Therapeutic efficacy of licorice and/or cisplatin against diethylnitrosamine and carbon tetrachloride-induced hepatocellular carcinoma in rats

Faried A. E. Hemieda, Hanaa M. Serag, El-Sayed El-Baz andSally M. E. Ramadan

Department of Zoology, Mansoura University, Faculty of Science, Mansoura, Egypt.
sally_mahmoud_2012@yahoo.com

Abstract: Complementary and alternative use of traditional herbs has raised hopes for finding curative options for liver diseases. In the present study, the curative effect of licorice extract against diethyl nitrosamine (DENA) and carbon tetrachloride (CCl_4)-induced hepatocellular carcinoma (HCC) in male rats in the presence or absence of the antineoplastic agent cisplatin was investigated. The results showed that treatment with DENA/CCl_4 caused oxidative stress as indicated by obtained increase in malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) in the liver. Meanwhile, reduction in the antioxidants including glutathione (GSH), superoxide dismutase (SOD) and glutathione-s-transferase (GST)] was observed. Also, the results showed induction of apoptosis as reflected by increase both p53 and caspases-3 along with decreased Bcl-2 in the liver. This was accompanied with changes in the hepatic function biomarkers which characterized by increased levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and total bilirubin; and decrease in total proteins and albumin content in the serum. Treatment of DENA/CCl_4 rats with cisplatin significantly reduced the changes in most parameters of oxidative stress, apoptosis and liver function, when compared to the untreated group of DENA/CCl4.Administration of licorice significantly alleviated the adverse effects induced by DENA/CCl4 in the presence or absence of cisplatin in all investigated parameters, compared to the untreated HCC group. In intact rats, administration of licorice alone had no side effects on mentioned parameters, while cisplatin caused adverse changes. Treatment with licorice in rats treated with cisplatin ameliorated induced hepatotoxicity by cisplatin. In conclusion, obtained results showed that treatment with licorice alone seemed to be more effective in attenuation of the hepatocarcinogenic action of DENA/CCl_4 than treatment of licorice pluscisplatin or cisplatin alone. Since licorice, unlike cisplatin, has no side effects, further studies on experimental animals are required in order to assess the strategy of cancer treatment using licorice extract and its active ingredients.

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1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common types of liver cancer in the world. It is the third-leading cause of mortality, with nearly 700,000 deaths each year worldwide **(El-Serag and Rudolph, 2007)**. In Egypt, HCC is the second most common malignancy in males and fifth in females **(Gouida, 2013)**. A variety of risk factors may cause HCC. Chronic liver disease sparticularly cirrhosis are considered the strongest predisposing factor for the development of HCC **(El-Serag and Rudolph, 2007)**. Contamination of food by some carcinogenic compounds such as aflatoxin B1 **(Parkin*et al*., 1999)** and N-nitrosamines **(Bartsch and Spiegelhalder, 1996)**was also considered as a risk factor for HCC.

Diethylnitrosamine (DENA) is one of N-nitrosamine compounds that have been known to be acute hepatocarcinogens in animals **(Jayakumar *et al*., 2012)**. In experimental induction of HCC in animal model, DENA is commonly used as a cancer initiator, while other factors such as CCl_4isintroduced as a promoter of carcinogenesis **(Dakshayani *et al*., 2005 and Subramanian *et al*., 2007)**. Production of reactive oxygen species resulting in oxidative stress and liver cell injury could be implicated in the pathogenesis of DENA-induced hepatocarcinogenesis **(Subramanian *et al*., 2007 and Singha *et al*., 2009)**. Moreover, DENA is a genotoxic compound which forms alkyl DNA adducts and induces several nuclear aberrations in the rat liver that eventually lead to the development of HCC **(Jagadeesh *et al*., 2009)**.

Cisplatin (cis-diaminedichloroplatinum) is an inorganic molecule which used in chemotherapy of various types of cancers **(Wang *et al*., 2004)**. Cisplatin can bindto DNA of cancer cell and thus causes cell death. It is also causing reduction in antioxidant status and increase in reactive oxygen species which lead to increased cytotoxicity **(Liao *et al*., 2008)**. Therefore, cisplatin can cause adverse

effects in a variety of normal tissues, so its clinical use is limited **(Florea and Büsselberg, 2011)**.

As cisplatin and other synthetic drugs have revealed serious side effects, several experimental studies have been carried out to assess the use of natural products as an alternative medicine in cancer therapy**(Ramakrishnan*et al*., 2006)**.In this regard, licorice plant (*Glycyrrhizaglabra*) has been found to exert clinical benefits in the treatment of numerous illnesses **(Hu *et al*., 2009 and Al-Razzuqi*et al*., 2012)**. In particular, treatment with licorice extract was found to exert a hepatoprotective effect against CCl_4 toxicity in rats **(Huo *et al*., 2011)**. The major bioactive component of licorice extract isthe glycyrrhizic acid which has been reported to inhibit the occurrence of HCC in DENA-treatedmice **(Shiota *et al*., 1999)**.

