

SYNERGISTIC ANTINEOPLASTIC ACTIVITY OF BERBERINE AND DOXORUBICIN ON A CHEMICALLY INDUCED HEPATOCELLULAR CARCINOMA IN RATS

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ABSTRACT

Background: Hepatocellular carcinoma (HCC) is the most common type of liver malignancies. Yet, the outcome of the traditional chemotherapeutic agents in the management of HCC still unsatisfactory, most probably due to their limited therapeutic efficacies. Thus, there is an urgent medical need for alternative therapeutic approaches for fighting HCC. **Aim:** our study aimed at evaluating the possible synergistic antitumor activity of the herbal alkaloid berberine (BER) with the conventional doxorubicin (DOX) in a DENA-induced HCC rat model. **Methods:** HCC was induced in male Wistar rats by oral DENA administration in their drinking water (100 mg/L) for 8 weeks. In addition to a positive and negative control groups, a group of animals (10 rats) was given DOX (4× 2.5 mg/kg, i.v., weekly). Another group (10 rats) was given BER (100 mg/kg) by oral gavages daily for one month. A third group (10 rats) was given a combination of both BER and DOX in the above described dose and schedule. **Results:** Combination between DOX and BER was superior over their corresponding individual administrations as indicated by significant improvement in the overall estimated indices of liver function. Also, in comparison to individual therapy, this combination was obviously more potent to reduce the levels of novel HCC related tumor markers including serum AFP-L3 and tissue levels of Golgi protein 73, and glypican 3. Furthermore, histological investigations and assessments of hepatic tissue levels of some oxidative stress markers strongly confirmed the advantageous effects of combined BER and DOX in fighting DENA-induced HCC. **Conclusion:** our study conclusively revealed that combining BER with DOX exhibited a promising preclinical anticancer efficacy and could be considered as a novel strategy to synergistically combat HCC in clinical practices.

KEY WORDS

Alphafetoprotein-L3; Apoptosis; Berberine; Doxorubicin; Hepatocellular carcinoma.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the main primary malignant tumor of the liver. Worldwide, it represents the third leading cause of cancer-related deaths in males and the fourth in females with a steadily increasing incidence of about 625000 new cases per year emerging around 600000 worldwide annual deaths [1]. Although the majority of the cases occur in Asia and Africa, the incidence has also been rising in the developed world. In Egypt, the burden of HCC has been increasing with a doubling in the incidence rate in the past 10 years [2]. The geographical variation in the incidence of HCC is explained by disparity in the prevalence of the major risk factors including chronic hepatitis B (HBV) or C virus (HCV) infection, alcohol-induced liver disease (ALD), non alcoholic fatty liver disease (NAFLD), primary biliary cirrhosis, exposure to

environmental carcinogens (particularly aflatoxin), and then type 2 diabetes and obesity [3, 4].

Unfortunately, the prognosis of HCC is usually very poor due to the lack of effective early diagnostic tools and the actual diagnosis is usually made at an advanced stage that being too late for any effective treatment [5]. Similarly, the outcome of HCC is not satisfactory yet because only 10 - 20 % of HCC can be completely surgically removed, and in case of inefficient surgical removal of the whole malignant tissues, the overall survival without treatment is only in the range of 3 to 8 months. Moreover, treatment with conventional chemotherapeutic agent is usually of limited benefit either due to the development of drug resistance or due to their unlimited chronic and acute systemic toxicities [6, 7]. During the past 30 years, no consistent survival benefits for these

approved anticancer agents in HCC have been recorded in around 100 randomized studies including systemic and intra-arterial chemotherapy (predominantly doxorubicin-based or platinum-based), various hormonal therapies (tamoxifen and antiandrogens), and immunotherapy (usually interferon alpha) [8, 9]. Therefore, there is a pressing medical need for the identification and discovery of alternative therapeutic strategies with lower drawbacks, to wage a more humane war against HCC and hence to combat the current morbidity and mortality associated with such malignancies.

Phytochemical remedies show promise in this area as their potential chemopreventive or chemotherapeutic actions in several types of cancer have been extensively indicated by epidemiologic and experimental studies [10]. For instance, herbal medicine has a long history of use in the treatment of cancer and it is significant that over 60% of the currently used anti-cancer agents are come from natural sources [11]. Naturally occurring drugs that are part of the war against cancer include vinca alkaloids (vincristine, vinblastine, vindesine, vinorelbine), taxanes (paclitaxel, docetaxel), podophyllotoxin and its derivative (etoposide, teniposide), camptothecin and its derivatives (topotecan, idarubicin) and others [12]. In the same respect, Berberine (BER), an isoquinoline derivative alkaloid that belongs to the camptothecin family of drugs, represents a new insight as a promising candidate of these natural compounds. It was initially isolated from the herbs *Rhizoma coptidis* (*Huang-Lian*) and was subsequently extracted from the roots, rhizome, and stem bark of a number of important medicinal plants such as *Berberis vulgaris* (barberry), *Berberis aquifolium* (oregon grape), *Berberis aristata* (tree tumeric), and *Tinospora cordifolia* [10]. The potential effectiveness of BER is indicated by its extensive use in traditional Indian and Chinese medicine for the treatment of several diseases such as diarrhea, rheumatic diseases, diabetes and some autoimmune diseases [13, 14].

