

EVALUATION OF HEPATOPROTECTIVE EFFECT OF PROPOLIS AGAINST SUB-CHRONIC DIAZINON INDUCED HEPATOTOXICITY

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ABSTRACT

Nowadays, the public health faces a major problem of increased pesticides use with appearing of various side effects. One of the most commonly used is diazinon. It exerts its effects on various tissues and cells including hepatocytes. This work aimed to study the tendency of diazinon to cause hepatic affection in rats and study the possible propolis protective role. We used 30 rats, divided into 4 groups; group Ia (-ve control); group Ib (+ve control, corn oil group); group II (propolis group; 400mg/kg/day orally); group III (diazinon 20 mg/kg/day orally); group IV (diazinon 20mg/kg/day + propolis 400mg/kg/day orally) for 8 weeks. We studied biochemical parameters: serum aminotransferases (ALT and AST), tissue oxidative stress markers (hepatic MDA, hepatic catalase and hepatic GPx activities). Liver will be examined by light microscope to evaluate histopathological changes and immunohistochemical reaction to caspase 3 to predict apoptosis. Results indicated: Significant increase in the serum levels of the ALT and AST as well as hepatic MDA of diazinon treated rats compared to control group, whereas both hepatic catalase and GPx activities were significantly decreased these results had significantly improved in both propolis and diazinon group. Light microscopic examination revealed disruption in hepatic histoarchitecture in diazinon group unlike that in group III which returned near normal; while immunohistochemical staining showed many positive reacted cells to active caspase 3 in diazinon group which became few in group III near to control. In conclusion, this study demonstrated that diazinon may have hepatotoxic effects which can be protected by co-administration of propolis in adult rats. Further studies about diazinon effect on liver and about propolis role in hepatoprotection are recommended.

Key words: Diazinon, liver, oxidative stress, propolis.

INTRODUCTION

Environmental pollution regarding pesticide residues is a main concern as a result of their wide agricultural use. Organophosphorus insecticides (OPs) are one of the main classes of pesticides commonly used. **Galloway and Handy, 2003** had revealed residuals of organophosphates in food stuffs as fruits, vegetables, grains and others as well as water and soil.

Diazinon (DZN) is one of the excessively utilized organophosphates agriculturally. Diazinon affects various organs ; such as liver, hematologic system, immune system, nervous system and kidney through cellular imbalance between over production of free radical and depleted tissue antioxidant mechanism (**Galloway and Handy, 2003 and Yilmaz et al., 2012**). Previous studies had reported that DZN might cause hepatotoxicity (**Shadnia et al., 2007**). One of the potential mechanisms of DZN induced hepatic affection was the overproduction of reactive oxygen species (ROS) (**Teimouri et al., 2006**).

Priyadarsini and Nagini, 2012 defined oxidative stress as imbalance between the compensatory reaction of endogenous anti-oxidants with increased production of reactive oxygen species.

Bee glue, Propolis, had been much familiar in the world for human health in traditional medicine and food supplementation (**Pereira et al., 2008**). It was believed that it could cure diabetes, heart diseases and even cancer. Several biological properties of propolis including antioxidant, antibiotic, anti-inflammatory and tumor cell arrest had also been reported (**Moreno et al., 2000**).

Kurek-G'orecka et al., 2014 and Tapas et al., 2008 explained that the principal antioxidant mechanism of propolis polyphenols can be summarized in the significant ability of "scavenger" reactive oxygen species and radical reactive nitrogen species so that reduction of xanthine oxidase reaction; chelate ion metals implicated in the sequence of free radical production , interrupt the reactions cascade of the lipids peroxidation and synergistic action added to different antioxidants.

So this work aimed to study the tendency of diazinon to cause hepatic affection in rats and study the possible propolis protective role

MATERIAL& METHODS

*** Material**

[A] Chemicals

1. Diazinon:

DZN (99% purity, CAS 333-41-5), soluble in petroleum or corn oil. It is colorless oil with very offensive odor. It was purchased from Sigma -aldrich Company.