Current study was conducted to investigate the potential curative impact of licorice aqueous extract and/or cisplatin on DENA/CCl_4-induced HCC in rats. The potential use of licorice as a beneficial supplement during cisplatin chemotherapy was assessed.

2. Material and Methods

Preparation of the aqueous extract of licorice

The licorice roots of *Glycyrrhizaglabra* were washed well with water, dried at room temperature in the dark, and then ground in an electric grinder to give a coarse powder. A known weight of this powder was suspended in a known volume of distilled water, and boiled for 30 minutes. The decoction obtained was centrifuged for 10 min, and the supernatant was collected. The supernatant was then filtered to remove cellulose fibers. The filtrate was concentrated and obtained residue was weighted then dried **(Huo *et al*., 2011)**. The resultant deposit was suspended in distilled water to prepare a doseof2g/kg body weight **(Lee *et al*., 2007)**.

Chemicals

DENA and CCl_4were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Cisplatin was a product of Hospira UK Limited, United Kingdom, Sigma-Aldrich Ltd., U.S.A. Cisplatin (50mg/50ml), each 50ml contain 50mg cisplatin, sodium chloride, mannitol, diluted hydrochloric acid and water. The applied dose from cisplatin was 1.5mg/Kg body weight **(Hamers *et al*., 1993)**.

Induction of hepatocellular carcinoma

DENA was dissolved in physiologic saline solution (0.9% NaCl) and injected intraperitoneally to each rat in a single dose of 200 mg/kg body weight **(Sarkar *et al*., 1997).** Two weeks later, animals received subcutaneous injections of CCl_4 once a week in a dose of 3 ml/kg body weight for 6 weeks to promote the carcinogenic effect of DENA **(Dakshayani *et al*., 2005**; **Subramanian *et al*., 2007 and Singha *et al*., 2009**).

Animals and treatment

Adult male Wistar albino rats (*Rattusnorvegicus*), weighing 100-120g were used in this experiment. Rats were obtained from Helwan Animal Farm, Cairo, Egypt. They were housed in stainless steel cages at a well-ventilated room, and fed on commercially standard diet. Tap water was given *ad libitum*. Care and use of the animals were conducted under supervision of the Animal Ethics Committee of Mansoura University, Egypt. After two weeks of acclimatization, rats were randomly divided into eight groups of six animals each, as follows:

I. Control group. Rats received no treatment.

II. Licorice extract-treated group. Animals received licorice extract alone orally at a dose of 2g/kg body weight daily for 3 weeks.

III. Cisplatin-treated group. Animals received cisplatin alone intraperitoneally in a dose of 1.5mg/kg body weight repeated twice a week for 3weeks.

IV. Licorice extract/Cisplatin-treated group. Rats received licorice extract plus cisplatin as described in groups 2 and 3 respectively.

V. DENA/CCl_4-treated group (HCC group). Rats received a single intraperitoneal dose of DENA (200 mg/kg body weight). Two weeks later, animals received subcutaneous injections of CCl_4 once a week in a dose of 3 ml/kg body weight for 6 weeks to promote the carcinogenic effect of DENA.

VI. DENA/CCl_4/Licorice extract-treated group. Animals received the same treatments described in group 5 for induction of HCC and then received licorice for additional 3 weeks as mentioned in group 2.

VII. DENA/CCl_4/Cisplatin-treated group. Animals received the same treatments described in group 5 for induction of HCC and then received cisplatin for additional 3 weeks as mentioned in group 3.

VIII. DENA/CCl_4/Cisplatin/Licorice extract-treated group. Rats received the same treatments described in group 5 for induction of HCC and then received both licorice extract and cisplatin for additional 3 weeks as mentioned in groups 2 and 3 respectively.

N.B. the total treatment period during the whole experimental study was 11 weeks.

Blood and liver sampling

At the end of the experimental period, overnight fasted animals were sacrificed by cervical dislocation and blood samples were collected in centrifuge tubes. After complete coagulation of the blood, tubes were centrifuged at 3000 rpm for 15 minutes. Sera were then separated and immediately frozen at -20°C for selected biochemical analysis. Meanwhile, animal were dissected and liver tissues were carefully removed and cleaned. Known portions of the liver

tissues were accurately weighed and homogenized in a 10 fold volume of distilled water. Liver homogenates were kept together with sera at-20°C for selected biochemical assays.

Biochemical analysis

The level of hepatic MDA was evaluated photometrically according to the procedure of **Ohkawa *et al.* (1979)**. The concentration of H_2O_2in liver homogenate was determined by the method of **Aebi (1984)**. Liver content of GSH was estimated colorimetrically by the method of **Prins and Loose (1969)**. The activities of hepatic SOD and GST were according to the methods of **Niskikimi *et al.* (1972) and Habig *et al.* (1974)** respectively. Enzymatic activities of AST and ALT in the blood serum were estimated photometrically by the method of **Reitman and Frankel (1957)**, while serum activity of ALP was determined according to the procedure of **Belfield and Goldberg (1971)**. Colorimetric methods of **Henry (1964), Doumas *et al.* (1971)** and **Walter and Gerade (1970)** were applied for the determination of serum contents of the total proteins, albumin and total bilirubin respectively.

