

TOXICOLOGICAL STUDIES ON TOPSIN M70 USED TO CONTROL WHITE MOLD DISEASE ON CUCUMBER

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ABSTRACT

Topsin M70 (thiophanate-methyl 70%) is recommended in Egypt to control many plant diseases such as root rot, powdery mildew, and fruits rot on different crops such as strawberry, mango, panama, apple, and grape. Latterly it was found to be superior to control cucumber stem and fruit white mold. Fungicide residues have been found on food for human consumption, mostly from post-harvest treatments all over the world. Cucumber fruits have short shelf life time and most of cucumber production is eaten fresh, or in salad. It was important to assess the risks of long term consumption of cucumber fruits contaminated with thiophanate-methyl specially their possible effect on liver functions and blood picture. In this respect, two groups of rats were fed with two concentrations of cucumber contaminated with the fungicide and the data were compared with third group which was fed on clean diet.

The tests showed higher level of each of Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST) Alkaline phosphates ((ALP) enzymes at both fungicide concentrations. Albumin content was decreased and in turn, total protein was decreased. On the contrary, increasing concentrations of cholesterol was recorded. As for red blood cell count (RBC), HCT and blood platelets they decreased. On the other hand white blood cells distractedly increased; the white blood cells increase side by side with the decreasing of the red blood cells invoke the suspicion that thiophanate methyl can badly affect bone marrow and/or has carcinogenic effect which may cause leukemia.

INTRODUCTION

Cucumber (*Cucumis sativus* L.) is one of the most important vegetable crops all over the world. In Egypt during the period from the year 2000 through the years 2009 the value of the total production of cucumber increased from 276 million LE. represent 80 million US\$ to 984 million LE. represent 178 million US\$ (Anonymous, 2012). The cucumber plastic-houses represent 55.55% of the total number of plastic houses in Egypt (Haggag, 1997).

In plastic houses as well as in open field cucumber is a subject to many fungal diseases (Leammelen, 2001). *Sclerotinia sclerotiorum* (Lib.) de Bary is the causal agent of white mold. Stem and fruit rot diseases is one of the most important diseases of greenhouse grown cucumbers in Egypt (Hanafy, 2011).

A number of fungicides, such as chlorothalonil, dichloran, iprodione, thiophanate-methyl, vinclozolin, and metam-sodium, that have excellent activity against *Sclerotinia* (Laemmelen, 2001). In addition to azoxystrobin, boscalid, thiophanate-methyl, and vinclozolin, benomyl, boscalid, fluazinam, prothioconazole, pentachloronitrobenzene, tebuconazole and trifloxystrobin

(Bradley, *et al.*, 2006). In a very recent study carried out by the authors Topsin M 70 was found to be superior for the control of white stem rot and white fruits mold of cucumber caused by *Sclerotinia sclerotiorum* under the Egyptian condition (Hanafi, 2011).

Topsin M70 (Thiophanate-methyl) is a wide spectrum systemic fungicide controlling a wide range of pathogens. It belongs to benzimidazole group which consists of thiophanate methyl, benomyl, carbendazim, and thiabendazole. It is absorbed by the roots and leaves of treated plants and has a protective and curative action (Saber, *et. al*, 2011). Thiophanate-methyl is registered in several countries (Canada, New Zealand, USA, UK and Australia.) for food and non-food uses. It is extensively used in industries, agricultures, home and gardens for many purposes. It is also used as a preservative in paint, papermaking, leather industry and as a preservative of fruits. It is effectively used against a wide variety of fungal diseases in vegetables and crops (Anonymous, 2010).

In Egypt Topsin M70 is recommended to control many plant diseases i.e. root rot, powdery mildew, and fruits rot on different crops such as cucumber, straw berry, mango, panama, apple and grape (Anonymous, 2011) Fungicide residues have been found on food for human consumption, mostly from post-harvest treatments (Brooks and Roberts, 1999).

Veneziano, *et al.* 2004 reported the occurrence of benzimidazole fungicides (benomyl and its metabolites carbendazim, thiabendazole and thiophanate-methyl) in 50 banana samples. Assessment of thiophanate-methyl residues in cucumbers in one trial in Denmark and 3 trials in the Netherlands gave residues at PHIs of 1-3 days ranging from <0.1 to 0.51 mg/kg., and five trials in the UK with foliar or drench treatments gave residues after 0 to 6 days from <0.2 to 0.3 mg/kg. (Banasiak, 2003).

