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Fungicidal Effect of Nano Copper Particles on *Alternaria solani* the Causal Organism of Tomato Early Blight Disease Compared with Other Copper Fungicides and Their Toxicity to Experimental Animals

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ABSTRACT

During the last few decades, nanotechnology has evolved very rapidly. Now nanoparticles are involved in most of our life branches from electronic industries, medicine, to kitchenware. Sulfur and copper are protective fungicides they are only the non-organic fungicides allowed under organic agriculture guidelines. Early blight of tomato, caused by the fungus *Alternaria solani*, is the most common foliar disease of tomatoes all over the world. In *vitro*, all the three tested copper fungicides showed remarkable fungicidal effects on mycelial growth. Copper nanoparticles (CuNPs) showed less fungicidal efficiency, compared with the two conventional copper fungicides, Adwicopper and Flacopper; however, there was remarkable increased efficacy of the (CuNPs) (copper sulfate pentahydrate Nps) compared with the bulk copper (copper sulfate pentahydrate). Calculating the disease severity and fungicidal efficiency in *vivo*, for each fungicide showed that Adwicopper resulted in the least disease severity and the highest fungicidal efficiency followed by Flacopper, then (CuNPs). Toxicological studies revealed that rats fed for six weeks on fodder mixed with the juice of tomatoes harvested from plants treated with any of the three tested fungicides showed a significant elevation in the liver enzyme alanine aminotransferase (ALT) level. Highest ALT level was found in case of Adwicopper, followed by Flacopper then (CuNPs). Only rats fed on fodder mixed with the juice of tomatoes harvested from plants treated with Adwicopper recorded significant elevated aspartate aminotransferase (AST) levels. The cholesterol level was significantly elevated in the blood of rats fed on fodder mixed with the juice of tomatoes harvested from plants treated with any of three tested copper fungicides; with significant difference among them. Adwicopper recorded the highest cholesterol level, followed by copper nanoparticles, then Flacopper. All three copper fungicides (copper nanoparticles, Adwicopper and Flacopper) resulted in significantly elevated levels of triglisroid; without significant differences among them, but with significant differences between any of them and the two control treatments. At the same time, feeding rats on any of the tested copper fungicides resulted in significant elevation of low density lipoprotein (LDL) levels. Adwicopper, produced in the highest LDL level, followed by copper nanoparticles then Flacopper. The three tested fungicides resulted in significant elevation of creatinine, and urea levels. Nanoparticles and Adwicopper resulted in the highest urea level in rat blood, followed by Flacopper.

INTRODUCTION

During the last few decades, the nanotechnology has evolved very rapidly, and nanoparticles are involved in most of our life branches from electronic industries, medicine, to kitchenware (**Filipina and Sutherland, 2013)**. Copper fungicides have been recommended and used on large scale since Millardet,1882 noticed that diseased grape vines that had been sprayed with a bluish-white mixture of copper sulfate and lime retained their leaves through the season **(Johnson, 1935), (Morton and Staub, 2008**). Sulfur and copper are protective fungicides, the only non-organic fungicides allowed under organic agriculture guidelines (**Letourneau and Van Bruggen, 2006**).On the year 2012, copper fungicides, recommended in Egypt to control different plant diseases comprised 29 traditional fungicides, including, 12 fungicides based on the active ingredient copper hydroxide, 11 fungicides based on the active ingredient copper oxychloride, 3 fungicides based on the active ingredient copper oxide and 3 fungicides based on the active ingredient copper sulfate. In addition, there were many other mixed fungicides in which copper was mixed with one or more other systemic active ingredient; nowadays there are growing concerns regarding the indiscriminate use of either copper or copper nanoparticles which can cause toxic effects to plants and other living organisms (**Kasana, *et.al.*, 2016**). Early blight of tomato, caused by the fungus *Alternaria solani*, is the most common foliar disease of tomatoes all over the world. This disease causes direct losses by the infection of fruits and indirect losses by reducing plant vigor. In addition, fruits from defoliated plants are also subject to sunscald. Agrochemicals (pesticides and fertilizers) are looked upon as a vehicle for improved crop production technology. This study was carried out to assess the fungicidal effect of (CuNPs) on *Alternaria solani* the causal of tomato early blight disease, and at the same time on determine its possible toxic side effects in humans, and compare it with other traditional copper fungicides.

