

Biochemical and molecular evidences for the antitumor potential of *Ginkgo biloba* leaves extract in rodents

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Hepatocellular carcinoma (HCC) is one of the deadliest primary cancers, with a 5-year survival rate of 10% or less. This study was undertaken to elucidate the underlying biochemical and molecular mechanisms in favor of N-nitrosodiethylamine-induced hepatocellular carcinoma. Furthermore, the aim of this work was extended to explore the efficacy of *Ginkgo biloba* leaves extract in deterioration of HCC in rats. In the current study, HCC group experienced significant downregulation of ING-3 gene expression and upregulation of Foxp-1 gene expression in liver. Treatment of HCC groups with *Ginkgo biloba* leaves extract resulted in upregulation of ING-3 and downregulation of Foxp-1 gene expression in liver. In addition, there was significant increase in serum alpha-fetoprotein (AFP), carcinoembryonic antigen (CEA) and glypican-3 (GPC-3) levels in HCC group versus the negative control group. In contrast, the groups with HCC subjected to either high or low dose of *Ginkgo biloba* leaves extract elicited significant reduction ($P < 0.05$) of AFP, CEA and GPC-3 in serum compared to the untreated HCC rats. Besides, histological examination of liver tissue sections of rats in HCC group revealed typical anaplasia. Interestingly, treatment with *Ginkgo biloba* leaves extract elicited marked improvement in the histological feature of liver tissue in HCC groups. In conclusion, this research indicated that the carcinogenic potency of N-nitrosodiethylamine targeted multiple systems on the cellular and molecular levels. In addition, the results of the current study shed light on the promising anticancer activity of *Ginkgo biloba* leaves extract in treatment of hepatocellular carcinoma induced chemically in the experimental model through its apoptotic and antiproliferative properties.

Key words: Hepatocellular carcinoma; *Ginkgo biloba*; AFP; CEA; GPC-3; ING-3; Foxp-1; histopathology

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Abbreviations: AFP, alpha-fetoprotein; ANOVA, one way analysis of variance; CEA, carcinoembryonic antigen; DNMT1, DNA methyltransferase-1; FADD, Fas-associated death domain; Foxp-1, Forkhead box transcription factor; GPC-3, glypican-3; HCC, Hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus; ING3, inhibitor of growth-3; NDEA, N-nitrosodiethylamine; SAH, S-adenosyl-L-homocysteine; SAM, S-adenosyl methionine; Skp2, S-phase kinase-associated protein-2; SPSS, statistical Package for the Social Sciences; STAT3, signal transducer and activators of transcription 3 and Tcf, transactivate T cell factor

INTRODUCTION

Hepatocellular carcinoma (HCC), like any other cancer, develops when there is a mutation in the cellular machinery that causes the cell to replicate at a higher rate and results in the cell growth avoiding apoptosis (Sapisochin & Charco, 2011). Among the primary liver cancers, HCC represents the major histological subtype, accounting for 70% to 85% of the total liver cancer burden worldwide (Wang & Shen, 2013). Globally, it is the fifth most common cancer and the second leading cause of cancer-related death (Zhang *et al.*, 2013). In developing countries, incidence rates are two- to three-folds higher than in developed countries (Harring *et al.*, 2011).

In Egypt, HCC contributes to 14.8% of all cancer mortality with a higher incidence in males (17.3%) than in females (11.5%). It is the second most frequent cancer type in Egyptian males after bladder cancer and the eighth most frequent in Egyptian females (Aleem *et al.*, 2012). Hospital-based studies from Egypt reported an overall increase in the relative frequency of all liver-related cancers in Egypt, from approximately 4% in 1993 to 7.3% in 2003 (El-Zayadi *et al.*, 2005). This rising incidence may be due to high prevalence of hepatitis C virus (HCV) and its complications and the fact that people born 20 years ago or earlier in Egypt have not been vaccinated against hepatitis B virus (HBV) (Lehman *et al.*, 2008). The strongest risk factors for HCC include chronic inflammations of the liver and subsequent cirrhosis due to hepatitis B and hepatitis C viral infections, alcohol abuse and metabolic liver diseases (diabetes and non-alcoholic fatty liver disease). Moreover, obesity, environmental pollutants, consumption of food contaminated with the fungal toxins such as aflatoxins produced by *Aspergillus flavus* in food grains as well as nitrosamine consumption are the common risk factors for HCC development (Farazi & De Pinho, 2006).

N-nitrosodiethylamine (NDEA) is an N-nitroso alkyl compound described as an effective hepatotoxin in the experimental animals, generating toxicity due to frequent administration (Jose *et al.*, 1998). Nutrients containing NDEA include smoked, salted and dried fish, cheese, cured meat, soybeans and alcoholic beverages (Liao *et al.*, 2001). NDEA is mostly metabolized in the liver by the action of cytochrome p450 enzymes, and its reactive metabolites are mainly responsible for its hepatotoxic effects. NDEA is activated to ethyldiazonium ion which alkylates DNA bases to form pro-mutagenic adducts such as O6-ethyldeoxyguanosine and O4 and O6-ethyldeoxythymidine and these bioactive molecules induce oxidative stress and cytotoxicity by damaging biomol-

ecules such as DNA, lipids and proteins (Verna *et al.*, 1996).

The tumor-suppressor ING3 gene plays a continuous role throughout the cancer advancement and development. The changes of this gene were shown to be involved in tumor transcriptional regulation, apoptosis and cell cycle arrest. Yang and coworkers (2012) demonstrated that the dysregulation of ING3 in HCC occurred at both transcription and translation levels. It was found that ING3 was significantly downregulated in HCC tissues compared with adjacent non-cancerous tissues. The immunohistochemical staining of tissue microarray data indicated a significant reduction of ING3 expression in 57.14% of HCC cases (64/112). Such downregulation was associated with the tumor differentiation stage and correlated with tumorigenesis, and the advancement of HCC (Lu *et al.*, 2012).