Hepatic content of p53, caspase-3, Bcl-2 was evaluated by flow cytometric analysis using the method of **Dean and Jett (1974)**. The flow cytometer used in this work is fluorescence activated cell sorter(FACS caliber) flow cytometer (Becton Dickinson, Sunnyvale, CA, USA), equipped with a compact air cooled low power 15 m watt argon iron laser beam (488nm). In brief, cell suspensions were prepared in a PBS/BSA buffer and then incubated with either anti-Bcl-2, anti-P53or anti-active caspase-3 fluorescein isothiocyanate at room temperature for 30 min. Cells were then washed with PBA/BSA, centrifuged at 400×g for 5 min, re-suspended in 0.5% paraformaldehyde in PBS/BSA and analyzed using flow cytometry. Fluorescence intensity was standardized using isotype-matched negative control antibodies. Data were analyzed using Cell Quest software (Becton Dickinson).

Statistical analysis

The results were presented as means ± SE. The statistical significance of differences between mean values of different animal groups were determined by one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests. *P* value ≤ 0.05 was considered the minimal level of significance. Statistical evaluation of the obtained data was performed using SPSS 17.5 software. Percent of changes in the treated groups with respect to control values were also calculated.

3. Results

Parameters of oxidative stress and antioxidants

Results arranged in table 1 show that administration of DENA/CCl_4 in rats produced significant increases in the levels of MDA and H_2O_2, the markers of oxidative stress, accompanied with marked decreases in the levels of antioxidants including SOD, GST and GSH in the liver, when compared to the control group. Post-treatment with licorice extract (2g/kg body weight daily for 3 weeks) in DENA/CCl_4-treated rats caused significant decreases in the hepatic contents of both MDA and H_2O_2, and significant increases in the hepatic levels of SOD, GST and GSH, as compared to untreated HCC rats. Post-treatment with cisplatin alone or cisplatin plus licorice administration in DENA/CCl_4-treated rats induced similar changes in all mentioned parameters in the liver, but the induced effects were less potent than those in case of treatment with licorice alone. Table 2 also exhibits that administration of licorice alone in intact rats did not produce any significant changes in mentioned parameters of oxidative stress and antioxidants. While, treatment of intact rats with cisplatin alone caused oxidative tissue damage as indicated by significantly increased levels of MDA and H_2O_2 associated with marked decreases in the levels of antioxidants including SOD, GST and GSH in the liver, when compared to the control group. Co-treatment of intact rats with licorice plus cisplatin ameliorated the adverse effects of cisplatin since the results showed restoration of hepatic measured parameters to almost the control levels, except the hepatic contents of GSH and GST which still significantly decreased in respect to the control results.

Biomarkers of liver function

In the table 2, DENA/CCl_4-treated group showed marked elevations in the serum activities of ALT, AST and ALP as well as serum total bilirubin content when compared to control group. Meanwhile, decreased serum concentrations of total proteins and albumin, compared to control group, were observed. However, treatment of HCC rats with licorice extract significantly reduced elevated serum levels of mentioned biomarkers of liver function, while it markedly raised the lowered serum contents of total proteins and albumin, in comparison with untreated HCC group. Cisplatin administration induced similar effects of licorice on the serum activities of hepatic markers enzymes, when compared to HCC group, but these effects appeared less potent. Administration of cisplatin in HCC rats also markedly increased the lowered concentration of serum albumin but it exerted no significant effects on the serum levels of total bilirubin and total proteins, in comparison with the untreated HCC. Post-treatment of DENA/CCl_4-treated rats with licorice plus cisplatin significantly ameliorated the alterations in measured hepatic

markers, when compared to the untreated HCC. However, the beneficial effect of treatment with licorice alone on measured liver function markers was more pronounced in respect to that of cisplatin alone or in combination with licorice. In intact rats, administration of licorice alone had no significant effects on the serum levels of investigated liver function markers when compared to control group. Unlike licorice, treatment of this group with cisplatin alone caused marked elevation in the serum activities of hepatic markers enzymes including ALT, AST and ALP, as well as serum level of total bilirubin, while it significantly lowered serum concentrations of total proteins and albumin comparing with control results. In intact rats treated with cisplatin, administration of licorice partially reduced cisplatin-induced changes in serum activities of hepatic enzymes, but the levels of these markers were still significant when compared to control group. However, licorice restored the serum concentrations of both total bilirubin and total proteins to almost the control levels in rats treated with cisplatin.

Table 1. Liver contents of MDA, H_2O_2, GSH, SOD and GST in different animal groups. Results are given in mean ± SE from 6 rats in each group. Values in parentheses represent % of changes from control.

