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ACCELERATED STORAGE AND SUBCHRONIC EFFECTS OF DIFENOCONAZOLE ON THE LIVER OF MALE ALBINO RATS

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ABSTRACT: The study objectives were to examine the effects of difenoconazole (Score 25%) being stored at 54°C for 21 days under accelerated conditions and estimation the impact the effects of subchronic exposure to fungicide on male albino rats. The findings showed that difenoconazole was reasonably stable while stored under accelerated conditions at 54±2°C for 21 days. Regarding the subchronic effects of difenoconazole, it was obvious that both doses 1/20 and 1/40 of LD50 increased the concentration of ALT, AST, cholesterol, and triglycerides, and decreased the total protein concentration. With respect to histopathological effect on liver, difenoconazole at dosage 1/20 induced severe dilatation and congestion in the portal vein with few inflammatory cells infiltration in the surrounding tissue of the portal area. Also, diffuse inflammatory cells, infiltration and few fibroblastic cells proliferation , degenerative changes in the hepatocytes with inflammatory cells infiltration surrounding the dilated central vein.

Keywords: Difenoconazole, Accelerated storage, Enzymes, Liver, Histopathological effects

INTRODUCTION

Pests, especially weeds, are managed by the use of pesticides. Herbicides and insecticides are included in the term pesticide (which may include insect growth regulator, termiticides, etc.) Molluscicides, nematicides, pesticides, rodenticides, bactericides, avicides, insecticides, animalicides, fungicides, antimicrobials, disinfectants (antimicrobials), and sanitizers are some of the chemicals used in modern medicine (Randall *et al.*, 2008).

The most of pesticides are designed to act as crop protection chemicals, which typically shield plants from weeds, fungus, and insects (Konstantinou *et al.*, 2001).

Fungicides are a type of pesticides, which are compounds of synthetic or natural origin that protect plants from fungal invasion or eliminate existing fungal infections (Oliver and Hewitt, 2014).

Fungicides significantly increased agricultural productivity and provided for the rising need for nutrients for humans. These fungicides, while their many benefits, there were number of concerns for humans, other animals, and the ecosystem as a whole. People presently deal with this as one of their problems (Bentley *et al.*, 2000).

Difenoconazole is a systemic fungicide belonging to the triazole group. It is applied to a variety of fungus-related illnesses affecting ornamental plants, fruits, and vegetables. Because it causes changes in the morphology and functionality of the fungal cell membrane, it is used to basidiomycetes, ascomycetes, and deuteromycetes. It is applied to control various fungi including *Septoria tritici*, Brown Rust, Light Leaf Spot, Leaf Spot, Pod Spot, Ring Spot and Stem canker (EFSA, 2013).

To get rid of undesirable plants, fungi, or insects today, pesticides are frequently utilised. It is widely acknowledged that any chemical compound could have a negative impact on health because of its toxicity. Pesticides have an immediate impact on both human and animal health (Mesnage and Seralini, 2018).

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The goal of this study is to estimated difenoconazole fungicide stability under accelerated temperature conditions and it´s toxicological effects on the liver in male albino rats.

MATERIALS AND METHODS

Difenoconazole is 1-((2-(2-Chloro-4- (4-chlorophenoxy) phenyl) -4-methyl-1,3-dioxolan-2-yl)methyl) -1H-1,2,4-triazole. The formulation of the fungicides (Score 25% EC) was provided by the Central Agricultural Pesticides Laboratory of the Agriculture Research Centre (ARC) of the Ministry of Agriculture, which came from Syngenta AGRO AG- Switzerland or its factories in Hungary.

1. Accelerated storage procedure

The storage was done according to CIPAC (1995) with some modification: About 50 ml of emulsifiable concentrate was placed in glass bottle. The bottles 125 ml were covered by caps and kept in the oven at 54 ±2℃ for 3,7,14 and 21 days. The bottles removed from the oven and allowed to reach room temperature and carried out to estimate the active ingredient.

