with chemotherapy drugs such as cisplatin.

DENA-CCl_4 caused a depletion in antioxidant enzyme activities (SOD, CAT, GPX) and GSH, which may be due to the DENA metabolism that led to the production of excessive reactive oxygen species (ROS) in the liver. Excessive production of ROS can modify a number of cellular targets and cause cell damage [45]. Oxidative stress generated during DENA metabolism leads to depressed levels of enzymatic and non-enzymatic antioxidants [62]. Hemieda *et al.* [44] reported that biotransformation of DENA in the rat liver by cytochrome P450 increased production of ethyl diazonium ion, which reacts with DNA forming adducts that is recognized as the initial step in DENA-induced carcinogenesis. Moreover, CCl_4 is metabolized by liver cytochrome P450 resulting in free radical production, which in turn reduce antioxidants causing promotion of carcenogenesis.

The co-administration of cisplatin with DENA-CCl_4 induced oxidative status as indicated by increased MDA and decreased GSH levels. It has been suggested that oxidative stress is an important mechanism of cisplatin induced toxicity possibly due to depletion of reduced glutathione GSH. Dasari & Tchounwou [60] showed that treatment with cisplatin increased levels of the oxidative stress marker (MDA) matched the reduction in total antioxidant activity in liver.

The hepatoprotective effect of BBR-NPs either alone or in combination with cisplatin may be attributed to the anti-inflammatory and antioxidant characters of berberine [63]. The current results were inconsistent with Sindhu *et al.* [37]. Li *et al.* [38] showed that the berberine treatment had an antioxidant effect in diabetic rats. BBR inhibited oxidative stress in a variety of tissues, including the liver, adipose tissue, kidney and pancreas. Furthermore, berberine exerts radical scavenging activity in cell based systems, by inhibiting ROS production, increasing the levels of non-enzymatic antioxidants and maintaining the activity of antioxidant enzyme activities [64].

DENA-CCl_4 caused an increase in the relative expression of ADAM metallopeptidase domain 17 (ADAM 17) and tumor necrosis factor α (TNF-α) as well as down regulation of protein 53 (P53) that may be due to the genotoxic damage of DNA induced by DENA which in turn induce oxidative stress to initiate hepatocytes necrosis resulting in the release of the proinflammatory cytokines. During the interplay of oxidative stress-

inflammatory pathway, a number of proinflammatory cytokines have been identified to drive genotoxically affected hepatocytes to undergo compensatory proliferation [65].

TNF-α has been given much attention because of its importance in preventing the formation of neoplastic lesions during DENA induction [66]. Furthermore, TNF-α level has been found to be associated with the increased ROS generation [67]. Moreover, pro-inflammatory cytokines (TNF-α) is able to up regulate of ADAM17 [68].

A previous report was consistent with the present results that showed the elevation in the relative gene expression of ADAM17 and TNF-α in DENA-CCl_4. Saile and Ramadori [69] reported that subjection of liver to DENA-CCl_4 results in hepatic inflammation that is initiated by parenchymal cell death and led to activation of resident macrophages that produce inflammatory cytokines as a result of chemokines release. Furthermore, Kupffer cells and natural killer cells secret large number of pro-inflammatory cytokine which includes ADAM17 and TNF-α.

Cisplatin treatment caused down regulation of ADAM 17 & TNF-α expressions. Furthermore, it upregulate the expression of P53 gene, which may be attributed to the anticancer properties of cisplatin that depends on its binding to DNA to form covalent platinum DNA adducts acting as DNA alkylator.

In the current study, treatment with BBR-NPs alone or in combination with cisplatin showed decreases in the concentration level of ADAM 17, TNF-α and an increase in the relative concentration of p53 as a result of antinflammatory properties of berberine [70]. The present results came accordance with Mahata *et al.* [71] who reported that berberine induces growth arrest and apoptosis in cervical cancer cells by increasing the concentration level of p53 gene expression. Similarly, Ghareeb *et al*. [63] observed the anti-inflammatory and antioxidant properties of berberine in treatment of Alzheimer and showed that berberine normalized the production of TNF-α, and ADAM 17.

5. CONCLUSION:

The present results of berberine nanoparticles treatment showed its potential therapeutic activity against the hepatocarcinogenesis *via* its effect as an antioxidant and anti-inflammatory. Finally, our study enhances the recommendation of berberine using as potential natural therapeutic agent for treatment of hepatic cancers.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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