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PROTECTIVE EFFECT OF LICORICE ON METIRAM FUNGICIDE INDUCED LIVER INJURY IN MICE

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ABSTRACT

Licorice (*Glycyrrhiza glabra*) is one of the oldest and most frequently used botanical treatments in many countries. The present study investigated the protective effect of licorice water extract against metiram fungicide induced liver toxicity in albino mice (*Mus musculus)*. Metiram is an ethylenebisdithiocarbamate fungicide used against a wide range of fungal diseases of field crops and fruits. Treating albino mice with metiram at a dose level of ½ LD_{50} (1240 mg/kg b.w.) for 10 days dissolved in distilled water induced various histological changes in the liver. These changes include congestion of blood vessels, leucocytic infiltration, cytoplasmic vacuolization of the hepatocytes and pyknosis. Metiram also caused an increase in PCNA expression in hepatocytes nuclei. The biochemical result revealed that there was significant elevation in serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Treating animals with licorice water extract for 10 days followed by metiram led to an improvement in the histological liver picture together with a decrease in the expression of PCNA in hepatocytes nuclei. Moreover, pretreatment with licorice reduced ALT and AST activity. The results of the present work proved that licorice extract revealed potent protective activity against metiram fungicide –induced hepatotoxicity in mice. It is suggested that this may be attributed to antioxidative action of one (e.g. glycyrrhizin) or more of licorice components.

Keywords: Metiram, licorice, liver, histology, PCNA.

INTRODUCTION

Herbal medicine is based on the premise that plants contain natural substances that can promote health and alleviate illness (Craig, 1999). Licorice (*Glycyrrhiza glabra*) is one of the oriental herbal medicines that have been most frequently prescribed for the treatment of various diseases (Wang and Nixon, 2001). Ancient Egyptian healers began using Licorice root (Glycyrrhiza) 4000 years ago and it has been continuously used by practitioners in both Eastern and Western medicine. Licorice root was utilized in Chinese medicine and it was recommended as a cure for injury, for swelling, for its detoxification effect, and for improving health and lengthening one's life span (Fukai *et al*., 2002). The root of licorice has been used for centuries as antidotes, demulcents, expectorants, and remedies for allergic inflammation, as well as flavoring and sweetening agents in Asia and Europe (Belinky *et al*., 1998). Licorice extracts were found to have a wide range of biological activities, including antimicrobial, antiatherosclerotic, antihepatitis, antinephritic, and cardiovascular protective activities (Fuhrman *et al*., 1997; Fukai *et al*., 2003; Kim *et al*., 2006). Fungicides are extensively used against a wide range of fungal diseases of many field crops fruits and ornamentals (Maloy, 1993). Disadvantages of fungicides include their toxicity to humans, animals, and useful plants, and the persistence (long life) of some of these chemicals in the environment. Moreover, these chemicals were shown to be present in fruits products prepared for human consumption (Cabras and Angioni, 2000). Metiram (polyram) is a non-systemically acting fungicide of dithiocarbamate group. Metiram was registered for use on food and ornamental crops to prevent crop damage in the field and to protect harvested crops from deterioration in storage or transport (Charls *et al*., 2000). The toxicity of metiram was studied by some investigators. Kornuta *et al*. (1996) reported that metiram is one of the pesticides which showed genotoxic effect. Dermal administration of metiram resulted in minimal to moderate exfoliation and ulcerative dermatitis in the skin of rabbits treated at the high-dose level (Ullmann *et al*., 1987). Sortwell *et al*. (1977) reported follicular hyperplasia in thyroid of female rhesus monkeys treated with metiram. The effect of the fungicides (maneb, metiram, and ziram) on human natural killer (NK) cells cytotoxic function was studied by Whalen *et al*. (2003). The results provide evidence of relative toxic potential for these compounds and the immunomodulatory effects on both T and NK lymphocyte function. The present work was aimed to study the possible role of licorice roots on histopathological as well as biochemical alterations induced by metiram fungicide in the liver of albino mice.

