Arch. Immunol. Ther. Exp. (2014) 62:511–520
DOI 10.1007/s00005-014-0292-z

ORIGINAL ARTICLE

Effect of Diethylcarbamazine Citrate and *Setaria equina* Excretory–Secretory Material on Rat Hepatocellular Carcinoma

Mahmoud Abdel-Latif · Thabet Sakran · Gamal El-Shahawi · Hoda El-Fayoumi · Al-Mahy El-Mallah

Received: 1 October 2013 / Accepted: 21 March 2014 / Published online: 31 May 2014

Abstract Diethylcarbamazine citrate (DEC) has been known for its efficacy to eradicate bancroftian filariasis in Egypt and other countries in the world. One of the known effects was to decrease the level of circulating filarial antigen in the patient's serum. The target of this study was to examine the effect of DEC, excretory–secretory (ES) material from the filarial parasite *Setaria equina* or a combination of both on the status of oxidative stress and pathogenesis of rat hepatocellular carcinoma (HCC) induced by diethylnitrosamine and 2-acetylaminofluorene. This could be tested in vitro using nitroblue tetrazolium reduction test for measuring the level of superoxide anion ($O_2^{\bullet -}$) released from rat peritoneal macrophages. For in vivo test, a single dose before induction of carcinogenesis or continually repeated doses with DEC, ES or DEC + ES was used. Exposure of macrophages to ES could lead to a significant decrease ($p < 0.01$) in $O_2^{\bullet -}$release, while DEC (200 μM) could modulate such effect with significant increase ($p < 0.05$). Pathogenesis of liver cancer and treatment were evaluated using histological investigation, level of antioxidant and liver function enzymes. Repeated ES doses could increase the activity of antioxidant enzymes, especially the catalase enzyme and show a protective effect on liver architecture. DEC could modulate the later effects when combined with ES. No significant effect on the liver function enzymes after treatment was observed. Nuclear factor κB was found to be localized only in the cytoplasm after single and repeated treatments with ES. This study could indicate the effect of *S. equina* ES as antioxidant against rat HCC, while DEC could modulate such effect when combined with it.

Keywords Diethylcarbamazine citrate · *Setaria equina* · Excretory–secretory material · Hepatocellular carcinoma

M. Abdel-Latif (✉) · T. Sakran · G. El-Shahawi · H. El-Fayoumi · A.-M. El-Mallah
Zoology Department, Faculty of Science, Beni-Suef University, Salah Salem Street, Beni-Suef 62511, Egypt
e-mail: mahmoud_1232000@yahoo.com

Introduction

Diethylcarbamazine citrate (DEC) has been known as one of the piperazine derivatives (*N*,*N*-diethyl-4-methyl-2-piperazine) which had a significance in anti-filarial chemotherapy (Gayral et al. 1989). It has also been known for its efficacy to eradicate bancroftian filariasis (BF) in Egypt and other countries in the world (Helmy et al. 2006; Raju et al. 2010). The mode of action for DEC was postulated as indirect on the parasite through inhibition of arachidonic acid metabolism in microfilariae and in host endothelial cells leading to an increased host innate immunity (Maizels and Denham 1992). DEC was found as a protective drug against liver injury and cancer development induced by leukotrienes because it is known as an inhibitor for its biosynthesis (Gonzàlez et al. 1994; Ikegami et al. 2005; Menna et al. 2010). Moreover, DEC was found to decrease oxidative stress in occult and chronic cases of filarial infection (Pal et al. 2006). It was postulated as an activator for natural killer (NK)-cell activity associated with increased interferon γ and interleukin 2 release after treatment to filarial infection (Pedersen et al. 1987). Furthermore, the drug was able to decrease the load of human immunodeficiency virus in co-infected patients with filariasis (Nielsen et al. 2007). This could give an indication for potent DEC action during filarial infection.

Adult *Setaria equina* is a filarial parasite commonly found floating free within the peritoneal cavity of the

equines in all parts of the world (Coleman et al. 1985). Worldwide, several surveys revealed high incidence of the parasite both in equines and vectors (Marzok and Desouky 2009). The source of excretory–secretory (ES) of adult female worm *S. equina* is the amniotic fluid which is the fluid located between the egg membrane and embryo in the uterine egg (Thilagavathy et al. 1990). ES is released along with microfilariae from adult female worms. Exposure of mice to ES resulted in an immunosuppression while treatment with DEC could initially potentiate the immune response followed by immunosuppression (Devi and Raj 1994). An immunomodulatory action of DEC after treatment to *S. equina* infected rats has been observed (El-Shahawi et al. 2010). This reversed effect on immune system was revealed by an increase in infiltrated leukocytes, cellular adherence and microfilaricidal effect.

The target of the current study was to examine the effect of DEC, ES or a combination of them on released superoxide anion ($O_2^{\bullet-}$) from rat macrophages and the pathogenesis of induced rat hepatocellular carcinoma (HCC). The results could show an antioxidant effect for ES, while DEC could modulate such effect.

Materials and Methods

Collection of *S. equina* Female Worms and Preparation of ES

Eighty female worms were collected from the peritoneal cavity of equines during slaughtering for lions feeding in Zoo. The worms were collected in peritoneal fluid and transferred to laboratory. Thereafter, worms were washed three times with Tyrode's solution (composition %w/v: NaCl 0.8 g, $CaCl_2$ 0.02 g, KCl 0.02, $MgCl_2$ 0.01 g, $NaHCO_3$ 0.015 g, Na_2HPO_4 0.05 g and glucose 0.01 g) supplemented with antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin), then divided as two worms/2 ml solution/well in six-well plate which was incubated for 6 h at 37 °C with changing the solution every 2 h. The solution was collected and centrifuged for 20 min at 3,000×*g*, aliquoted and frozen at −85 °C till use. The protein content in prepared ES was determined using Bradford (1976) method.