Cucumber fruits had short shelf life and most of cucumber production is eaten fresh, or in salad. This work aimed to assess the risks of eating cucumber fruits contaminated with thiophanate-methyl and their possible effect on liver functions and blood picture.

MATERIALS AND METHODS

Topsin M70, common name thiophanate- methyl, [dimethyl ((1,2-phenylene) bis (iminocarbonothioyl)) bis (carbamate)] was sprayed 3 times on cucumber plants cv. (Beta alfa) grown in plastic house located in Elgarbeia Governorate to control stem and fruit white mold. After three days of the third spray, five healthy cucumber fruits weighted (about 500 g) were collected from sprayed plants, in addition to five healthy fruits collected from unsprayed plants (control treatment) were preserved in clean new polyethylene bags and labeled, then kept at -4°C .

White albino healthy female rats (a pure strain) of 3-4 months old with the mean average weight of 180-200 gm were purchased from Medicine Faculty, Tanta Univ. The rats were reared under the laboratory conditions ($25 \pm 5^{\circ}\text{C}$ and $65 \pm 5\%$ RH) in metallic cages, fed daily with balanced diet consisted of bread and healthy, fungicide free cucumber fruits (from the control treatment), then the rats were fasted for 4 days.

Cucumber fruits, treated with fungicide and untreated, were macerated separately, and homogenized. Three types of diet were prepared; (0.5 : 1 w/w) contaminated fruits and bread, (1 : 1 w/w) contaminated fruits and bread, and (1 : 1 w/w) uncontaminated fruits and bread respectively.

The rats were divided into three groups (3 rats/group) equal in age and weight, each was fed on one diet type.

After 21 days, the rats were sacrificed, the blood from each group was collected in two tubes, one containing coagulate substance (EDTA), centrifuged at 4500 rpm for 15 min to separate the serum from the plasma. The serum samples were kept at -4°C for the measurement of different parameters.

I- Effect on liver functions:

a-Transaminases determination:

Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT) activity were determined using diamond-diagnostics kits based on the method of Reitman and Frankel (1957) in which:



The pyruvate or the oxaloacetate formed is measured spectrophotometrically using 5010 chemistry analyzer as its derivative, 2, 4-dinitrophenyl hydrazone at 550 nm.

b- Alkaline phosphates ((ALP) determinations:

Alkaline phosphatase activity was determined using Kind and King (1954) method in which the reagent P-nitrophenyl phosphate is split by the enzyme into phosphate and the yellow colored indicator P-nitrophenol measured at 500 nm.

c- Albumin (Alb) determination:

Albumin was determined according to Doumas and Watson (1971), which is based on the production of a green colored complex as a result of the reaction of the albumin with bromocresol green. The color density is proportional to the amount of albumin in the samples and can be measured at 623-630 nm.

d- Determination of total protein (TP):

Determination of total protein amount in the blood was determined using diamond diagnostic kit according to the method of Gornall *et al.* (1949). In this method alkaline cupric sulphate reacts with the protein to produce a violet color, which can be measured at 550 nm.

e- Determination of cholesterol:

Cholesterol was determined using diamond diagnostic kit according to Watson (1960), which is based on the measurement of color, formed as a result of reaction of cholesterol with acetic acid and sulfuric acid, at 578 nm.

II-Haematotoxicity of certain tested treatments on albino rats:

The blood was collected in small glass vials containing EDTA as an anticoagulant for hematological studies. Then measurements of blood constituents were done. These parameters have been assessed in respect of complete blood count (CBC), comprised red blood cell (RBC's), Hemoglobin

concentration (HGB), Hematocrit (HCT), white blood cell count (WBC's), total platelet count (PLT). CBC was measured by Haematology analyzer system. Liver function and blood picture disruption percentages caused by feeding rats on cucumber fruits contaminated with fungicide were calculated referring to the rats fed on clean fruits.

RESULTS AND DISCUSSION

Effect of Topsin M70 fungicide on some enzymes activities and biochemical substances in blood serum of rats fed on cucumber fruits contaminated with this fungicide for 21 days compared with rats fed on non-contaminated fruits was studied.

a- Liver enzymes and function:

Studying the effect of feeding rats on two concentrations (0.5 and 1.0 : 1w/w) of Topsin-M70 on liver enzymes and function (Table 1 and Figure 1) revealed that, as for the activity of AST enzyme, the fungicide at the two concentrations resulted in higher activity (200 and 277 IU/l) respectively compared with 30 IU/l in blood of rats which did not receive any fungicide. These increase of activities represent 566.67 and 823.33 % .