Some years ago, cumulative evidences, in both *in vitro* and *in vivo* studies, have demonstrated that BER exhibits a promising potential to suppress growth, invasion, and metastasis of various lines of cancers such as glioma, lung cancer, nasopharyngeal carcinoma, and melanoma cell mostly via induction of apoptosis and cell cycle arrest with little resistance and low toxicity to normal cells [15-18]. More recently, other studies explored the chemosensitizing

influence of BER in combination with approved chemotherapeutic agents as an alternative approach for cancer control and treatment [19]. In this regard, emerging evidences confirmed that BER exhibited a synergistic anticancer activity with DOX, most likely through its own capability to enhance apoptosis and cell cycle arrest in cancer cells. Accordingly, this combinatorial approach can strongly inhibit mitogenic and survival signaling to inhibit cell proliferation and eventually induce cell death [19, 20]. In light of the above knowledge, we hypothesized that combining DOX with BER could be considered as a novel strategy to increase the *in vivo* antitumor efficacy of DOX against certain malignancies. The current study was therefore performed to test this hypothesis in a chemically induced HCC rat model.

MATERIALS AND METHODS

Chemicals

Berberine (BER) and diethylnitrosamine (DNA) were purchased from Sigma-Aldrich GmbH (Munich, Germany). Commercially available doxorubicin (DOX) vials (10 mg adriamycin hydrochloride) were purchased from Pharmacia Italia S.P.A. Gruppo Pfizer Inc. (Nerviano Italy). Alpha-Fetoprotein Lens Culinaris Agglutinin 3 (AFP-L3) rat specific ELISA assay kit was purchased from Glory Science. Co., Ltd (Hangzhou, China). Rabbit polyclonal anti-GP73 antibody and alkaline phosphatase-conjugated goat anti-rabbit secondary antibody were purchased from Novus Biologicals, LLC, Littleton (CO, USA). Mouse monoclonal anti β -actin antibody was purchased from Santa Cruz Biotechnology, Inc. (CA, USA). Total RNA purification kit was purchased from Jena Bioscience GmbH (Jena, Germany). RevertAid M-MuL V Reverse Transcriptase kit was purchased from Thermo Fisher Scientific inc. (MA, USA). Protease inhibitor cocktail was purchased from Cell Signaling Technology, Inc. (MA, USA). BCIP/NBT substrate detection kit was purchased from Genemed Biotechnologies, Inc. (CA, USA). Molecular screening agarose gel was purchased from Roche Diagnostics, GmbH (Mannheim, Germany). PCR primers were custom-made by Vivantis Technologies Sdn. Bhd. (Selangor Darul Ehsan, Malaysia) to amplify the rat GPC3 and β -actin cDNA (Table 1). All other chemicals, reagents, and solvents were of analytical grade that obtained from standard suppliers and were used without further purification.

Table-1: The primers used for amplification of GPC3 and β -actin in RT-PCR detection

Primer	Amplified Product (bp)	Sequence	Gen Bank Number	Accession	Annealing Temp. °C
GPC3	603	FORWARD: 5'-GGCAAGCTGACCACCACTAT-3' REVERSE: 5'-CTTGTTCCCTGGAGCATCGT-3'	NM_012774.1		53 °C
β -actin	243	FORWARD: 5'-CCACCATGTACCCAGGCATT-3' REVERSE: 5'-ACGCAGCTCAGTAACAGTCC-3'	NM_031144.3		54 °C

Animals and experimental design:

Animal experiments were performed after approval by the Institutional Animal Care and Use Committee of the Faculty of Medicine, Assiut University, Assiut, Egypt. All experiments were performed using 15-16 week old healthy male Wistar rats (weighting 130-140 g) that were purchased from the laboratory animal colony, Assiut University, Assiut, Egypt. Rats were housed (5 per cage) in wire-floored cages at a regulated environment (temperature, $22 \pm 2^\circ\text{C}$; humidity, $50 \pm 5\%$; night/day cycle, 12 hours) with free access to standard pellet diet and tap water *ad libitum*. Animals' weights were taken every 3 days and animal behaviors were monitored daily. After two weeks acclimatization period, rats were randomly divided into two groups of 10 and approximately 40 rats, respectively. Animals in the first group (control group) were received only a suspending vehicle that consist of a mixture of 1% sodium Carboxymethyl cellulose and 1% Tween-80 (daily by oral gavages) throughout the experimental period. The animals of the second large group received DENA, given in their drinking water (100 mg/L) for 8 weeks [21]. The DENA solution was administered in dark bottles (additionally covered with aluminum foil) since the solution is light-sensitive and was prepared as a fresh solution every other day. One month after the end of DENA administration, the 40 rats of the second group were allocated to one control group (DENA group) which received the above mentioned suspending vehicle and 3 other treated subgroups of 10 animals each. Rats in the first subgroup (DENA+DOX group) were given doxorubicin 4×2.5 mg/kg (i.v., weekly) via tail vein [19]. Rats in the second subgroup (DENA+BER group) were given daily BER by oral gavages (100 mg/kg) suspended in the above mentioned suspending vehicle for four weeks [19]. Rats in the third subgroup (DENA+DOX+BER group) were given a combination of both DOX and BER in the same above

described doses and schedule. One week after the last treatment, animals were sacrificed by cervical decapitation under isoflurane anesthesia after blood collection from all animals via retro-orbital vein plexus for serum preparation. Subsequent to autopsy, the livers were excised, purified from adhering fat and connective tissues, washed in ice-cold isotonic saline and then divided into three parts; the first part was stored in formalin (10%) solution and subjected for histopathological examination and the remaining two parts were instantly flash frozen in liquid nitrogen and stored separately at -70°C for subsequent western blotting and RT-PCR assays.