Isolation and Activation of Rat Peritoneal-Exudate Macrophages

All the experimental animal work was carried out according to the guidelines and the standard regulations of the Faculty of Science, Beni-Suef University. Rat peritoneal macrophages were isolated as previously described (Leiro et al. 2003). Briefly, white albino rats (200–300 g) were injected intraperitoneally (i.p.) with 1 ml of 3 % thioglycollate broth (Sigma, St. Louis, USA), and peritoneal exudate was extracted 5 days later. The abdomen of the rat was soaked with 70 % ethanol for disinfection, a midline incision was then made with scissors, and the abdominal skin retracted. Thirty milliliters of DMEM (Sigma, St. Louis, USA) was then injected into the peritoneal cavity using a syringe with a 19-G needle. After gentle abdominal massage, about 30 ml of peritoneal fluid was extracted using the same syringe and transferred to 50-ml sterile polypropylene tubes on ice. A 20-µl aliquot was then extracted for cell counting in a hemocytometer and the cells were washed once by centrifugation at 1,500×*g* and resuspended to a concentration of 10^6 cells/ml. The number of viable cells was estimated by the trypan blue (Sigma, St. Louis, USA) exclusion test. Aliquots of 100 µl of the cell suspension were added to the wells of 96-well microculture plates (Corning, MA, USA) and left for 90 min in a humidified incubator (37 °C, 5 % CO_2) to allow adhesion. Non-adherent cells were then removed by gently washing with DMEM.

Nitroblue Tetrazolium Reduction Assay

DEC (Sigma, St. Louis, USA), ES and combination of both were added in duplicates to the plate to test their effect on released $O_2^{\bullet-}$ from isolated rat macrophages. One hundred micrometers of DEC (0, 50, 100, 200 and 400 µM), ES (50 µg) or combination of former DEC concentrations with ES was added to the plate and left for 1 h at 37 °C, 5 % CO_2. One hundred micrometers of 0.1 % nitroblue tetrazolium (NBT; Park, Northampton, UK) in phosphate-buffered saline (PBS) and 10 µl of opsonized zymosan were added to the wells and the plate was incubated for 30 min (Hisadome et al. 1990). For photometrical quantification, cells were fixed in 100 % methanol (15 min) and washed twice in 70 % methanol and plates were dried at room temperature. The fixed cells were homogenized in 62.5 µl 2 M KOH (Rasayan laboratories, Gujarat, India) and 75 µl dimethyl sulfoxide (Sigma, St. Louis, USA) per well. The OD_{650} of 100 µl of the cell lysate was determined (Helming et al. 2005). Mean ± SD was calculated from four independent experiments.

Hepatocarcinogenesis Model and Regimen of Treatment

The experimental hepatocarcinogenesis was initiated by diethylnitrosamine (DEN) and promoted by 2-acetylaminofluorene (2-AAF) according to the protocol described with some modifications (González de Mejía et al. 2004). Hepatocarcinogenesis was evaluated by histological observations for the rat liver. Eighty male white albino rats (180–200 g weight, 2 months of age) were randomly allocated into eight groups (Fig. 1) of ten animals each and

acclimated to the laboratory environment at 23 °C and exposed to 12 h of light and 12 h of dark photoperiod. The first group was a normal control while other groups were induced for HCC. For the induction of hepatocarcinogenesis, rats were injected i.p. with a single dose of DEN (Sigma, St. Louis, USA) dissolved in water (100 mg/kg body weight). They also received via gavage a solution of 2-AAF (20 mg/kg body weight) in 1 % Tween 80 (Sigma, St. Louis, USA) on days 7, 8, 9 after DEN injection for promoting carcinogenesis. One of these seven groups acted as a tumor control group (HCC) because it was only exposed to DEN and 2-AAF without any treatments but received the vehicle solutions for treatments in other groups. Rats in the normal control group (G1) were given an i.p. injection of the same volume of water, Tyrode's solution and 1 % Tween 80 in saline as those in HCC group (G2). The other six rat groups were divided into three for single dose treatment and three for continually repeated dose treatment. The first three groups (G3, G4, G5) were pretreated i.p. with 100 μg ES (Chenthamarakshan et al. 1996) in Tyrode's soln, 100 mg/kg DEC (Mitsui et al. 1996) in saline or DEC + ES two days before DEN injection. The second three groups (G6, G7, G8) were pretreated as in the first three groups but also post-treated with the same materials. Post-treatments were given per month for a period of 4 months. G1 was also injected with saline and Tyrode's soln at the time of pre- and post-treatments. After one week of last injection, animals were euthanized where liver tissue and blood samples were taken.

Tissue and Sample Preparations

Parts of liver tissue were fixed in 10 % buffered formalin (Sigma, St. Louis, USA), dehydrated in alcohol series and then embedded in paraffin wax for histological analysis. Sections (6 μm) were then stained with hematoxylin and eosin (Sigma, St. Louis, USA) and examined under light microscope (Olympus optical corp., Tokyo, Japan). Other parts of tissue were frozen to be homogenized in ice-cold Tris–HCl buffer (150 mM, pH 7.4). The w/v ratio of the tissue to the homogenization buffer was 1:10 w/v. Liver homogenates were centrifuged at 1,500×*g* at 4 °C for 20 min and supernatants were taken. Aliquots were prepared and used for determination of different biochemical markers. Collected blood samples were centrifuged at 1,500×*g* for 20 min at 4 °C to obtain serum.

