Hepatoprotective Effect of Curcumin against Diethyl Nitrosamine Induced Hepatocellular Carcinoma in Albino Rats

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Curcumin is widely used traditionally as an old plant in traditional medicine, this work was aimed to investigate its possible protective effect against chemically induced Hepatocellular carcinoma (HCC) in rats. Fifty male albino rats, weighing 120±20g each, were used. Rats were divided to five groups (n=10) for each group, control group received a single dose of normal saline, diethylnitrosamine (DENA) bearing non-treated group received a single intra-peritoneal dose of DENA (200mg/Kg body weight), the 3rd, 4th and 5th groups were DENA bearing treated group daily administrated curcunine (CUR) via intra-gastric intubation in doses of 300,200 and 100 mg Kg[-1] b.wt. respectively for 20 weeks. Serum, and liver samples were used for determination of alpha feto-protein (AFP), Interleukine-2 (IL-2), Interleukine-6 (IL-6), serum liver enzymes (AST, ALT, ALP and GGT) levels as well the activities and gene expression of glutathione peroxidise (GPx), glutathione reductase (GR), catalase (CAT) and super oxide dismutase (SOD). Curcumin was significantly lower the serum level of AFP, IL-2 and IL-6 , ALT, ALT malondialdehyd (MDA) level as well the level of gene expression of IL-2 and IL-6 in contrast increase the levels of gene expression and activities of Gpx, GRD, CAT and SOD. The protective effect of CUR against DEN-induced hepatocarcinogenesis in albino rats was proven.

Key words: Hepatocellular carcinoma; Curcumin; antioxidant.

Hepatocellular carcinoma (HCC) is an aggressive cancer, it is one of the most frequent primary cancer of the liver at which its incidence rate has been increased to become the fifth most common malignancy worldwide (Zhong *et al*., 2014). The HCC incidence rate is one million cases every year (Liovet, *et al*., 2008). The pathogenesis of HCC is multifactorial, highly associated with many risk factors but it is mainly develops after exposition of the cellular machinery to a mutation that results in replication of the cell in higher rate and/or avoiding of apoptosis (Youns *et al*., 2013). The main way for HCC treatment is surgical resection and liver transplantation (Zekri *et al*., 2013; Zhang *et al*., 2013; Zhu *et al*., 2013). For unresectable HCC other methods were developed such as chemotherapies by interferon or 5 Flouro-uracil (Parker *et al*., 2004). Bad prognosis and reoccurrence of HCC in most cases directed the most of recent studies toward the prevention of HCC by any method especially the use of natural foods or natural food additives as Curcumin, which has been used traditionally in medicine in India and other Asian countries (Zhaohui *et al*., 2013). It has a wide spectrum of biological and

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pharmaceutical activities, as anti-inflammatory, Anti-infectious, anti-fungal, anti-viral, anti-mutagenic, anti-parasitic and anti-carcinogenic (Gopal *et al.*, 2014). Many studies have demonstrated the anticancer effect of curcumin in a verity of tumors as pancreatic, breast, gastrointestinal and hepatic cancer (Preetha *et al.*, 2008). Those data are now attracting the interest of many researchers for developing this agent as chemo-preventive or even chemotherapeutic drugs (Corson and Crews, 2007).

MATERIALS AND METHODS

DENA (Diethyl nitrosamine) and Carbon tetrachloride were obtained from Sigma-Aldrich Co. (St. Louis, Missouri, USA). Curcumin, obtained in commercial form as capsules from Vitacost, USA. DENA was diluted in normal saline while carbon tetrachloride was diluted in corn oil

Experimental Design

All experimental procedures were approved by the Medical Research Ethics Committee of Zagazig University, Egypt.