2. Propolis: Propolis was produced by Sigma Pharmaceutical Industries (Cairo, Egypt) for International Business Establishment Co. (IBE pharma). It was existed in capsules of Biopropolis, each capsule contained 400 mg.

[B] Animals:

The study was done on 30 healthy adult male albino rats weighing 150-200g. Rats were got from the animal house of Zagazig Faculty of Medicine. We gave the rats balanced food consisting of milk, barley and bread, this food should be rich in all substances necessary to preserve their health before and during administration of the treatment. We gave them water

in a different clean utensils. The experimental work was done according to the basic instructions advised through the Institutional Research Board for the use and care of experimental animals.

[C]Experimental Design:

Rats were randomly divided into four groups: **Group I a** (negative control group): No intervention was done to rats in this group for adjusting the basic parameters, and allowed drinking water *ad libitum*. **Group I b** (positive control group): Rats received 1ml corn oil orally for 8 weeks. **Group II** (Propolis group): Rats received propolis (400mg/kg/day) dissolved in corn oil orally for 8 weeks (**Rahvar et al., 2018**). **Group III** (diazinon treated group): The group was Orally received diazinon dissolved in corn oil. A daily dose of 20 mg/kg body weight which equals 1/20 of DZN LD50 (**Gokcimen et al., 2007**). **Group IV** (diazinon and Propolis): The group was administered diazinon (20 mg/kg) and 400mg /kg Propolis orally once daily for 8 weeks.

* Methods:

Twenty- four hours after the end of experimental duration, the rats were subjected to sampling of blood and liver tissue as the follows:

- Collection of blood samples;

Under light ether anesthesia, venous samples from the retro-orbital plexus were obtained by capillary glass tubes as described by **Schemere (1967)**, to measure:

Alanine- aminotransferase (ALT) and Aspartate-aminotransferase (AST) Assay. The estimation was performed according to the method primarily introduced by **Bergmeyer et al., 1978 and Saris, 1978**.

- Tissue parameters:

Liver was immediately dissected out and grossly inspected to assess any gross abnormalities then washed with cold normal saline and used for:

* Estimation of catalase activity following the method of **Luck (1974)**, and glutathione peroxidase activity in liver tissue following **Paglia and Valentine (1967)**.

* Determination of the level of malondialdehyde in liver using the demonstrated method by **Jain et al. (1989)**.

* Light microscope examination:

Fixation of the liver in 10% formalin saline then the tissues were firmed in blocks of paraffin and 5 μ thickness sections were prepared then subjected for Hematoxylin and Eosin stains (**Horobin and Bancroft, 1998**) and then examined by light microscope.

* Immuno-histochemistry for caspase 3:

According to **Ramos-Vara (2005)** the site where antigen was present was clearly revealed by brown colouration in the cytoplasm. Collected results were tabulated and statistically analyzed using appropriate methods by Statistical Package of Social Science (SPSS), software version 22.0 (**SPSS Inc., 2013**). ANOVA with least significant difference Post hook test were used to find difference between the studied groups. Statistically significance was considered when *P* value was <0.05. Histological studies were presented as photomicrographs and scientifically interpreted.

RESULTS

Biochemical results:

The results of this study had been appropriately summarized, tabulated and subjected to statistical analysis before completion and graphic illustration. ANOVA test was used in

this study to compare between means of different groups.

* As regard the groups; group Ia (-ve control), group Ib (+ve control) & group II (Propolis): There were a non significant differences ($p > 0.05$) between groups all over the the study period using test of ANOVA (Table 1) as regard:

1- Serum ALT & AST
2- Catalase & GPx activity in hepatic tissues.

3- MDA in hepatic tissues.

So the negative control group was selected to be compared with the results of the treated groups; group III (diazinon) and group IV (diazinon + Propolis).