Immunohistochemistry

Antiserum for the p65 subunit of nuclear factor (NF)-κB was obtained from Abcam (MA, USA). The liver specimens were

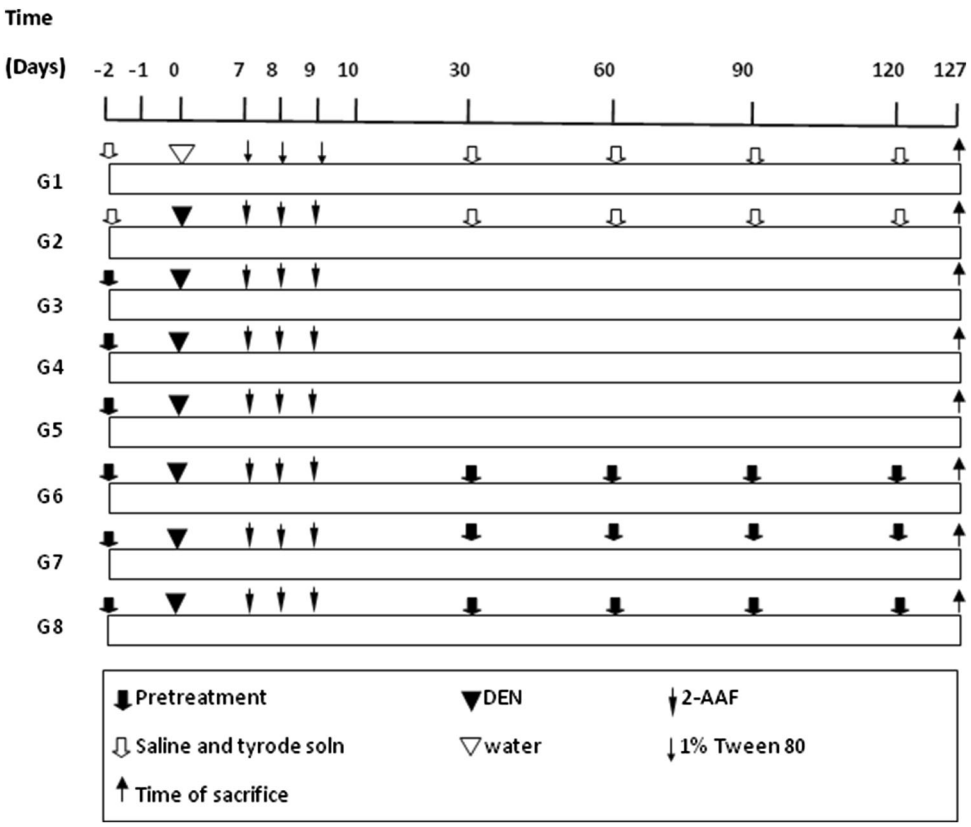

Fig. 1 Experimental schedules for administration of DEN, 2-AFF, DEC and ES. Rats ($n = 80$) were divided into the following eight groups: G1 rats received vehicle solutions; G2 received DEN and 2-AFF; G3, G4 and G5 received single dose with ES, DEC and DEC + ES, respectively, before DEN and 2-AFF injections; G6, G7 and G8 received additional doses per month

incubated with blocking buffer (PBS containing 0.5 % bovine serum albumin, 0.3 % Triton X-100, and 0.2 % horse serum). The primary antibody was used at a 1:50 dilution in PBS containing 0.5 % bovine serum albumin plus 0.2 % horse serum for NF-κB. The primary antibodies were incubated with the specimens for 1 h at 25 °C followed by washing with PBS. The immunoreactivity was visualized using the avidin and biotinylated horseradish peroxidase macromolecular complex and diaminobenzidine tetrahydrochloride system (Invitrogen, CA, USA). Specimens were counterstained with hematoxylin using standard techniques.

In Vivo Antioxidant Status

Lipid peroxidation in liver tissue homogenates was assayed according to Preuss et al. (1998). Briefly, the homogenate and 1, 1, 3, 3, tetramethoxypropane (standard) were precipitated with 0.15 ml of 76 % trichloroacetic acid (TCA). The precipitate was mixed with 0.35 ml of 0.7 % thiobarbituric acid and incubated at 80 °C for 30 min. Further 0.5 ml of 90 % TCA was added and absorbance was read at 532 nm. Other antioxidant enzymes like catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPX) were measured in liver homogenates according to the methods of Cohen et al. (1970), Marklund and Marklund (1974) and Kar and Mishra (1976), respectively.

Serum Markers of Liver Damage

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined in serum according to the colorimetric method described in instructions of commercial kits (Biodiagnostic Co., Cairo, Egypt).

Statistical Analysis

SPSS (version 20) statistical program (SPSS Inc., Chicago, IL, USA) was used to carry out a one-way analysis of variance (ANOVA) on our data. Analysis of differences between the means of the treated and control groups was performed using Dunnett's *t* test.

Results

In Vitro and in Vivo Antioxidant Properties of ES

In vitro test for $O_2^{\bullet-}$ release from rat peritoneal macrophages after DEC, ES or DEC + ES exposures was performed (Fig. 2). Each of DEC (at a dose of 400 μM) and ES could decrease the superoxide release significantly ($p < 0.05$ and $p < 0.01$, respectively). Surprisingly, a combined exposure for both DEC at 200 μM and ES could increase the $O_2^{\bullet-}$ release significantly ($p < 0.05$) compared to ES alone.

In vivo experiments did not show any effect of vehicle solution in control groups on liver tissue. Quantitative measurement for malondialdehyde (MDA) as a product of liver lipid peroxidation could show a significant increase in G2 (HCC) group ($p < 0.001$) compared to G1 or normal control (Fig. 3A). The lipid peroxidation in the liver tissue of G6 was decreased ($p < 0.01$) compared to HCC group. In contrast, lipid peroxidation increased significantly ($p < 0.05$) in G8 when compared to G6.