Fifty male albino rats, weighing 120±20g each, were used. Animals were acclimatized under controlled conditions for two weeks before starting the experimental procedures. Rats were divided to five groups (n=10) for each group, control group received a single dose of normal saline, DENA bearing non-treated group received a single intra-peritoneal dose of DENA (200mg/Kg body weight) (Al-Rejaie, *et al.*, 2009), the 3rd, 4th and 5th groups were DENA bearing treated group daily administrated curcunine via intra-gastric intubation in doses of 300,200 and 100 mg Kg^{-1} b.wt. respectively for 20 weeks.

Biochemical analysis

At the end of experimental period, rats from all groups were sacrificed by decapitation under ether anesthesia; blood and liver samples were collected, serum was isolated and used for investigation of AFP using ELFA (Enzyme Linked Fluorescent Assay) technique using VIDAS ® AFP kits (Catalogue number 30 413, BioMerieux, France), IL-2 and IL-6 that were determined using Abcam's IL-2 Rat ELISA kit (ab100769) and Abcam's IL6 Rat ELISA Kit (ab119548) respectively. Serum ALT, AST, ALP and β-GT were determined using spectrum Kits (Egyptian Company for

Biotechnology, Cairo, Egypt REF: 265 002, 261 002, 263002 and 264002) following the manufacture instructions. Hepatic tissue samples were used for homogenate preparation, for estimation of Malondialdehyde (MDA), CAT, SOD, GR and GPx activities (Ali *et al.*, 2014).

Hestophathological analysis

Small parts of hepatic tissue were fixed in formalin buffered neutral solution, gradually dehydrated in ethanol (70-100%), then cleaned with xylene finally embedded in paraffin. Paraffin sections were prepared and stained with hematoxylin and eosin (HE) dyes (Bancroft and Gamble, 2008).

Molecular analysis

Total RNA was isolated from liver samples using the RNeasy Mini Kit Qiagen, Cat. No.74104. The amount of extracted RNA was quantified and qualified using NanoDrop® ND-1000 Spectrophotometer, NanoDrop Technologies, Wilmington, Delaware USA. The purity of RNA was checked and ranged between 1.8 and 2. 0.5µg of total RNA was used for production of cDNA using QIAGEN LongRange 2Step RT-PCR Kit, Cat. No.205920. The PCR reaction was placed in a gradient T Professional thermo-cycler (Biometra, Germany). PCR conditions were a denaturation step at 95 °C for 4 min followed by 28 cycles of 95°C, 1 min; 55°C, 1 min; 72°C, 1 min for all genes except for IL-2 annealing temperature was 60°C. 10 ul of PCR products were analyzed on a 2% agarose gel stained with ethidium bromide in 1X Tris acetate EDTA buffer (TAE) pH 8.3-8.5 (Stock solution was 50X from Bioshop® Canada Inc. Burlington, ON. L7L6A4. Lot No. 1L22854). The electrophoretic picture was visualized and analyzed by gel documentation system (Bio Doc Analyze, Biometra, Germany). The Primer sequences (Table 1) of rat GPx, CAT, CuZnSOD, GR and ß-actin were obtained from the published sequences (Ali *et al.*, 2014), IL-2 and IL-6 (Olivier *et al.*, 1995). The primers design was optimized for Polymerase Chain Reaction (PCR) with EugeneTM version 2.2 (Daniben Systems, Cincinnati, OH).

Statistical analysis

The obtained data were analyzed and graphically represented using the statistical package for social science (SPSS, 18.0 software, 2011) for obtaining means and standard error. Duncan's test was used for making a multiple

comparisons among the groups for testing the inter-grouping homogeneity.

RESULTS

In this work, we tested the antitumor effects of CUR against DEN inducerd hepatic tumorigenesis. Liver hestopathological changes, hepatic tumor markers and proinflamatory markers were tested and had been compared for the different animals groups.