MATERIALS AND METHODS

(CuNPs) sample tested in this work was kindly donated by Prof. Dr., Mahmoud AbdElhalim AbdElgawad; Prof. of Nanotechnology and Head of Chemical Engineering Department, Faculty of Engineering-Elmenia Univ.

Geochemical Instrumentation and Analysis:

Examination of physicochemical properties of the tested copper nanoparticles was performed in the Nanotechnology& Advanced Material Central Lab. (NAMCL), Agriculture Research Center (ARC) using:

1- Transmission Electron Microscope (TEM):

High resolution transmission electron microscope (HR-TEM, Tecnai G20, Super twin, double tilt, with magnification range: up to 1,000,000 X and elemental analysis "qualitative and semi-quantitative analysis. Two different modes of imaging were employed; the bright field at electron accelerating voltage 200 kV using a lanthanum hexaboride (LaB6) electron source gun and diffraction pattern imaging. An Eagle CCD camera with (4k*4k) image resolution was used to acquire and collect transmitted electron images. TEM Imaging & Analysis (TIA) software was used for spectrum acquisition, analysis of EDX peaks and the estimation of crystalline structure, morphology and mean size of nanoparticles. In addition, the average size of nanoparticles was determined.

2- X-Ray Diffraction

X-Ray Diffraction was carried out using XPERT–PRO–PANalytical– Netherland with the following parameter, X-Ray Position [°2Th.]: 79.9900, Step Size [°2Th.]: 0.0200, Scan Step Time [s]: 0.5000, Scan Type: Continuous, Offset [°2Th.]: 0.0000, Divergence Slit Type: Fixed, Divergence Slit Size [°]:0.8709, Specimen Length [mm]:10.00, Receiving Slit Size [mm]: 0.1000, Measurement Temperature [°C]: 25.00, and Anode Material: Cu (40Kv-30mA).

A. Fungicidal efficacy:

The fungicidal efficacy of (CuNPs) compared with two traditional copper fungicides and on *Alternaria solani the* causal of tomato early blight disease, was investigated in *vitro* and in *vivo.*

A-1. In *vitro* studies.

Serial concentrations (5, 10, 25, 50, 100, 200, 300, 400 and 500 ppm) of (CuNPs), and three other traditional copper fungicides i.e., copper sulphate ($CuSO_4.5H_2O$), Flacopper (copper hydroxide $Cu(OH)_2$ 88%) and Adwicopper (copper oxychloride $CuCl_2.3Cu(OH)_2$ 84%), were prepared by mixing a weight of copper from each fungicide equivalent to the desired concentration with PDA, just before solidification under continues shaking. The media with different copper concentrations were poured in Petri dishes; and after solidification, a 3mm disk of *Alternaria solani* from the edge of a 7- day- old culture was mounted on the media in the center. *Alternaria solani* culture was kindly provided by the Department of Plant Diseases and Fungi Survey and Identification Research; Plant Pathology Research Ins, ARC. Egypt. Media which contained no fungicide served as control.

The Petri dishes were incubated at 25˚C until the fungus growth reached the edge of a dish, during which, the fungal growth was periodically observed and linear growth in each dish was recorded. The mean of linear growth for each treatment was calculated and fungicidal efficacy and EC_{50} of each treatment was calculated.

A-2.In *vivo* studies.

The greenhouse experiments were carried out during 2015 and 2016 growing season at plant pathology research Ins., A.R.C. Egypt. Tomato transplants (Vr. Castel rock) 5 weeks old were planted under greenhouse conditions in 60 pots (50Ǿ cm) containing sandy loam soil. Four transplants were planted in each pot. The pots were divided into 12 groups (5 pots per each). The groups were arranged in 3 rows (replicates) of 4 groups each. Each group represented one treatment. When the plants were well established, they were artificially infected by spraying with a spore suspension (1 x 10^5 spore/ml) from *Alternaria solani* that was grown on V8 juice agar-$CaCO_3$ media 6.5 pH, at 25±1 C^o, with alternating 12 hour periods of light and darkness (**Tatiana 2010**).