2. HPLC Determination of difenoconazole

The active ingredient percentage for difenoconazole was determined by using HPLC before and after storage according to the method of Lamkhanter *et al.*, (2021) with some modification. Methanol: Acetonitrile with a ratio of (1:9) was used mobile phase, at the flow rate 1 ml/min, and wavelength detector 210 nm. At this condition the retention time ((RT) of difenoconazole was 3.161 min.

3. Animals and Treatments

From the Helwan farm of the Egyptian Organisation for Vaccine and Biological preparation (Vaccera), 35 adult male albino rats *(Rattus Norvegicus* Sprague Dawley strain), with body weights ranging from 150-170 g, were collected. Male albino rats were kept in the Centre for Excellence in Toxicology Testing at the Central Agricultural Pesticides Laboratory (CAPL), located in Giza, Dokki, Egypt. These animals were housed in a clean laboratory setting for two weeks prior to the experimentation. The animals were fed a regular basal diet consisting of a mixture of starch (60%), salt (4%), casein (20%), cotton seed (10%), and cellulose (5%), as well as access to water and drinking from specially made glasses.

4. Determination of median lethal dose LD_{50}

Four groups of 20 male albino rats were separated into their own cages. Score 25%, a fungicide, was administered orally to each group at various amounts (666,666 - 1000 - 1500 – 2250) mg/kg body weight, respectively). According to Weil (1952), deaths were monitored for three days.

$$\text{Log } LD_{50} = \text{Log } D_a + d\,(f+1) \text{ for } K = 3$$

Where:

$\text{Log } D_a$ = the log of the lowest of the four dosage levels used

d = the logarithm of the constant ratio between dosage levels

f = it is taken from the table

5. Experimental design of subchronic effect of difenoconazole

A total of fifteen mature albino male rats were divided into three groups, with a total of 5 rats for each group, as follows: Rats in Group 1 (Control) had a regular diet, access to tap water, and no dosages of fungicides. Rats in group 2 (Score 25% EC) received the highest dosage of score (equivalent to 1/20 of LD50). Rats in Group 3 (Score 25% EC) received the smallest score dose (1/40 of LD50).

6. Sampling

At the end of experiment, after a period of 65 days, the control and treated rats in each group were weighed. The animals were anestheized and scarified, then dissection and the heart was removed. Blood samples were collected from the eye in special tubes and left for 30 minutes at room temperature, then centrifuged at 3000 rpm for 15 minutes, after that the serum was

separated and kept at 20°C until needed. Liver was removed; livers weighed and placed in 10% formalin solution for histopathological examination through a light microscope.

7. Liver enzyme level estimation

7.1. Determination of AST and ALT levels

Colorimetric analysis was used to measure the enzymes alanine aminotransferase (ALT/GPT) and aspartate aminotransferase (AST/GOT). AST and ALT determination method: in a test tube put 0.5 ml of reagent 1 (R1 buffer) and 100 µl of serum were mixed. Then, 0.5 ml of reagent 2 added, along with mixed tube contents, and should be incubated for exactly 20 minutes at 20 to 25°C. Finally, 5 millilitres of sodium hydroxide was added, along with mixed tube contents. The reagent blank was made as described previously without add the serum. Using an enzyme analyzer (JASCO V-630 spectrophotometer), compare the specimen's absorbance at 546 nm to a blank after 5 minutes (Reitman and Frankel, 1957).

7.2. Determination of total protein

Total protein was calculated using a colorimetric technique. One milliliter of reagent (R) was placed firstly on test tube (1) and one milliliter of reagent (R). 20 µl of standard placed in test tube (2). In test tube (3) 20 µl of sample and 1 ml of reagent (R) for the sample (serum) were added. Then, all of these test tubes were incubated for 10 minutes at room temperature. The enzyme analyzer (JASCO V-630 spectrophotometer) at 540 nm was used to determine. The absorbance of the sample and the standard against the reagent blank within 30 minutes (Kaplan and Szalbo, 1983).

