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LICORICE AMELIORATES CADMIUM CHLORIDE INDUCED HEPATOTOXICITY IN ALBINO RATS VIA B-CELL LYMPHOMA 2 UP REGULATION

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Received: 17 September 2017; **Accepted:** 3 October 2017

ABSTRACT

Cadmium (Cd) is a widespread toxic pollutant of occupational and environmental concern because of its diverse toxic effects including hepatotoxicity, nephrotoxicity and reproductive toxicities. There is an increasing demand for herbal medicines. Licorice is a plant used in traditional medicine across the world effectively used as an anti-oxidant. **Aim:** Investigate the protective role of licorice against cadmium chloride (CdCl2) induced hepatotoxicity. **Methods:** Forty male albino rats were randomly divided into four groups each consisting of 10 animals and were treated as follows: Untreated control group (G1), Licorice treated group (300mg/ kg b.w) intragastric daily for 21 days (G2), CdCl2 treated group (3mg/kg b.w) intraperitoneal 5 times per week for 3 weeks (G3) and a group treated with the licorice (300mg/ kg b.w) intragastric for 3days before CdCl2 injection and continued along with CdCl2 injection (3 mg/kg b.w) 5 times per week for 3 weeks (G4). Specimens from liver were taken and examined histopathologically besides estimation of hepatic enzymatic functions. Also, immunohistochemical expression of B-cell lymphoma 2 (Bcl-2) in the livers of all experimental rats was investigated. **Results:** Significant increase of hepatic enzymes levels in Cd treated rats when compared with control group with low levels of albumin and total protein. While, co-treatment of CdCl2 with licorice reduces the elevation of enzymatic activities. Histopathologically, the liver sections from Cd group showed severe changes including marked degenerative changes of hepatic cells, dark stained nuclei with condensed cytoplasm indicating nuclear pyknosis accompanied with marked kupffer cells proliferation as well as focal areas of hepatic necrosis with mononuclear cells infiltration. Combined administration of Licorice with CdCl2 improves the biochemical and histopathological changes induced by cadmium intoxication. Down-regulation of Bcl-2 in hepatic cells induced by Cd was restored by co-administration of licorice. **Conclusion:** Licorice has a protective effect against hepatotoxicity of cadmium.

Key words: Cadmium, protective, licorice, hepatotoxicity, and Bcl-2.

INTRODUCTION

Cadmium (Cd) is a widespread environmental heavy metal pollutant, not biodegradable and persists in the environment. Despite efforts by many countries and international agencies to reduce the usage of cadmium, it continues to be a major public health problem, especially in emerging industrial nations where environmental controls are still being developed (Satarug *et al.,* 2003; Nordberg, 2004; Teeyakasem *et al.,* 2007; Jarup and Akesson, 2009). River Nile water is seriously contaminated with heavy metals, pesticides, and hydrocarbons as a result of increasing discharge of untreated industrial wastes and agricultural irrigation wastewater (Badawy *et al.,* 1995). High concentrations of heavy metals, including cadmium, are among the pollutants in the water. Plants and fish grown in this water are also contaminated with heavy metals (Abdel-Sabour 2001; Abou-Arab and Abou Donia, 2000), which can in turn accumulate in humans and animals that feed on these contaminated foods (Osfor *et al.,* 1998). Long term exposure to Cd leads to an increase in lipid peroxidation and causes inhibition of superoxide dismutase (SOD) activity indicating oxidative damage in liver, kidney and testes (Casalino *et al.,* 1997; El-Missiry and Shalaby, 2000). Trends on applying nutritional antioxidants in diseases related to oxidative stress have gained immense interest in recent years. Plant products are known to exert their protective effects by scavenging free radicals and modulating antioxidant defense system. Licorice (Glycyrrhizae radix) is one of the oldest and most frequently used botanical treatments in East Asia. Licorice has been recommended for its life-enhancing properties, detoxification and as a cure for digestive disorders and swelling (Wang and Nixon, 2001). Herbal medicines containing licorice showed stimulatory effects in the immune systems (Kiyohara *et al.,* 2004 and Gao *et al.,* 2005). Licorice has also

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been reported to attenuate free radical-induced oxidative damage in the kidney (Yokozawa *et al.*, 2005) and prevent carcinogenesis induced by toxicants or hormones (Mori *et al.*, 2001).