MATERIALS AND METHODS

Healthy adult male albino mice (*Mus musculus*) approximately three months old and weighting 20±5 g were used in this study. Animals were kept in the laboratory under constant condition of temperature 25◦C

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for at least one week before and throughout the experimental work. They are provided with a commercial rodent chow (Egyptian Company of Oils and Soaps, Egypt) and water was given *ad libitium*. Animals were divided into 4 groups. Animals of the first group (25mice) were orally given licorice extract (50mg/kg b.w.), daily for 10 days. Licorice (*Glycyrrhiza glabra*) extract was obtained from Albatros Apotheke pharmacy, as product of Caelo Company, Germany. This extract is yellow powder dissolved in distilled water and given orally to mice (Al-Qarawi *et al*., 2002). Animals of the second group (25 mice) were orally given ½ LD_{50} (1240 mg/kg b.w.) of metiram for 10 days dissolved in distilled water. It consists of 80% active ingredients [zinc ammoniate ethylenebis (dithiocarbamate)-poly (ethylenethiuram disulfide)] and 20% inert ingredients. Animals of the third group (30mice) were orally given licorice extract for 10 days followed by metiram for another 10 days. Animals of the fourth group (25 mice) were saved as normal control (untreated). The treated animals and their controls were killed by cervical dislocation after 1, 2 and 3 weeks, quickly dissected and small pieces of liver were fixed in 10% neutral buffered formalin. For histopathological examination, specimens were dehydrated, embedded in wax and 5 micrometers thick sections were stained with haematoxylin and counterstained with eosin.The degenerative liver regions showing necrosis and other histopathological alterations was calculated and expressed as %/mm^2 of hepatic parenchyma. For immuno-histochemical localization of proliferating cell nuclear antigen (PCNA), fixed wax sections were stained using the avidin-biotin peroxidase method (Hsu *et al*., 1981). To evaluate PCNA –labeling index, the percentage of number of stained cells were counted in 10 liver sections from each animal group. For enzymes determination, blood was collected by means of a heart puncture and serum was obtained by centrifugation. Serum was stored at -20Cº until assayed for the biochemical parameters. Serum alanine aminotransferase (ALT) and asparate aminotransferase (AST) were determined on the basis of Gella *et al*. (1985). The results were analyzed statistically using Student's "t" test.

RESULTS

Histological results

Examination of liver of control animals and those treated with licorice extract showed normal architecture. Examination of liver specimens treated with metiram fungicide for one week showed impaired structural organization of the hepatic lobules, the characteristic cord-like arrangement of the normal liver cells was lost. In addition, central and portal veins were enlarged, congested with blood and surrounded by leucocytic infiltration (Fig. 1). The histopathological changes of the liver were more pronounced after two weeks of treatment with metiram. In these specimens, the intrahepatic the central and portal veins were congested with blood. The sinusoidal spaces were widen and filled with activated kupffer cells and there were masses of leucocytic infiltrations (Fig. 2). After 3 weeks of metiram treatment, the inflammatory leucocytic infiltration was increased and cytoplasmic vacuolization appeared in most of the hepatocytes and their nuclei were pyknotic. In addition, severe necrosis around central veins was observed (Fig. 3). Examination of liver sections from mice treated with licorice followed by metiram for 1 and 2 weeks revealed somewhat normal hepatic strands with widen sinusoidal spaces but the portal and central veins were congested with blood (Fig. 4). Specimens examined after 3 weeks post-treatment showed few inflammatory leucocytic cells, mild cytoplasmic vacuolization, and activated kupffer cells (Fig. 5). The percentage of degenerative hepatic regions was 69% in animals treated with metiram for 3 weeks in comparison with 3% in controls and 2% licorice treated mice. In contrast, these regions decreased to 32% in animals given licorice and metiram (Fig. 6).

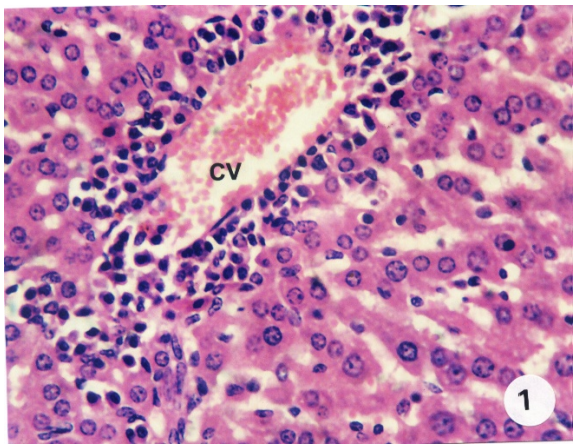

Fig. 1. Section of liver of a mouse treated with metiram for 1 week showing congested central vein (CV), (H&E., X400).