The plants received all the normal recommended cultural practices except that daily, early in the morning the floor of the greenhouse was sprayed with water to provide enough air humidity. Copper nanoparticles, Flacopper and Adwicopper were suspended in water at the rate of 0.5, 2.5 and 2.5 gr./1 water respectively. Three groups were sprayed with one of the three fungicides, three times at 2 week intervals; the fourth treatment received only water. Disease incidence and disease severity were recorded 2 weeks after each application on scale which consisted of six categories ranging from 0 to five (0 = no infection, 1= scattered spots of infection less than 10% of the leaf area, 2 = 10% and <20%, 3 = 20% and <30%, 4 =30%, and < 40% and 5 = >40%.

Disease severity was calculated using the equation developed by (**Townsend and Heuberger 1943)**

$$DS (\%) = \Sigma(nV) / NV \times 100$$

Where,

n – degree of infection according to the scale;

v – number of samples per category;

V – total number of samples screened;

N – highest degree of category.

Then efficacy of fungicides was calculated using Abbott’s formula (**Abbott, 1925 :**

$$Efficacy (\%) = (X – Y) / X \times 100$$

Where,

X – disease severity in the control;

Y – disease severity in the treatment

Two weeks after the third application, the area under the disease progress curve, during the application period, was calculated using the following equation, according to **(Campbell and Madden1990):**

$$AUDPC = \sum_{i=1}^{n-1} \frac{Yi+Yi+1}{2} x (t_{i+1} - t_i)$$

Since:

AUDPC is the area under disease progress curve

Yi is the disease severity assessed at the beginning of treatment and after two weeks after each treatment

ti is time (two weeks for each disease severity assessment)

n is the total number of observations (4).

Disease severity data were statistically analyzed as complete randomized block design and least significant differences (L.S.D 0.5) were calculated according to **(Fisher 1948**) and **(Snedecor and Cochran 1967**) and Multiple range and multiple F test **(Duncan, 1955),** using the Web Agri Stat Package computer program (WASP).

B. **Toxicological studies:**

The fruits from each treatment were separately collected in clean polyethylene bags. The bags were closed, marked up and kept in a deep freezer to be used in the further toxicological studies. For the toxicological studies, 16 male Sprague-Dawley albino rats four weeks old weighting (100 to120 g) were obtained from the Animal House of the Food Technology Research Institute, Agricultural Research Center, Giza, Egypt (ARC). Before carrying out the toxicological studies, all the rats were kept for an adaptation period of two weeks long; feeding on a basal diet which consisted of corn starch 70%, casein 10%, corn oil 10%, cellulose 5%, salt mixture 4% and vitamin mixture 1% as described by (**Peter and Person 1971).** After the adaptation period, the rats were divided into 5 groups (4 rats each). The first group fed for 28 days on basal feed mixed with 10% juice of tomato fruits collected from the plants which had previously been treated with nano copper. The second group fed for the same period on basal feed mixed with 10% juice of tomato fruits collected from the plants which had previously been treated with Adwicopper. The third group fed for the same period on basal feed mixed with 10% juice of tomato fruits collected from the plants which had previously been treated with Flacopper. The fourth group feed for the same period on basal feed mixed with 10% juice of tomato fruits collected from the plants which were not treated with any fungicide (positive control) and the last group fed for the same period on basal feed only (negative control).At the end of the feeding period, blood samples were collected from the *Retro-orbital venous plexus* in centrifuge tubes by carefully inserting a capillary tube 15 ^{o} – 30^{o} ^{angle} near the nose of the rat. The serum was separated and kept at -2^{o}C for analysis; then the weight of body, brain, liver and kidney were registered for each rat separately. All the biochemical analyses in this work were carried out using spectrum diagnostic kits produced by MDSS GmbH (Hannover, Germany)which were supplied by the Egyptian Company for Biotechnology, according to the methods described in the user manual provided with each kit using a JENWAY 6400 spectrophotometer as follows:

Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT) activities were calorimetrically determined according to the method of **(Bergmeyer and Harder 1986)** which depends on the reaction with α-oxoglutrate to produce oxaloacetate and pyruvate which can be determined spectrophotometrically in the form of hydrazone, which is produced by reaction with 2,4-dinitrophenylhydrazine in an alkaline medium. Serum total cholesterol (High Density Lipoprotein cholesterol HDL and low density LDL and triglycerides) were determined as follows:

Cholesterol was determined using the CHOD-PAP–enzymatic colorimetric method which depends upon the reaction of cholesterol esterase with the reagent (IFUFCC08) to produce cholesterol + fatty acids which in turn is transformed to Quinoneimine dye. High Density Lipoprotein (HDL) was determined using precipitation method in which the LDL and VLDL in the sample precipitate with phosphortungstate and magnesium ions; after precipitation, the HDL fraction, which remains in the supernatant is determined using the reagent (IFUFCC26). Triglycerides were determined using GPO-PAP-enzymatic method, which depends upon the series reactions with the reagent (IFUFCC39) to form at the last step Quinoneimine dye.

LDL was calculated according the equation

$$\mathrm{LDL} = \mathrm{cholesterol} - \{\frac{\mathrm{triglycerides}}{5} + \mathrm{HDL}\}$$

Urea was assessed using urease-colorimetric method by reaction of the sample with the reagent (IFUFCC40) to hydrolyze the urea in the presence of water and urease to produce ammonia and carbon dioxide.

Creatinine was determined using the Buffered Kinetic jaffe reaction without deproteinization method, using the reagent (IFUFCC09) in which creatinin reacts with picric acid under alkaline conditions to form a yellow-red complex.

Albumin was determined using modified bromocresol green method which is based on its binding with the reagent bromocresol green (IFUFCC52) in 4.1 pH.

Statistical analysis:

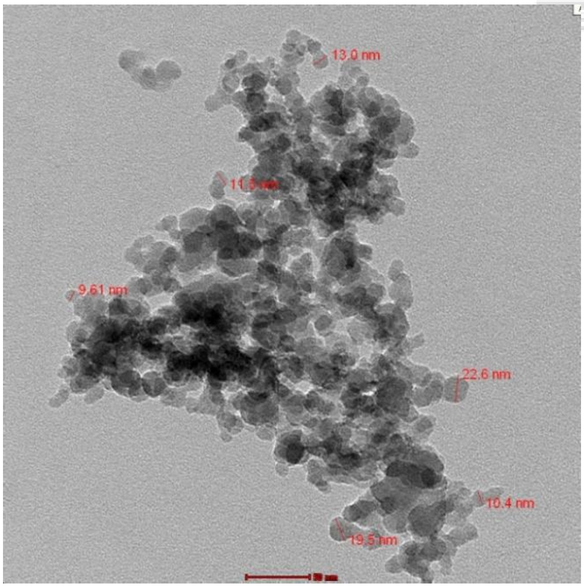
The analysis of variance (ANOVA) was performed then critical values of Duncan's new multiple range test **(Duncan,1955)** were calculated and the data were represented as mean ± SEM (standard error of the mean) and category letter.

RESULTS AND DISCUSSION

Geochemical Instrumentation and Analysis:

Examination of physicochemical properties of the tested copper nanoparticles using a Transmission Electron Microscope (TEM) and X-Ray Diffraction (XRD) revealed: The sample is copper sulphate pentahydrate (Cu SO_4 (H_2 O)$_5$) with empirical formula: $CuH_{10}O_9S$. with the following crystal1 graphic parameters:

Crystall system:anorthic, space group: P –l, space group number: 2, a (A): 6.113 4, b (A): 10.711 6, c(A): 5.958 4, Alpha (°): 82.376 0, Beta (°): 107.323 0 and Gamma (°): 102.594 0,volume of cell (10^6 prn-^3): 3 62.5 6, Z: 2 . 0 0, RIR: 0.9 9, and nanoparticles size ranged from 9.61 to 22.6 nm with very high purity (fig.1 and 2)