Forkhead box transcription factor (Foxp-1) gene, a member of F box family of ubiquitously expressed transcription factors, plays a critical role in cancer development. The expression of FOXP1 mRNA and protein in hepatic cancer cells is much higher than in normal hepatic cells. The comparison of clinicopathological characteristics and immunohistochemistry showed that the high expression of FOXP1 in HCC was related to large tumor diameter, high serum α -fetoprotein levels, and later stage grouping with tumor node metastasis classification. It was shown that high FOXP1 expression correlates with an aggressive malignant phenotype and may constitute a novel prognostic factor for HCC (Zhang *et al.*, 2012).

Natural products are non-toxic extracts or compounds that compared with synthetic equivalents, generally produce less side effects (Genoux *et al.*, 2011). *Ginkgo biloba* belongs to the botanical family of *Ginkgoaceae*, one of the best known examples of a living fossil. The constituents of *Ginkgo biloba* leaves include high levels of flavonoids (kaempferol and quercetin), terpenoids (ginkgolides and bilobalide) and Biflavonoids (ginkgetin) (Puttalingamma, 2015). *Ginkgo biloba* leaves extract has powerful anticancer properties through its antioxidant, gene-regulatory and antiangiogenic properties (Sagar *et al.*, 2006). *Ginkgo biloba* leaves extract could regulate cell proliferation and induce apoptosis, therefore, it might have protective effects against hepatocarcinogenesis (Chao & Chu, 2004).

The current study was designed to elucidate the biochemical and molecular mechanisms of N-nitrosodiethylamine-induced HCC in rats. In addition, our objective was extended to investigate the potency of *Ginkgo biloba* leaves extract in inhibiting the HCC progression with special concern for its mechanism of action.

MATERIALS AND METHODS

Materials

Chemicals and drug. N-nitrosodiethylamine (NDEA) (CAS no. 55-18-5) was purchased from Sigma-Aldrich Chemicals Co. (St Louis, MO, USA). Doxorubicin was supplied from Pharmacia Italia S.P.A. Milan, Italy. All other chemicals used in the present study were of high analytical grade and were purchased locally (Egypt).

Plant materials. *Ginkgo biloba* leaves were purchased from local market, Cairo, Egypt. The plant was authenticated by Professor Ibrahim El-Garf, Professor of Plant Taxonomy, Department of Botany, Faculty of Science, Cairo University. Voucher Specimens were kept in the

herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Cairo University, Cairo, Egypt.

Preparation of *Ginkgo biloba* leaves methanolic extract. *Ginkgo biloba* leaves methanolic extract was prepared by adding 2000 ml of 70% methanol to 1 kg of *Ginkgo biloba* leaves and leaving the mixture for 72 hours, then the extract was filtered using filter paper. Then the filtrate was evaporated under reduced pressure at 45–50 °C using rotary evaporator to give a dry residue of crude aqueous methanolic extract (4.5% from dry leaves) (Van Beek, 2002).

Animals and experimental protocol. A total number of 60 adult female albino rats of Wistar strain weighing 120–150 g were used in the current study. The rats were obtained from the Animal House Colony of the National Research Centre, Giza, Egypt. The animals were housed in polypropylene cages in an environmentally-controlled clean air room with a temperature of $25 \pm 1^\circ\text{C}$, an alternating 12 h light/12 h dark cycle, a relative humidity of $60 \pm 5\%$ and free access to tap water and a standard rodent chow (Wadi El Kabda Co., Cairo, Egypt). The animals were allowed to adapt to these conditions for 2 weeks before the beginning of the experimental protocol. The animal experimental protocol was approved by the Ethical Committee for Medical Research, National Research Centre, Egypt. Following the acclimatization period, the animals were divided into 5 groups (12 rats/group) as follows: (1): Normal healthy rats received 1 ml/rat normal saline orally and served as negative control group, (2): Hepatocellular carcinoma (HCC) group consisted of rats with orally administered NDEA (dissolved in 0.9% normal saline), in a dose of 20 mg/kg b.wt. five times/week for four weeks and 10 mg/kg b.wt. for another week (Karimov *et al.*, 2003). HCC tumor model was established after the fifth week of NDEA administration and confirmed by pathological investigation. (3): Doxorubicin-treated group (HCC+Doxo) in which the rats were treated with doxorubicin intraperitoneally (i.p.) as reference drug in dose of 0.72 mg/rat which is equivalent to the human dose of 20 mg/m² according to Barnes and Paget (Barnes & Paget, 1965) once weekly for 9 weeks. (4): *Ginkgo biloba* (high dose)-treated group (HCC+ *Ginkgo biloba* H) of rats with orally administered 0.675 g/kg.b.wt/day of *Ginkgo biloba* leaves extract five times a week for 9 weeks and (5): *Ginkgo biloba* (low dose)-treated group (HCC+ *Ginkgo biloba* L) of rats with orally administered 0.337 g/kg.b.wt/day of *Ginkgo biloba* leaves extract five times a week for 9 weeks. The treatment with doxorubicin or *Ginkgo biloba* leaves extracts was started following the induction of HCC.

Blood sampling. After the completion of the treatment (14 weeks), all animals fasted for 12 hours then the blood samples were collected from the retro-orbital venous plexus under diethyl ether anaesthesia. Blood samples were left to clot and the sera were separated by cooling centrifugation at $1800 \times g$ for 10 min at 4°C and stored immediately at -20°C in clean Eppendorf prior to the biochemical analysis.

Liver tissue sampling. After blood collection, the animals were sacrificed by cervical decapitation, dissected and the whole liver of each rat was rapidly excised and thoroughly washed with isotonic saline. Later, whole liver of each rat was divided into two portions; the first portion was fixed in formalin saline (10%) for 24 h for histological examination whereas, the second portion was snap-frozen directly in liquid nitrogen and

stored at -80°C prior to RNA isolation for gene expression analysis.