Activities of antioxidant enzymes like CAT and SOD could indicate a significant ($p < 0.05$) increase in G6 compared to HCC group (Fig. 3B, C). However, enzyme activity for GPX increased significantly ($p < 0.05$) only in G3 (Fig. 3D). The CAT activity decreased in G8 when compared to either G6 or G7 ($p < 0.001$ and $p < 0.05$, respectively). Moreover, the enzyme activity decreased ($p < 0.05$) in G8 when compared to G5. Significant increase in the activity of SOD was observed in G5 when compared with HCC ($p < 0.01$), G3 ($p < 0.001$), G4 ($p < 0.01$) or G8 ($p < 0.01$). Comparison of enzyme activity between G6 and G3 revealed an increase in SOD ($p < 0.01$) and decrease in GPX ($p < 0.05$) for the former. Also, GPX was increased ($p < 0.05$) in G5 compared to G4.

Histological Observations

Liver of G1 represented normal liver tissue with normal architecture (Fig. 4A). Hepatocytes were normal, arranged in hepatic strands and with normal hepatic vein. Liver tissue of G2 indicated abnormal architecture with irregularly-shaped and dysplastic hepatocytes including polymorphic nuclei and vacuolated cytoplasm (Fig. 4B). Hepatocytes of G3 showed

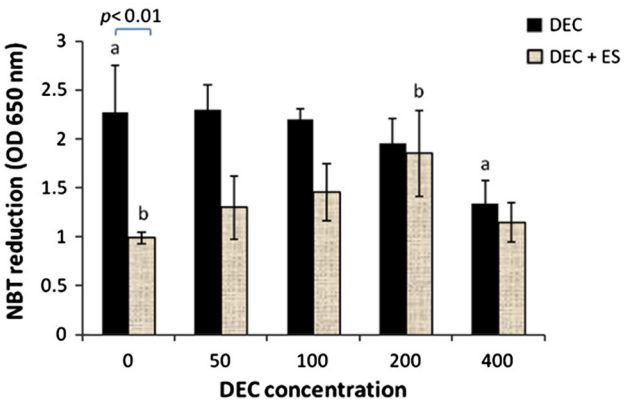

Fig. 2 In vitro effect of DEC concentrations (0, 50, 100, 200 and 400 μM), ES (50 μg/ml) or DEC + ES on $O_2^{\bullet-}$ release (NBT reduction) from rat peritoneal macrophages. *a* Difference between control and DEC at 400 μM ($p < 0.05$), *b* difference between ES and DEC (200 μM) + ES ($p < 0.05$)