	C	LIC	CIS	LIC+CIS	HCC	HCC+LIC	HCC+CIS	HCC+LIC+CIS
MDA (nmol/g)	20.5 ±0.28	20.3 ±0.25 (-0.98)	25.23 ±0.26[a] (+23.07)	22.20 ±0.29 (+8.43)	35.16 ±1.6[a] (+71.51)	26.07 ±1.03[a,b] (+21.17)	30.00 ±0.42[a,b] (+46.34)	28.50 ±0.68[a,b] (+39.02)
H_2O_2 (mM/g)	5.20 ±0.07	5.00 ±0.60 (-3.80)	5.80 ±0.14[a] (+11.50)	5.50 ±0.20 (+5.70)	7.70 ±0.15[a] (+48.07)	6.20 ±0.14[a,b] (+19.23)	7.00 ±0.18[a,b] (+34.61)	6.70 ±0.18[a,b] (+28.84)
GSH (mg/g)	4.2 ±0.05	4.3 ±0.06 (+2.38)	3.5 ±0.14[a] (-16.67)	3.8 ±0.08[a] (-9.5)	2.2 ±0.15[a] (-47.61)	3.0 ±0.04[a,b] (-28.57)	2.6 ±0.08[a,b] (-38.09)	2.8 ±0.05[a,b] (-33.30)
SOD (U/g)	213 ±1.65	215 ±1.45 (+0.93)	185 ±1.25[a] (-13.15)	190 ±1.46 (-10.79)	150 ±1.59[a] (-29.50)	170 ±1.29[a,b] (-20.18)	160 ±1.23[a,b] (-24.80)	163 ±1.51[a,b] (-21.59)
GST (mol/g)	1.16 ±0.011	1.21 ±0.024 (+4.31)	0.82 ±0.059[a] (-29.31)	0.91 ±0.037[a] (-21.50)	0.423 ±0.057[a] (-63.50)	0.706 ±0.058[a,b] (-39.13)	0.506 ±0.06[a] (-56.37)	0.607 ±0.041[a,b] (-47.67)

[a], [b] significant changes at $P \leq 0.05$ comparing to control and HCC groups respectively. **C**: Control, **LIC**: Licorice, **CIS**: Cisplatin**, HCC**: Hepatocellular carcinoma (DENA & CCl_4).

Table 2. Serum levels of biomarkers of liver function in different animal groups. Results are given in mean ± SE from 6 rats in each group. Values in parentheses represent % of changes from control.

	C	LIC	CIS	LIC+CIS	HCC	HCC+LIC	HCC+CIS	HCC+LIC+CIS
ALT (U/L)	21.70 ±0.18	21.00 ±0.22 (-3.22)	25.00 ±0.20[a] (+15.20)	23.30 ±0.26[a] (+7.37)	40.00 ±0.57[a] (+84.22)	32.00 ±1.39[a,b] (+47.56)	35.50 ±0.93[a,b] (+63.59)	34.00 ±0.44[a,b] (+56.68)
AST (U/L)	45.00 ±1.28	43.5 ±1.27 (-3.30)	55.50 ±1.65[a] (+23.30)	52.00 ±1.07[a] (+15.50)	70.00 ±2.88[a] (+55.50)	58.00 ±1.23[a,b] (+28.80)	63.00 ±1.86[a,b] (+40.00)	61.20 ±1.30[a,b] (+36.60)
ALP (U/L)	53.40 ±0.24	51.60 ±0.44 (-3.37)	65.10 ±1.25[a] (+21.91)	58.50 ±1.29[a] (+9.55)	73.2 ±1.15[a] (+42.69)	58.50 ±1.12[a,b] (+9.55)	66.40 ±1.39[a,b] (+24.34)	60.20 ±1.33[a,b] (+12.73)
Total Bilirubin (mg/dl)	0.43 ±0.02	0.45 ±0.03 +3.48	0.52 ±0.04[a] +21.12	0.49 ±0.07 +15.02	0.72 ±0.03[a] +69.01	0.60 ±0.02[a,b] +39.53	0.66 ±0.07[a] +52.56	0.64 ±0.03[a,b] +49.30
Total protein (mg/dl)	7.50 ±0.13	7.80 ±0.11 +4.00	6.53 ±0.14[a] -12.93	7.18 ±0.15 -4.26	4.60 ±0.18[a] -38.60	5.50 ±0.12[a,b] -26.60	4.80 ±0.21[a] -36.00	5.20 ±0.13[a,b] -30.60
Albumin (mg/dl)	3.71 ±0.05	3.80 ±0.04 (+3.77)	3.00 ±0.02[a] (-22.39)	3.50 ±0.03[a] (-6.62)	2.05 ±0.04[a] (-44.74)	2.50 ±0.05[a,b] (-28.17)	2.19 ±0.03[a,b] (-40.90)	2.30 ±0.05[a,b] (-38.01)

[a], [b] significant changes at $P \leq 0.05$ comparing to control and HCC groups respectively. **C**: Control, **LIC**: Licorice, **CIS**: Cisplatin**, HCC**: Hepatocellular carcinoma (DENA & CCl_4).