Methods

Biochemical analyses. Serum alpha-fetoprotein (AFP) was determined by ELISA using kit purchased from Glory Science Co., Ltd (USA), according to the manufacturer's instructions provided with AFP assay kit. Serum carcinoembryonic antigen (CEA) was quantified by ELISA using kit purchased from Glory Science Co., Ltd (USA), according to the manufacturer's instructions provided with CEA assay kit. Serum glypican-3 (GPC-3) was detected by ELISA using kit purchased from Glory Science Co., Ltd (USA), according to the manufacturer's instructions provided with GPC-3 assay kit.

Molecular genetic analysis. Isolation of total RNA. Total RNA was isolated from the liver tissue of rats with TRIzol[®] Reagent (Invitrogen, USA). The isolated RNA was treated with 1 U of RQ1 RNase-free DNase (Invitrogen, USA) to digest any DNA residues and then re-suspended in DEPC-treated water. The purity of RNA was assessed by 260/280 nm ratio (between 1.8 and 2.1). Additionally, the integrity was assured by the analysis of 28S and 18S bands using formaldehyde-containing agarose gel electrophoresis.

Reverse transcription (RT) reaction. The Poly(A)+ RNA isolated from the rat liver tissues was reverse transcribed into cDNA in a total volume of 20 μL using RevertAid[™] First Strand cDNA Synthesis Kit (MBI Fermentas, Germany) according to the manufacturer's instructions provided with the kit.

Semi-quantitative real time-polymerase chain reaction (sqRT-PCR). A Rotor-Gene Q – Qiagen (USA) was used to determine the rats cDNA copy number. The subsequent PCR was performed using a 25 μL reaction mixtures containing 12.5 μL 1 \times SYBR[®] Premix Ex Taq[™] (TaKaRa, Biotech. Co. Ltd.), 0.5 μL of 0.2 μM forward primer, 0.5 μL of 0.2 μM reverse primer, 6.5 μL distilled water, and 5 μL of cDNA template. The cycling conditions were summarized in Table 1. Each experiment included a distilled water control. The semi

Table 1. sqRT-PCR reaction program

Step 1: (1 cycle)	Temperature	Time
a: Initial denaturation step Step 2: (30 cycles)	95.0 $^{\circ}\text{C}$	03.00 min
a: denaturation step	95.0 $^{\circ}\text{C}$	00.15 sec
b: annealing step	55.0 $^{\circ}\text{C}$	00.30 sec
c: extension step Step 3: (71 cycles)	72.0 $^{\circ}\text{C}$	00.30 sec
a: melting curve analysis Then increased about every 0.5 $^{\circ}\text{C}$ 10 sec. up to 95 $^{\circ}\text{C}$	60.0 $^{\circ}\text{C}$	00.10 sec

quantitative values obtained in RT-PCR (sqRT-PCR) for the studied genes were normalized against the expression value of β -actin gene (house-keeping gene).

Primer sequences were as follows: ING3, F: 5'-AGT GGC AGG AAG AGC AAA AA-3', R: 5'-TCC CAC CAT CTC TCC ATA GG-3', Foxp-1, F: 5'-CAG GCA GAT CCC CTA TGC AA-3', R: 5'-GGA CAG AGG GCC TTC AGC TT-3' (Shu *et al.*, 2007) and β -actin, F: 5'-CCC CAT CGA GCA CGG TAT TG-3', R: 5'-ATG GCG GGG GTG TTG AAG GTC-3' (Eshak *et al.*, 2010). At the end of each sqRT-PCR, a melting curve analysis was performed at 95.0 $^{\circ}\text{C}$ to check the quality of the used primers.

Calculation of gene expression. The amplification efficiency (Ef) was calculated from the slope of the standard curve using the following formula: (Bio-Rad Laboratories Inc, 2006).

$$\text{Ef} = 10^{-1/\text{slope}}$$

$$\text{Efficiency (\%)} = (\text{Ef} - 1) \times 100$$

The relative quantification of the target gene compared to the reference gene was determined by using the ΔCT method if E for the target (ING3 and Foxp-1) and the reference primers (β -actin) are the same:

$$\text{Ratio}_{(\text{reference}/\text{target gene})} = \text{Ef} C_{1(\text{reference})} - C_{1(\text{target})}$$

Histological examination. After the fixation of the second portion of the liver tissue in formal saline (10%) for 24 hours, The tissue were subjected to dehydration using series of alcohols (methyl, ethyl and absolute ethyl alcohol) followed by embedding in paraffin wax at 56 $^{\circ}\text{C}$. After that, the wax tissue blocks were sectioned by sliding microtome and then prepared for the histological slide examination under the light electric microscope (Olympus Cx21 with attached digital camera) after staining with hematoxylin and eosin stain (Drury & Wallington, 1980).

Statistical analysis. In the present study, the results were expressed as Mean \pm S.E. of the mean. Data were analyzed with one way analysis of variance (ANOVA) using the Statistical Package for the Social Sciences (SPSS) program, version 14 followed by least significant difference (LSD) to compare significance between groups (Armitage and Berry, 1987). Difference was considered significant when P value was <0.05 .

RESULTS

Biochemical analyses

The results in Table (2) show that there was significant increase ($P<0.05$) in serum AFP, CEA and GPC-3 levels in HCC group versus the negative control group (Ahmed *et al.*, 2015). Insignificant decrease ($P>0.05$) in serum AFP level was recorded in HCC rats treated with doxorubicin as compared to untreated HCC rats. Significant decreases ($P<0.05$) in serum CEA and GPC-3 levels were detected in HCC rats treated with doxorubicin in comparison to the untreated HCC rats (Ahmed *et al.*, 2015). Similarly, the groups with HCC treated with either high (0.675 g/kg.b.wt.) or low (0.337 g/kg.b.wt.) dose of *Ginkgo biloba* leaves extract elicited significant reduction ($P<0.05$) in serum AFP, CEA and GPC-3 compared to the untreated HCC rats (Table 2).