Biochemical estimations

Serum AFP-L3 was assayed by rat specific ELISA assay kit. Serum Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), and Alkaline Phosphatase (ALP) activities were assayed by kinetic procedures using corresponding kits according to the manufacturer's instructions. Serum total bilirubin was determined using colorimetric kit according to the manufacturer's instructions. Lipid peroxidation was determined spectrophotometrically in liver tissues as thiobarbituric acid reacting substance (TBARS) and is expressed as equivalents of malondialdehyde (MDA) [22]. Reduced glutathione (GSH) was assayed spectrophotometrically in liver tissues using Ellman assay method [23]. Superoxide dismutase (SOD) activity in liver tissues was estimated using the xanthine oxidase method [24]. Nitric oxide (NO) was assayed spectrophotometrically in liver tissues by measuring its stable metabolites, in particular, nitrite and nitrate [25].

Investigation of golgi protein 73 (GP73) protein expression by western blotting technique

Liver tissue homogenates were prepared in ice-cold Tris-HCl lysis buffer, pH 7.4 containing 1% protease inhibitor cocktail using Potter-Elvehjem rotor-stator homogenizer (glass/teflon homogenizer), fitted with a

Teflon pestle. Proteins in each corresponding homogenate were denatured at 95 °C for 5 minutes in 2× Laemmli buffer. SDS-PAGE electrophoresis was achieved by loading 50 µg protein per lane at 75 volts through resolving gel 12% followed by 125 volts during approximately 2 hours and transferred to a PVDF membrane using T-77 ECL semidry transfer unit (Amersham BioSciences UK Ltd) for 2 hours. Immunoblotting was performed by incubating the PVDF membrane in TBS buffer containing 0.1% Tween and 5% defatted milk for one hour at 4°C, followed by overnight incubation at 4°C with rabbit polyclonal anti-GP73 antibody at 1:2000 dilution. After being washed three times with TBST buffer, each membrane was incubated for one hour at room temperature with an alkaline phosphatase-conjugated goat anti-rabbit secondary antibody at 1:5000 dilution. After being washed four times in TBST, the membrane bound antibody was detected with a commercially available BCIP/NBT substrate detection kit. Equivalent protein loading for each lane was confirmed by stripping and re-blotting each membrane at 4°C against mouse monoclonal anti β -actin antibody at 1:5000 dilution. The analysis was repeated to assure reproducibility of results.

Detection of glypican 3 (GPC3) mRNA using reverse transcriptase PCR (RT-PCR)

In order to obtain a maximum intact RNA yield, a part of liver was harvested in a specific lysis buffer supplied in total RNA purification kit using a Potter-Elvehjem rotor-stator homogenizer according to the manufacturer's instructions. To avoid RNA destruction during or after procedure by active RNases, outer stationary glass tube and inner turning teflon shaft of the homogenizer were washed with 0.1% diethylpyrocarbonate-treated water (DEPC-treated water), incubated overnight at 37°C, and then autoclaved for 15 minutes to eliminate residual DEPC. The purity (A260/A280 ratio) and the concentration of the isolated RNA were determined using a GeneQuant 1300 spectrophotometer (Uppsala, Sweden). RNA quality was subsequently confirmed by gel electrophoresis. Then the first-strand cDNA was synthesized from 4 µg of total RNA using an Oligo (dT) 18 primer and RevertAid M-MuL V Reverse Transcriptase kit. This mixture was incubated at 42 °C for one hour, followed by incubation at 70 °C for 5 minutes to terminate the reaction. The resulting cDNA was amplified by PCR. Numbers of cycles and reaction temperature conditions were estimated to be optimal to provide a linear relationship between the amount of input template and the amount of PCR

product generated over a wide concentration range: from 1 to 20 µg of total RNA. In brief, cDNA was first denatured for 3 minutes at 95 °C then amplified for 33 cycles consisting of: denaturing for 30 second at 95 °C; annealing for 30 second at 53 °C for GPC3; and 54 °C for β -actin; primer extension for 30 second at 72 °C followed by one cycle of primer extension for 5 minutes at 72 °C. RT-PCR of β -actin was performed in parallel as an internal control. The RT-PCR products were analyzed by electrophoresis using 2% molecular screening agarose gel, stained with ethidium bromide and visualized by UV light.

Histopathological examinations

For histopathological examination, liver tissue samples of all lobes were fixed in 10% formalin, embedded in paraffin and routinely stained with hematoxylin and eosin (H&E). Washing was carried out in sterile tap water, then in serial dilutions of alcohols (methyl, ethyl and absolute ethyl) which was used for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56 °C in a hot air oven for 24 hours. Paraffin bees wax tissue blocks were prepared for sectioning at 4 microns thickness with a sledge microtome and the obtained tissue sections were placed on glass slides, deparaffinized and stained by H&E stain and then examined under an electric light microscope for assessment of histopathological changes [26].

Statistical analysis

Statistical analyses of the data were carried out using Graphpad prism version 5.0 (Graph pad software San Diego, USA). Data comparisons were performed using analysis of variance (ANOVA) followed by Tukey's t-test. The levels of significance were accepted with $p < 0.05$ and all relevant results were graphically displayed as mean \pm SEM.