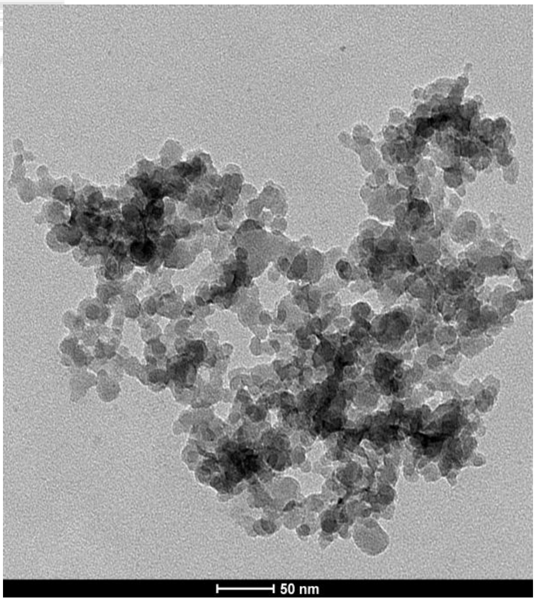


Figure 1: TEM pictures show the geochemical properties of Cu nanoparticles, their shape and size which ranged from 9.61 to 22.6 nm.

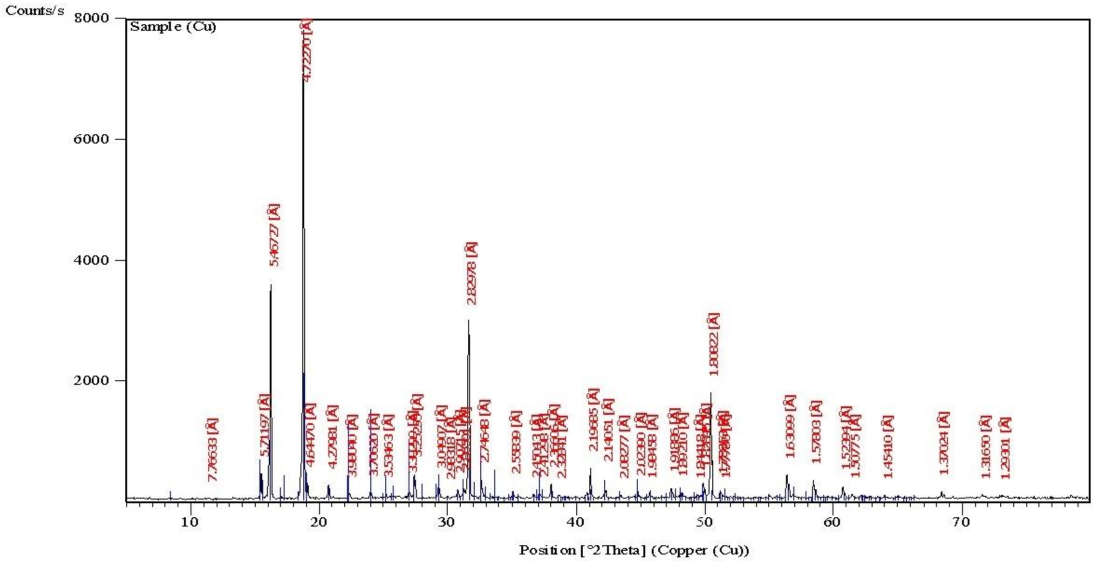


Figure 2: XRD diagram shows Xr diffraction through Cu SO_4 (H_2 O)$_5$ nanoparticles crystals at the positions, 16, 19, 31.8 and 50.5 ^{o}2theta, absent of any other sharp picks confirms the high purity of sample.

A- Fungicidal efficiency:

A-1.In *vitro* studies.

Growing the fungus *Alternaria solani* on media containing different concentrations of the four copper compounds separately, and calculating the mean of fungus linear growth and the fungicidal efficacy (Tab. 1 and Fig 3) indicated that, *Alternaria solani* showed remarkably different sensitivity to the different fungicides. Copper oxychloride was the most efficient one. On media which contained 200 ppm only 1.2 cm linear growth was recorded, calculating EC_{50} for this fungicide was found to be 50 ppm.