Calculation

Serum protein conc (g/dL) =

$$^{A}\text{specimen} / {}^{A}\text{standard} \times 6$$

Where:

^{A}specimen= Measure absorbance of specimen

^{A}standard= Measure absorbance of standard

7.3. Determination of triglycerides

Using a colorimetric method, triglycerides were determined. Triglycerides are calculated as follows: The first test tube should contain one milliliter of reagent (R), the second test tube should have one milliliter of reagent (R) and 10 µl of standard and the third test tube should contain 10 µl of sample (serum) and 1 ml of reagent (R). These test tubes are incubated collectively for 5 minutes at 37 °C. Determine the absorbance of the sample and the standard against the reagent blank in less than 30 minutes using an enzyme analyzer (JASCO V-630 spectrophotometer) operating at 546 nm (McGowan *et al.*, 1983).

Calculation

Serum triglycerides conc (mg/dL) =

$$^{A}\text{specimen} / {}^{A}\text{standard} \times 200$$

Where:

^{A}specimen= Measure absorbance of specimen

^{A}standard= Measure absorbance of standard

7.4. Determination of cholesterol

Cholesterol was measured using a colorimetric technique. Firstly one milliliter of reagent (R) was placed in the first test tube, then one milliliter of reagent (R) and 10 µl of standard placed in the second test tube, and 10 µl of sample (serum) was mixed with 1 ml of reagent (R) in the third test tube (R). After that, all test tubes were incubated at 37 °C for 5 minutes. The enzyme analyzer (JASCO V-630 spectrophotometer) operating at 546 nm was used to determine the absorbance of the sample and the standard against the reagent blank in less than 30 minutes (Ellefson and caraway, 1976).

Calculation

Serum cholestrol conc (mg/dL) =

$$^{A}\text{specimen} / {}^{A}\text{standard} \times 200$$

Where:

^{A}specimen= Measure absorbance of specimen

^{A}standard= Measure absorbance of standard

8. Histopathological Examination for Testes and livers

For the length of the histological analysis, testes and livers were fixed in neutral buffered formalin at 10%, and then were subsequently rehydrated in 70% ethanol. The tissue was

immersed in paraffin after being sliced into 5-mm slices and mounted on slides. Light microscopy was utilised to analyse the slide slices using the Hematoxylin and Eosin (H&E) stain (Bancroft *et al.*, 2013).

9. Statistical analysis

All statistical comparisons between groups were performed using SPSS 16.0 (SPSS, USA). The study's data were statistically used to determine the mean value and related standard deviation (SD) for each group. In order to statistically assess differences between means, one-way analysis of variance (ANOVA) was performed. The posthoc least significant difference (LSD) tests for multiple comparisons were used to determine the statistical significance between the groups at the p0.05 level of significance if the F-ratio was significant.

RESULTS AND DISCUSSION

1. Accelerated storage procedure of difenoconazole

The data in Table (1) illustrate the effect of accelerated storage on difenoconazole formulation.

The obtained data indicated that the loss % of the tested fungicide was 0.51%, 0.66%, 1.88% and 2.23% when stored for 54 °C at 3 days, 7 days, 14 days and 21 days, respectively.

According to the previous findings, the formulation of difenoconazole are very stable after the storage test, where the loss % after 21 days of storage at 54 °C was 2.23%, the obtained data are in accordance with FAO (2019), which stated that the content of propiconazole (Triazole group) after storage should be 95% of the content that was found before storage.

Additionally, these findings are in line with Zhong *et al*., (2009), who found that stability in the physical and chemical properties existed and that the rate of loss in a mixture of propiconazole and difenoconazole (30%) did not surpass 5% over the course of two weeks of storage at a temperature of 54 ± 2° C.

2. Toxicological effect of difenoconazole

2.1. LD_{50} of difenoconazole

The results showed that LD_{50} of difenoconazole (Score 25%) was 1412.53 mg/kg. For each set of treated rats, the doses of 1/20 and 1/40 of LD_{50} were administered separately. The dose of difenoconazole at 1/20 of LD_{50} was 70.626 mg/kg b.w., while at 1/40 of LD_{50} it was 35.31 mg/kg b.w.

2.2. Body Weight and Clinical Indicators

Male albino rats were given difenoconazole orally for 65 days; it was clear that no deaths happened during this time, and no more signs of general toxicity were noticed. Weight gain in the body and in the organs is a sensitive sign of potentially dangerous drugs (Bailey *et al.*, 2004).