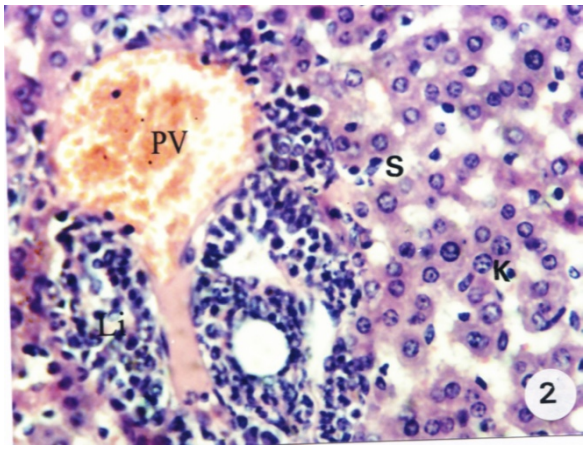

Fig. 2. Section in the liver of a mouse treated with metiram for 2 weeks showing congested portal vein (PV), widen sinusoidal spaces (S), activated kupffer cells (K) and mass of leucocytic infiltrations (Li), (H&E., X400).

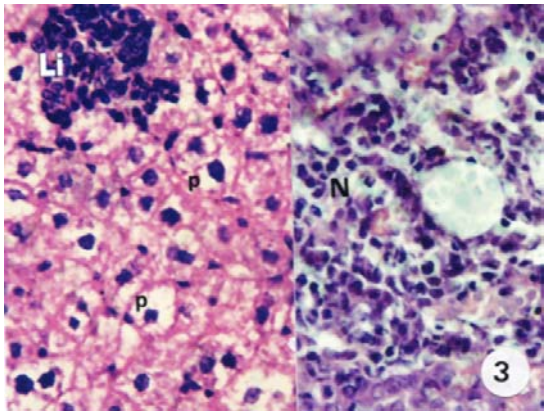

Fig. 3. Section of liver a mouse treated with metiram for 3 weeks showing cytoplasmic vacuolization of the hepatocytes with pyknotic nuclei (P), leucocytic infiltration (Li) and necrotic area (N), (H&E., X400).

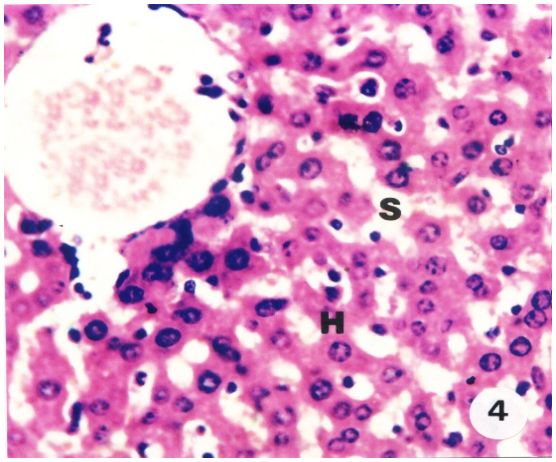

Fig. 4. Section of liver of a treated mouse with licorice and metiram for 2 weeks showing congested central vein, hepatic strands (H) and wide sinusoidal spaces (S) (H&E., X400).

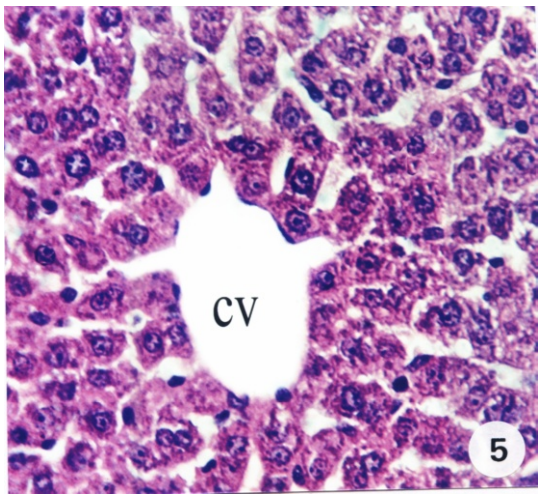

Fig. 5. Section of liver of a mouse treated with licorice and metiram for 3 weeks showing improvement in hepatic tissue, (H&E., X400).