The present work was designated to investigate the induced hepatotoxicity of cadmium chloride (CdCl2) in albino rats (biochemical and histopathological changes and immunohistochemically). The possible protective role of licorice against the toxic effect of cadmium was also investigated. In the available literature, no previous report of Cd/ licorice on Bcl-2 immunohistochemical expression was found.

MATERIALS AND METHODS

Experimental Animals:
Forty male albino rats 4 weeks old weighing 90-100 grams were used in the present study and obtained from the animal house colony, National Research Center, Giza, Egypt. Rats were acclimatized for one week to laboratory conditions, provided with commercial balanced diet and tap water adlibitum throughout the experiment.

Chemicals:
A. Cadmium chloride (CdCl2) was purchased from Sigma (St.Louis, MO, USA).

B. Aqueous extract of licorice prepared from licorice root in concentration 3:1 after washing, grinding, boiling, centrifugation and filtration (Andalos Company, China).

Treatment of experimental animals and schedule of sacrifice:
Rats were randomly divided into four groups each consisting of 10 animals and were treated as follows: Untreated control group (G1), Licorice treated group (300mg/ kg b.w) intragastric daily for 21 days (G2), CdCl2 treated group (3mg/kg b.w) intraperitoneal 5 times per week for 3 weeks (G3) and a group treated with the licorice (300mg/ kg b.w) intragastric for 3days before CdCl2 injection and continued along with CdCl2 injection (3 mg/kg b.w) 5 times per week for 3 weeks (G4). At the end of the experiment all animal were sacrificed by cervical decapitation.

Blood Chemistry:
At the end of the experimental period, blood was collected from all of the experimental animals directly from the retro-orbital venous plexus under light ether anesthesia and serum was separated from clotted blood by centrifugation at 3,000 rpm for 10 min. Serum was used for estimation serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), gamma glutamyl transferase (GGT), total protein (TP) and albumin (Alb) according to the kinetic method of (Reitman and Frankle, 1957; Wootton, 1964; Doumans *et al.*, 1981 and Reinhold, 1953).

Histopathology:
Tissue sections of the liver of each animal were excised and processed for histopathological examination. The sections were directly fixed in 10% formalin solution, dehydrated in alchols, cleared in xylene and embedded in paraffin blocks. Sections of 5 µm thickness were obtained and stained with hematoxylin and eosin (Bancroft *et al.*, 1991).

Immunohistochemistry:
Immunohistochemical staining of Bcl-2 was performed on the livers of all tested groups using 4-µm thick paraffin-embedded sections. The sections were dewaxed in xylene and rehydrated in graded ethanol. For antigen retrieval, the sections were immersed in (EDTA solution, PH 8), then treated with hydrogen peroxide 0.3%. To prevent the binding of non-specific proteins, the sections were incubated with Protein Block Serum Free. Immunolabelling of Bcl-2 was performed on all samples, using anti-Bcl2 (Santa Cruz, at 1:100 dilution). The slides were washed three times with PBS, and incubated with anti-mouse IgG secondary antibodies (EnVision + System HRP; Dako) for 30 minutes at room temperature. The sections were washed with phosphate-buffered saline (PBS) and visualised with EnVision+ System, HRP-labelled polymer anti-rabbit (Dako®). After washing 3 times with PBS, 3,3-diaminobenzidinetetrahydrochloride (Liquid DAB + Substrate Chromogen System, Dako®) was added to the sections. The sections were then washed in distilled water, counterstained with Mayer's haematoxylin, dehydrated in an alcohol gradient, cleared with xylene, and mounted for examination under light.

Statistical analysis:
A one way ANOVA followed by Post hoc tests were used to compare the significance among different experimental groups and a P value < 0.05 was considered statistically significant. All data were tabulated as Means± SD.

RESULTS

Biochemical findings:
Rats given Cd showed a significant increase in activities of AST, ALT, ALP, LDH, GGT levels. However, Cd treatment resulted in lowering levels of total protein (TP) and albumin (Alb) as compared to control. Rats administered only with licorice showed non-significant difference when compared with control rats. However, in Cd intoxicated rats and treated with licorice extract there was significant decrease in AST, ALT, ALP, LDH, GGT levels when compared with Cd treated rats. While TP and Alb levels were normalized when received combined administration of licorice and Cd as shown in (Table 1 and 2).

Table 1: Effect of licorice on liver enzymatic functions in cadmium intoxicated rats.