Hestopathological examination of liver with HE

The control non-treated rats hepatic histological sections displayed the normal hepatic lobules organization, normal hepatocytes and sinusoidal architecture (Figure 1a). Although, DENA treated rats displayed Focal replacement of portal area with extensive fibrous C.T with islets of cholangioadenocarcinoma represented mainly by large biliary cells with destructed membrane, scanty basophilic cytoplasm and large vesicular nuclei beside numerous mitosis with pressure atrophy of adjacent hepatocytes (Figure1 b,c). Compared with DEN-bearing non-treated rats , the DEN-bearing CUR-treated rats showed disappearance of cholangioadenocarcinoma, but the hepatic tissue still has a diffuse hydrophobic degeneration in portal area with scanty fibrous C.T this lesions correlated with the CUR dose (Figure 1d,e, f).

Biochemical analysis

The index of liver function in control non-treated group was normal. DEN-bearing non-treated rats had an increased ALT, AST, ALP, GGT, AFP, IL2 and IL6 in comparing to the other groups.CUR treated rats displayed a significant decrease in these parameters when compared to DEN-bearing non-treated rats in a dose dependant (table 2).

Antioxidant enzymes activities and MAD levels

Table 1. Primer sequences of rats GPx, CAT, CuZnSOD, GR, IL-2, IL-6 and β-actin.

Gene		Oligonucleotide sequences	Size (bp)	Gene ID
GPx	F	5'-CACAGTCCACCGTGTATGCC-3'	292	S50336.1
	R	5'-AAGTTGGGCTCGAACCCACC-3'		
CAT	F	5'-GTCCGATTCTCCACAGTCGC-3'	272	AH004967.1
	R	5'-CGCTGAACAAGAAAGTAACCTG-3'		
SOD	F	5'-ATGGGGACAATACACAAGGC-3'	225	Z21917.1
	R	5'-TCATCTTGTTTCTCGTGGAC-3'		
GR	F	5'-CCATGTGGTTACTGCACTTCC-3'	171	NM_053906
	R	5'- GTTCCTTTCCTTCTCCTGAGC-3'		
IL-2	F	5'AACAGCGCACCCACTTCAA3'	400	NM_053836.1
	R	5'TTGAGATGATGCTTTGACA3'		
IL-6	F	5'CTTCCCTACTTCACAAGTC3'	457	NM_012589.2
	R	5 'CTCCATTAGGAGAGCATTG3'		
β-actin	F	5'-TCACTATCGGCAATGTGCGG-3'	260	NM_007393
	R	5'-GCTCAGGAGGAGCAATGATG-3'		

Table 2. The effect of Curcumin on serum AFP, IL-2, IL-6, ALT, AST, ALP and GGT levels.

	Control	DENA group	CUR group (300mg/kg)	CUR group (200mg/kg)	CUR group (100mg/kg)
α-FP (IU/ml)	3.49± 0.5	268±17***	46±2*##	76±3**##	114±4**#
IL-2 (ng/ml)	3.52±0.5	21±0.6***	11±0.3*##	15±0.5**##	17±0.5**#
IL-6 (ng/ml)	3.1±0.2	22±0.6***	10±0.4*##	14±0.4**##	18±0.3**#
ALT (U/L)	54.47±4	130±2***	60±1##	70±2*##	99±3**#
AST (U/L)	180±10	284±3***	185±3##	209±12*##	251±5**#
ALP (U/L)	142.66±5	217±2***	150±2##	187±4*##	190±6**#
GGT (U/L)	18.59±2	100±2***	20±1##	40±2*##	71±5**#

Data is represented as Mean± S.D. *$P<0.05$, **$P<0.01$, ***$P<0.001$ (statistical significant compared to the control group); #$P<0.05$, ##$P<0.01$, ###$P<0.001$ (statistical differ from DEN group)

Table 3. The effect of Curcumin on hepatic MDA and GSH
concentrations and hepatic GPx, GR, SOD and CAT activities