Similar result was found in case of ALT enzyme, since the enzyme activity increased in blood of rats fed on concentrations of (0.5 and 1.0 :1w/w) of the fungicide to 265 and 300 IU/L, respectively, compared with 39.0 IU/l in blood of rats which did not receive any fungicide. These increase of activities represent 579.49 and 669.23 % respectively.

As for ALT enzyme reaction, the effect of feeding the rats on both fungicide doses (0.5 and 1.0 : 1w/w) were at the same trend ; since the recorded ALP reaction was 479 and 605 IU/l compared with 160 IU/l and disruption of 199.68 and 278.13% respectively .

As well as the effect on the liver enzymes, feeding rats on (0.5 and 1.0 :1w/w) contaminated fruits resulted in gradually increased level of cholesterol in blood; this increase reached 280 and 420 g/dl respectively representing 14.75 and 72.13% disruption.

In contrast to the liver enzymes and the cholesterol, albumin concentration in blood of rats fed on contaminated fruits decreased gradually as the fungicide concentration increased; recording 3.2 and 2.2 g/dl respectively, compared with 6.8 g/dl in blood of rats which did not receive any fungicide. These decreases of albumin concentration represent -36.36 and -54.55 % respectively.

For the total protein similar data were obtained, since total protein albumin concentration in blood of rats fed on contaminated fruits decreased gradually as the fungicide concentration increased; recording 2.8 and 2.0 g/dl respectively, compared with 4.4 g/dl in blood of rats did not receive any fungicide. These decreases of albumin concentration represent -52.49 and -67.65 % respectively.

Table 1: liver function and disruption percentages of female rats fed on cucumber fruits collected from plants treated with Topsin M70.

Treatments	liver function											
	AST		ALT		ALP		Albumin		T. protein		Cholesterol	
	Activity (IU/l)	Disruption %	Activity (IU/l)	Disruption %	Activity (IU/l)	Disruption %	Activity (g/dl)	Disruption %	Activity (g/dl)	Disruption %	Activity (g/dl)	Disruption %
0.5 : 1 (w/w)	200	566.67	263	579.94	479	199.38	2.80	-36.36	3.20	-52.49	280	14.75
1.0 : 1 (w/w)	277	823.33	300	669.23	650	306.25	2.00	-54.55	2.20	-67.65	420	72.13
Control	30	-	39	-	160.00	-	4.40	-	6.80	-	244	-

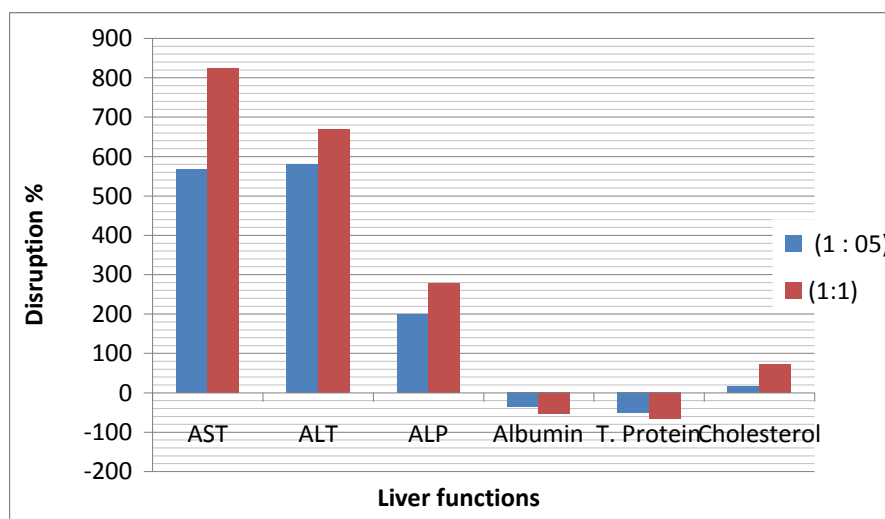


Figure1: Liver function disruption percentage caused by feeding rats on cucumber fruits contaminated with Topsin M70 referred to rats fed on fungicides uncontaminated fruits.

b- Blood picture (indices of plasma).

Concerning the blood picture (Table 2 and Figure 2), five indices of plasma were assessed (Hb. conc., RBC's., HCT, Platelets Count, and WBCs. Count).