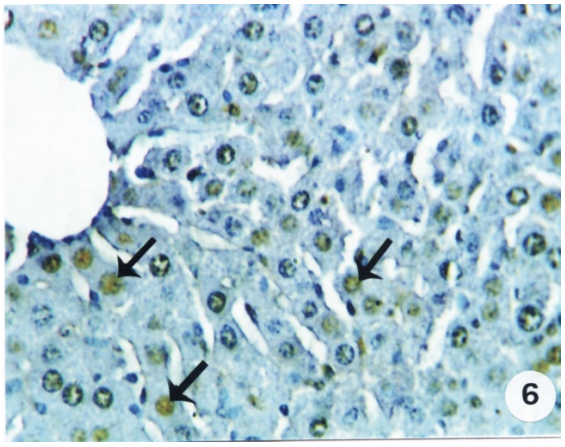

Fig. 6. Section of liver of a mouse treated with metiram for 3 weeks showing strong staining of PCNA in most of hepatocytes, (arrows), (PCNA immunohistochemical stain., X400).

Immunohistochemical results

Some of hepatocytes displayed faint stain of PCNA in controls and in animals treated with licorice. Animals treated with metiram for 1 and 2 weeks showing stimulation of DNA synthesis and increased PCNA expression in most of hepatocytes when compared with control animals. After 3 weeks the expression of PCNA in all hepatocytes was strong (Fig. 6). Treating Animals with licorice and metiram for 1 and 2 weeks showing moderate PCNA expression in some of hepatocytes .At the 3rd week the expression of PCNA in hepatocytes nuclei decreased (Fig. 7). PCNA –labeling index in different groups after 3 weeks is presented in figure 9. This index is significantly higher ($P<0.05$) in animals given metiram compared with those treated with licorice and metiram.

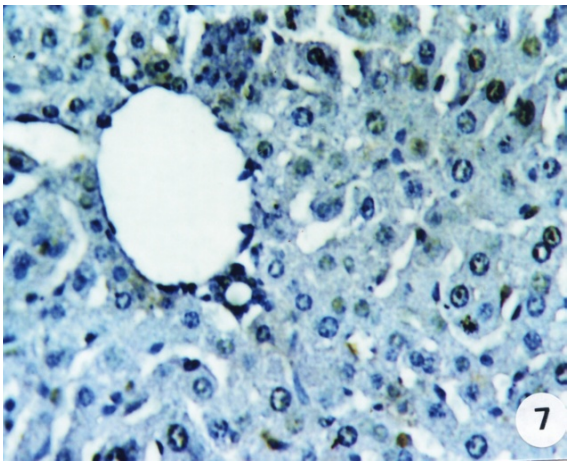

Fig. 7. Section of liver of a mouse treated with licorice and metiram for 3 weeks showing few cells have PCNA expression, (PCNA immunohistochemical stain., X400).

Biochemical results

As shown in table 1, animals treated with metiram showed significant increase in serum AST activity in all

periods of treatment. The group of animals treated with licorice and metiram showed significant decrease in AST activity in comparison with those given metiram alone. Similarly, animals treated with metiram showed significant increase in ALT activity (Table 2) while those treated with licorice followed by metiram had a noticeable decrease in ALT activity in compare with metiram treated animals.

DISCUSSION

Results obtained in the present study revealed that treating mice with metiram induced many histopathological alterations in mice liver. These alterations include leucocytic infiltrations, congestion of blood vessels, cytoplasmic vacuolization of hepatocytes and necrosis. Such lesions were previously observed in the liver of animals exposed to different types of fungicides (Deveci *et al*., 1997). Dithiocarbamates (DTCs) fungicides (e.g. metiram) have toxic effects on liver, kidney and testis (Szepvolgyi *et al*., 1989). Özbay *et al*. (1991) studied the pathological changes in male and female mice given the fungicides, maneb and zineb. Internal organs (liver and kidneys) of the experimental mice were found to be heavier and darker in color than those of the controls. Vein congestion and mononuclear inflammatory cell infiltrations were observed in these organs. Thompson *et al*. (2002) reported that metam sodium fungicide can damage the liver. A single oral dose of metam sodium caused liver injury and inflammation. Sakr (2007) reported that treating albino rats with mancozeb fungicide induce various histological changes in the liver. These changes include congestion of blood vessels, leucocytic infiltration, cytoplasmic vacuolization of the hepatocytes and pyknosis. Mancozeb also caused significant elevation in serum ALT and AST enzymes.