Table 1: Statistical comparison among groups (Ia & Ib & II) as regard serum ALT&AST, hepatic catalase, GPx activity and hepatic MDA along the period of the study by ANOVA test:

Group Parameter	-ve control group(Ia) Mean \pm SD	+ve control group(Ib) Mean \pm SD	Propolis group (II) Mean \pm SD	F	P
Serum AST (mg/dl)	14.13 \pm 3.49	14.15 \pm 3.28	13.9 \pm 2.88	0.011	> 0.05
Serum ALT (mg/dl)	25.3 \pm 3.40	24.9 \pm 3.14	24.1 \pm 3.01	0.220	> 0.05
Gpx (U/g protein)	28 \pm 4.62	27.84 \pm 4.53	28.36 \pm 5.43	0.018	> 0.05
Catalase (U/g protein)	66.31 \pm 4.35	67.01 \pm 3.98	68.44 \pm 8.28	0.205	> 0.05
MDA (n mol/mg protein)	98.35 \pm 8.99	99.07 \pm 9.4	99.95 \pm 10.33	0.042	> 0.05

N.B: Number of rats = 6 rats for each group. SD = Standard Deviation. $p > 0.05$ = non significant

* As regard the treated groups; group III (diazinon) & group IV (diazinon + Propolis):

The result of this study showed significant difference between these groups in all parameters when compared with -ve control group. Using LSD it was found that there was significant increase of serum ALT & AST levels and MDA in hepatic tissue with significant decrease of catalase

and GPx activity in hepatic tissue in group III (diazinon) when compared with group Ia (-ve control). Also these differences was found between group III (diazinon) and group IV (diazinon + Propolis).No difference was found between group I (control) and group IV (diazinon + Propolis).So intake of Propolis with diazinon can significantly prevent these changes (table 2).

Table (2): Statistical comparison among the negative control (Ia), diazinon (III) and diazinon and propolis (IV) groups as regard ALT & AST, hepatic GPx, and catalase enzymatic activity and MDA levels in hepatic tissues by ANOVA test.

Group Parameter	-ve control (group Ia) Mean ± SD	Diazinon (Group III) Mean ± SD	Diazinon+Propolis (Group IV) Mean ± SD	F	P
Serum AST (mg/dl)	14.13±3.49 ^a	95.29±3.47 ^b	15±4.13 ^a	947.300	<0.001**
Serum ALT (mg/dl)	25.3±3.40 ^a	105.77±17.44 ^b	24.8±4.71 ^a	115.702	<0.001**
GPx (U/g protein)	28±4.62 ^a	10.62±1.6 ^b	29.14±5.77 ^a	33.901	<0.001**
Catalase (U/g protein)	66.31±4.35 ^a	26.70±4.48 ^b	64±6.72 ^a	105.723	<0.001**
MDA (nmol/mg protein)	98.35±8.99 ^a	402.34±4.97 ^b	93.38±5.18 ^a	4,258.846	<0.001**

N: Number of sacrificed rats for every group was 6 rats. **SD :** Standard Deviation.

****:** Highly-significant (P<0.01). **LSD:** Groups with different letters are statistically highly different (P<0.01).

II)-Histopathological changes:

Light microscopic examination of sections from the liver of groups I & II showed the same structures. They showed normal architecture of classic hepatic lobule with arranged bands of hepatocytes ramifying from the central vein to the periphery of the lobules which contain the portal tracts. The periportal and pericentral hepatocytes were polyhedral containing central vesicular nucleus surrounded with acidophilic cytoplasm. They were separated by narrow blood sinusoids (**fig., 1**).

While the liver of rats treated with diazinon showed venous congestion, many hepatocytes with vacuolated cytoplasm and deeply stained shrunken nuclei, dilated sinusoids and areas of cellular infiltrates (**fig. 2**), these changes are less in group IV (**fig., 3**).

Immunohistochemical staining for caspase 3 of liver in groups I & II showed little positive reaction (brown

color) of few hepatocytes for activated caspase 3 (**fig., 4**).