Table 1: Fungicidal effect of copper nanoparticle on *Alternaria solani* linear growth and EC_{50} compared with other copper fungicide.

Fungicides	0.0 ppm	5 ppm		10 ppm		25 ppm		50 ppm		100 ppm		200 ppm		300 ppm		400 ppm		500 ppm		EC_{50}
	LG	LG	Eff	LG	Eff	LG	Eff	LG	Eff	LG	Eff	LG	Eff	LG	Eff	LG	Eff	LG	Eff	
Nano Copper	8.6	8.1	5.8	7.6	11.6	7.2	16.3	6.5	24.5	2.8	67.4	2.3	73.3	2.3	73.3	1.5	81.3	1.5	81.3	63 ppm
Copper S	8.6	8.5	1.2	8.3	3.5	8	7	7.6	11.6	6.2	30	4.3	50	2.4	72	2	76.7	1	88.4	200 ppm
Adwico-pper	8.6	7.5	12.8	7	18.6	6.6	23.2	4.3	50	3.2	62.8	1.2	86	0	100	0	100	0	100	50 ppm
Flacopp-er	8.6	8.2	4.7	7.2	16.3	5.6	35	5.2	40	2.6	70	1.5	81.3	0	100	0	100	0	100	53 ppm

LG = Linear growth, Eff = Fungicidal efficacy and EC = Effective active material concentration able to inhibit 50% of linear growth.

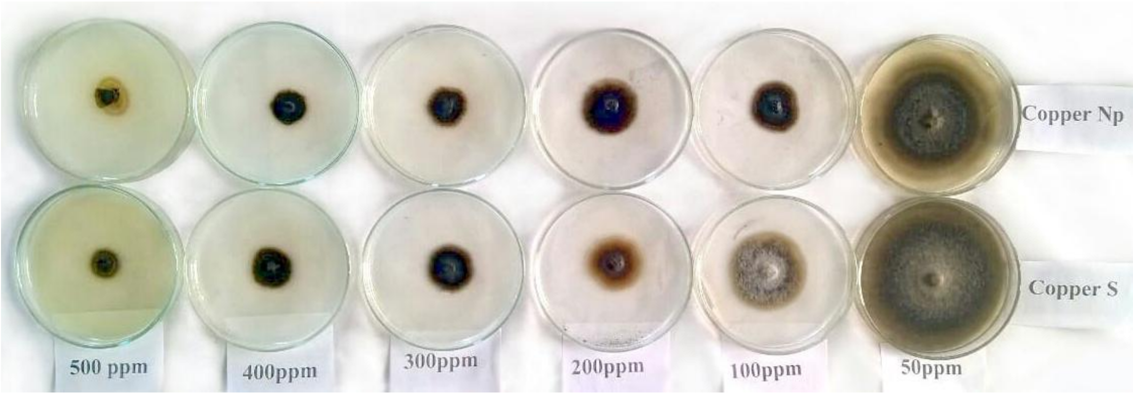


Fig 3: Comparison between the linear growth of *Alternaria solani* on media amended with copper nanoparticles and copper sulfate.

Copper hydroxide at concentration of a 200 ppm resulted in 1.5 cm linear growth, with an EC_{50} of 53 ppm. (CuNPs) ranked third, with an EC_{50} of 63 ppm. Copper sulphate was the least effective one; it could grow on media contained 500 ppm resulting in 1 cm linear growth; and an EC_{50} of 200 ppm.

A-2.In *vivo* studies.