The immunohistochemical results indicated that treatment with metiram fungicide stimulates DNA synthesis and increased PCNA expression in liver hepatocytes. Similarly, Mangipudy *et al*. (1995) reported that proliferating cell nuclear antigen (PCNA) elevated in hepatocytes of male Sprague-Dawley rats injected intraperitioneally with a 12-fold dose range of thioacetamide fungicide. Waterson (1994) observed that after initiation of treatment with dose of folpet fungicide, more animals receiving folpet showing a greater degree of PCNA staining in the duodenum than control. The effects of captafol fungicide and expression of proliferating cell nuclear antigen (PCNA) in the rat's kidney were investigated by (Kim *et al*., 1997). The PCNA-labelling indices of renal tubule cells were elevated in rats treated with captafol.

In the present work, the biochemical analysis indicated that treatment with metiram induced a significant increase in activity of serum ALT and AST. This result is in agreement with that of Reena-Kackar *et al*. (1999) who reported that oral administration of mancozeb to male rats induced changes in the activities of ALT and AST throughout the period of the study in a dose- dependent manner. Lavric *et al*. (1990) reported that the fungicide bithionol sulfoxide at high doses (50,500 and 1000 mg/kg) caused hepatotoxicity including an increase in

Table 1. Effect of metiram and licorice on serum aspartate aminotransferase (AST) (U/L).

Treatment period	Control group	Licorice	Metiram	Licorice and Metiram
	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD
1st week	32.4±1.64	31.2±3.4	77.4±9.2*	37.0±6.3
2nd week	32.4±1.66	30.2±3.1	80.4±9.4*	35.4±3.6
3rd week	32.5±1.69	30.0±3.0	67.4±8.6*	45.0±5.0

n= 5 animals for each group.
*: Significant increase ($p<0.05$) in comparison with control animals.

Table 2. Effect of metiram and licorice on serum alanine aminotransferase (ALT) (U/L).

Treatment period	Control group	Licorice	Metiram	Licorice and Metiram
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
1st week	37.8±1.89	36.6±1.8	100.4±7.6*	41.0±2.3
2nd week	37.8±1.91	37.6±2.3	105.2±8.4*	44.8±5.5
3rd week	37.8±1.9	34.8±3.8	113.6±8.3*	68.0±11.5

n= 5 animals for each group
*: Significant increase ($p<0.05$) in comparison with control animals.

serum AST. Sakr and Lamfon (2005) observed that metalaxyl fungicide induced many histological changes in liver of mice and increased liver enzymes (transaminases). Many authors had elucidated that hepatocellular damage could be correlated with the disturbance in enzyme activities. Martin *et al*. (1983) announced that hepatic tissues lose their enzymes (e.g., transaminases) in case of liver damage. This ultimately leads to their raised levels in the sera of those animals. Hence they suggested that the higher value of these enzymes, whenever they are detected in the blood sera, should be taken as an indicator of various causes of liver damage. Metiram was found to affect liver enzymes. This result together with the histopathological observations indicated that metiram caused liver injury in mice.

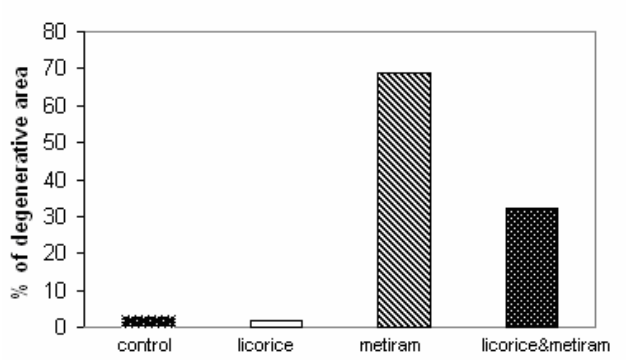

Fig. 8. Percentage of degenerative area in experimental groups.

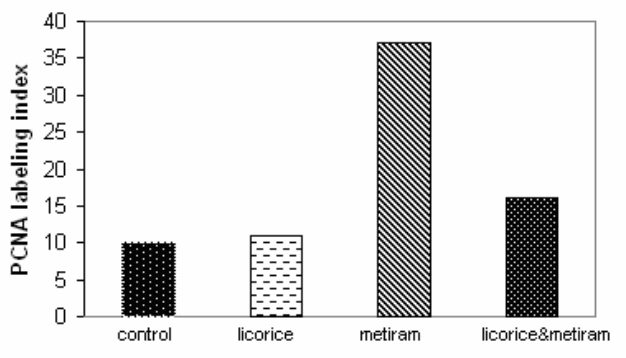

Fig. 9. PCNA labeling index in hepatocytes of experimental groups.