In diazinon treated group, there was extensive positive reaction to caspase 3 (**fig., 5**). Which became less in group IV (**fig., 6**).

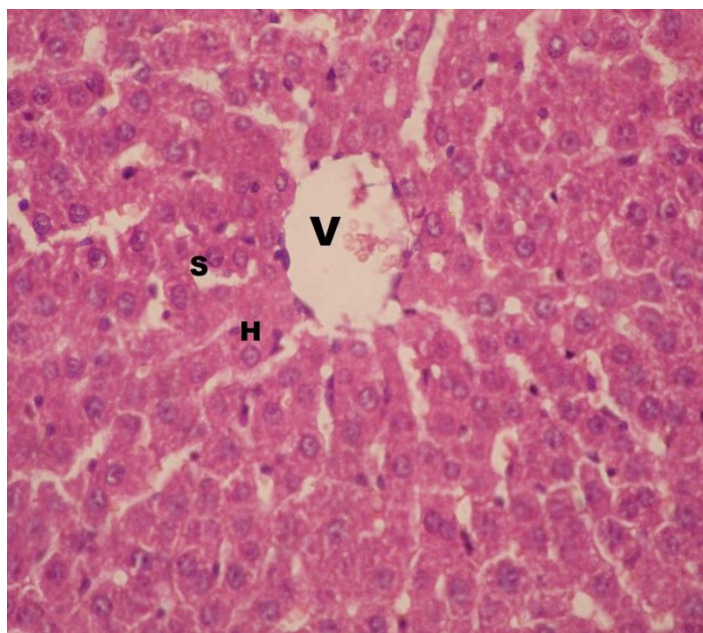


Figure (1): A photomicrograph of a section from the liver of a control group showing hepatocytes (H) arranged in plates radiating from the central vein (V) and separated by blood sinusoids (S); hepatocytes are polygonal in shape, with central rounded vesicular nuclei and acidophilic cytoplasm. (H&E X 400)

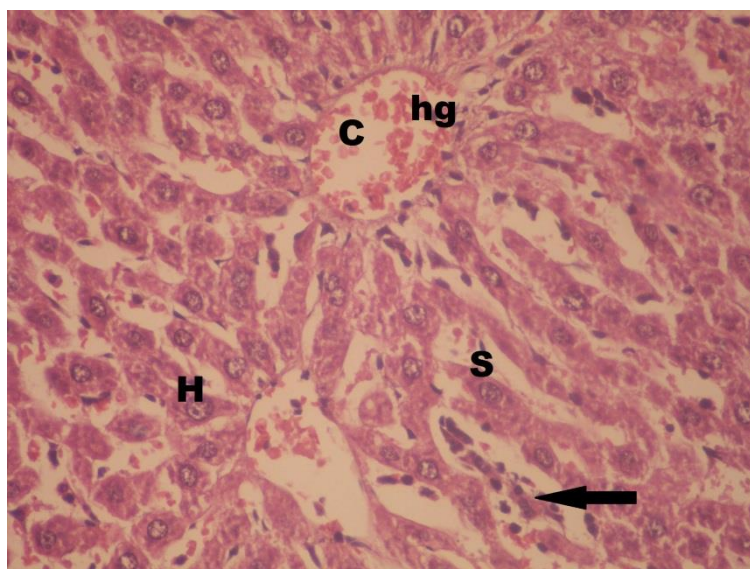


Figure (2): A photomicrograph of a section of liver of diazinon treated group showing congested (hg) central vein (C), dilated sinusoids (S), deeply stained shrunken nuclei of hepatocytes (H) and cellular infiltrations (arrow). (H&E X 400)