	Control	DENA group	CUR group (300mg/kg)	CUR group (200mg/kg)	CUR group (100mg/kg)
MDA (µmol/L)	13± 1	26±0.5***	12±0.5###	18±0.4*##	20±0.6**#
GSH (mg/gm tissue)	19±1	6±0.5***	18±0.5###	14±0.7*##	8±0.7**#
GPx(µmol NADPH / mg protein)	59±3	18±1***	50±2*###	45±2**##	28±1**#
GR (U/ gm tissue)	18±1	7±0.6***	17±0.8###	13±0.8*##	10±0.5**#
SOD (eu/mg protein)	2.5±0.1	0.9±0.01***	2.33±0.03###	1.7±0.02*##	1.2±0.08***##
CAT (µmol H_2O_2 decomposed /gm tissue	148±10	115±4***	145±5###	130±8*##	125±8**#

Data is represented as Mean± S.D. *P<0.05, **P<0.01, ***P<0.001 (statistical significant compared to the control group); #P<0.05, ##P<0.01, ###P<0.001 (statistical differ from DEN group).
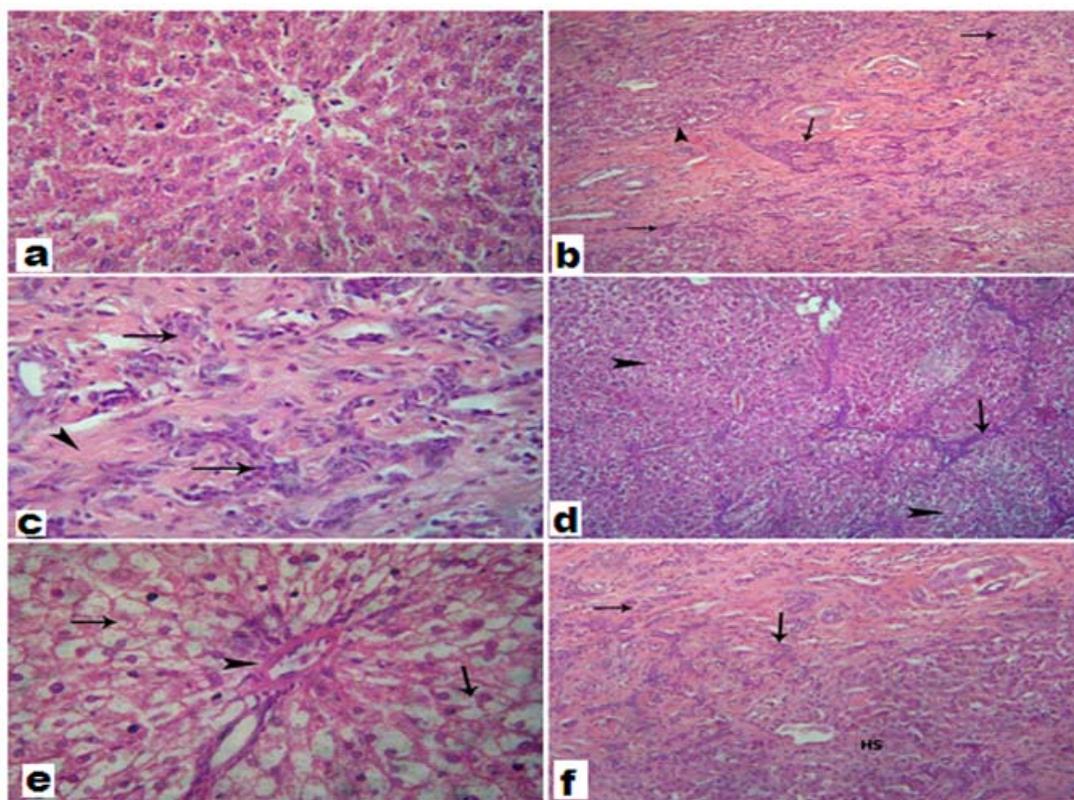