Groups	AST	ALT	ALP	LDH	GGT
Control	192±43.38	28.89±1.96	315±138	410.67±104.7	1.17±0.55
Cadmium	527.6±107.75*	48.13±7.07*	570.6±71.6*	1003±194.5*	6.45±0.68*
Licorice	197±25	29.6±1.5	283±37.6	309±90.2	1.33±0.39
Cd/licorice	392±51.3*	35.50±2.77*	438.8±94.32*	618±105.5*	5.31±0.54*

*Significant difference (P value <0.05) when compared with control or cadmium treated group

Table 2: Effect of Licorice on serum biochemical parameters of total protein and albumin in Cd treated rats.

Groups	TP	Alb
Control	6.46±0.43	4.01±0.27
Cadmium	5.90±0.26*	3.52±0.12*
Licorice	6.40±0.40	3.96±0.13
Cd/ licorice	6.39±0.52*	3.81±0.14*

*Significant difference (P value <0.05) when compared with control or cadmium treated group.

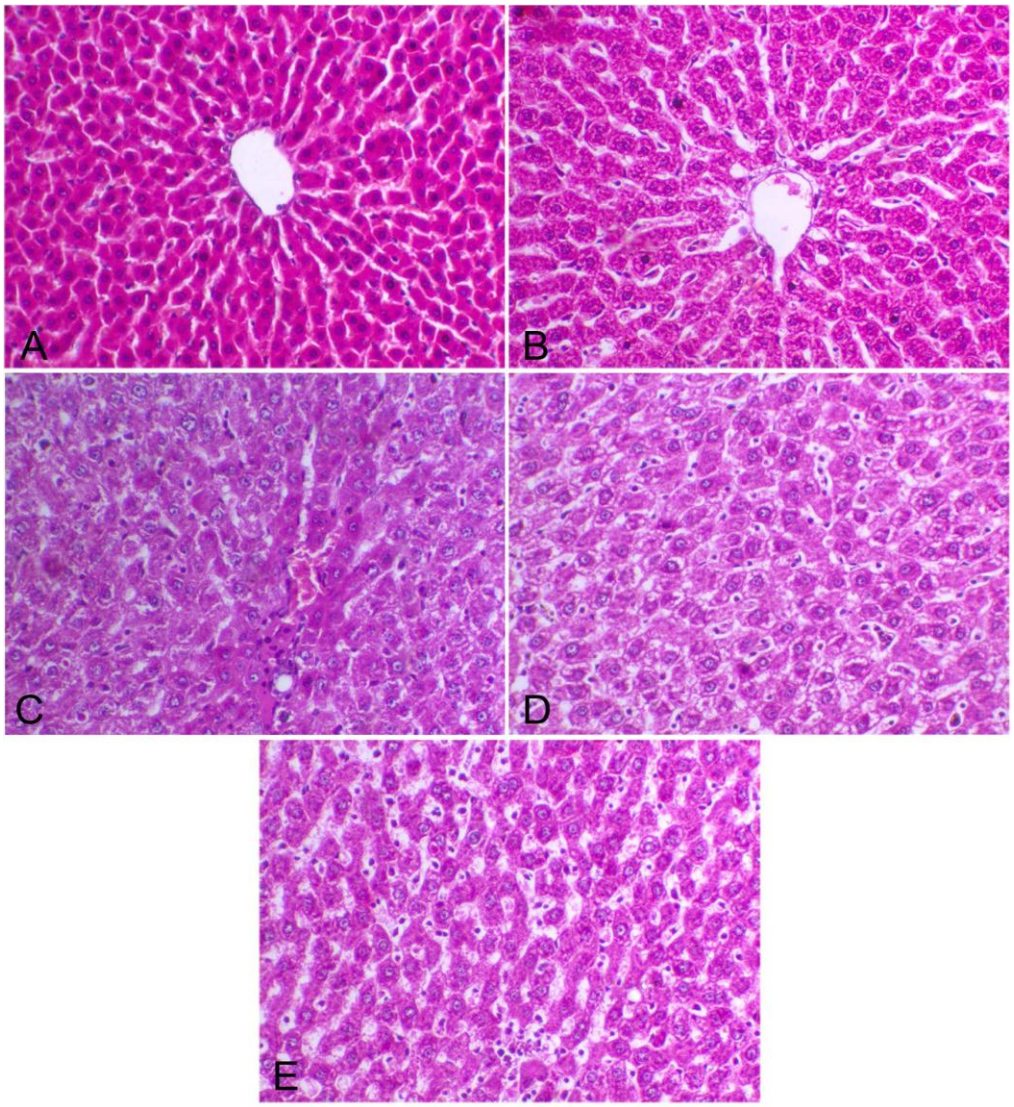

Figure (1): Histopathological changes induced in the livers of cadmium (Cd) intoxicated rats.
(A): Liver of control rats showing normal hepatic architecture (HE, 10x10). **(B):** Liver of licorice treated group showing mild vacuolar degeneration and nuclear pyknosis (HE, 10x10). **(C):** Liver