Concerning hemoglobin concentration (Hb.), clear decrease was found between the amount in the blood of the rats fed on (0.5 and 1.0 :1w/w) of the Topsin M70 contaminated fruits and uncontaminated fruits. Hb. concentration gradually decreased to record 8.2 and 7.0 Gm/dl respectively compared with 14.0 in blood of control rats and representing -41.43 and -50.00 %. As for RBC's count, it was found to be decreased too. RBC's count in blood of the rats fed on with 0.5 and 1.0 ml/100g of Topsin M70 contaminated fruits was found to be $3.2 \times 10^3/\text{cml}$ and $2.3 \times 10^3/\text{cml}$

respectively compared with $5.3 \times 10^3/\text{cml}$; this count represented -37.74 and -56.60%). This decrease was reflected on HCT; since the HCT decreased from 43.0% in the blood of rats fed on uncontaminated fruits to 33.0 and 29.3% in blood of rats fed on (0.5 and 1.0 :1w/w) Topsin M70 contaminated fruits respectively representing disruption of -23.26 and -31.86%.

On the other hand platelets count, slightly increased from 256×10^3 c/ml in the blood of rats fed on uncontaminated fruits to 275×10^3 c/ml in the blood of rats fed on fruits contaminated with fungicide at the rate of 0.5 ml/100g, representing disruption of 7.42%. This increase was more obvious when the fungicide was more concentrated (1.0 :1 w/w) since platelets count increased to 355×10^3 representing disruption of 38.67%.

Markedly, WBCs count increased from 4.5×10^3 c/mm in the blood of rats fed on uncontaminated fruits to 28.0×10^3 c/ml and 42.0×10^3 c/ml in the blood of rats fed on (0.5 and 1.0 :1w/w)100g of Topsin M70 contaminated fruits respectively representing 533.22 and 833.33%.

Table 2: Blood picture and disruption percentages of female rats fed on cucumber fruits collected from plants treated with Topsin M70.

Treatments	blood picture indices of plasma									
	Hemoglobin		Red blood cells		Hematocrit		Platelets		White blood cell	
	Hb Conc. (Gm/dl)	Disruption %	RBC's Count $\times 10^3$ (c/ml)	Disruption %	HCT %	Disruption %	Platelets Count $\times 10^3$ (c/ml)	Disruption %	WBCs. Count $\times 10^3$ (c/ml)	Disruption %
0.5 : 1 (w/w)	8.2 \pm 0.8	-41.43	3.3 \pm 208	-37.74	33.0 \pm 2.4	-23.26	275 \pm 4.8	7.42	28.0 \pm 0.2	522.22
1.0 : 1 (w/w)	7.0 \pm 0.55	-50.00	2.3 \pm 0.0	-56.60	29.3 \pm 3.1	-31.86	355 \pm 2.0	38.67	42.0 \pm 0.0	833.33
Control	14 \pm 0.62		5.3 \pm 0.17		43 \pm 0.7		256 \pm 1.0		4.5 \pm 0.11	

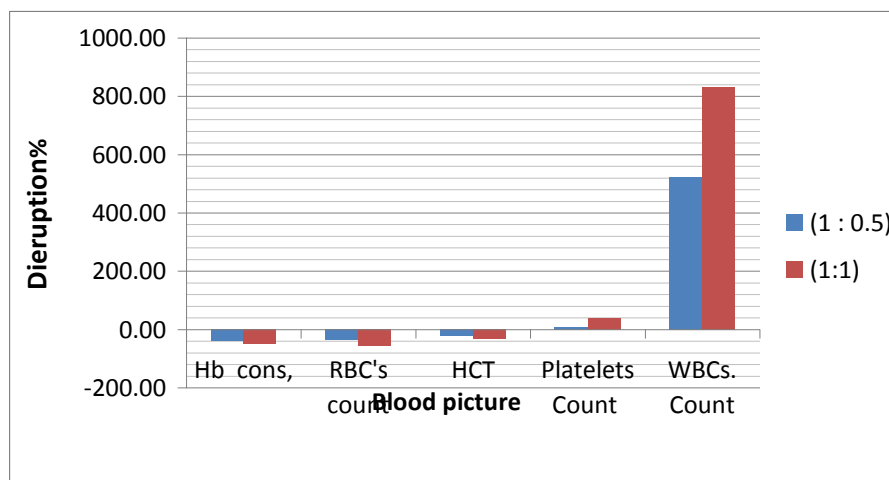


Figure 2: Blood picture disruption percentages caused by feeding rats on cucumber fruits contaminated with Topsin M70 referred to rats fed on fungicide uncontaminated fruits.