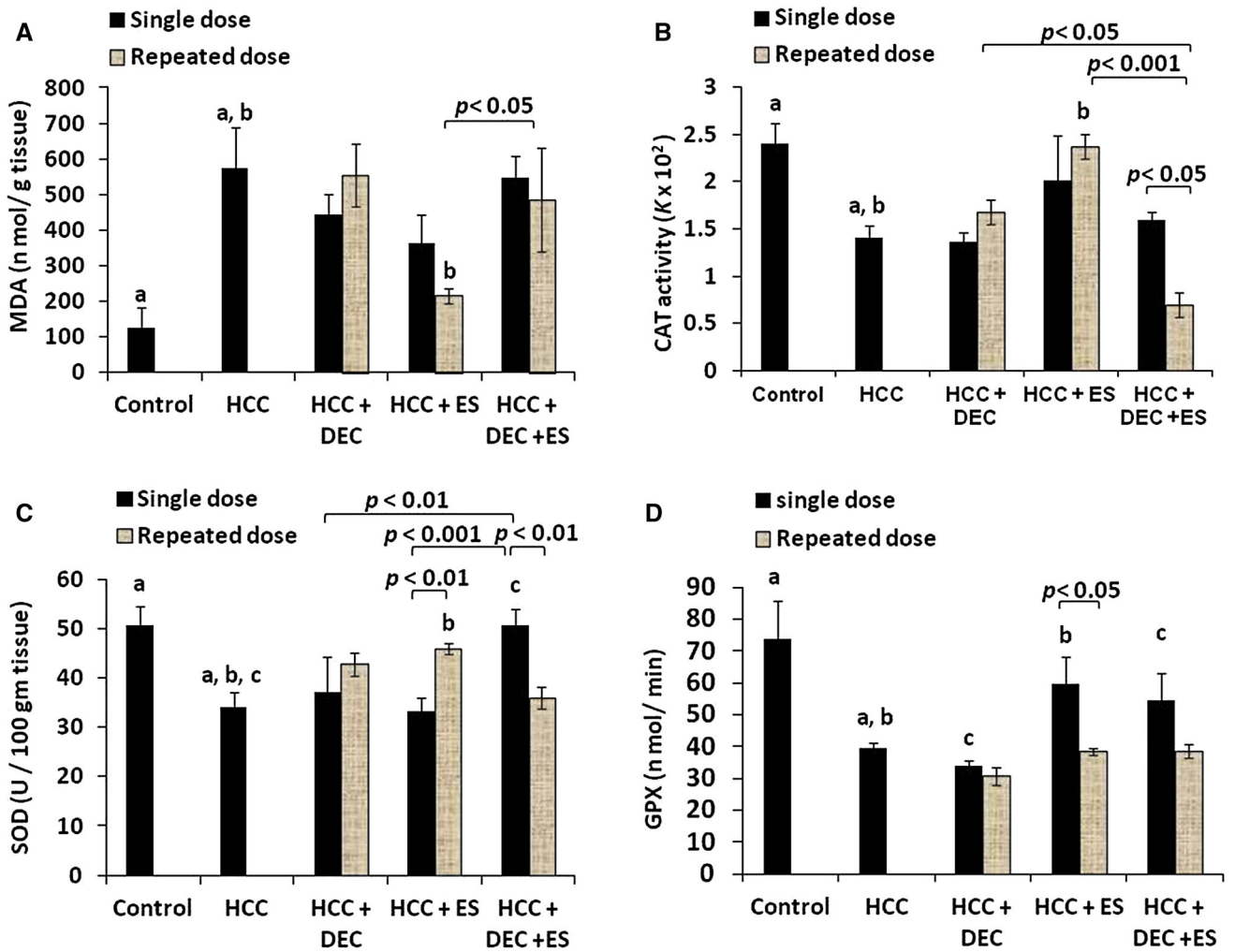

Fig. 3 In vivo effect of DEC (100 mg/kg), ES (100 µg) or combination of both on lipid peroxidation through quantitative assessment for MDA (**A**), activity of catalase (CAT; **B**), superoxide dismutase (SOD; **C**) and glutathione peroxidase (GPX; **D**) enzymes after single or repeated doses before and after DEN injections, respectively. HCC is an indication for induced hepatocarcinogensis. **A**: *a*, $p < 0.001$, *b*, $p < 0.01$; **B**: *a*, $p < 0.05$, *b*, $p < 0.05$; **C**: *a*, $p < 0.01$, *b*, $p < 0.05$, *c*, $p < 0.01$; **D**: *a*, $p < 0.01$, *b*, $p < 0.05$, *c*, $p < 0.05$

the same observation of G2 in addition to the presence of cellular infiltration (Fig. 4C). G4, G5, G7 and G8 (Fig. 4D, E, G, H, respectively) indicated an abnormal architecture with dilated congested sinusoids and central vein, but some nuclei in G5 were enlarged with greater nuclear/cytoplasmic ratio. Hyperemia and cellular infiltration were observed in G4, while G6 showed normal hepatic architecture except enlargement of nuclei and irregularity of sinusoid (Fig. 4F).

Location of NF-κB in Hepatocytes

In G1, G4 and G5 animals (Fig. 5A, D, E), the NF-κB expression was not found either in the cytoplasm or in the nucleus. It appeared slightly expressed in both of cytoplasm and nucleus of G2 (Fig. 5B). Its expression in other groups was found in the cytoplasm and not in the nucleus. Stained NF-κB in the cytoplasm was more abundant in G3, G6 and G7 (Fig. 5C, F, G) than G8 (Fig. 5H).

Liver Function

Serum markers for liver function like AST and ALT indicated a significant increase in G2 ($p < 0.01$ and $p < 0.05$, respectively) when compared to G1 (Fig. 6A, B). No significant difference in AST level between G2 and treatment groups was observed. ALT level in G3 and G8 could decrease in comparison to G5 ($p < 0.01$ and $p < 0.05$, respectively).

Discussion

It has been shown from in vitro and in vivo experiments that the filarial ES could significantly decrease the $O_2^{\bullet-}$ release from rat macrophages and lipid peroxidation, respectively, in the liver tissue. In vitro experiment has shown the downregulating effect of ES on $O_2^{\bullet-}$ production