From the (table 2) it is clear that, after the first spray, disease severity on the plants treated with any of the three copper compounds was very low; since it ranged from 6.4 on plants treated with Adwicopper to 8.8 on plants treated with (CuNPs), without significant difference, compared with37.1 on control plants with very high significant difference. After second spray, however, disease severity on control plants rose to reach 70.0, disease severity, on the sprayed plants ranged from 8.6 on plants treated with Adwicopper to 14.6 on plants treated with (CuNPs) with significant differences within the three copper treatments, and with highly significant differences between any of the three copper treatments on one side, and the control treatment on the other side. After the second spray, the three copper treatments (copper nanoparticles, Adwicopper and Flacopper resulted in 71.4, 82.8 and 78.5% efficacy. After the third spray, disease severity on control plants rose to reach 85.0 compared with 20.8, 11.6 and 15.0 on plants sprayed with copper nanoparticles Adwicopper and Flowcopper respectively recording 82.4, 90.6 and 87.0% efficacy respectively.

Table 2: Early blight disease severity and fungicide efficacy on tomato plants treated three times with copper nanoparticles compared with plants treated with traditional two fungicides Adwicopper and Flacopper.

Treatments	After 1st treatment		After 2nd treatment		After 3rd treatment		Area under disease progress curve	
	Disease severity	Fungicide efficacy	Disease severity	Fungicide efficacy	Disease severity	Fungicide efficacy	Disease severity	Fungicide efficacy
Copper(CuNPs)	8.8[a]	76.9	14.6[c]	71.4	20.8[c]	82.4	13.45[b]	75.89
Adwicopper	6.4[a]	83.2	8.6[a]	82.8	11.6[a]	90.6	8.25[a]	85.21
Flacopper	6.8[a]	82.2	11.4[b]	78.5	15.0[b]	87.0	10.92[ab]	80.42
Control	37.1[b]	-	70.0[d]	-	85.0[d]	-	55.78[c]	-
LSD 5%	5.26	-	1.95	-	2.31	-	3.21	-

Calculating the area under disease progress curve during the period from the first spray till two weeks after the third spray indicated that no significant difference was found between disease severity on plants sprayed with the Adwicopper and Flacopper. These two fungicides fall into one category (a).On the side, Flacopper and copper nanoparticles fall into one category (b) with a significant difference between Adwicopper and copper nanoparticles. All three fungicides, copper nanoparticles, Adwicopper and Flacopper, showed highly significant differences compared with the control treatment; recording mean the fungicide efficacy under disease progressive curve during the experimental period of 75.9, 85.2 and 80.4% respectively.

B. Toxicological studies:

Blood chemoanalysis of rats fed on fodder mixed with juice of tomato harvested from plants treated with any of the three tested fungicides (Table 3) showed a significant elevation in liver enzymes alanine aminotransferase (ALT) level compared with the two control treatments (control + control -). The highest ALT level was found in case of Adwicopper with 33.25 U/l followed by Flacopper with 29.25 U/l, then copper nanoparticles with (24.25 U/l), while the two control treatments (control + control -) Recorded only 21.0 U/l for each.

Regarding to aspartate aminotransferase (AST), only rats fed on fodder mixed with juice of tomato harvested from plants treated with Adwicopper recorded a significantly elevated aspartate aminotransferase (AST) level, with 72.21 U/l, compared with the two other copper fungicides copper nanoparticles and Flacopper, which recorded 53.95 and 58.51 U/l respectively, and the two control treatments which recorded 56.03 for each.

Regarding cholesterol, chemoanalysis showed a significant increase of its level in blood of rats fed on fodder mixed with juice of tomato harvested from plants treated with any of three copper fungicides (Copper nanoparticles, Adwicopper and Flacopper) with significant differences among them, on one side and between any of them and the two control treatments (control+ and control-) on the other side; recording 134.5, 148.3 115.3, 99.25 and 103.75mg/Dl respectively. The same data indicated that Adwicopper recorded the highest cholesterol level elevation followed by copper nanoparticles, then Flacopper.

Similar data were obtained in case of triglyceride, since all the three copper fungicides (Copper nanoparticles, Adwicopper and Flacopper) resulted insignificantly elevated levels of triglyceride; without significant differences among them, but with significant difference between any of them and the two control treatments, recording 106.25±6.02[a], 109.25±5.60[a],107.75±7.92[a],78.75±3.08[b] and 86.25±3.54[b] mg/Dl respectively.