Animals treated with both licorice and metiram revealed an improvement in histopathological and biochemical alterations when compared with animals given metiram alone. This proved the effectiveness of licorice in prevention of metiram hepatotoxicity. Licorice contains flavonoids and pentacyclic triterpene saponins including liquiritigenin, liquiritin, isoliquiritigenin, liquiritin apioside and glycyrrhizin (Kamei *et al*., 2003). Some of these components have been reported to exhibit hepatoprotective activities. Nose *et al*. (1994) reported that glycyrrhizin, a major component of licorice, is a well-known hepatoprotective compound against CCl_4-induced liver injury in rats ß-Glycyrrhetinic acid, the aglycone found in glycyrrhizin, is also a potent hepatoprotective compound in CCl_4-induced hepatotoxicity (Jeong *et al*., 2002). Pretreatment with 18ß-Glycyrrhetinic acid reduced ALT and AST in serum, and also reduced hepatic lipid peroxidation caused by CCl_4. Kim *et al*. (2006) reported that Liquiritigenin pretreatment significantly reduced ALT and LDH activities induced by acetaminophen and also reduced liver necrosis in rats. Liquiritigenin, an aglycone of liquiritin, shows cytoprotective effects against cadmium-induced toxicity in a rat-derived hepatocyte cell line (Kim *et al*., 2004). Yoshida *et al*. (2007) investigated the effects of glycyrrhizin isolated from licorice root on acute hepatitis induced by lipopolysaccharide and d-galactosamine in mice. Serum ALT activity and levels of cytokines such as TNF, IL-6, IL-10 and IL-12 reached a maximum after treatment with LPS/GalN. Increases in ALT levels were reduced by an administration of glycyrrhizin. However, glycyrrhizin had no effect on the production of TNF-alpha, IL-6, IL-10 and IL-12, whereas it significantly inhibited IL-18 production. Abe *et al*. (2008) reported that treatment with glycyrrhizin suppressed increases in serum levels of ALT and AST in mice treated with LPS/GalN. Furthermore, glycyrrhizin inhibited levels of both mRNA and protein for MMP-9.

The effects of glycyrrhizin on liver injury caused by ischemia-reperfusion in rats were determined by Nagai *et al*. (1991). In the liver ischemia-reperfusion model, levels of serum AST, ALT and LDH, lipid peroxides in the liver tissue, and blood superoxide dismutase activity were significantly increased. Administration of glycyrrhizin suppressed the elevation of these enzymes. Tang *et al*. (2007) reported that glycyrrhizin, pre-administered before hepatectomy, prolonged the survival of Male Wistar rats submitted to partial hepatectomy and lipopolysaccharide (LPS) injection, compared with saline controls and also up-regulated the expression of proliferating cell nuclear antigen, implying that it might be able to promote regeneration of livers harmed by LPS**)**. When rats were pretreated with either glycyrrhizin and 18beta-glycyrrhetinic acid for three consecutive days prior to retrorsine exposure, the elevated serum GOT and GPT levels induced by retrorsine were significantly reduced and no extensive hepatocellular damages were observed (Lin *et al*., 1999). Lee *et al*. (2007) observed that ALT, AST and LDH increased in serum of rats after i.v. injection of cadmium. In contrast, pretreatment with licorice water extract reduced the levels of these enzymes and decrease the necrosis and degenerative hepatic regions.

It was reported that the oxidative stress is the principle manifestations of fungicides-induced toxicity (Kaloyanova *et al*., 1991) and there are evidences suggesting that fungicides-induced liver damage is closely

associated with increase in lipid peroxidation and the decrease in the antioxidant enzymes (Calviello *et al*., 2006). In this concern, Sakr *et al*. (2007) recorded a significant increase in malondialdhyde which is lipid peroxidation marker and a significant decrease in the level of serum antioxidant enzyme, superoxide dismutase activity in mancozeb fungicide-treated rats. Recent studies showed that dietary antioxidants and some plant extracts can attenuate hepatotoxicity induced by different toxicants. Licorice water extract contains high levels of glycyrrhizin, liquiritin, liquiritin apioside, liquiritigenin, isoliquiriti and isoliquiritin apioside (Kamei *et al*., 2003). These components exhibit antioxidative (Rahman *et al*., 2006) superoxidative scavenging (Nagai *et al*., 1991) and anticarcinogenic activity (Lee *et al*., 2007). The present study proved that licorice extract revealed potent protective activity against metiram fungicide –induced hepatotoxicity in mice. It is suggested that this may be attributed to antioxidative action of one (e.g. glycyrrhizin) or more of licorice components.

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