The data in Table 2 revealed that the treatment of HCC rats with high dose of *Ginkgo biloba* leaves extract induced significant reduction ($P<0.05$) in serum AFP and GPC-3 levels compared to doxorubicin-treated group. Meanwhile, treatment of HCC rats with high dose of *Ginkgo biloba* leaves extract produced insignificant reduction ($P>0.05$) in serum CEA level in comparison with doxorubicin-treated group (Table 2). Treatment of HCC rats with low dose of *Ginkgo biloba* leaves extract elicited insignificant reduction ($P>0.05$) in serum AFP and CEA levels, and significant depletion ($P<0.05$) in serum GPC-

Table 2. Effect of treatment with *Ginkgo biloba* leaves extract and doxorubicin on serum AFP, CEA and GPC-3 levels in hepatocellular carcinoma rat model

Parameters Groups	AFP (ng/mL)	CEA (ng/mL)	GPC-3 (pg/mL)
Negative control	19.1 ± 1.5	0.12 ± 0.01	9.4 ± 0.2
HCC	40.1 ± 2.9 ^a	0.6 ± 0.06 ^a	28.6 ± 0.6 ^a
HCC + Doxo	36.9 ± 1.8	0.45 ± 0.04 ^b	22.4 ± 0.3 ^b
HCC + <i>Ginkgo biloba</i> H	28.5 ± 2.0 ^{bc}	0.39 ± 0.04 ^b	17.4 ± 0.4 ^{bc}
HCC + <i>Ginkgo biloba</i> L	32.5 ± 2.3 ^b	0.41 ± 0.03 ^b	19.8 ± 0.3 ^{bc}

All data are expressed as means ± standard error S.E. for 8 animals/group. HCC: hepatocellular carcinoma. Doxo, Doxorubicin. *Ginkgo biloba* H: *Ginkgo biloba* leaves extract (high dose 0.675g/kg.b.wt). *Ginkgo biloba* L: *Ginkgo biloba* leaves extract (low dose 0.337g/kg.b.wt). ^aSignificant change at $P < 0.05$ in comparison with the negative control group. ^bSignificant change at $P < 0.05$ in comparison with HCC group. ^cSignificant change at $P < 0.05$ in comparison with HCC + Doxo group.

3 level in comparison with doxorubicin-treated group (Table 2).

Molecular genetics analysis

As illustrated in Fig. 1, the current results revealed that there was a significant downregulation ($P < 0.05$) of the expression level of ING3 gene in the liver tissue of HCC rats compared to the negative control group (Ahmed *et al.*, 2015). Significant upregulation ($P < 0.05$) of the expression level of ING3 gene was detected in liver tissue of HCC rats and treated with doxorubicin versus the untreated HCC rats (Fig. 1) (Ahmed *et al.*, 2015). Similarly, the treatment of HCC rats with *Ginkgo biloba* leaves extract in either high (0.675 g/kg. b. wt.) or low (0.337g/kg. b. wt.) dose elicited significant upregulation ($P < 0.05$) of the expression level of ING3 gene in the liver tissue as compared to the untreated HCC rats.

In comparison to doxorubicin-treated group, treatment of HCC rats with high dose of *Ginkgo biloba* leaves extract resulted in significant change ($P < 0.05$) in the expression level of ING3 gene in the liver tissue, whereas, insignificant upregulation ($P > 0.05$) of the expression level of ING3 gene in the liver tissue was detected in HCC rats treated with low dose of *Ginkgo biloba* leaves

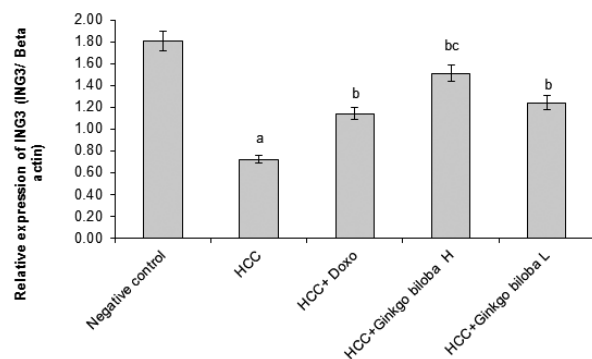


Figure 1. Effect of treatment with *Ginkgo biloba* leaves extract and doxorubicin on hepatic ING3 gene expression level in hepatocellular carcinoma rat model.

HCC: hepatocellular carcinoma. Doxo, Doxorubicin. *Ginkgo biloba* H: *Ginkgo biloba* leaves extract (high dose 0.675g/kg.b.wt). *Ginkgo biloba* L: *Ginkgo biloba* leaves extract (low dose 0.337g/kg.b.wt). ^aSignificant change at $P < 0.05$ in comparison with the negative control group. ^bSignificant change at $P < 0.05$ in comparison with HCC group. ^cSignificant change at $P < 0.05$ in comparison with HCC + Doxo group.

extract as compared to doxorubicin-treated group (Fig. 1).

As represented in Fig. 2, the present results showed that there was significant upregulation ($P < 0.05$) of the expression level of Foxp-1 gene in the liver tissue of HCC rats with respect to the negative control group (Ahmed *et al.*, 2015). Treatment of HCC rats with doxorubicin elicited significant downregulation ($P < 0.05$) of the gene expression level of Foxp-1 in the liver tissue compared to the untreated HCC rats (Fig. 2) (Ahmed *et al.*, 2015). In the same manner, the treatment of HCC rats with either high or low dose of *Ginkgo biloba* leaves extract induced significant downregulation ($P < 0.05$) of the expression level of Foxp-1 gene in the liver tissue in comparison with the untreated HCC rats.

In comparison with doxorubicin-treated group, the treatment of HCC rats with high dose of *Ginkgo biloba* leaves extract resulted in significant downregulation ($P < 0.05$) of the expression level of Foxp-1 gene in the liver tissue. On the other hand, insignificant downregulation ($P > 0.05$) of the expression level of Foxp-1 gene in the liver tissue was recorded in HCC rats treated with low dose of *Ginkgo biloba* leaves extract as compared to doxorubicin-treated group (Fig. 2).