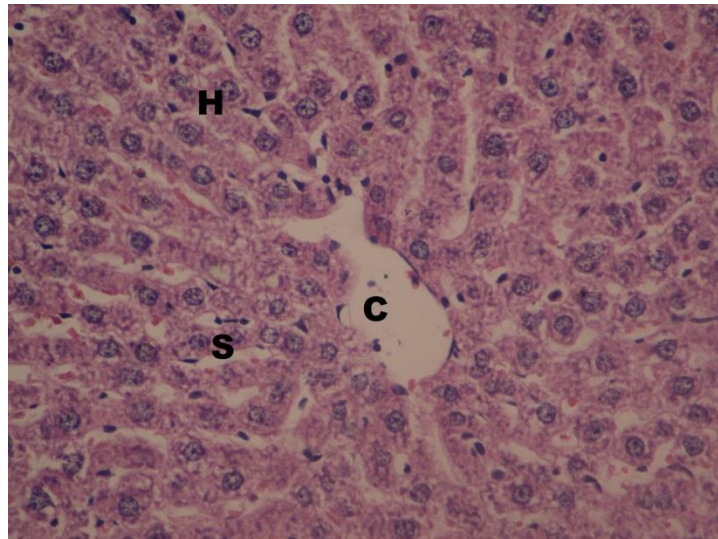


Figure (3): A photomicrograph of a section of liver of group IV (diazinon and propolis) treated group showing central vein (C), less dilated sinusoids (S) and hepatocytes (H) (H&E X 400).

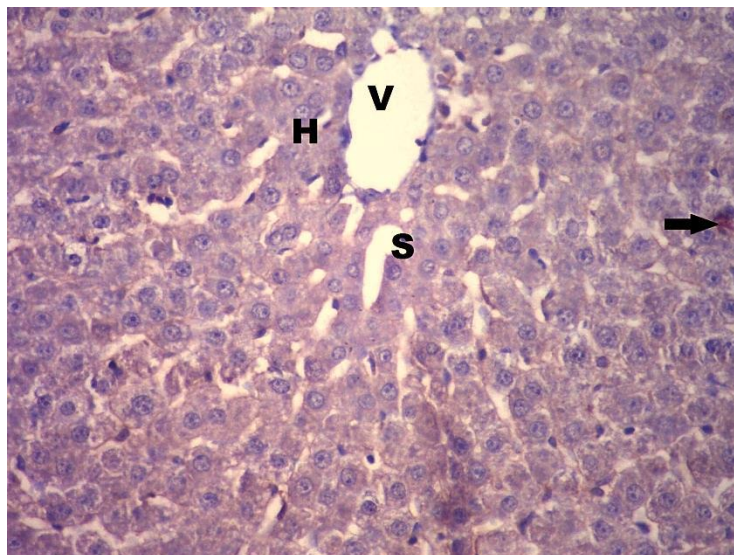


Figure (4): photomicrograph of a section from the liver of a control group showing few positive reaction for activated caspase 3 in few hepatocytes (arrow) (Caspase 3 immunostaining X 400).

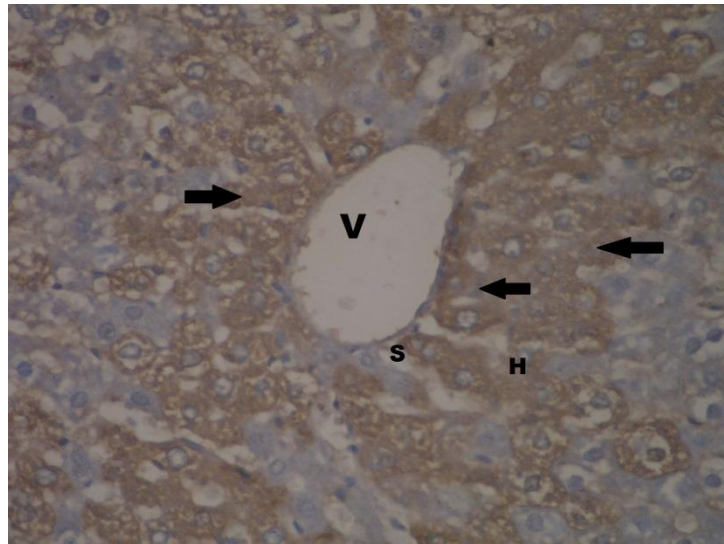


Figure (5): A photomicrograph of liver from diazinon treated group showing many positive reacted cells for activated caspase 3 (arrows) in cytoplasm of hepatocytes (H) (Caspase 3 immunostaining X 400).