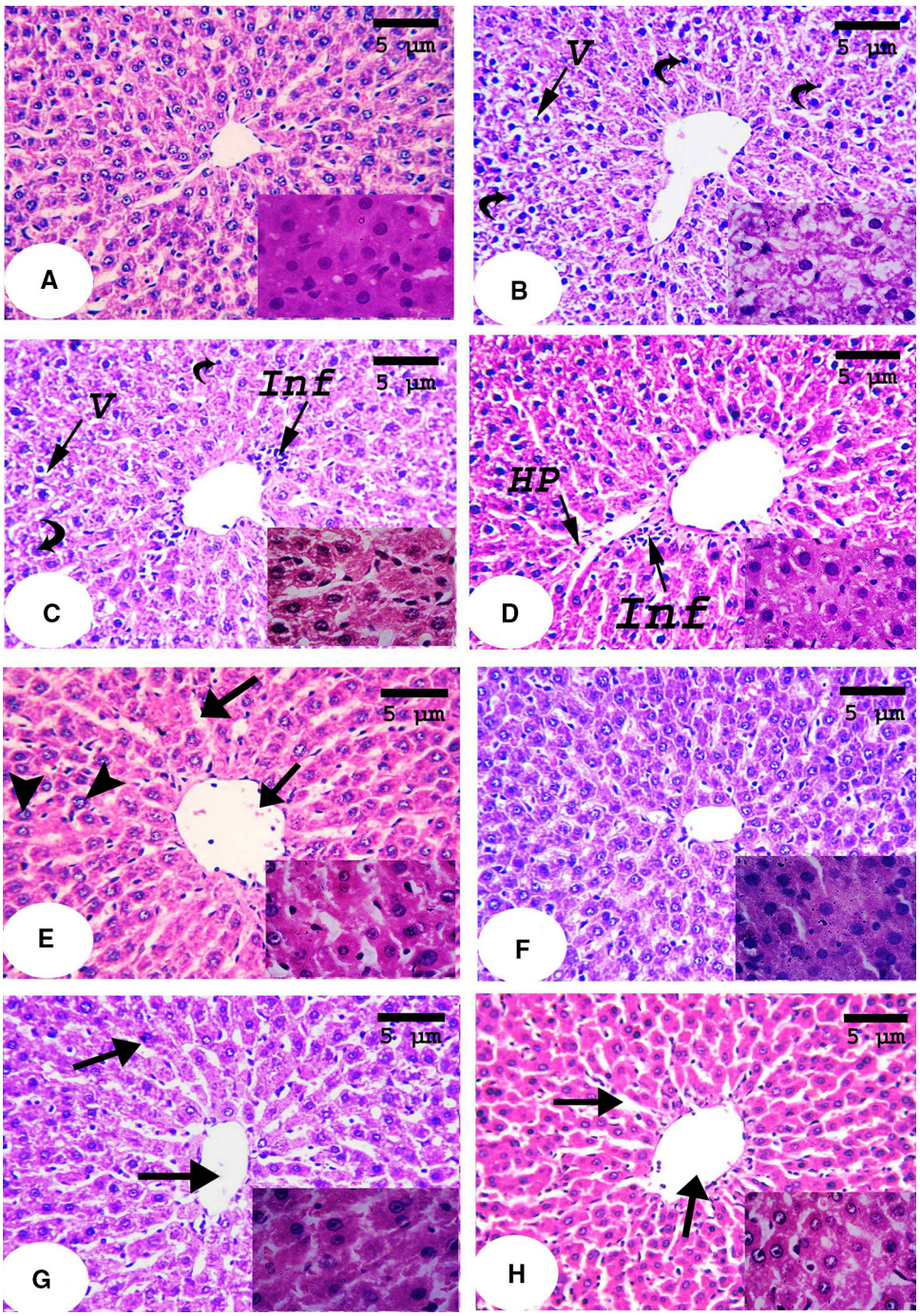

Fig. 4 Histopathological observations for liver of different rat groups (H&E) showing **A** normal control group; **B** HCC group; **C–E** HCC + single dose of ES, DEC, DEC + ES, respectively; **F–H** HCC + repeated doses of ES, DEC, DEC + ES, respectively. Vacuolated cytoplasm (V); cellular infiltration (Inf); hyperemia (HP); enlarged nuclei showing greater nuclear/cytoplasmic ratio (*arrow head*); polymorphic nuclei (*curved arrow*); dilated congested sinusoids or central vein (*straightly coarse arrow*). *Scale bars* are presented

which has been known as one of the major reactive oxygen species responsible for oxidative stress and carcinogenesis (Klaunig and Kamendulis 2004). The results of in vivo experiment were related to what observed on intraperitoneal macrophages where ES acted as an antioxidant in both experiments. The application of single and repeated doses in this study was to investigate which of them is enough to mount a beneficial effect against development of hepatocarcinoma cells. This effect was obvious after repeated ES compared to single ES administration. Therefore, the beneficial effect of ES as an antioxidant in hepatocarcinoma was dependent on repeated doses.

ES could increase the activity of antioxidant enzymes (CAT, SOD and GPX) compared to HCC group. Furthermore, repeated administrations with ES could suppress any histological alterations in the liver except the existence of a large nuclei and irregular sinusoides. This may be