of Cd treated group showing marked degenerative changes of hepatocytes (HE, 10x20). **(D):** liver of Cd treated rats showing marked vacuolar degenerative changes of hepatocytes with kupffer cells activation and nuclear pyknosis (HE, 10x20). **(E):** liver of Cd treated rats showing marked kupffer cells activation with sinusoidal dilatation (HE, 10x20).

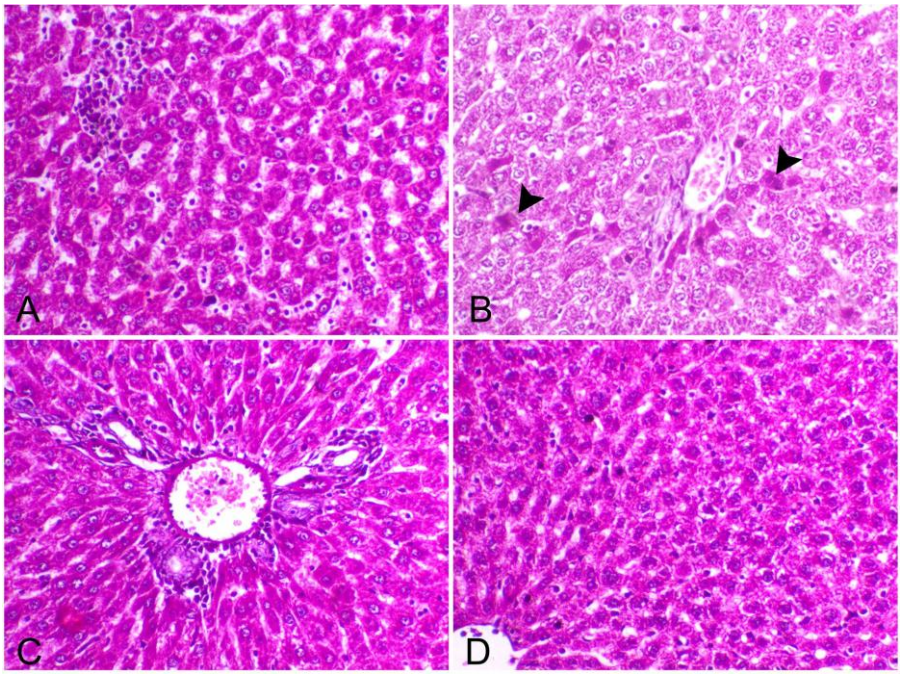

Figure (2): Histopathological changes induced in the livers of cadmium (Cd) intoxicated rats and the protective role of licorice. (A): Liver of Cd treated group showing focal hepatic necrosis accompanied with mononuclear cells infiltrations along with kupffer cells activation (HE, 10x20). **(B):** Liver of Cd treated group showing nuclear pyknosis with darkly stained cytoplasm indicating necrosis, arrow head (HE, 10x20). **(C):** Liver of Cd treated group showing mild bile ducts hyperplasia (HE, 10x20). **(D):** Liver of licorice and Cd treated rats showing mild degenerative changes with mild kupffer cells activation (HE, 10x20).