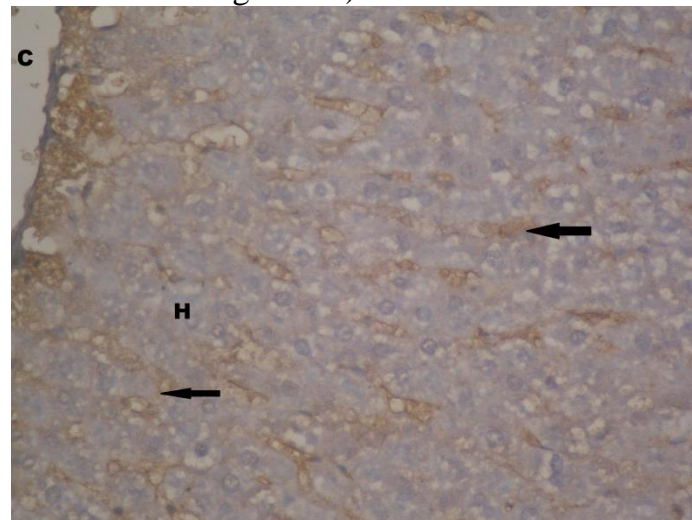


Figure (6): photomicrograph of a section from the liver of diazinon and propolis group showing less positive reaction for activated caspase 3 in few hepatocytes (arrows) (Caspase 3 immunostaining X 400).

DISCUSSION

Agricultural extensive use of pesticides and its resulting residuals is considered a universal bother. One of the main classes of pesticides used is organophosphorus compounds which were detected in food, soil and drinking water as residuals (Costa, 2006).

Diazinon is considered one of the globally used organophosphates. Impact of low levels chronic exposure to DZN on human health was reported in only few studies. Hepatotoxicity had

been reported to be induced by DZN. It was reported that DZN could elevate liver transaminases; AST & ALT and alkaline phosphatase. Also; it was studied that DZN could induce dose-dependent liver affection both biochemically and histopathologically (Azmi et al., 2006; Gokcimen et al., 2007 and Kalender et al., 2005).

In the current study, a high significant elevation in AST and ALT mean values in DZN administered group was detected as opposite to their

corresponding ones in control group. A significant decrease in these enzymes mean values was observed after administration of propolis with DZN when compared with the corresponding results in DZN administered group.

Alfahdawi, 2011 considered that aminotransferases are most useful in hepatocellular disease diagnosis and monitoring.

Srivastava and Raizada, 1999 explained that the increase in these enzyme activities reflected active liver destruction. While **Gaskill et al. (2005)** declared that, ALT release from the cytoplasm of hepatic cells could have occurred secondary to cellular necrosis.

In agreement with these results **Sarhan and Al-Sahhaf (2011)** reported that, AST and ALT showed a significant increase in serum of rabbits that received diazinon 20 mg/kg for 4 weeks.

In the existing study, we estimated hepatotoxicity resulting from chronic DZN exposure in rat and its possible mechanism as lipid peroxidation and apoptosis and the potential effect of propolis in its protection.

The present study also showed a significant rise in hepatic MDA in DZN treated group when correlated with control group while a significant decrease in its mean value was observed after administration of propolis with DZN when compared with the corresponding results in DZN administered group.

Also, the present study reported a significant decrease in hepatic GPx and catalase activities with significant increase in MDA in DZN treated group when compared with control group. Administration of propolis with DZN caused a significant improvement in

these mean values as compared to their corresponding values in DZN administered group.

These results were matched with **Caughlan et al., 2004; Kaur et al., 2007; Li, 2007; Oral et al., 2006; Rush et al., 2010 and Tenn and Wang, 2007** who supposed that the elevation of reactive oxygen species level was the core mechanism of chronic organophosphates toxicity.