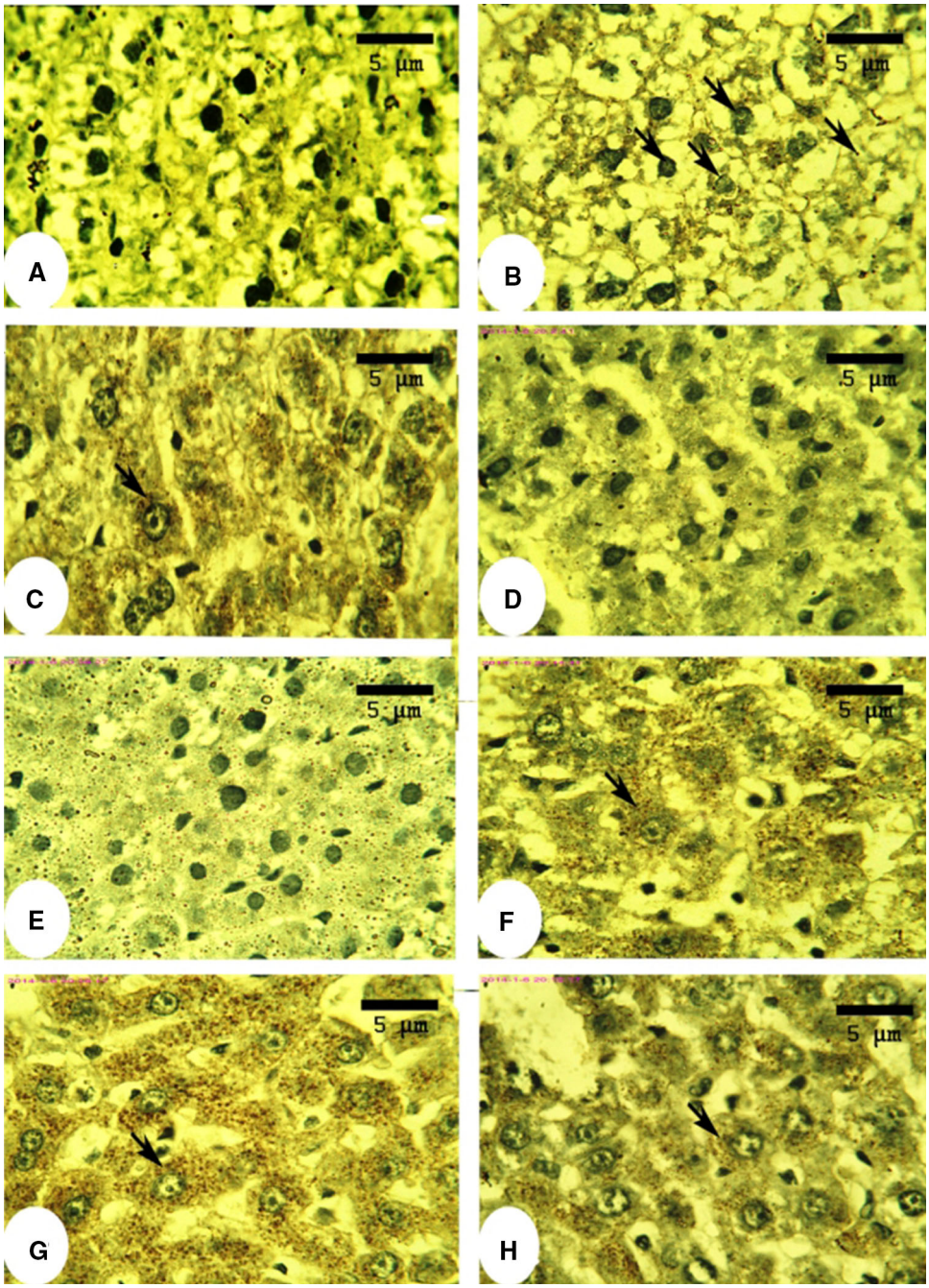

Fig. 5 Immunohistochemical staining for NF-κB p65 showing its localization in the hepatic cells of different rat groups. **A** Normal control group; **B** HCC group; **C**–**E** HCC + single dose of ES, DEC, DEC + ES, respectively; **F**–**H** HCC + repeated doses of ES, DEC, DEC + ES, respectively. Stained NF-κB in the cytoplasm was more abundant in G3, G6 and G7 (**C**, **F** and **G**) than G8 (**H**). *Scale bars* are presented

compatible with the previous reports about the induced cellular changes by ES like nuclei hypertrophy and muscle bundles becoming rounded and separated from each other (Ko et al. 1994; Li et al. 1999). The decrease of serum markers for liver function after ES treatment did not decrease significantly in comparison to HCC group. ES from filarial parasites contains antioxidant substances which can downregulate the release of reactive oxygen species or oxidative stress (Prince et al. 2013). This downregulating effect of ES is to protect the parasite from host immune cellular attack and release of reactive oxygen species like $O_2^{\bullet -}$ for killing. Secreted material from *Brugia malayi*, *Dirofilaria immitis* and *Onchocerca volvulus* was found to contain CuZn superoxide dismutases, selenocysteine-independent glutathione peroxidase and glutathione *S*-transferase (Selkirk et al. 1998). Moreover, investigation for the components of *Setaria digitata* ES indicated an enrichment with ubiquinones which are antioxidants (Mohan et al. 2001). ES products of other parasitic worms were studied for their properties where the antioxidant activity due to the presence of antioxidant enzymes was found (Farahnak et al. 2013). However, ES from *Toxocara canis*

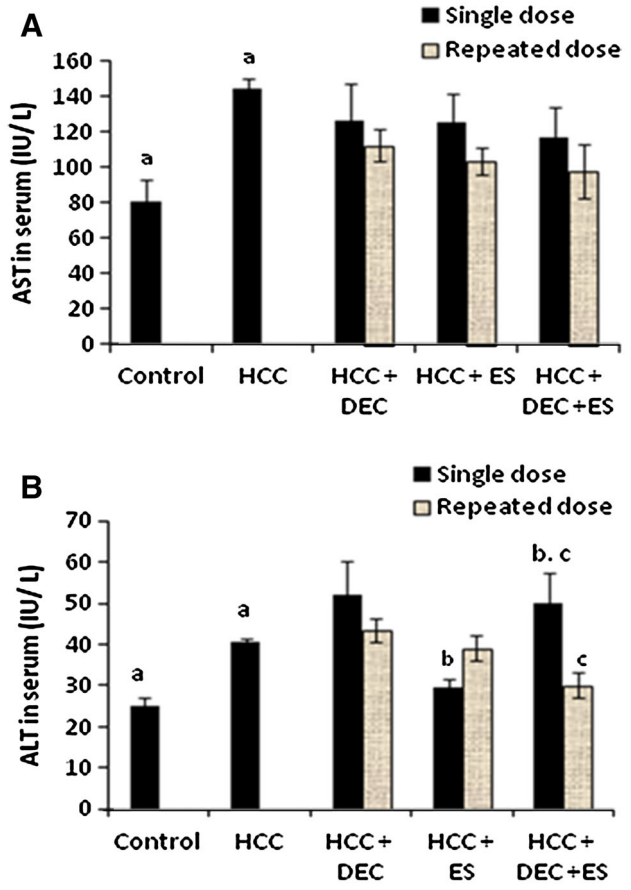

Fig. 6 Effect of DEC (100 mg/kg), ES (100 µg) or combination of both on serum markers of liver function (AST and ALT). **A**: *a*, $p < 0.01$; **B**: *a*, $p < 0.05$, *b*, $p < 0.01$, *c*, $p < 0.05$

was found to increase nitric oxide production through intracellular signaling pathways and induce apoptosis in astrocytes (Espinoza et al. 2002; Hsiao et al. 2013). Exposure of rats to a single dose of ES could elevate GPX but histopathological investigations could show infiltrated immune cells in the tissue. The repeated exposure to ES could elevate the antioxidant enzymes except GPX but there were no infiltrated cells in the hepatic tissue. It seemed that the level of later enzyme increases during tissue inflammation (Ishibashi et al. 2002). In addition, high level of selenium glutathione peroxidase was found in ES of *Setaria cervi* after in vitro cultivation (Singh and Rathaur 2005). These selenium containing compounds were found to protect against hepatocarcinogenesis (Novoselov et al. 2005). The infiltrated cells after first dose of ES might be responsible for the non-significant decrease of liver function enzymes in spite of antioxidant effect. Furthermore, ES has been used as crude material which contains a mixture of different components and not a purified one. Therefore, purification of antioxidant components from ES material is needed.