Histological examination

Histological examination of liver tissue section of rat in the negative control group stained with hematoxylin and eosin (H&E) showed normal histological structure of the central vein and the surrounding hepatocytes and no histopathological alteration was observed (Fig. 3a) (Ahmed *et al.*, 2015). On the contrary, microscopic investigation of liver tissue section of rat in HCC group showed anaplastic hepatocytes activities indicated by polarity, pleomorphism and hyperchromatic nuclei and the ratio between the nucleus and cytoplasm was 1:1 which is associated with acinar glandular structure formation (Fig. 3b) (Ahmed *et al.*, 2015). Photomicrograph of liver tissue section of HCC and doxorubicin-treated rat showed thickening and fibrosis with inflammatory cell infiltration and dilated blood capillaries in liver capsules. Also, many liver cells showed karyomegaly and pyknotic nuclei

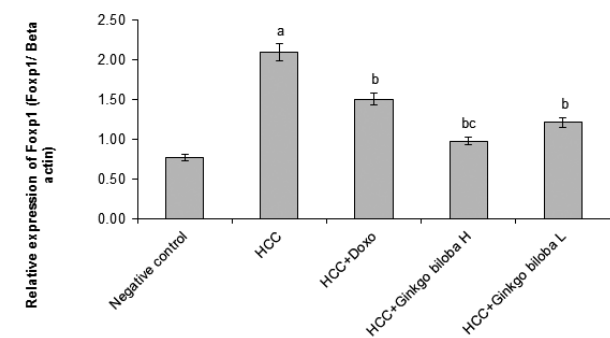


Figure 2. Effect of treatment with *Ginkgo biloba* leaves extract and doxorubicin on hepatic Foxp-1 gene expression level in hepatocellular carcinoma rat model.

HCC: hepatocellular carcinoma. Doxo, Doxorubicin. *Ginkgo biloba* H: *Ginkgo biloba* leaves extract (high dose 0.675 g/kg.b.wt). *Ginkgo biloba* L: *Ginkgo biloba* leaves extract (low dose 0.337 g/kg.b.wt). ^aSignificant change at $P < 0.05$ in comparison with the negative control group. ^bSignificant change at $P < 0.05$ in comparison with HCC group. ^cSignificant change at $P < 0.05$ in comparison with HCC + Doxo group.

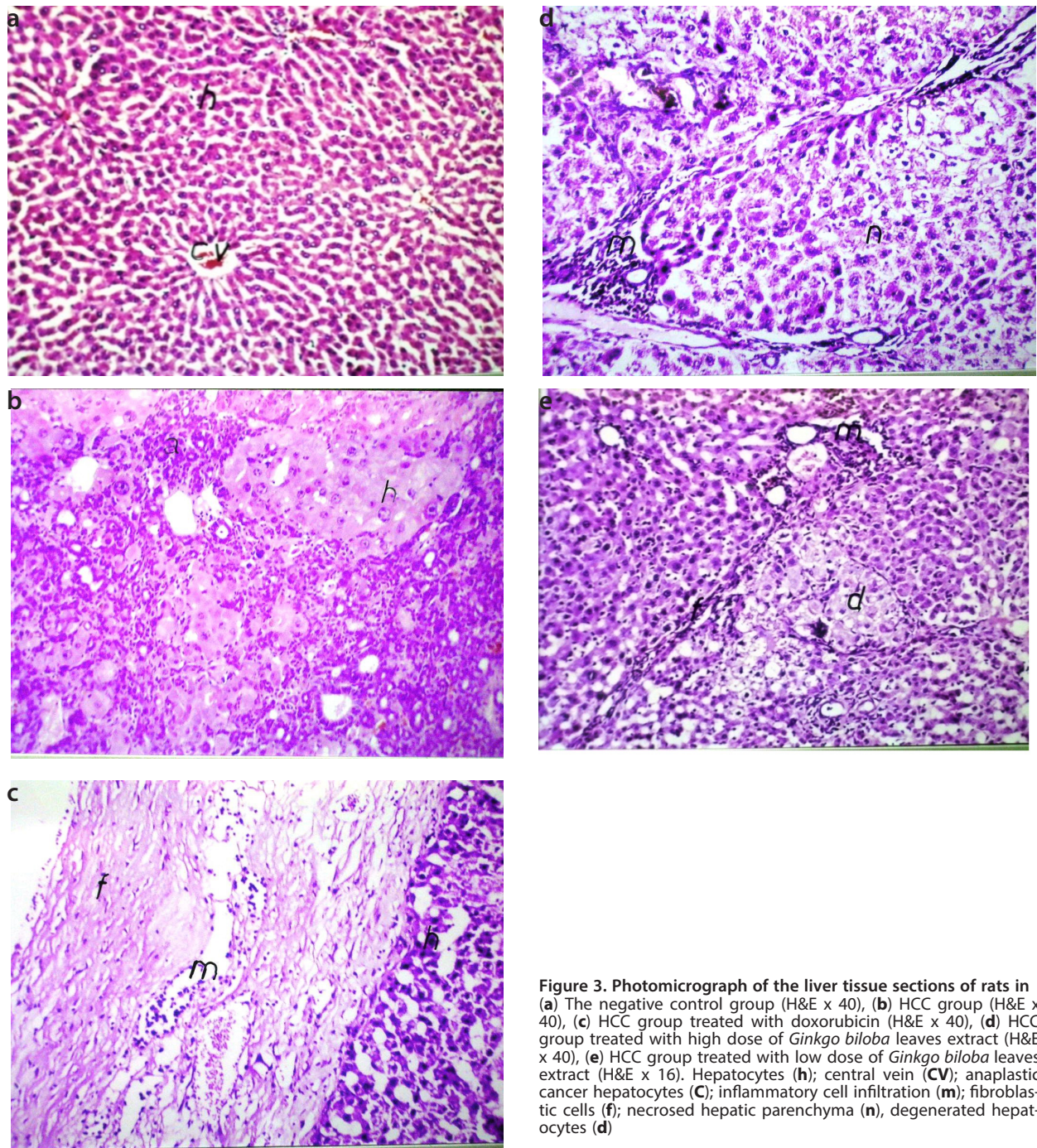


Figure 3. Photomicrograph of the liver tissue sections of rats in (a) The negative control group (H&E x 40), (b) HCC group (H&E x 40), (c) HCC group treated with doxorubicin (H&E x 40), (d) HCC group treated with high dose of *Ginkgo biloba* leaves extract (H&E x 40), (e) HCC group treated with low dose of *Ginkgo biloba* leaves extract (H&E x 16). Hepatocytes (h); central vein (CV); anaplastic cancer hepatocytes (C); inflammatory cell infiltration (m); fibroblastic cells (f); necrosed hepatic parenchyma (n), degenerated hepatocytes (d)