RESULTS AND DISCUSSION

Hepatocellular carcinoma (HCC) is a major worldwide public health concern that represents globally the fifth most common type of cancer [27]. Despite recent advances, there has been a little success in improving the survival of HCC patients, partly due to lack of effective therapy, tumor recurrence, and because the diagnosis is generally made at an advanced stage of the disease [28]. Therefore, the prevalence of HCC is expected to worsen in the coming years, plateauing between 2015 and 2020 [29].

In the last few decades, treatment with conventional chemotherapeutic agents such as doxorubicin (DOX) enjoyed considerable popularity as one of the most

practiced strategies in the management of unresectable HCC [30]. Nowadays, this approach is no longer widely used in clinical applications as a result of the limited efficacy of DOX, most likely due to the elevation of drug resistance, in addition to its undoubted implication in the appearance of numerous dose related side effects that leading eventually to unsatisfactory outcomes [31]. Accordingly, improving both the therapeutic efficacy and index of DOX is considered as an unmet golden goal to achieve a more efficient fighting potential against tumors. In this regard, a lot of evidences indicated that combined therapy with multiple drugs or modalities is a common practice in cancer treatment, which can achieve better therapeutic effects than a single drug or modality and can reduce the side effects and resistance to drugs [32]. As an option, chemosensitizing phytochemicals in combination with approved chemotherapeutic agents are being explored as an alternative more efficient approach for cancer control and treatment [33]. In the light of this knowledge, we aimed here in the current study to evaluate the potential of berberine (BER), a botanical alkaloid of the protoberberine type, to improve the *in vivo* anticancer efficacy of the

conventional DOX against HCC in a chemically induced rat model.

In the current study, we have observed that, combinatorial treatment with both DOX and BER was superior over their corresponding individual use in respect to signs of toxicity as was manifested for example by decrease in body weight gain. Our results have shown that the increase in the animal body weight was obviously attenuated in all DENA drinking animals during the course of the experiment before starting treatment, suggesting primary tumor burden or the metastatic spread of the tumor as a possible cause. A similar observation was also reported in previous works using this model [34]. As illustrated in Figure-1, following treatments, the induced decreases in body weight gain were hampered to large extent in the combination group (DENA+DOX+BER) and to less extent in the BER-treated group (DENA+BER) demonstrating decreased side effects which, on the contrary, was more pronounced in the DOX-treated group (DENA+DOX), most likely due to the direct toxic effects of DOX on the animal intestinal mucosa as well as its indirect actions arising from reduced food intake causing a decrease in secretion of internal hormones and resulting in decreased trophic effects to the mucosa [35].

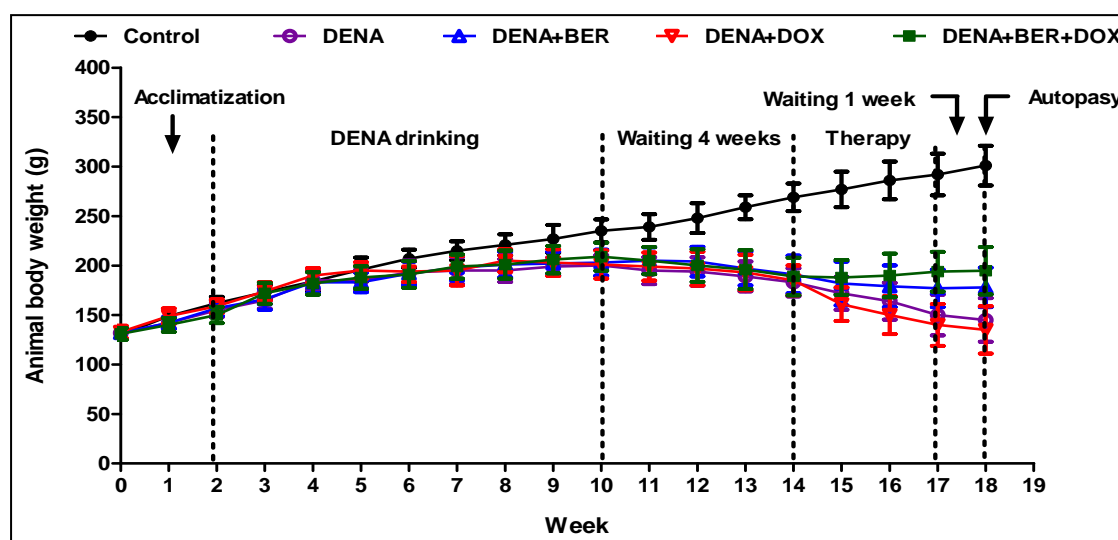


Figure-1: illustration of the changes in the animal's body weights during the experiment.

We have also observed that representative macroscopic views of livers from the DENA group showed abnormal morphological features with several macroscopic tumor nodules scattered throughout the liver with an irregular rough surface, as appear in Figure-2. Similar morphological observations have also previously been reported in

several experiments in which DENA was used as a carcinogen for animal models of HCC [34, 36]. In a comparatively similar style, livers of rats treated with individual DOX and BER macroscopically showed altered morphological features with irregular rough pale surface incorporating numerous scattered macro and micronodules of different sizes throughout the

liver – see Figure-2. On the contrary, livers in the combination group (DENA+BER+DOX) showed relatively better morphological appearance with very

few micro-nodules. Ideally livers in the control group showed normal morphological aspects as seen in Figure-2.