In Egypt Topsin M70 is recommended to control many plant diseases i.e. root rot, powdery mildew, and fruits rot on different crops such as straw berry, mango, panama, apple and grape (Anonymous, 2011). The author found Topsin M 70 to be superior for control white steam rot and white fruits mold of cucumber caused by *Sclerotinia sclerotiorum* (Hanafi, 2011). Thiophanate-methyl residues was found in cucumbers in one trial in Denmark and 3 trials in the Netherlands gave residues at PHIs of 1-3 days ranging from <0.1 to 0.51 mg/kg., and five trials in the UK with foliar or drench treatments gave residues after 0 to 6 days from <0.2 to 0.3 mg/kg. (Banasiak, 2003). Zang Zhiyongl *et al.* (2012) could retrieve from 87.3% to 96.0% of thiophanate-methyl from cucumber fruits treated with 0.01, 0.05, 0.1 and 1.0 mg/kg. Douch (1974) incubated mouse liver preparations with 1 mmol/litre thiophanate-methyl, and he could identify thiophanate-methyl, in addition to 11 metabolites: 1-(3-methoxycarbonyl-2-ureido) -2-(3-methoxycarbonyl -2-thioureido) 2-(3- methoxycarbonyl-2-thioureido)aniline, benzene, 1,2 - bis(3-methoxycarbonyl -2- ureido)benzene, 2-(3-methoxycarbonyl-2-ureido)aniline, 1-thioureido-2-(3-methoxycarbonyl-2-thioureido)benzene, 1-(2-ureido) -2 - (3-methoxy - 2 - thioureido) benzene , 1 - (2 - ureido) - 2 -(3-methoxycarbonyl-2-ureido)benzene, and 2-aminobenzimidazole, 5-hydroxy-2-aminobenzimidazole, methylbenzimidazole-2-ylcarbamate (carbendazim), and methyl 5-hydroxybenzimidazol -2- ylcarbamate (5-hydroxy - carbendazim).

In this work, feeding rats on cucumber fruits contaminated with Topsin M70 fungicide for 21 days compared with rats fed on non-contaminated fruits resulted in excessive increase of AST, ALT and ALP enzymes in blood. This enzymes increasing may be due to the injured liver cells and the mononuclear cell infiltration that was found by Singh *et al.* (1987) when they studied the effect of different doses of topsin on rats; especially, albumin content was decreased, which influenced the plasmatic colloidal osmotic pressure. Since albumin is synthetic in liver, and since albumin is the main constituent of the protein (60%), decreasing of albumin was reflected on the total protein level which in turn was decreased. This decrease of albumin and total protein confirm the opinion that, liver cells were injured and so affected the liver synthetic functions. Albumin levels are decreased in chronic liver diseases, such as cirrhosis, or it is decreased in nephrotic syndrome, where it is lost through the urine. On contrast, cholesterol was increased. Cholesterol is an important component for the manufacture of bile acids, steroid hormones, and vitamin D. All animal cells manufacture cholesterol with relative production rates varying by cell type and organ function. About 20–25% of total daily cholesterol production occurs in the liver; other sites of higher synthesis rates include the intestines, adrenal glands, and reproductive organs (Espenshade and Hughes, 2007). Cholesterol high levels in blood circulation is strongly associated with progression of arteriosclerotic vascular disease, depending on how it is transported within lipoproteins, however, cholesterol synthesis can be turned off when cholesterol levels are high, as well. HMG CoA reductase contains both a cytosolic domain (responsible for its catalytic function) and a membrane domain. The membrane domain functions to sense signals for its

degradation. Increasing concentrations of cholesterol (and other sterols) cause a change in this domain's oligomerization state, which makes it more susceptible to destruction by the proteasome. This enzyme's activity can also be reduced by phosphorylation by an AMP-activated protein kinase. Because this kinase is activated by AMP, which is produced when ATP is hydrolyzed, it follows that cholesterol synthesis is halted when ATP levels are low (Tymoczko, *et al.*, 2002); since in this study, ATP level was high, thus, cholesterol synthesis was out of control, and its level increased.