(Fig. 3c) (Ahmed *et al.*, 2015). Photomicrograph of liver tissue section of rat with HCC and the one treated with high dose of *Ginkgo biloba* leaves extract showed regenerative reaction with inflammatory cells infiltration and dividing the degenerated and necrosed hepatic parenchyma into nodules (Fig. 3d). Photomicrograph of liver tissue section of rat with HCC and the one treated with low dose of *Ginkgo biloba* leaves extract showed little fibroblastic cells proliferation with inflammatory cells infiltrating in between the degenerated hepatocytes (Fig. 3e).

DISCUSSION

Chemical induction of HCC with special carcinogens such as N-nitrosodiethylamine (NDEA) is a model close

to human disease. It is widely accepted that metabolic activation of nitrosamines by cytochrome P450 enzymes to reactive electrophiles is required for their cytotoxic, mutagenic and carcinogenic activity. Because of its relatively simple metabolic pathway and potent carcinogenicity, NDEA has been used as an effective experimental model in the field of carcinogenesis and chemoprevention (Ramakrishnan *et al.*, 2009).

The present data revealed that serum AFP level in rats with HCC was significantly elevated. This finding comes in line with other studies that reported that NDEA injection in rats caused significant increase in AFP level in serum (Sadiknah *et al.*, 2008; Sivaramakrishnan *et al.*, 2008) and the gene expression in the liver tissue (Lazarovich, 2000). Zhou *et al.* (2006) demonstrated that the high serum concentration of AFP in HCC patients might

be due to the tumor excretion of this protein. Furthermore, some clinical research indicated that the high percentage of AFP is closely related to poor differentiation and biologically malignant characteristics especially portal vein invasion of HCC (Oka *et al.*, 2001). The possible explanations for the reinitiation of AFP synthesis by neoplastic hepatocytes include either increased transcription of AFP gene or post-translational modification affecting AFP production. In rats exposed to chemical carcinogens or those with HCC, AFP production is roughly proportional to the amount of transplantable mRNA present (Motaleb *et al.*, 2008). Steady state AFP transcription may begin prior to the development of histologically or symptomatic hepatocellular carcinoma (Premalatha & Sachdanandam, 1999).

In view of the present data, serum CEA level in rats with HCC showed significant increase. The elevation in serum CEA levels upon NDEA administration was presumably associated with production rates of tumor, its location, stage, size, differentiation and vascularity (Zimmer & Thomas, 2001). Macnab and coworkers (1978) demonstrated that the tumor could cause a release of CEA from damaged liver cells adjacent to it. Other studies revealed that CEA is normally removed from circulating plasma by the liver (Thomas & Hems, 1975). Thus, the increased serum levels of CEA might therefore result from impaired hepatic uptake of CEA or CEA-like glycoproteins.

The efficacy of *Ginkgo biloba* extract in decreasing the serum levels of AFP and CEA might be explained by its antioxidant properties (Maccocci *et al.*, 1994). The expression of Bcl-xL, Bcl-xS, and Bax could be differently regulated by quercetin, suggesting that the balance in the expression of these proteins might be involved in the control of the apoptotic process. Quercetin decreased the Bcl-xL:Bcl-xS ratio. Herrera and coworkers (2001) proposed that Bcl-xL is a caspase substrate. Quercetin also increased Bax translocation from the cytosol to the mitochondrial membrane, an event that promotes apoptotic death. Quercetin inhibited the growth of tumor cells (Middleton *et al.*, 2000).

The decline in serum level of GPC-3 after treatment with *Ginkgo biloba* leaves extract might be attributed to the inhibitory effect exerted by quercetin towards Beta-catenin/transactivate T cell factor (Tcf) signaling pathway (Park *et al.*, 2005) and consequently towards c-myc gene as the Beta-catenin/Tcf signaling pathway activates c-myc gene (He *et al.*, 1998) which is a key regulator of GPC3 gene. Thus, the inactivation of c-myc gene might lead to the observed depletion in serum GPC-3 level.

The current results revealed that NDEA administration led to significant increase in serum glypican-3 (GPC-3) level. Glypican-3 is a heparan sulfate proteoglycan that has an important role in cell growth and differentiation, and its function in carcinogenesis is tissue-dependent (Li *et al.*, 2012a). Moreover, the expression of GPC-3, its receptor, and other growth factors coordinates signal transduction pathways that regulate cellular morphology and a variety of cellular behaviors. GPC-3 is differentially expressed during the invasive growth of liver cancer, so its expression might be involved in the initiation and development of liver cancer (Filmus & Capurro, 2008). Capurro and coworkers (2003) recorded overexpression of GPC-3 mRNA and protein in both serum and tissues of early-stage HCC. Also, Capurro and coworkers (2005) stated that GPC-3 could enhance the growth of HCC cells by stimulating the Wnt pathway through facilitating the interaction between the Wnts and their signaling receptors. Chan and coworkers (2004) suggested that the

upregulation of GPC-3 in some HCC tumors might be attributed to c-Myc gene amplification as it was demonstrated that c-Myc transcriptionally regulates GPC-3 expression by direct interaction with the GPC-3 promoter. Together, these data indicate that the transcription factor c-Myc is a novel regulator of GPC-3 gene and may accelerate the proliferation of liver cancer cells at least in part by upregulating the GPC-3 pathway. Moreover, the exogenous overexpression of c-Myc increased the endogenous mRNA and protein levels of GPC-3 (Li *et al.*, 2012a). In accordance with our results, Suzuki and coworkers (2010) indicated that circulating levels of GPC-3 protein were significantly elevated in patient with HCC and the expression of mRNAs for GPC-3 was markedly elevated in the liver carcinoma tissues. Therefore, GPC-3 showed potential as a tumor biomarker for HCC.