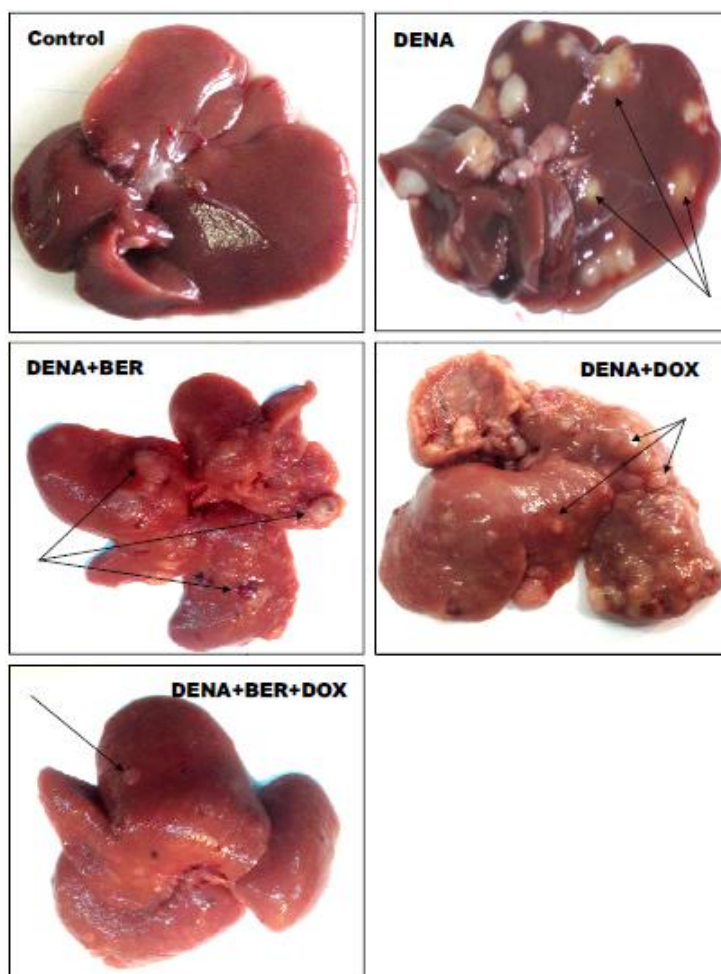


Figure-2: Effect of tested compounds on the morphological aspect of the liver. Representative macroscopic views of livers from the DENA, DENA+BER and DENA+DOX groups showed abnormal morphological features with several macroscopic tumor nodules of different sizes (arrows) scattered throughout the livers with an irregular rough surface. Conversely, livers in the combination group (DENA+BER+DOX) showed relatively better morphological appearance with very few micro-nodules. Livers in the control group showed normal morphological aspects.

Regarding hepatic performance, it was clearly observed in our study that in comparison to the healthy control group, animals in DENA group suffered from marked indications of impaired liver functions including significant hypoalbuminemia ($p < 0.001$), in addition to significant elevations in the serum activities of ALT ($p < 0.001$), AST ($p < 0.001$), GGT ($p < 0.001$) and ALP ($p < 0.001$) as well as total serum bilirubin level ($p < 0.001$) – see table-2. These observed DENA-forced alterations in serum indices of

liver functions could be secondary events following lipid peroxidation of hepatocyte membranes with the consequent increase in the leakage of ALT, AST, GGT, ALP and total bilirubin from damaged liver tissues as have been previously reported in many models of DENA-induced hepatocellular degeneration [37, 38]. Of note that neither BER nor DOX individual administration was able to significantly restore any of these impaired indices to its corresponding normal levels. Fortunately, the overall estimated liver

function indices were obviously improved in the group treated with BER and DOX combination (DENA+BER+DOX) as illustrated in table-2. In comparison to the DENA group, animals in the combination group showed significant increase in the serum albumin level ($p < 0.05$), in addition to significant decreases in the serum activities of ALT ($p < 0.001$), AST ($p < 0.001$), GGT ($p < 0.01$), and ALP ($p < 0.01$) as well as total level of serum bilirubin ($p < 0.01$). Notably, in comparison to individual

therapeutic agent in DENA+BER, and DENA+DOX groups, combination therapy revealed much more superiority in the form of significant increase in the serum albumin level ($p < 0.05$ and $p < 0.01$ respectively), besides significant decreases in the serum activities of ALT ($p < 0.001$ and $p < 0.001$ respectively), AST ($p < 0.001$ and $p < 0.001$ respectively), GGT ($p < 0.05$ and $p < 0.05$ respectively) and ALP ($p < 0.05$ and $p < 0.05$ respectively) as well as total level of serum bilirubin ($p < 0.05$ and $p < 0.05$ respectively).