Apoptotic markers

Table 3and figure 1(a, b and c) exhibit that treatment of rats with DENA/CCl_4induced apoptosis. Obtained results demonstrated increased hepatic concentrations of p53 and caspase-3 and decreased hepatic level of Bcl-2 in HCC animals, when compared to control group. However, treatment of HCC rats with licorice and cisplatin separately or together significantly decreased the elevated hepatic contents of p53 and caspase-3, while it markedly

raised the lowered hepatic level of Bcl-2, compared to the untreated HCC group. In intact rats, administration of licorice alone caused increases in the hepatic apoptotic markers p53 and caspase-3, while the anti-apoptotic Bcl-2 appeared unchanged. Treatment of the intact rats with cisplatin alone caused increases in the hepatic apoptotic markers p53 and caspase-3, and decrease in the anti-apoptotic Bcl-2 which clearly suggested incidence of apoptosis. Administration of licorice in cisplatin-treated intact rats partially inhibited the apoptotic response of cisplatin in the liver.

Table 3. Liver contents of p53 %, caspase-3 % and Bcl-2 % in different animal groups. Results are given in mean ± SE from 6 rats in each group. Values in parentheses represent % changes from control.

	C	LIC	CIS	LIC+CIS	HCC	HCC+LIC	HCC+CIS	HCC+LIC+CIS
p53 (%)	33.50 ±0.46	39.42 ±0.09[a] (+17.6)	54.84 ±1.90[a] (+63.7)	46.60 ±0.33[a] (+39.10)	66.30 ±0.96[a] (+98.8)	55.94 ±0.86[a,b] (+66.86)	58.41 ±0.16[a,b] (+74.35)	56.37 ±0.43[a,b] (+68.2)
Caspase-3 (%)	23.08 ±0.52	32.80 ±0.39[a] (+42.11)	38.40 ±1.29[a] (+63.8)	35.70 ±0.39[a] (+54.6)	45.90 ±1.53[a] (+98.8)	38.49 ±1.12[a,b] (+66.76)	41.00 ±0.58[a,b] (+77.64)	40.20 ±1.14[a,b] (+74.17)
Bcl-2 (%)	48.30 ±0.36	48.99 ±0.45 +1.43	39.58 ±0.54[a] -18.06	42.12 ±0.45[a] -12.79	27.29 ±0.67[a] -43.49	38.66 ±0.57[a,b] -19.95	32.52 ±0.58[a,b] -32.67	36.17 ±0.71[a,b] -25.11

[a], [b] significant changes at $P \leq 0.05$ comparing to control and HCC groups respectively. **C**: Control, **LIC**: Licorice, **CIS**: Cisplatin, **HCC**: Hepatocellular carcinoma (DENA & CCl_4).

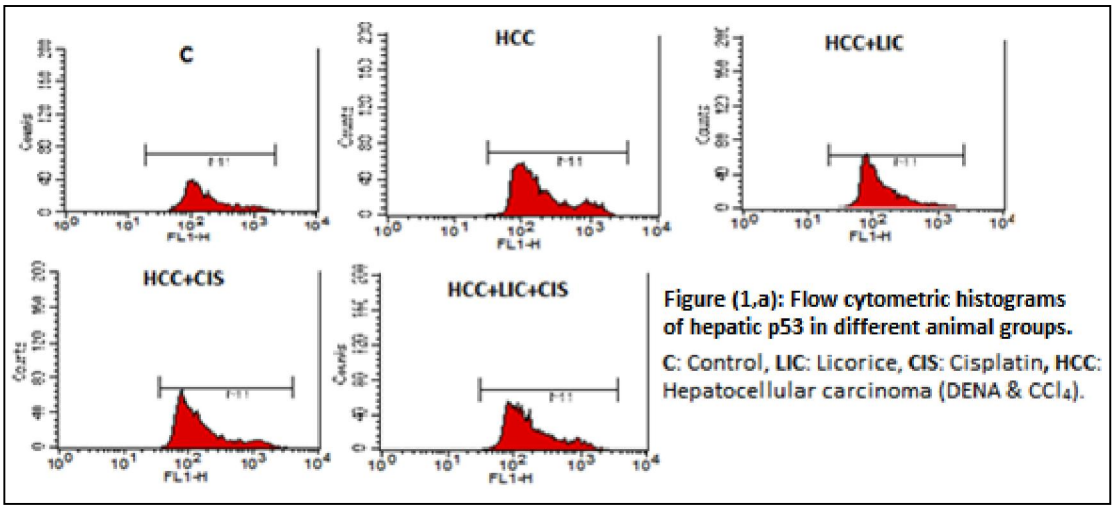

Figure (1,a): Flow cytometric histograms of hepatic p53 in different animal groups.

C: Control, **LIC**: Licorice, **CIS**: Cisplatin, **HCC**: Hepatocellular carcinoma (DENA & CCl_4).