NF-κB was not activated by DEC, ES or combined treatment. This was obvious because NF-κB was not found or translocated in the nucleus in comparison to HCC group which showed slightly expressed NF-κB in the nucleus. The higher cytoplasmic expression of NF-κB was found after single or repeated treatments with ES, while none or low reaction was found after single or repeated treatments with DEC, respectively. The activation of NF-κB or its translocation into the nucleus was found to correlate positively with cancer cell growth and survival (Guo et al. 2001; Mauriz et al. 2003). NF-κB activation is generally controlled by reactive oxygen species, so that its activation can be inhibited by antioxidants (Tunon et al. 2003). The current results confirm the antioxidant effect of *S. equina* ES against cancer cells. Single and repeated treatments with DEC could decrease the expression of NF-κB in both of G5 and G8 when compared to G3 and G6, respectively. This confirms the results of Santos Rocha et al. (2012) where DEC could decrease the expression of NF-κB and inflammatory cytokines in alcohol-induced liver injury.

In vitro effect of DEC on $O_2^{\bullet -}$ release could show a dose-dependent decrease and statistical significance at higher concentration (400 µM). However, this dose-dependent decrease was reversed to dose-dependent increase when ES was combined with DEC with statistical significance at 200 µM. This means that the combination of both DEC and ES could modulate the effect of ES for suppressing the $O_2^{\bullet -}$ release from rat macrophages. This can be one of the effective mechanisms for DEC to kill the filarial parasites by dampening the ES action of suppressing the host immune response (Muthian et al. 2006). In consistence, previous studies have indicated the reversed status of immune reactivity after DEC treatment (Bhattacharya et al. 1997; Devi and Raj 1994; Lammie et al. 1992). It is apparent that the exposure of antioxidants in ES to DEC could lead to their suppression. This may explain the necessity for supplying DEC in repeated doses to treat filariasis (Helmy et al. 2006).

Comparison between the level of lipid peroxidation in the liver tissue after ES and ES + DEC repeated administrations could indicate an obvious increase in the later group. This can confirm the in vivo suppressing effect of DEC on ES anti-oxidative effect. In contrast, activity of CAT enzyme was found to decrease in DEC + ES compared to that in ES group. The same result was also found in SOD measurement; however, the difference was not significant. Repeated administration with DEC + ES increased the oxidative stress through increased lipid peroxidation and decreased CAT while histological observation for the liver tissue could show many dilated sinusoids which are type of sinusoidal blood vessels. This also appeared in other groups which have been treated with DEC. The effect of this drug on liver architecture was

shown through a study involving its effect on microfilaremic dogs infected with *D. immitis,* where the dilation of blood vessels and lymphatics together with increased inflammatory cell infiltrates was apparent (Sutton et al. 1985).

It can be concluded that ES could protect against lipid peroxidation in the liver tissue by increasing the activity of CAT after repeated injections or GPX after single one. Histological observations were in line with the results of oxidative stress where the rat group repeatedly administered with ES could show normal appearance compared to single ES or HCC groups. Although DEC is an inhibitor for lipoxygenase pathway which contributes to inflammatory responses and subsequently decreased oxidative stress, co-administration of DEC with ES could rather increase the oxidative stress in the liver tissue (Etienne et al. 1985). Although DEC was found to decrease liver damage induced by alcohol through histological investigation and decreased AST (Santos Rocha et al. 2012), the later was not significantly decreased after DEC or ES treatments in the current study. This may be related to a technical procedure or the used carcinogen for inducing liver damage.

It was evident that repeated exposure for DEC + ES was more effective to reduce CAT activity in comparison to single exposure of DEC + ES or repeated exposure to DEC alone. This may be related to more potent stimulation for liver innate immunity or inflammatory responses after repeated exposure to DEC + ES rather than single DEC + ES or repeated DEC alone (Seki et al. 2011). This can be also consistent with the outlines of previous studies about the potent effect of the drug to increase the immune response and eradicate the virus infection during filarial co-infection (Nielsen et al. 2007; Pedersen et al. 1987). Nevertheless, administration by DEC + ES for once could increase the activity of SOD and GPX in comparison to single treatment by DEC or ES. However, this was not enough to downregulate the lipid peroxidation. Therefore, a single exposure to both of them did not have antioxidant effect. ALT level after single DEC + ES was found to increase as compared to both single ES and repeated DEC + ES. Furthermore, histological investigation for single DEC + ES group indicated both hyperemia and red blood cells in sinusoids. This was consistent with the previous observation for hemorrhage around the central vein in the dog liver after DEC treatment (Sutton et al. 1985). Single or repeated treatment with DEC plus ES did not confer antioxidant activity. However, DEC therapy was found to decrease oxidative stress in filarial infection where this was related to decrease of circulating antigen level in blood (Pal et al. 2006).

Anti-*S. cervi* ES antibodies were found to identify circulating filarial antigen in infected human serum with BF (Mustafa et al. 1996). In addition, an apparent cross reaction of BF-infected human serum with *S. equina* ES antigen could be observed (Bahgat et al. 2011). Therefore, treatment of infected human with DEC leading to a decrease in the level of circulating filarial antigen and enhancement of immunity might be related to binding of such drug to the circulating filarial antigen. In this study, DEC was not able to reduce the hepatocarcinogenesis but further studies should be carried out to investigate the effect of DEC binding to different ES components on enhancement of innate immunity, especially NK activity.

In conclusion, ES from filarial worms can downregulate the oxidative stress by increasing the activity of antioxidant enzymes, especially the catalase enzyme. DEC which had been known as an effective antifilarial drug could modulate the ability of ES to decrease the oxidative stress or the inflammatory response. Isolation of antioxidant components in ES can be more effective to treat hepatocarcinogenesis without unpleasant effects, such as non-significant effect on liver enzymes, nuclei hypertrophy or irregular sinusoids.

Acknowledgments This work was supported by the Advanced Science Research Institute (ASRI) of Beni-Suef University, Beni-Suef city, Egypt. The authors had no commercial interest in the study.

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