Table 1. Effect of accelerated storage procedure on difenoconaole formulations

Temperature 54 ± 2°c / Time (days)	Difenoconazole	
	Score 25%	
	Content (w/v) %	Loss%
Zero time	25.53	0
3	25.4	0.51
7	25.36	0.66
14	25.05	1.88
21	24.96	2.23

The effects of difenoconazole on body weight gain for each group were displayed in Table (2). The results showed that there was no appreciable variation in body weights between the difenoconazole exposure groups.

The results also showed that male albino rats exposed to 1/20 and 1/40 LD_{50} of difenoconazole did not have any overall adverse effects. In comparison to the control group, the weight of the mice increased during the experiment at normal rates. These findings are in line with those of (Shahat, 2013) who reported that difenoconazole (Score) treatment of mice for 8 weeks had no effect on their weight.

3. Effect of difenoconazole on male albino rats liver enzymes

The data in Table (3) displays the blood's concentrations of cholesterol, ALT, AST, triglycerides, and total protein. The acquired data demonstrated that, in comparison to the control group.

The results show that difenoconazole 1/20 and 1/40 of LD50 increased the concentration of ALT, AST, cholesterol, and triglycerides, and led to a decrease in the total protein concentration, and this is consistent with (Shahat, 2013) who found that difenoconazole (score) increased ALT and AST enzymes, triglycerides, and total lipids while the total protein levels declined noticeably.

4. Histopathological examination of the liver

Difenoconazole's effect on the liver was seen in a dose-dependent manner in rats after histopathology investigation. The control rats revealed no histological abnormalities that were noted in (Fig.1), and the usual histopathology structure of the central vein and surrounding hepatocytes in the parenchyma were seen.

The rats administered difenoconazole at 1/20. Figure (2) showed that there was Sever dilatation and congestion in the portal vein with few inflammatory cells infiltration in the surrounding tissue of the portal area (A). The portal area showed also diffuse inflammatory cells infiltration and few fibroblastic cells proliferation (B), there were degenerative changes in the hepatocytes with inflammatory cells infiltration surrounding the dilated central vein (C). In contrast, the rats administered difenoconazole at 1/40 of LD50 showed that no histopathological alteration was observed (Fig.3).

These results are consistent with (Mohamed *et al.,* 2021) who reported that the foremost unmistakable histopathological findings were watched within the liver of difenoconazole-treatment rats were degenerative changes within the hepatocytes encompassed and adjoining the widened central vein of rats taking after treatment in expansion, the entry region appeared oedema and periductal fibrosis encompassing the bile channel, as well as hyperplasia in other bile conduits was identified.

Table 2. The effects of difenoconazole on body weight gain for 65 days of treatment

Treatment	Body weight (g) ± SD		Body weight gain	
	Initial	final	(g)	%
Controle	157±3.78[a]	348±15.17[a]	191	100
DIF 1/ 20	160±1.52[a]	350±14.46[a]	190	99
DIF 1/ 40	159±4.16[a]	346±28.51[a]	187	98

Results are presented as means ± SD.

([a]) Indicate not significant difference at P <0.05 compared with the control group, respectively.

Table 3. Effect of difenoconazole on male albino rats liver enzymes

Serum enzymes levels	Treatment		
	Control	**DIF 1/20**	**DIF 1/40**
ALT (U/L)	$3.05^{a}\pm 0.064$	$5.73^{b}\pm 0.096$	$3.31^{c}\pm 0.1$
AST (U/L)	$10.22^{a}\pm 0.17$	$28.13^{b}\pm 0.23$	$12.33^{c}\pm 0.21$
Chelestrol (mg/dL)	$62.75^{a}\pm 0.66$	$105.45^{b}\pm 4.01$	$81.79^{c}\pm 3.14$
Total protein (g/dL)	$5.70^{a}\pm 0.115$	$5.36^{b}\pm 0.034$	$5.30^{c}\pm 0.028$
Triglycerides (mg/dL)	$106.06^{a}\pm 1.36$	$134.18^{b}\pm 2.90$	$120.43^{c}\pm 2.10$

Results are presented as means ± SD.