MDA was deemed to be one of the indicators of oxidative stress occurrence or lipid peroxidation (**Nair et al., 2008**). Many researchs had reported peroxidative effects and lipid peroxidation of different organophosphate compounds (**Bhatti et al., 2010; El-Shenawy, 2010; Ghafour-Rashidi et al., 2007 and Spodniewska and Zasadowski, 2008**).

El-Shenawy et al., 2010 studied that coadministration of vitamin E had alleviated the oxidative stress of DZN after 14 days diazinon exposure of 32.5 mg/kg that caused elevated oxidative stress biomarkers and diminished the antioxidant defenses like catalase, glutathione and superoxide dismutase activities.

Also; **Mehta et al., 2009** studied that the organophosphate insecticide; chlorpyrifos created abnormal changes in the rat liver levels of nitrate, hydrogen peroxide, and nitrite.

In agreement with the previous; **Jasprica et al. (2007) and Newairy et al., (2009)** explained that propolis caused significant decrease in TBARS levels which are indicators of lipid peroxidation and increase in CAT, GPx, and SOD activities.

On the other hand, the above mentioned biochemical results obtained in the present study had been supported by findings in light microscopic

histopathologic examination and immunohistochemical staining of liver sections.

The histological results showed that DZN induced several histopathological alterations in the liver. Many hepatic lobules showed dilatation of the sinusoidal spaces, inflammatory cellular infiltration, congested veins, shrunken hepatocytes with darkly stained nuclei.

These findings were in agree with **Sarhan and Al-Sahhaf (2011)** who reported leucocytic infiltrations, congestion of veins, cytoplasmic vacuolation of the hepatocytes and fatty degeneration in liver samples of rats received DZN.

In the present work, adding propolis with DZN to rats showed improvement in the form of preserved normal hepatic lobular architecture and uncongested veins. Less dilated sinusoidal spaces and few cellular infiltration were observed.

Also, we studied the activated form of caspases-3. Our study showed that DZN induced apoptosis in hepatocytes of the rats treated with DZN through activation of caspases -3.

Caspases are deemed to be the key moderator of apoptosis. Caspase-3 is known to be the a final main implementer of apoptosis. It is responsible for the splitting of the key cellular proteins, leading to the known morphological modulations observed in apoptotic cells (**Budihardjo et al., 1999 and Fischer et al., 2003**).

Many studies had shown the apoptotic effects of variable organophosphate compounds like **Caughlan et al. (2004)** who determined that the chlorphyrifos organophosphorus compound had

induced apoptosis in rat cortical neurons.

Kaur et al. (2007) showed that long term exposure to the organophosphorus compound; dichlorvos even in low levels resulted in neuronal apoptosis with mitochondrial functional impairment.

Furthermore, the activation of caspase-3 was alleviated in DZN and propolis group significantly to a near level of control group.

Antecedent studies had demonstrated the action of propolis as anti-apoptosis. This is because its high content of flavonoids that had been shown to be able to scavenge free radicals and so protect the cell membrane from lipid peroxidation (**Pinchuk and Lichtenberg, 2002**).

According to **El-Masry et al., 2011** propolis contains twelve different flavonoids including rutin, luteolin, naringenin, quercetin and others; in addition to phenolic acids, caffeic and cinnamic acid. In addition to that, **Haro et al., 2002** reported that propolis was considered an eminent provenance of substantial trace elements, like iron, magnesium, copper and others which might provide optimum trace elements responsible for antioxidant enzymes reactivation.

Kanbura et al., 2009 assumed that the first mechanism of propolis effect might need the free radicals scavenging that motivate lipid peroxidation. The second mechanism might inhibit xanthine oxidase, which is known to cause generation of free radicals.

Added to the above, **Nirala et al., 2008** reported that propolis was fit to protect the liver against paracetamol hepatotoxicity in mice and **Omar et al. (2016)** also found that propolis had induced a reduction in the elevated

serum ALT and AST activity in doxorubicin treated rats.