Table-2: Liver function tests:

	Control	DENA	DENA+BER	DENA+DOX	DENA+BER+DOX
Albumin (g/dL)	4.06 ± 0.20	2.23 ± 0.24 ***	2.26 ± 0.14 ***	2.02 ± 0.20 ***	3.15 ± 0.20 *,†,§,‡
sALT (IU/L)	63 ± 1.41	165 ± 3.53 ***	160 ± 2.82 ***	155 ± 1.41 **,†	75 ± 2.12 *,††,§§,‡‡
sAST (IU/L)	115 ± 7.07	320 ± 14.14 ***	300 ± 10.61 ***	280 ± 7.07 ***	170 ± 1.21 *,††,§§,‡‡
sGGT (IU/L)	29.4 ± 4.49	118.0 ± 15.34 ***	101.9 ± 11.91 ***	100.4 ± 12.21 ***	54.9 ± 8.44 ††,§,‡
ALP (IU/L)	75 ± 3.53	160 ± 14.14 ***	155 ± 7.07 ***	150 ± 3.53 ***	115 ± 7.07 *,††,§,‡
Total Bilirubin (mg/dL)	0.38 ± 0.01	1.60 ± 0.21 ***	1.50 ± 0.07 ***	1.46 ± 0.13 ***	0.90 ± 0.14 ††,§,‡

Data are presented as mean ± SEM (n = 10). *,†,§, and ‡ indicate significant changes from control, DENA, DENA+BER, and DENA+DOX groups respectively. *,†, § and ‡ indicate significant change at $P < 0.05$; **,††,§§, and ‡‡ indicate significant change at $p < 0.01$; ***,†††,§§§ and ‡‡‡ indicate significant change at $p < 0.001$.

Additional evidences that clearly support this superiority of BER and DOX combination over either BER or DOX individual administration were presented by the outcome of assessments of a panel of novel HCC tumor markers including AFP-L3, GP73 and GPC3. These contemporary markers have been recently used by several investigators for the early detection and monitoring treatment response as well as poor differentiation, recurrence, and/or malignant invasion of HCC and also as surrogate markers of its clinicopathological variability with relatively satisfactory sensitivity and specificity [39-41]. As

appear in our results, serum AFP-L3 level was significantly increased in DENA group in comparison to normal healthy control group ($p < 0.001$). As obvious in Figure-3, while both BER and DOX solitary administration failed to significantly decrease the elevated AFP-L3 circulating levels, their combinatorial administration was more efficiently able to significantly reduce the elevated AFP-L3 levels earning approximately 56% ($p < 0.001$), 52% ($p < 0.01$), and 46% ($p < 0.05$) corresponding decreases in comparison to DENA, DENA+BER and DENA+DOX groups respectively.

Table-3: Hepatic tissue levels of oxidative stress markers

	Control	DENA	DENA+BER	DENA+DOX	DENA+BER+DOX
MDA (nmol/g wet tissue)	3.42 ± 0.51	11.51 ± 1.00 **	13.33 ± 2.33 ***	13.35 ± 1.34 ***	14.09 ± 1.74 ***
NO (μmol/g wet tissue)	2.66 ± 0.43	11.85 ± 1.95 **	13.21 ± 2.30 **	15.61 ± 2.10 ***	18.95 ± 1.88 ***
GSH (μmol/g wet tissue)	10.61 ± 1.44	5.35 ± 0.83 **	4.89 ± 0.64 **	5.62 ± 1.22 *	4.92 ± 0.73 **
SOD (U/mg wet tissue)	10.81 ± 1.40	4.99 ± 0.92 **	4.24 ± 0.92 **	4.10 ± 1.00 **	4.35 ± 1.50 **

Data are presented as mean ± SEM (n = 10). * indicates significant changes from control group. * indicate significant change at $P < 0.05$; ** indicate significant change at $p < 0.01$; *** indicate significant change at $p < 0.001$.

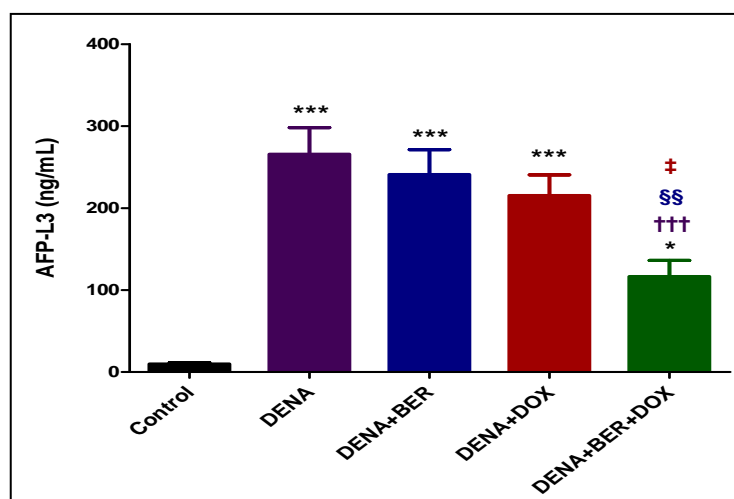


Figure-3: Serum levels of AFP-L3 as a circulating tumor markers of HCC in different groups. Data are presented as mean \pm SEM (n = 10). *, †, §, and ‡ indicate significant changes from control, DENA, DENA+BER, and DENA+DOX groups respectively. *, †, §, and ‡ indicate significant change at $P < 0.05$; **, ††, §§, and ‡‡ indicate significant change at $p < 0.01$; ***, †††, §§§, and ‡‡‡ indicate significant change at $p < 0.001$.

Additionally, western blotting assessments of GP73 as well as RT-PCR detection of GPC3 mRNA as HCC-related molecular markers augmented the previously observed beneficial role of combined BER and DOX therapy, but not their individual use, in fighting HCC. As shown in figure-4, DENA administration induced strong expression of both GP73 and GPC3 genes, proving the occurrence of premalignant liver changes in this group. These results are in agreement with some previous researches which reported that GP73 and GPC3 are upregulated in HCC tumor tissues

compared with normal and benign liver diseases and contributed to promoting the growth of HCC by stimulating Wnt signaling [42, 43]. Auspiciously, combined BER and DOX therapy was able markedly to attenuate this elevated expression to be asymptotic to its expression pattern in the healthy control group, meanwhile singular BER or DOX administration were unable to hinder the elevated expressions of any of these molecular markers – See Figure 4.