($^{b,\ c}$) Indicate a significant difference at $P < 0.05$ compared with the control group, respectively.

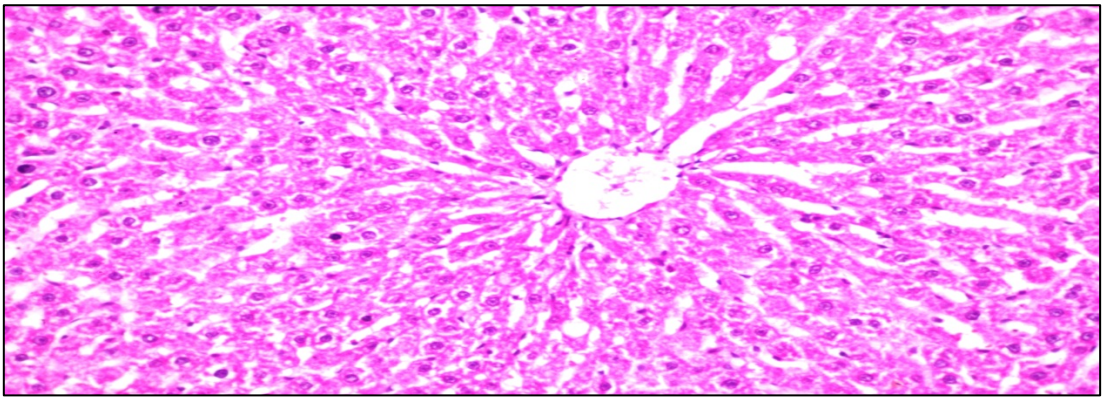

Fig. 1. A photomicrograph of liver of male rats form control group

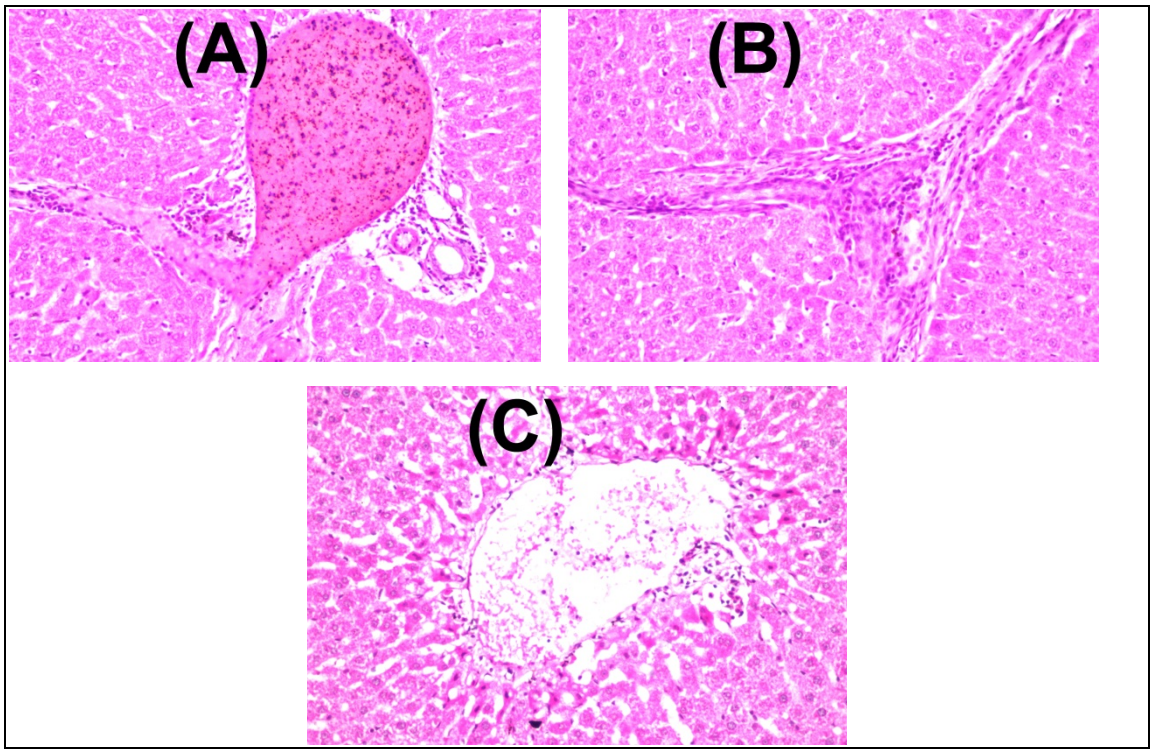

Fig. 2. A photomicrograph of liver of male rats form high dose of difenoconazole 1/20 LD_{50}