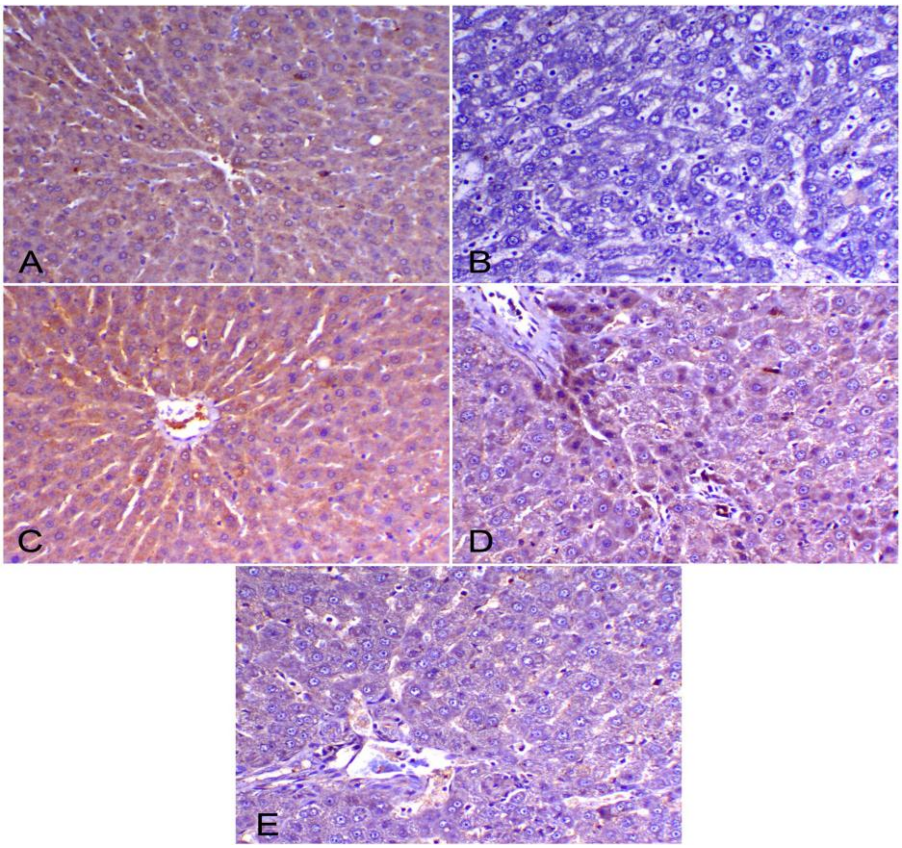

Figure (3): Immunohistochemical expression of Bcl-2 in the livers of Cd intoxicated rats.
(A): Liver of control group showing mild immunoreactivity of Bcl-2 in the cytoplasm of some hepatocytes (IHC, 10x10). **(B):** Liver of Cd treated group showing negatively stained hepatocytes for Bcl-2 (IHC, 10x20). **(C):** Liver of licorice/ Cd treated rats showing marked cytoplasmic immunoreactivity of Bcl-2 in most of hepatocytes (IHC, 10x10). **(D):** Liver of licorice/ Cd treated rats showing cytoplasmic immunoreactivity in the hepatocytes especially around blood vessels along with staining of some inflammatory cells (IHC, 10x20). **(E):** Liver of licorice/ Cd treated rats showing immunoreactivity of Bcl-2 in the lining endothelium of blood vessels and blood sinusoids (IHC, 10x20).

Histopathology:
Healthy control rats showed normal lobular architecture with central vein and radiating hepatic cells **(Fig1. A)**. No obvious histopathological changes were observed in the livers of licorice treated rats except of mild degenerative changes and some nuclear pyknosis **(Fig1. B)**. However, in Cd-treated rats severe changes were observed including congestion, marked degenerative changes of hepatic cells **(Fig1. C, D)**, marked kupffer cells proliferation **(Fig1. E)** with focal areas of hepatic cells necrosis accompanied with mononuclear cells infiltration with dilation and congestion of blood sinusoids **(Fig2. A)**. Also, perivascular and periductal mononuclear cells infiltration was observed. In addition to, many hepatocytes with dark stained nuclei with condensed cytoplasm indicating necrosis **(Fig2. B)** and mild bile duct hyperplasia **(Fig2. C)**. Combined administration of licorice resulted in restoration of hepatic architecture accompanied with mild degenerative changes of hepatocytes **(Fig2. D)**; however mild kupffer cells proliferation, nuclear pyknosis were observed.

Immunohistochemistry:
Immunolabelling of Bcl-2 was observed in some hepatocytes of control rats **(Fig3. A)**. Negative expression of Bcl-2 was observed in Cd intoxicated rats **(Fig3. B)** which up regulated in rats received licorice along with Cd administration **(Fig3. C, D)**. Positive labelling of endothelial cells of blood vessels and some proliferating bile ducts epithelial cells **(Fig3. E)** was also observed.