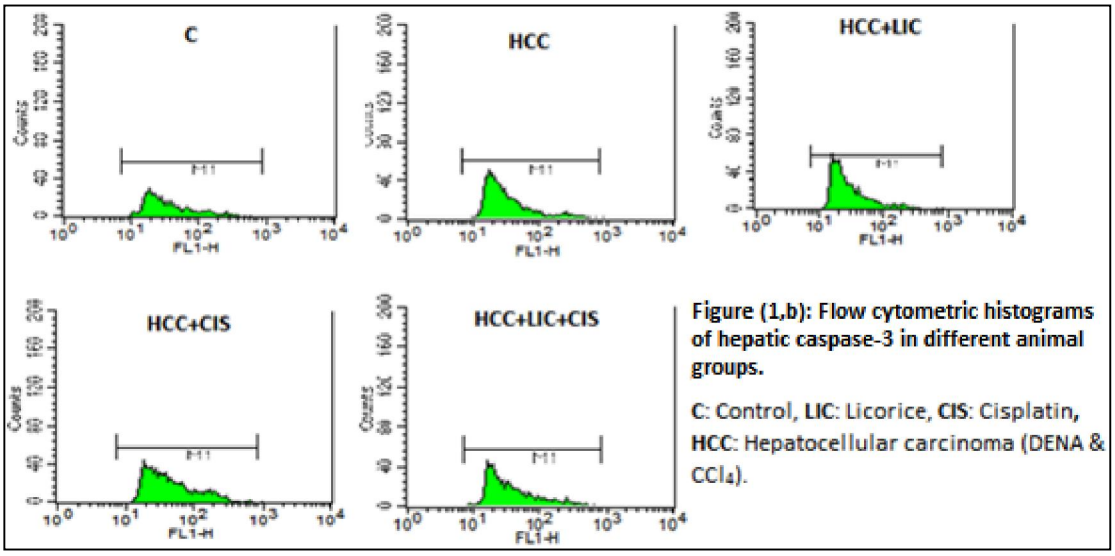

Figure (1,b): Flow cytometric histograms of hepatic caspase-3 in different animal groups.

C: Control, **LIC**: Licorice, **CIS**: Cisplatin, **HCC**: Hepatocellular carcinoma (DENA & CCl_4).

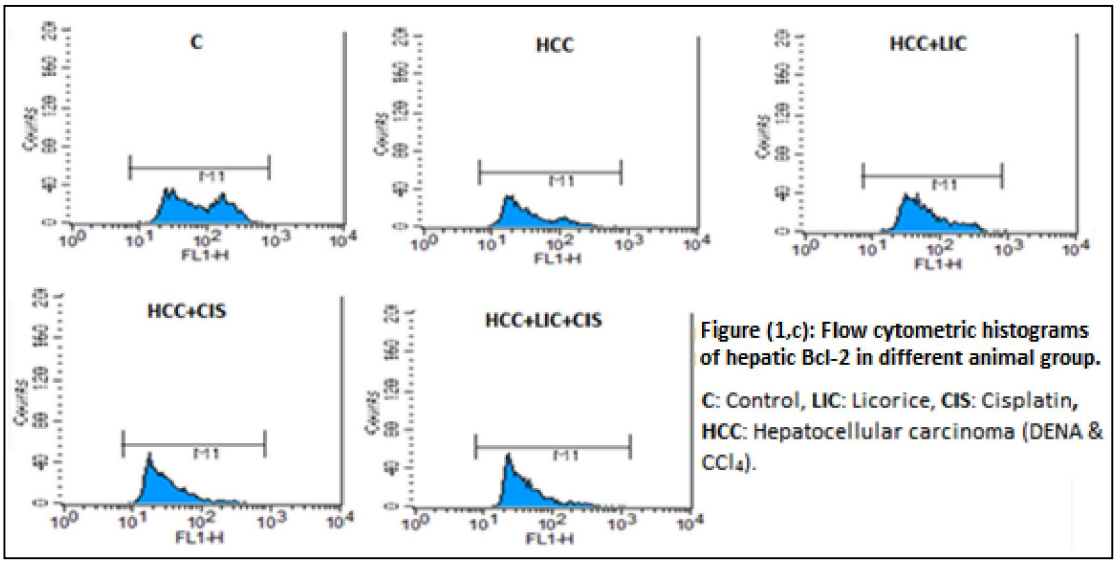

Figure (1,c): Flow cytometric histograms of hepatic Bcl-2 in different animal group.

C: Control, **LIC**: Licorice, **CIS**: Cisplatin, **HCC**: Hepatocellular carcinoma (DENA & CCl_4).

4. Discussion

It has been reported that, reactive oxygen species and lipid peroxidation can play a major role in tumor promotion **(Kensler and Trush, 1984)**. In case of DENA as a tumor initiator, oxidative stress is produced and can contribute to the hepato carcinogenesis **(Qi *et al*., 2008)**. Moreover, biotransformation of DENA in the rat liver by cytochrome P450 showed production of a reactive product, ethyl diazonium ion, which reacts with DNA forming adducts that is recognized as the initial step in DENA-induced carcinogenesis **(Verna *et al*., 1996)**. On the other hand, CCl_4 is metabolized by liver microsomal cytochrome P450 producing free radicals which deplete hepatic antioxidants and damage macromolecules leading to promote tumor genesis **(Weber *et al*., 2003)**. In agreement, present study displayed hepatic oxidative stress as indicated by increased production of MDA and H_2O_2, accompanying with decreased hepatic levels of antioxidants including GSH, SOD and GST in rats treated with DENA/CCl_4.Concurrently, apoptotic markers including p53 and caspase-3 proteins were significantly increased along with deceased level of anti-apoptotic Bcl-2 protein in the liver of treated rats, suggesting damage of DNA and genotoxicity. These findings could suggest incidence of HCC in rats treated with DENA/CCl_4, In this regard, several reports similar to the present study have demonstrated the incidence of HCC in rats treated with DENA/CCl_4 **(Dakshayani *et al*., 2005; Subramanian *et al*., 2007** and **Singha *et al*., 2009**). It has also been reported that during rat liver carcinogenesis induced by chemicals, a number of initiated hepatocytes undergo apoptotic cell death **(Schulte-Hermann *et al*., 1995)**. In addition, **Aiub *et al*. (2011)** demonstrated dose-