On the other hand, Red blood cell count (RBC) decreased, this decrease was negatively correlated with the increase of fungicide level administered; consequently, HCT was decreased too. At the same, blood platelets count decreased, too. Since red blood cell and blood platelets are produced in bone marrow, it is to suggest that, bone marrow may be damaged. On the other hand white blood cells distractedly increased; this white blood cells increase side by side with the decreasing of the red blood cells invoke the suspicion that thiophanate methyl can have carcinogenic effect and may cause leukemia. This data are in agreement with some other data obtained by different investigators and disagree with others. However, in long term study on the toxicity of thiophanate methyl to mice carried out by Kosaka (1973), the body-weight gain of males was reduced during this study, no signs of toxicity were seen, no macroscopic or histopathological alterations were seen, and there was no evidence of carcinogenicity; that may be due to that, the organ weights, clinical chemical and hematological parameters were not investigated. On the contrary, histopathological examination carried out by Tompkins (1992) revealed that the liver masses seen grossly were adenomas. The incidence of hepatocellular adenomas was significantly increased in males and females. Hepatocellular carcinomas were seen, one male had a hepatoblastoma, which is a relatively rare tumor and the incidence of atrial thrombosis was increased. In other long term study of carcinogenicity in mice fed different dietary levels, there was no evidence of any carcinogenic response. However, effects were seen on the testes (mainly hypospermatogenesis), thyroid gland, thyroid hyperplasia and thyroid tumors were seen at higher doses, together with parathyroid hyperplasia, nephrotoxicity, hepatotoxicity, and lipidosis of the adrenal cortex (Anonymous 1995). Thiophanate-methyl was found to be carcinogenic to male and female mice, it caused significant increase liver adenomas, and combined adenomas, carcinomas and/or hepatoblastomas in addition to increase in liver and thyroid weight and histological changes in liver, thyroid and heart; thus the CARC classified as "likely to be carcinogenic to humans" (Anonymous, 1999). It can be concluded that administration of diets contaminated with thiophanate-methyl cause disruption for liver function, blood picture and may disrupt bone marrow resulting in leukemia.

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دراسات على سمية مبيد التوبسن م70 المستخدم لمقاومة مرض العفن الأبيض على الخيار

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مبيد التوبسن م 75 أحد المبيدات الفطرية الموصى به فى مصر لمقاومة عديد من الأمراض النباتية مثل أعفان الجذور وأمراض البياض الدقيقى وأعفان الثمار على عديد من المحاصيل مثل الفراولة والمانجو والتفاح والعنب وأخيرا على الخيار؛ كما وجد أنه من أكفأ المبيدات لمقاومة مرض العفن الأبيض على ساق وثمار الخيار. هذا ولقد وجدت آثار للمبيدات فى عديد من الأغذية فى مختلف أنحاء العالم خاصة تلك التى تم معاملتها لمقاومة أمراض ما بعد الحصاد. ونظرا لأن ثمار الخيار لا يمكن تخزينها لفترات طويلة, ونظرا لأنها تؤكل طازجة كما تدخل فى عمل السلطات , لذلك كان من المهم دراسة مخاطر التغذية على ثمار الخيار المعاملة بمبيد التوبسن م70 وتأثيرها على وظائف الكبد وصورة الدم.

تم تغذية مجموعتين من الفئران على عليقتين تحتوى على تركيزين من ثمار خيار مجموعة من صوب تم معاملتها بمبيد توبسن م70 وتم مقارنة النتائج بمجموعة ثالثة تم تغذيتها على عليفة تحتوى على ثمار خيار لم تعامل بأى مبيد.

أثبتت التقديرات أزيادة نشاط أنزيمات الألانين ترانس أمينيز و أسبارات ترانس أمينيز والألكالين فوسفاتيز كنتيجة للتغذية على كلا التركيزين . أنخفض محتوى الدم من الألبومين والمحتوى الكلى من البروتين؛ وبالعكس ارتفع محتوى الكوليستيرول . فى حين أنخفض تعداد الكرات الدموية الحمراء والصفائح الدموية فى الوقت التى أرتفعت فيه تعداد الكرات الدموية البيضاء أرتفاعا شديدا . وهذا الأرتفاع الشديد فى تعداد الكرات الدموية البيضاء مع انخفاض الكرات الدموية الحمراء يؤدى الى الاعتقاد بأن مبيد التوبسن م 70 له تأثير سئ على نخاع العظام وقد يكون له تأثير مسرطن على الدم مما قد يؤدى الى لوكيميا.

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