Signal transducer and activators of transcription 3 (STAT3) are involved in oncogenesis through the upregulation of genes encoding apoptosis inhibitors (Bcl-xL, Mcl-1 and survivin), cell-cycle regulators (cyclin D1 and c-Myc (DNA binding protein involved in the regulation of nucleic acid metabolism and the response to growth factor)), and inducers of angiogenesis (vascular endothelial growth factor). It was found that STAT3 has an indirect role in GPC-3 expression *via* upregulation of c-myc; and the poor control of the pathway mediated by STAT3 plays a role in GPC-3 overexpression (Suzuki *et al.*, 2010). Therefore, STAT3 recently drew attention as a novel target for cancer therapy (Fuks *et al.*, 2007).

The current data revealed that the treatment with Dox in HCC rats resulted in marked decrease in AFP, CEA and GPC-3 serum levels. This finding could be attributed to the powerful apoptotic property of Dox. Dox induces apoptosis *via* p53/Fas/Caspase-8 system (Friesen *et al.*, 1999). This drug stimulates transcriptional activity of the proapoptotic molecule p53, that triggers expression of the death ligand (Fas L) leading to activation of the Fas receptor. The latter binds, *via* its cytosolic domain, to adapter proteins like the Fas-associated death domain (FADD) and the apoptosis initiator, caspase-8 (Cohen, 1997). Activated caspase-8 cleaves Bid (Kim *et al.*, 2000), producing truncated Bid, which interacts with mitochondria to cause the release of cytochrome *c*. The latter activates caspase-9, conveying apoptotic signals to the nucleus through downstream targets such as caspase-3 and endonucleases, eventually triggering DNA fragmentation and apoptosis (Cohen, 1997).

The results of the current study showed that the expression of inhibitor of growth-3 (ING3) gene in liver tissues of NDEA treated rats was significantly downregulated. It was revealed that ING3 is degraded by S-phase kinase-associated protein-2 (Skp2) mediated ubiquitin proteasome system (Chen *et al.*, 2010). In addition, there was a correlation between the downregulation of ING3 protein and tumor grades as it was shown that the low expression of ING3 protein is correlated with more aggressive behavior of the tumor (Yang *et al.*, 2012). Furthermore, the lower expression of ING3 is also associated with elevated AFP serum levels (Caruso & Valentini, 1999). It has been reported that p53 negatively regulated AFP gene expression through alteration of chromatin structure at the core promoter (Ogdene *et al.*, 2001). Studies of Nagashima and coworkers (2003) and Luo and coworkers (2009) demonstrated that ING3 gene regulated p53. Thus, it is possible to postulate that ING3 could regulate the AFP gene through modifying p53 function. ING3 was shown to modulate p53-mediated transcription, cell cycle control and apoptosis. Thus, ING3 cooperates with p53 to regulate apoptosis death

receptor/extrinsic apoptotic pathways (Nagashima *et al.*, 2003). In addition, ING3 was found to upregulate Fas expression at both mRNA and protein levels (Wang & Li, 2006).

Treatment of HCC rats with *Ginkgo biloba* leaves extract resulted in significant upregulation in the expression level of liver ING3 gene. Russo and coworkers (2012) reported that quercetin can be efficient in treating cancer by inducing cell death or cell cycle arrest preferentially in cancer cells than in their normal counterparts through a process involving the down-regulation of selective oncogenes, or the up-regulation of tumor suppressor genes (p53, p21), which, in turn, enhance selective pathways leading to the elimination of cancer cells. Quercetin induced apoptosis and cell cycle arrest in HepG2 cells (Tanigawa *et al.*, 2008). Subsequently, quercetin induced p53 activation resulting in increase of p21 and suppression of cyclin D1 expression in favor of cell cycle arrest as well as in increase of Bax/Bcl-2 ratio in favor of apoptosis (Tanigawa *et al.*, 2008). Also, Chang and coworkers (2006) showed that quercetin-induced cell cycle arrest of HA22T/VGH hepatocarcinogenic cells was due to production of reactive oxygen species.

The present findings revealed that the treatment with Dox in HCC rats caused remarkable upregulation of the expression of ING3 gene in the liver tissue. Deficiency of Fas expression is one of the mechanisms involved in the immune evasion by tumors. Several antitumor drugs, such as Dox causes apoptosis *via* Fas-mediated activation of caspase-8 *in vitro* (Nakamura *et al.*, 2000). However, the significance of Fas expression *in vivo* is still unclear. Dox was found to be able to induce activation of caspase-8. The role of caspase-8 appeared to be restricted to apoptosis mediated by death receptors, such as CD95, TNF-R1, and the TRAIL receptors. But, it could be postulated that Dox induces apoptosis through molecular expression of ING3 gene and in turn activation of caspase-8 (Pan *et al.*, 1997).