response increase in the apoptosis rate in the liver of rats treated with DENA alone or that pre-treated with phenobarbital. There is also evidence of increased apoptotic markers p53 and caspase-3 and decreased anti-apoptotic Bcl-2 in the liver of rats treated with DENA **(De Miglio *et al*., 2000 and Zahran *et al*., 2014)**. It could be suggested that, incidence of apoptosis during hepatocarcinogenesis induced by high dose of genotoxic DENA is a compensatory mechanism for slowing but not preventing the proliferation of tumor cells, by the removal of part of cells containing damaged DNA.

In the present study, DENA/CCl_4-induced oxidative stress can induce liver tissue damage and hence modify its function. Obtained results, therefore, showed increased activities of ALT, AST and ALP; and level of total bilirubin in serum, along with decreased serum content of total proteins and albumin in DENA/CCl_4-treated rats which suggested hepatocellular damage and impairment of liver function. These findings were comparable to other published data which reported incidence of HCC in rats given the same treatment regimen using DENA/CCl_4 **(Dakshayani *et al*., 2005; Subramanian *et al*., 2007** and **Singha *et al*., 2009**). Available literatures also showed disturbances in the serum levels of biomarkers of liver tests such as transaminases in many models of DENA-induced HCC **(Kartik *et al*., 2010 and Mohammed *et al*., 2014)**. Moreover, **Abdel-Hamid *et al*. (2011)** reported that DENA significantly increased serum ALT, AST and γ-glutamyl transferase activities in rats.

Cisplatin is an important antineoplastic drug which can able to kill cancer cells by forming DNA adducts **(Wang *et al*., 2004 and Florea and Büsselberg, 2011)**. In the present study, post-treatment with cisplatin in rats injected with DENA/CCl_4 suppressed partially the oxidative stress as indicated by significantly decreased hepatic content of MDA and H_2O_2 and increased content of GSH and SOD in the liver, compared to the untreated HCC group. This was accompanied with partial improvement in both hepatic apoptotic markers (p53, caspases-3 and Bcl-2) and serum levels of hepatic function biomarkers including ALT, AST, ALP and albumin. However, post-treatment with cisplatin had no beneficial effect on serum levels of total proteins and bilirubin in DENA/CCl_4-treated rats. Available data showed that post-treatment with cisplatin in rats treated with a single sub-necrogenic dose of DENA (125 mg/kg) improved liver histopathological alterations and serum activities of the liver enzymes **(Abdel-Hamid *et al*., 2011)**.The mechanism through which cisplatin partially prevented the adverse changes in HCC rats is not fully understood. However, the ability of cisplatin to kill cancer cell might be followed by increased healing and regeneration rate of the liver tissue, which might lead to partial improvement of the liver structure and function.

Because of the cisplatin adverse side effects, the strategies of cancer treatment using combined agents that frequently involves antioxidants such as natural products are considered more promising. In the present study, potential curative efficacy of licorice extract alone or in combination with cisplatin in the suppression of HCC was investigated. Obtained results showed that administration of licorice alone or in combination with cisplatin in DENA/CCl_4-treated rats markedly increased the levels of antioxidative parameters including GSH, SOD and GST accompanying with significantly decreased H_2O_2, MDA and apoptosis (as reflected by decreased levels of p53 and caspases-3, and increased level of Bcl-2)in the liver, when compared to the untreated HCC group. Generally, treatment with licorice alone appeared to be more effective than treatment with licorice plus cisplatin or cisplatin alone. Thereason beyond this could be attributed to the presence of bioactive compounds such as flavonoids in licorice extract which exhibit antioxidative, free radicals scavenging and anti-carcinogenic activities **(Chin *et al*., 2007; and Asl and Hossein zadeh, 2008)**. In particular, the ability of licorice to suppress the oxidative stress and to improve the antioxidant status in the liver of DENA/CCl_4-treated rats might afford protection against further DNA damage leading to reduced apoptosis. **El-Tahawy *et al*. (2011)** reported that, treatment of rat model of acute hepatitis with glycyrrhizin, the sweet-tasting constituent of licorice root, revealed anti-apoptotic activity.