Fig. 1. The histopathological finding (a) liver of control group, show normal hepatocytes and sinusoidal architecture. (b&c) livers of DENA-treated group, with H&E (100X) and H&E (400X) showing: Focal replacement of portal area with extensive fibrous C.T (arrow) with islets of cholangioadenocarcinoma represented mainly by large biliary cells with destructed membrane, scanty basophilic cytoplasm and large vesicular nuclei beside numerous mitosis (arrow heads) with pressure atrophy of adjacent hepatocytes (small HS).liver of DENA bearing CUR treated rats (100, 200 and 300 mg/kg) showed diffuse hydrophobic degeneration, portal area shows hyalinization in the wall of blood vessels with no evidence of carcinogenesis (d). Scanty fibrous C.T proliferation (arrows) well developed in the portal tract forming incomplete bridge with no evidence of carcinogenesis (e), and limited extension of carcinogenesis and cholangioadenocarcinoma (large hepatic spare HS) (f)

The hepatic tissue of DEN-bearing non-treated rats had an increased MDA levels and decreased GPx, GR, SOD and CAT activities in comparing to the other groups.CUR treated rats displayed a significant decrease in MDA and increase of GPx, GR, SOD , CAT, GSH when compared to DEN-bearing non-treated rats in a dose dependant (table 3).

Molecular analysis

The hepatic tissue of DEN-bearing non-treated rats had an increased AFT, IL2 and IL6 and decreased GPx, GR, SOD and CAT mRNA levels in comparing to the other groups.CUR treated rats displayed a significant decrease in AFT, IL2 and IL6 and increase GPx, GR, SOD and CAT mRNA levels when compared to DEN-bearing non-treated rats in a dose dependant (figure 2).
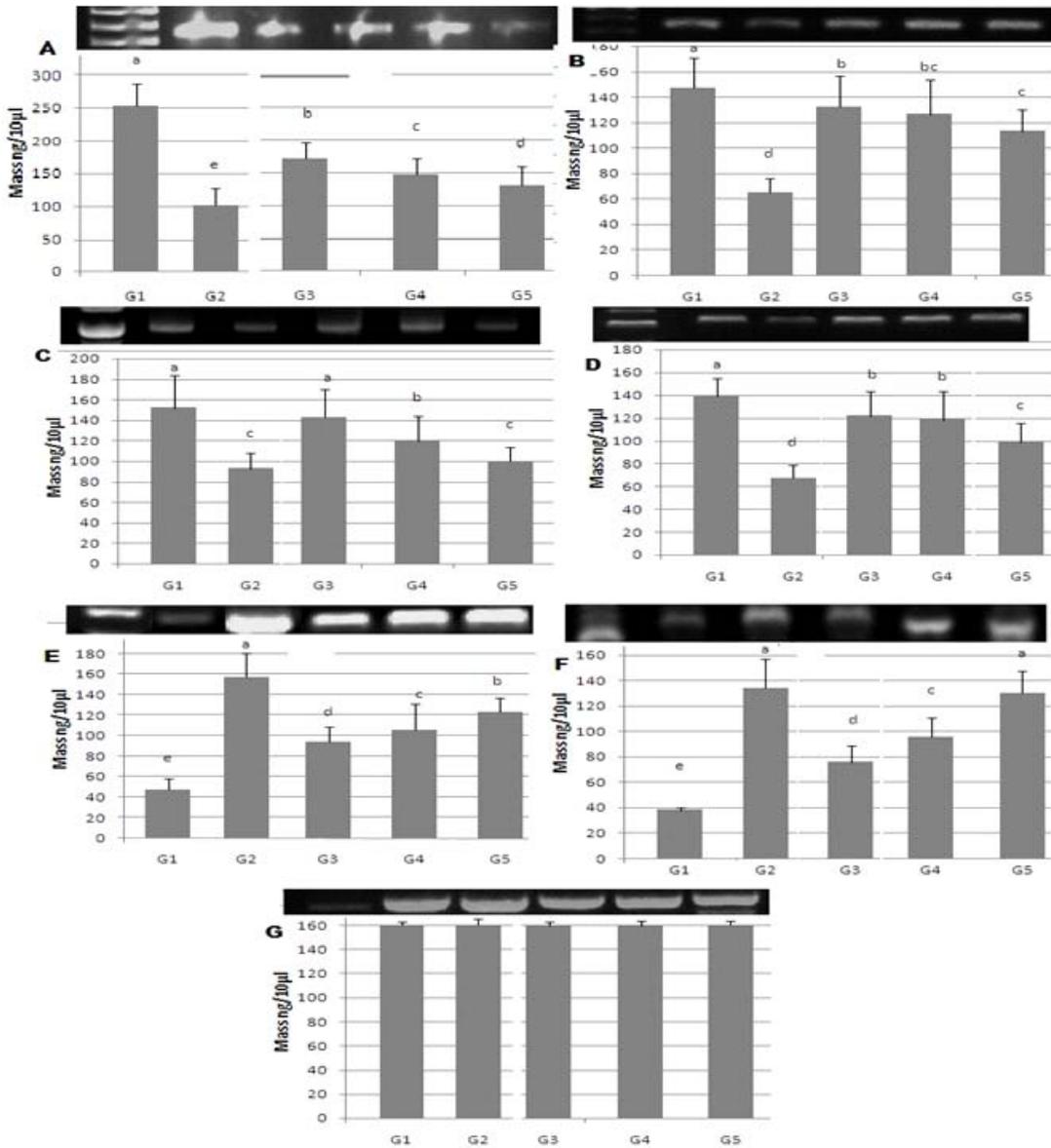
DISCUSSION