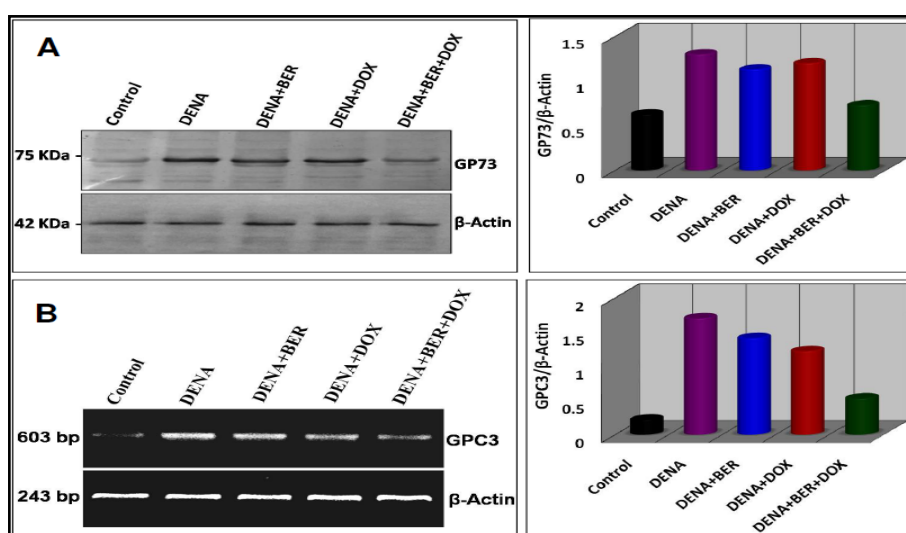


Figure-4: illustration of the western blotting assessments of GP73 (A) and RT-PCR detection of GPC3 mRNA fragments (B) as HCC-related molecular markers in liver tissue homogenates. β -Actin was used in parallel as an internal control. The right panels represent corresponding quantification of each analysis measured by Image J software and expressed as a β -actin ratio.

As well, histological examinations of liver tissues presented another strong evidence for the combined BER and DOX advantageous effects to fight DENA-induced HCC. The representative photomicrographs of liver sections obtained from animals in DENA group showed hepatocellular carcinoma with apparent hepatic tissue alterations that reflect severe dilatation of central vein and degeneration in the surrounding adjacent hepatocytes incorporating several micronodular lesions characterized by focal necrosis in the hepatic parenchyma associated with fibroblastic cells proliferation and inflammatory cells infiltration beside hyperplasia and marked atypia as seen in figure 5. Similar histological observations have been previously reported in published DENA-induced rat models [44, 45]. Combination of BER and DOX, on

the other hand, showed relatively normal hepatic histological structures that were approximately comparable to those observed in the hepatic tissues of the healthy control group with only mild dilatation in the portal vein – see figure 5. These observations firmly suggest the tumor suppressing potential of the blend incorporating both BER and DOX against DENA-induced HCC. Meantime, individual BER or DOX administration failed to completely buffer the DENA induced histological alterations in the hepatic tissues and notable dilatation and congestion of the portal vein with focal hepatic hemorrhage and periductal inflammatory cells infiltration surrounding the bile ducts as well as fibroblastic cells proliferation and malignant foci are still observed in these tissues – see figure 5.