DISCUSSION

Metals such as lead, mercury and cadmium are examples of heavy metals which are very toxic even at minute quantities (Cockerham and Shane, 1994). Cadmium, one of the most common toxic heavy metals, can induce and bind to metallothionein, which concentrates Cd up to 3000-fold (Klassen *et al.,* 1999). Although chronic Cd intoxication mainly results in renal disease, acute exposure to toxic Cd doses primarily results in liver damage (Rikans and Yamano, 2000). The liver is the most important target organ because Cd primarily accumulates in the liver (Swiergosz- Kowalewska, 2001). Exposure to CdCl2 leads to a decrease in the activities of antioxidant enzymes, such as superoxide dismutase and catalase (Jurczuk *et al.,* 2004). At the cellular level, Cd depletes glutathione and protein-bound sulfhydryl groups, leading to increased lipid peroxidation and enhanced intracellular oxidized states (Koyu *et al.,* 2006). Cd also causes apoptosis and necrosis. Micro molar Cd induces apoptosis irrespective of sulfhydryl deficiency, whereas submolecular Cd, in conjunction with sulfhydryl deficiency, causes non-apoptotic cell death (Kim *et al.,* 2003). Licorice reduced apoptosis via inhibition of Bad protein translocation from cytosol to the mitochondrial membrane (Kim *et al.,* 2004). In the current study, i.p injection of Cd resulted in congestion, marked degenerative changes of hepatic cells, kupffer cells proliferation with focal areas of hepatic cells necrosis accompanied with mononuclear cells infiltration with dilation and congestion of blood sinusoids. Also, perivascular and periductal mononuclear cells infiltration, in addition to, many hepatocytes with dark stained nuclei with condensed cytoplasm indicating necrosis and mild bile duct hyperplasia which reflected on liver enzymatic function tests and causes significant elevation of AST, ALT, ALP, LDH, and GGT levels when compared with control group ($p<0.05$). Serum levels of total protein and Alb were significantly decreased when compared with control group. Combined treatment of licorice and cadmium resulted in mild to moderate amelioration of hepatic toxic effects of cadmium accompanied with lowering the levels of AST, ALT, ALP, LDH, and GGT when compared with cadmium treated group only with mild improvement when compared with control group. Levels of total protein and Alb were normalized by co-administration of Cd and licorice. These findings agree with Renugadevi and Prabu, (2010) who showed that liver damage induced by cadmium (5mg/kg orally) for 4 weeks was clearly shown by increased activities of AST, ALT, ALP, LDH, GGT and TP. Also Jurczuk *et al.* (2004) reported that acute exposure to cadmium caused hepatotoxicity which indicated by swelling of hepatocytes, fatty changes, focal necrosis, hepatocytes degeneration and impaired functions of biomarkers of liver function. Bamidele *et al.* (2012) administered 2.5 mg/kg b.w of cadmium s.c every other day regularly for six weeks. They observed significant reduction in total proteins and albumin levels while ALT activity was significantly increased and histopathologically the liver showed fatty degeneration, cytoplasm vacuolization with focal and diffused hepatocellular necrosis. Also, Srinivasan and Ramprasath, (2012) showed that administration of 3 mg/kg body weight of cadmium subcutaneously to rats for three weeks showed significant ($p<0.05$) increase in activities of AST, ALP, LDH and lipid peroxidation with significant decrease in the levels of anti-oxidant in the liver accompanied with change in liver architecture via macrovasicular fatty changes in hepatocytes, peri-portal inflammation and cell infiltration. Single intravenous injection of Cd at 4 mg/kg elevated levels of ALT, AST and LDH and the liver showed central lobular necrosis around central veins, peripheral hemorrhage around portal triads and pretreatment with 50, 100 mg/kg/day of licorice extract normalized ALT, AST and LDH levels with decreasing of the central necrosis around central veins, the peripheral hemorrhage around portal triads, the percentage of degenerative hepatic regions and the number of degenerative hepatic cells accompanied with inhibition of the increment of Bad (a BH 3 domain containing protein) translocation by cadmium in liver