On the other hand, **Yousef et al., (2003a& b)** explained that administration of constituent of propolis; isoflavones had minimized cypermethrin toxicities and this might be due to the isoflavone antioxidant properties. Moreover, the results of **Yousef et al., (2004a& b)** studied that isoflavone doses of 2.5 or 5 mg/kg, had beneficial effect on plasma lipid and lipoprotein concentrations, and antioxidant activities in rabbits.

The results of the current study also go parallel with **Eraslan et al., (2008)** who reported the antioxidant protective action of propolis against cypermethrin treated rats.

CONCLUSION

Taking the previous findings together, it can be concluded that hepatic apoptosis through oxidative stress pathway can be induced by sub-chronic DZN exposure which can be alleviated by propolis coadministration.

RECOMMENDATIONS

Further studies about diazinon effect on liver and about propolis role in hepato-protection are recommended.

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الملخص العربي:

تقييم الدور الوقائي لصمغ العسل ضد التسمم الكبدي الناتج من التعرض دون المزمّن لمادة الديازينون

يعتبر تقييم التأثيرات السمية الخاصة بمبيدات الآفات مثل الديازينون مصدر قلق كبير للصحة العامة حيث زاد استخدامه و يلعب دوراً هاماً في مجال الزراعة. ويؤثر الديازينون تأثيراً ساماً على عدد كبير من الأنسجة والخلايا المتنوعة بما في ذلك خلايا الكبد. كان الهدف من هذا العمل هو دراسة ميل الديازينون إلى التسبب في اضطراب وظائف الكبد في الجرذان والدور الوقائي المحتمل لصمغ العسل. تم تقسيم عدد 30 من الفئران إلى 4 مجموعات. المجموعة الأولى a (ضابطة سالبة)، المجموعة الأولى b (ضابطة موجبة) المجموعة الثانية (مجموعة صمغ العسل؛ 400 مجم / كجم / يوم عن طريق الفم)؛ المجموعة الثالثة (دايزنون 20 مجم \ كجم \ اليوم عن طريق الفم)؛ المجموعة الرابعة (دايزنون 20 مجم \ كجم \ اليوم مع صمغ العسل 400 مجم \ كجم \ اليوم عن طريق الفم) لمدة 8 أسابيع. تمت دراسة العوامل البيوكيميائية: انزيمات الكبد (ALT و AST)، علامات الإجهاد التأكسدي في الأنسجة (مالوندايالدهيد، نشاط كل من الكاتلاز والجلوتاثيون بيروكسيداز بانسجة الكبد). تم فحص الكبد بواسطة المجهر الضوئي لتقييم التغيرات النسيجية والتفاعلية المناعية لـ caspase 3 للتنبؤ بالموت المبرمج للخلايا. أشارت النتائج إلى أن: مستويات ALT و AST بالإضافة إلى مالوندايالدهيد الكبدي للجرذان المعالجة بالديازينون أظهرت زيادة ذات دلالة احصائية مقارنة بالمجموعة الضابطة، في حين أن نشاط كل من الكاتلاز والجلوتاثيون بيروكسيداز انخفضت بشكل احصائي ملحوظ وقد تحسنت هذه النتائج بدلالة احصائية في مجموعة كل من صمغ العسل مع الديازينون. كشف الفحص المجهرى عن اضطراب في بنية نسيج الكبد في مجموعة الديازينون على عكس المجموعة الثالثة التي عادت قرب المستوى الطبيعي. في حين أظهر الفحص المناعي الكيميائي العديد من الخلايا المتفاعلة الإيجابية إلى caspase 3 النشطة في مجموعة الديازينون التي أصبحت قليلة في المجموعة الثالثة مقارنة للمجموعة الضابطة السالبة. نستنتج في هذه الدراسة أن التأثير السام للديازينون على الكبد يمكن تحسنه عن طريق صمغ العسل.