Fig. 2. The electrophotograph of GPx (A), GR (B), SOD(C), CAT (D), IL-2 (E), IL-6 (F) and β-actin (G) gene expression in hepatic tissue. Columns with different litters are significant differ at P≤ 0.05.

In the present study we aimed to investigate the protective effects of CUR against the DENA induced HCC in rats. The hestopathological examination of hepatic tissue, displayed the HCC lesions in rats administrated DENA. The same observation was documented by many authors (Bendong *et al*., 2012; Zhao *et al*., 2014). The hestopathological features suggested that CUR is effective in reducing DEN-induced hepatocarcinogenesis in a dose dependant manner (Chuang *et al*., 2000; Sreepriya and Bali, 2006; Zhao *et al*., 2014).

It is known that the AST, ALT, ALP, GGT serum levels are indicative for hepatic function, their increase is correlated with the hepatic injury (Zhao *et al*., 2014). DEN hepatic injury is related to the disturbance in hepatocytes membrane instability and metabolism resulting in alterations of the serum levels of these enzymes. The increase of ALT and AST serum levels are specific to hepatocellular disturbance (Al-Rejaie *et al*., 2009). ALP is a liver function enzymes that related to the membrane lipid in canalicular ducts. ALP increase in serum reflects the biliary flow disturbance. So, the extra or intra-hepatic interference with the bile flow leads to elevation of ALP serum levels (Zhao *et al*., 2014). GGT is a membrane-bound enzyme, present mainly in the canalicular ducts. GGT serum levels are ultared by many pathologic and physiological factors, such as carcinogenesis and development (Yao *et al*., 2004). In our results the increase of serum GGT level in DENA group may be attributed to its liberation from the cellular membrane into blood indicating the cellular membrane damage as a result of carcinogenesis (Zhao *et al*., 2014). The decrease of ALT, AST, ALP and GGT serum levels in CUR treated rats may be attributed to the decrease of cellular damage (Sreepriya and Bali, 2006; Zhao *et al*., 2014).