Also, plant extract partially prevented elevated serum levels of ALT, AST, ALP and total bilirubin; and decreased serum concentration of total proteins and albumin in DENA/CCl_4-treated rats. The ability of licorice extract alone to improve partially the liver status in HCC rats raised its potential medicinal use in cancer therapy. Current study also showed that combined treatment with licorice plus cisplatin in DENA/CCl_4-treated rats resulted in partial amelioration of mentioned parameters of oxidative stress, antioxidant, apoptosis and liver function markers. Taken together, the resultant beneficial efficacy of various types of treatment regimens against the adverse effects in DENA/CCl_4-treated rats followed this order: licorice alone > licorice plus cisplatin>cisplatin alone. Published data in this concern reported that treatment with glycyrrhizin appeared to be effective in lowering the incidence of HCC either by chronic hepatitis C or by DENA **(Shiota *et al*., 1999)**. Another study demonstrated that administration of licorice extract enhanced hepatic antioxidant enzymes activities and GSH level, reduced

MDA content and improved serum levels of markers of liver function in CCl_4-treated rats **(Huo *et al*., 2011)**. Also, *in vitro* studies showed that licochalcone A (a bioactive component of licorice extract) has the ability to protect from oxidative stress mediated by reactive oxygen species in UVA-irradiated human dermal fibroblasts (**Kühnl *et al*., 2015**). In clinical studies, treatment with glycyrrhizin improved hepatic function tests including transaminases and γ-glutamyltransferase in patients with chronic active hepatitis **(Suzuki *et al*., 1983)**. In mouse model of colon carcinoma, administration of the licorice extract significantly inhibited tumor growth. The administration of the licorice extract plus cisplatin recovered the adverse changes in the functional indices of the liver to almost the control levels. Also, the combination of the licorice extract and cisplatin diminished the therapeutic efficacy of cisplatin but promoted considerably antitumor activity of the licorice extract **(Lee *et al*., 2007)**.

Although cisplatin is an important anticancer drug, its serious side effects and resistance greatly limited its clinical uses **(Chirino *et al*., 2004 and Gosepath *et al*., 2005)**. Previous findings demonstrated that cisplatin-induced hepatotoxicity in the rats which was associated with suppression in the hepatic activities of antioxidant enzymes (CAT and SOD) and an increase in MDA, the marker of lipid peroxidation **(Palipoch and Punsawad, 2013).** Treatment of rats with cisplatin also caused hepatic oxidative tissue damage as manifested by increased level of liver MDA associated with significantly reduced activities of the antioxidant enzymes including SOD and glutathione peroxidase **(Abou zeinab, 2013)**. In agreement, present study on intact rats treated with cisplatin alone showed moderate hepatotoxicity as reflected by increased production of MDA and H_2O_2, and reduced levels of antioxidant parameters including GSH, SOD and GST in the liver tissue. Treatment with cisplatin also led to produce apoptosis in the intact liver as reflected by obtained increase in both p53 and caspase-3, and decrease in Bcl-2. Potentially, the ability of cisplatin to cause damage to DNA, the molecular target of this metal, and to produce oxidative stress could activates various signal transduction pathways which stimulate overexpression of p53 proteins and caspases leading to DNA damage repair, cell-cycle arrest and apoptosis, that prevent some of cisplatin cytotoxicity **(Siddik, 2003)**. Cisplatin-induced oxidative stress can cause liver dysfunction and hepatocellular damage with consequent leakage of the enzymes from the hepatocytes. In this regard, obtained results displayed significant increase in the serum levels of ALT, AST, ALP and total bilirubin along with significantly decreased serum content of total proteins and albumin following cisplatin treatment alone in intact rats as compared to control group. These results are in agreement with previous reports which displayed that the administration of cisplatin in rats impaired liver function as indicated by an increase in the serum activities of transaminases **(Palipoch and Punsawad, 2013)**.

Interestingly, treatment with licorice extract in intact rats treated with cisplatin significantly reduced cisplatin-induced increase in hepatic contents of MDA, $H_2O_2$to almost the control levels. Also, it ameliorated the levels of SOD, GST and GSH by elevating their hepatic contents in cisplatin-treated rats, the hepatic activity of SOD reached to near the control level. Furthermore, administration of licorice extract plus cisplatin in intact rats caused reduction in the apoptosis induced by cisplatin. Moreover, licorice extract revealed potent protective effect against cisplatin-induced liver dysfunction in the intact rats, since it partially reduced the adverse effect of cisplatin on measured markers of liver function, compared to control group. These results are consistent with published data of **Lee *et al*. (2007)** who reported that the administration of licorice extract plus cisplatin significantly reduced cisplatin-induced oxidative stress and recovered the changes in functional indices of the liver induced by cisplatin to almost the control levels.

In conclusion, current study provided evidence that the therapeutic efficacy of cisplatin alone or in combination with licorice is less effective in comparison with that of licorice alone in case of the treatment of DENA/CCl_4-induced HCC in rats. Since licorice treatment alone has no side effects, while cisplatin treatment frequently is associated with serious adverse effects, the strategy of cancer treatment using licorice is considered more promising and needs further investigations.

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