In the present study, NDEA administration resulted in overexpression of liver forkhead box transcription factor (Foxp-1) gene in rats. Foxp-1 is distinctive as it can act as an oncogene and tumor suppressor depending upon the tissue type (Koon *et al.*, 2007). Significant upregulation of Foxp-1 protein in human primary HCC suggests its potential role in hepatic tumorigenesis (Datta *et al.*, 2008). Foxp-1 was identified as a potential target of miR-1 and the upregulation of miR-1 target Foxp-1 in hepatocellular carcinomas. This further explains the growth regulatory functions of miR-1 in the liver and probably in other tissues by predisposing these tissues to neoplastic transformation due to loss of miR-1. Recently, several studies showed the deregulation of miR-1 in many types of tumor, such as hepatocellular (Li *et al.*, 2012b) and renal (Kawakami *et al.*, 2012) cancer. Numerous observations indicated an epigenetic deregulation of miRNAs expression in different tumors, including HCC. MiR-1 downregulation in human HCC is associated with promoter methylation (Datta *et al.*, 2008). Treatment of primary HCC cultures with the hypomethylating agent caused miR-1 reexpression, which in turn led to downregulation of miR-1 oncogenic targets, including Foxp-1 which was overexpressed in HCCs, causing inhibition of cell cycle progression and cell survival (Datta *et al.*, 2008). As *miR-1* has specific targets including Foxp-1 that have roles in tumor formation, *miR-1's* ability to reduce the expression of many tumor promoting genes could have a global influence on the suppression of tumor development (Datta *et al.*, 2008; Reid *et al.*, 2012).

Treatment of HCC rats with *Ginkgo biloba* leaves extract resulted in significant downregulation in the expression of liver Foxp-1 gene level. Certain polyphenols such as bioflavonoids (quercetin) exert a profound inhibitory effect on DNA methylation and DNA methyltransferase-1 (DNMT1) activities (Yang *et al.*, 2008). Quercetin may indirectly inhibit DNMTs and thus DNA methylation, by changing S-adenosyl methionine (SAM) and S-adenosyl-L-homocysteine (SAH) concentrations inside the cell (Lee *et al.*, 2005).

The current results showed that the treatment with Dox in HCC rats resulted in observable downregulation in Foxp-1 gene expression in the liver tissue. Suggesting that DNA methyltransferase-1 (DNMT1) is one of the targets for Dox-induced apoptosis in cancer cells, it was proposed that the expression levels of DNMT1 in tumor cells might be important criteria that should be taken into account to evaluate the selective cytotoxicity of the drug (Pratt *et al.*, 1994) and to determine the optimal dose regimen. Therefore, the interaction between DNMT1 and Dox might be represented as alternative mechanism for induction of apoptosis in cancer cell by Dox (Yokochi & Robertson, 2004).

Photomicrographs of liver tissue sections of rats in NDEA group (HCC group) showed anaplastic activity of hepatocytes characterized by polarity, pleomorphism and hyperchromatic nuclei. In addition, the ratio between the nucleus and cytoplasm was 1:1 with the presence of acinar glandular structure formation. Scherer and Emmelot (1975) stated that in NDEA administered rats, carcinomas are preceded by the development of so-called altered foci and hyperplastic nodules. Also, Burr and coworkers (1996) reported that there was necroinflammation with perivenular inflammatory cells and macrophages in the liver tissue after NDEA administration. Gupta and coworkers (2010) found that NDEA administration caused vacuole formation, loss of normal hepatocyte architecture and presence of pycnotic nuclei. These outcomes might be caused by NDEA being metabolized in the liver and the reactive generated metabolites are known to damage hepatocytes. NDEA is an N-nitroso alkyl compound described as an effective hepatotoxin in experimental animals, causing toxicity after frequent administration (Jose *et al.*, 1998).

The microscopic investigation of liver tissue section of rats with HCC and treated with high dose of *Ginkgo biloba* leaves extract showed congestion in the central veins while the bile ducts showed proliferation and cystic dilatation with polyps formation. In addition, regenerative reaction with inflammatory cells infiltration dividing the degenerated and necrosed hepatic parenchyma into nodules was observed. These beneficial effects of *Ginkgo biloba* leaves extract could be partially explained by its antioxidant properties and by inhibition of CYP2E1 that metabolizes NDEA in the liver (Verna *et al.*, 1996). However, regarding the histopathological alterations due to treating HCC bearing rats with low dose of *Ginkgo biloba* leaves extract, the microscopic investigation of liver tissue sections showed proliferating cystic bile ducts associated with regenerative reactive tissue dividing in the degenerated parenchyma into nodules. Nuclear mitotic activity was noticed in few hepatocytes. In addition, few fibroblastic cells proliferation with inflammatory cells infiltration in between the degenerated hepatocytes was observed.

Histological investigation of liver tissue sections of rats with HCC showed inflammatory cells forming granulomatous lesions and periportal fibrosis after Dox administration. Dox has been shown to induce accumula-

tion of inflammatory cells (Saad *et al.*, 2001). Moreover, it was reported that Dox was able to cause accumulation of collagen fibers mainly in the portal area, manifested as an early sign of fibrosis (El-Sayyad *et al.*, 2009). Furthermore, microscopic examination of liver tissue sections of rats with HCC and treated with Dox revealed that many hepatocytes showed karyomegaly and pyknotic nuclei indicating apoptosis. Apoptosis is a common feature of hepatotoxicity induced by many anti-carcinogenic drugs. It may precede necrosis or it may occur concurrently with necrosis (Faber, 1994). The influence of Dox on hepatocellular carcinoma comes from its interference with the synthesis of macromolecules, DNA cross-linking and covalent DNA binding, inhibition of topoisomerase II, arresting of tumor cell cycle progression in G2 phase, induction of apoptosis and generation of reactive oxygen radicals (Lubgan *et al.*, 2006).

In summary, hepatocellular carcinoma rat model induced by NDEA represents a well established model for this disease. This was evidenced by significant elevation in serum AFP, CEA and GPC-3 levels, downregulation of ING-3 and overexpression of FOXP-1 genes in liver tissue, in addition to typical anaplasia illustrated by histopathological investigation. The treatment with *Ginkgo biloba* leaf extracts elicited significant reduction in serum tumor markers and downregulation of oncogene besides upregulation of tumor suppressor gene in liver tissue. Furthermore, *Ginkgo biloba* leaf extracts restrain the anaplastic features of HCC.

CONCLUSION

The results of the present study shed light on the underlying biochemical and molecular mechanisms by which N-nitrosodiethylamine could induce hepatocellular carcinoma. Moreover, the present research provided experimental evidences about the potent anticancer activity of *Ginkgo biloba* leaves methanolic extract in regression of hepatocellular carcinoma in rats.

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