It is well documented that, DENA induces hepatic dysfunction through the induction of disturbances in antioxidant defense systems, increases the reactive oxygen species (ROS) and membrane lipid peroxidation and consequently vital bio-membranes damage (Mohamed *et al*., 2010; Ismail *et al*., 2011). The anti-oxidative defense system includes enzymatic and non-enzymatic components as GPx and SOD which consider the key enzymes in elimination of free radicals. SOD act upon the toxic superoxide anions (O^{2-}) converting it to O_2 and H_2O_2, which reduced by GSH-Px in presence of GSH to H_2O and produced the oxidize glutathione (GSSG) (Subudhi and Chainy, 2010), that converted back to GSH by GR .While CAT directly breakdown of H_2O_2 to O2 and H_2O (Kaushik and Kaur, 2003). In the present study, DENA treated rats had a significant decrease in the activity and gene expression of GPx, GR, SOD and CAT and the level of GSH and a significant increase in the level of MDA (Bendong *et al*., 2012). In the same line of the present study results, several studies exhibits that, the anti-carcinogenic effect of CUR is produced through its protective effect against oxidative damage and its antioxidant property exerting as a powerful scavenger for oxygen free radicals and its ability to increase intracellular glutathione concentration, in addition by protecting the level of lipid peroxidation (Ciftci *et al*., 2012; Bendong *et al*., 2012).

Alpha fetoprotein is a fetal specific glycoprotein secreted from fetal liver and yolk sac, rapidly falls few weeks after birth (Mallikarjun *et al*., 2013).AFP is the most important serum marker for diagnosis of HCC (Wen *et al*., 2013). In the same line of our results Soresi *et al* (2003), reported that, AFP serum level had been elevated in DENA treated rats. CUR treatment has an ameliorative effect on AFP (Oscar *et al* 2007; Ahmed *et al*., 2014).

IL-6 is an immunoregulatory cytokines that produced by cancer cells and associated macrophages, its high serum level is associated with specific immune and metabolic alterations that lead to cancer cachexia, one of the main causes of death in cancer patients. IL-6 involved in cancer cells growth through induction of matrix metalloproteinase (MMP)-9 productions (Lane *et al*., 2011), also induction of tumor angiogenesis (Rabinovich *et al*., 2011), IL-2 and Il-6 represents the main factors of the cell-mediated immune response. The production of IL-2 and its receptor on cytotoxic T lymphocytes is induced by IL-6. IL-2 is the key cytokine in the regulation of the antineoplastic immunity (Lo *et al*., 2011). IL-6 was increased in the serum patient with cancer and may be considered an indicator of the inflammatory and oxidative status of these patients (Mantovani *et al*., 2003). Our data indicated the significant increase of the serum levels and hepatic gene expression of IL-2 and IL-6 in experimental HCC-

induced rats than in other experimental groups. Some authors in the same line demonstrated that the IL-6 concentrations were higher in cancer patient than healthy controls (Katriina *et al.*, 2008). The administration of CUR suppress the IL-2 and IL-6 gene expression as well decrease there levels in serum, this explain the antiiflamatory activity of curcumin. This effect can be produced by the ability of CUR to modulate a variety of molecules that have an important role in cancer progression such as cytokines (Fujioka *et al.*, 2003). CUR has been found to interrupt the cell cycle, acting as anti-proliferative, cytotoxic and apoptotic for HCC cell line (Vietri *et al.*, 2003). CUR also inhibits IL-6 production (Chen *et al.*, 2003) which explains the reduction of IL-2&6 in CUR treated groups. CUR has been shown as potent immunomodulatory agent that down regulates the expression of various proinûammatory cytokines including TNF,IL-1,IL-2, IL-6, IL-8, IL-12 (Ganesh *et al.*, 2007). In conclusion, based on the present study results, we suggest the protective effects of CUR against DEN-induced hepatocarcinogenesis in albino rats. CUR had been modulated the hepatic pathological alteration, liver function enzymes serum levels, induced the hepatic antioxidant system and suppressed the proinflamatory cytokines.

ACKNOWLEDGMENTS

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