cells Lee *et al.* (2009) which is partially agree with the current results where licorice water extract resulted in decreasing the elevated serum AST and ALT levels along with decreasing the intensity of hepatic tissue damage on the histological level. The members of the B- cell lymphoma 2 (Bcl-2) protein family are known to regulate the release of apoptosis-activating factors that the ratio to determine cell survival or cell death Oltvai *et al.* (1993). Bcl-2 is localized to the outer membrane of mitochondria, where it plays an important role in promoting cellular survival and inhibiting the actions of pro-apoptotic proteins Hardwick and Soane, (2013). Damage to the Bcl-2 gene has been identified as a cause of a number of cancers, including melanoma, breast, prostate, chronic lymphocytic leukemia, and lung cancer and also is a cause of resistance to cancer treatments Renner *et al.* (2017). In the present study, Bcl-2 protein expression was negatively expressed in the livers of Cd treated rats. However, strong cytoplasmic immunoreactivity of Bcl-2 was observed in many of the hepatic cells in the rats supplemented with licorice a long with Cd which indicated the role of licorice in up regulation of Bcl-2 and minimize cellular death. Currently, there is no previous report on the effect of Cd /licorice on the immunohistochemical expression of Bcl-2. These findings in our study are similar to those of a study on sub-chronic lead and cadmium co-induce apoptosis protein expression in liver and kidney of rats where immunohistochemical tests showed increased numbers of positive cells under Bax and caspase-3 protein detection and decreased Bcl-2 protein expression Yuan *et al.* (2014).

CONCLUSION

Hepatic damages induced by Cd intoxication which manifested by histopathological and immunohistochemical changes associated with a significant changes in hepatic biochemical markers, were ameliorated by administration of licorice. To the best of our knowledge, there is no previous report on the effect of licorice/ Cd on Bcl-2 expression.

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العرقسوس وتحسين الآثار السامة المحدثة بكلوريد الكادميوم علي الكبد في الجرذان البيضاء

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يعد الكادميوم واحدا من الملوثات البيئة الشائعة، لما له من مضار مهنية وبيئية متنوعة، نظرا لزيادة الاهتمام باستخدام الاعشاب بديلا الدواء، واتسع نطاق العلاج فيما يعرف بالطب التقليدي، ومن بين هذه النباتات العرقسوس والمستخدمة بفاعلية كأحد مضادات الاكسدة. من هذا المنطلق كان الهدف من هذه الدراسة هو التحقق من الدور الوقائي للعرقسوس ضد سمية الكبد الناجمة عن التسمم بالكادميوم في صورة كلوريد الكادميوم. اجريت التجربة على ٤٠ جرذ ابيض، تم تقسيمها الى أربع مجموعات، بكل مجموعة عشرة جرذان:
١- المجموعة الاولى: استخدمت كمجموعة ضابطة. ٢- المجموعة الثانية: تم تجريعها بواسطة انبوبة اللى المعدي بالعرقسوس بجرعة ٣٠٠مجم/كجم وزن لمدة ٢١ يوم فقط. ٣- المجموعة الثالثة: تم حقنهم بجرعة ٣ مجم/كجم وزن بكلوريد الكادميوم ٥ مرات/اسبوع لمدة ثلاثة اسابيع في التجويف البطني. ٤- المجموعة الرابعة: تم تجريعها بواسطة انبوبة اللى المعدي بالعرقسوس بجرعة ٣٠٠مجم/كجم وزن لمدة ثلاث ايام قبل واثناء الحقن بكلوريد الكادميوم. وقد أظهرت النتائج زيادة معنوية في مستويات الانزيمات الكبدية مع انخفاض مستويات الزلال والبروتين في المجموعة المعاملة بالكادميوم مقارنة بالمجموعة الضابطة. المجموعة المعالجة بالعرقسوس مع الكادميوم انخفضت فيها مستويات الانزيمات المرتفعة مقارنة بالمجموعة المعاملة بالكادميوم. الفحص الهسوباثولوجي أظهر تغييرات نسيجية في كبد المجموعة المعاملة بالكادميوم مثل نخر في الخلايا وتغييرات تكيسية مع زيادة انتشار الخلايا وحيدة النواة. لوحظ أن حقن العرقسوس أدى الى تحسن هذه الظواهر. أظهرت نتائج التحليل الهستوكميائي قلة تعبيرات Bcl-2 في كبد المجموعة المعاملة بالكادميوم مقارنة بالمجموعة الضابطة، ومع حقن العرقسوس لوحظ عودة التعبيرات الى أعلي من معدلاتها. نستخلص من الدراسة أن العرقسوس يحمي ضد الاثار السامة للكادميوم علي نسيج الكبد.