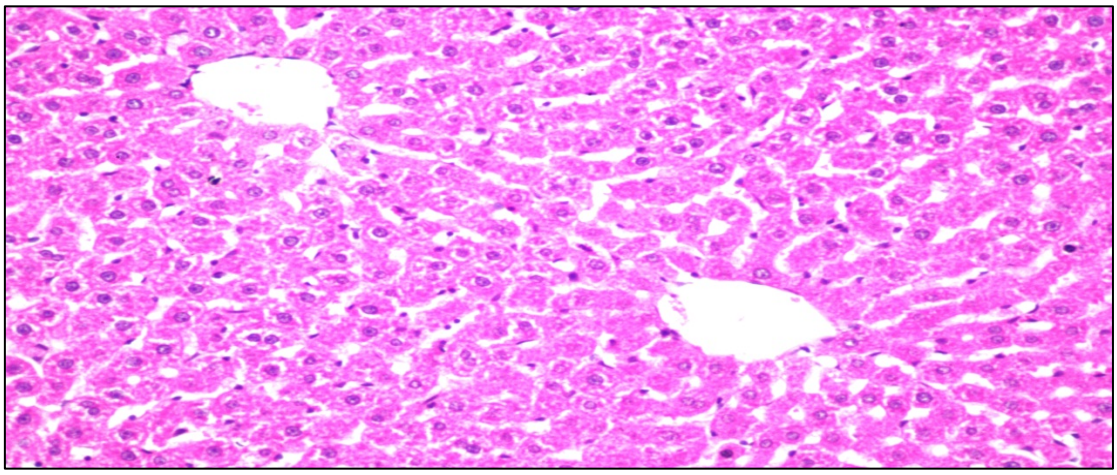

Fig.3. A photomicrograph of liver of male rats form low dose of difenoconazole 1/40 LD_{50}

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التخزين المعجل والتأثيرات تحت المزمنة للديفينوكونازول على كبد ذكور الجرذان البيضاء

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الملخص العربى

الهدف من هذه الدراسة هو فحص تأثير تخزين مبيد الديفينوكونازول (سكور 25%) على درجة حرارة 54 درجة مئوية لمدة 21 يوماً فى ظل ظروف التخزين المعملى المحكم وتقدير تأثير التعرض تحت المزمن للمبيد الفطرى على ذكور الفئران البيضاء. أظهرت النتائج أن الديفينوكونازول كان مستقرًا بشكل مقبول أثناء تخزينه في ظل ظروف التخزين المعملى عند درجة حرارة 54 ± 2 درجة مئوية لمدة 21 يومًا. فيما يتعلق بالتأثيرات تحت المزمنة للديفينوكونازول, كان من الواضح أن كلا الجرعتين 20/1 و 40/1 من LD_{50} تزيد من تركيز ALT ، AST، الكوليسترول ، والدهون الثلاثية ، وتقلل من تركيز البروتين الكلي. فيما يتعلق بالتأثير النسيجي المرضي على الكبد، فإن ديفينوكونازول بجرعة 20/1 يسبب توسعًا شديدًا واحتقانًا في الوريد البابي مع تسلل عدد قليل من الخلايا الالتهابية في الأنسجة المحيطة بمنطقة المدخل. أيضا انتشار تسلل الخلايا الالتهابية وقليل من تكاثر الخلايا الليفية ، والتغيرات التنكسية في خلايا الكبد مع تسلل الخلايا الالتهابية المحيطة بالوريد المركزي المتوسع.

الكلمات المفتاحية: ديفينوكونازول ، التخزين المعجل، إنزيمات ، كبد ، تأثيرات نسيجية