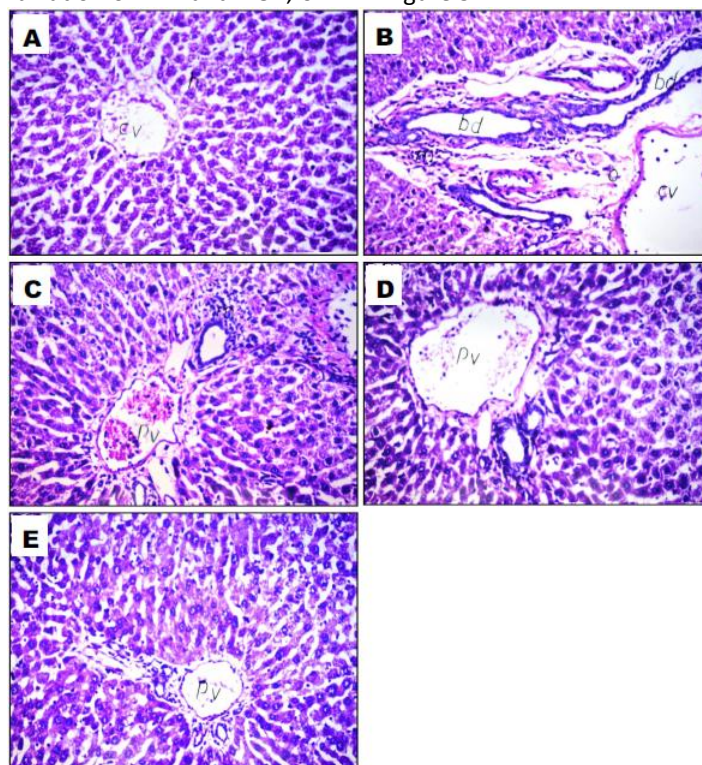


Figure-5: Representative photomicrographs of liver sections from different groups. [A] Liver tissues of the control group showing normal histological structure of hepatic lobule; [B] Liver tissues of the DENA group showing hepatocellular carcinoma distorting the normal trabecular structure of the liver with severe dilatation in the portal vein and focal necrosis, fibroblastic cells proliferation and inflammatory cells infiltrations in the hepatic parenchyma; [C] Liver tissues of the DENA+BER group showing dilatation in the portal vein with periductal inflammatory cells infiltration and fibroblastic cells proliferation as well as dilatation and congestion of hepatic sinusoids; [D] Liver tissues of the DENA+DOX group showing hepatocellular carcinoma with marked dilatation in the portal vein and focal hepatic hemorrhage as well as inflammatory cells infiltration and fibroplasia around bile duct; [E] Liver tissues of the DENA+BER+DOX group showing normal intact hepatic histological structure.

Additionally, signs of intense perturbations in the oxidant/antioxidant balance were reported clearly in our current investigation which revealed serious increases in oxidative and nitrosative damages in liver tissues of DENA group in the form of significantly increased lipid peroxidation products MDA ($p < 0.001$) and NO levels ($p < 0.001$) associated concomitantly with significant depletion in the GSH content ($p < 0.001$) and SOD activity ($p < 0.001$) in comparison to the healthy control. Increased generation of reactive oxygen species (ROS) and decreased antioxidant enzymes in liver tissues have been reported in many models of DENA induced HCC [46]. These studies concluded that oxidative stress and free radicals play a major role in tumor promotion through interaction with critical macromolecules including lipids, DNA, DNA repair systems, and other enzymes leading eventually to induction of DNA damage and lipid peroxidation [47]. As expected, individual DOX administration in DENA+DOX group clearly potentiates the DENA-induced oxidant/antioxidant disruption, most likely due to the attendant recognized prooxidant activities of DOX itself [48].

Unexpectedly, in comparison to the DENA group, administration of BER, either in individual or combination therapeutic protocols, failed to calm-down the abovementioned DENA-induced oxidative stress, rather, it aroused more disruptions in the levels of the estimated biomarkers of oxidative stress than their corresponding values in DENA group in the form of insignificant increases in hepatic tissue levels of MDA and NO with concomitant insignificant decreases in hepatic tissue GSH content and SOD activity. These data contradicted with results of several studies which established inhibitory effects of BER on chemically induced lipid peroxidation and oxidative stress in CCl₄-induced toxic liver damage [49]. A possible accommodation for this contradiction is presented by the results of a recent report which attributed the BER-induced cytotoxicity in malignant HepG2 cells to the BER-forced ROS overproduction that may be the upstream event to procaspases activation and could be the critical initiator of apoptotic cell death through a mitochondrial/caspases pathway [15]. It is worth noting that, in the contrary, such BER-forced cell death is inapplicable in normal hepatocytes mainly due to its no influence on ROS production in these cells. Additional support for this hypothesis was proposed by another study which concluded that

enhancement of ROS production represents the key element in the selective BER-induced apoptosis in human prostate cancer cells, but not in the normal prostate epithelial cells [17]. This inconsistent cytotoxicity of BER in prostate cancer cells and prostate epithelial cells strongly support the hypothesis that the resistance to BER-induced oxidative stress of normal cells but not their corresponding malignant cells appears non-specific to certain tissues.

Finally, all the aforementioned results in our study strongly suggest that neither BER nor DOX singular administration exhibited a reproducible anticancer activity against chemically induced HCC in rat model. Their combinatorial use, on the other hand, revealed indubitable *in vivo* antineoplastic activity against this kind of cancer as confirmed herein through assessment of some biochemical, molecular and histological proofs. These results are in agreement with other research which reported that BER alone and DOX alone did not show any considerable effects on murine melanoma tumor growth; however their combination strongly inhibited the *in vitro* cell growth and induced cell death, and caused G2/M arrest in cell cycle together with a decrease in Kip1/p27. In a corresponding *in vivo* experiment, this combination significantly decreased murine B16F10 xenograft tumor volume (85%, $p < 0.005$) and tumor weight (78%, $p < 0.05$) as compared to control indicating inhibition of proliferation and an increase in apoptosis [19]. Also, these results were in agreement with the study which reported that BER was able significantly to enhance the anticancer effect of DOX in A549 and HeLa cells *in vitro*, possibly mediated by inducing apoptosis [18]. Moreover, another recent study presented an additional testimony for the synergistic anticancer potential of BER and DOX combination in the treatment of lung cancer. The authors concluded that BER was able to sensitize lung cancer cells to the cytotoxic effect of DOX by suppressing the DOX-mediated activation of the signal transducer and activator of transcription 3 (STAT3) which plays critical roles in malignant transformation and progression and was found to be constitutively activated in a variety of human cancers [50].

CONCLUSION

In conclusion, our study revealed that combining BER with DOX could resemble a novel strategy to

synergistically generate *in vivo* anticancer effects against HCC supposedly due to their dual apoptotic endeavor. Nevertheless, more investigations are definitely required to translate this promising preclinical efficacy of the BER and DOX combination into clinical practice for treating cancer patients.

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Conflict of interest:

The authors declare that there is no conflict of interests.

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