No significant differences in HDL level was recorded among any the tested three copper fungicides (Copper nanoparticles, Adwicopper and Flacopper) on one side and any of them and the two control treatment on the other side. HDLs were 56.50±0.56, 56.50±0.75, 54.25±0.41, 55.56±0.56 and 58.25±0.74a mg/Dl respectively.

Table 3: Blood chemoanalysis of rats fed on fodder mixed with tomato juice from plants treated with Copper (NPs), Adwicopper, Flowcopper or juice free of any fungicide (control +) or without any juice (control-)

Analysis test	Copper (NPs)	Adwicopper	Flowcopper	Control +	Control –
ALT U/l	24.25± 0.96[b]	33.25± 0.41[c]	29.25± 1.63[c]	21.0±0.89[a]	21.0±0.0[a]
AST U/l	53.95± 1.50[a]	72.21± 1.38[b]	58.51± 0.53[a]	56.03±2.15[a]	56.03±1.50[a]
Cholesterol mg/Dl	134.5± 3.08[c]	148.3± 3.45[d]	115.3± 2.16[b]	99.25±8.10[a]	103.75±3.3[a]
Triglyceride mg/Dl	106.25±6.02[b]	109.25±5.60[b]	107.75±7.92[b]	78.75±3.08[a]	86.25±3.54[a]
HDL mg/Dl	56.50±0.56[a]	56.50±0.75[a]	54.25±0.41[a]	55.56±0.56[a]	58.25±0.74[a]
LDL mg/Dl	56.75±2.16[c]	70.00±4.68[d]	39.50±0.75[b]	24±0.50[a]	25.75±1.08[a]
Albumin mg/Dl	4.85± 0.33[a]	6.45± 0.53[b]	4.65± 0.08[a]	4.05±0.39[a]	3.725±0.24[a]
Creatinin mg/Dl	0.295±0.004[b]	0.296±0.004[b]	0.317±0.008[b]	0.224±0.011[a]	0.218±0.006[a]
Urea mg/Dl	19.25±0.22[c]	19.00±0.00[c]	16.75±0.65[b]	12.0±0.82[a]	11.5±0.75[a]

* Data representing body weight mean ± SEM (standard error of the mean) and category letter

** Treatments with same letter showed no significant difference

In respect to LDL, feeding rats feed exposed to the tested copper fungicides resulted in significant elevation of LDL levels. Adwicopper, resulted in the highest LDL with 70.00 mg/Dl followed by copper nanoparticles then Flacopper recording 56.75 and 39.50 mg/Dl, compared with 24 and 25.75 mg/Dl recorded in the two control treatments (control+ and control-) respectively.

With regard to albumin, only Adwicopper resulted in a significantly elevated albumin level. No significant difference in albumin level was recorded between the two tested fungicides, copper nanoparticles and Flacopper on one side, or between any of them and the two control treatments (control+ and control-). Albumin levels were 4.85, 6.45, 4.65, 4.05 and 3.72 mg/Dl respectively; compared with 0.224 and 0.218 mg/Dl for the two control treatments (control+ and control-) respectively.

For creatinin, feeding rats on fodder mixed with juice of tomato harvested from plants treated with any of the three tested fungicides (CuNPs, Adwicopper or Flacopper) resulted in a significant elevation of creatinin level in blood without any significant difference among any of them. They recorded, 0.295, 0.296 and 0.317 mg/Dl respectively; compared with 0.224 and 0.218 mg/Dl in the blood of the two control treatments (control+ and control-) respectively.

Regarding the urea level, feeding rats on fodder mixed with juice of tomato harvested from plants treated with any of the three tested fungicides (**(CuNPs)**, Adwicopper or Flacopper) resulted in a significant elevation of urea level in blood; these levels were 19.25, 19.00 and 16.75 mg/Dl respectively compared with 12.0 and 11.5 mg/Dl in the blood of the control rats (control+ and control-) respectively. Copper nanoparticles and Adwicopper resulted in the highest urea level in rates blood, followed by Flacopper.

Feeding rats on fodder which contained 10% tomato juice from plants which had been treated with copper fungicides for six weeks, and recording the body weight of each rate weekly (Tab.4), revealed that, after one week of feeding on fodder mixed with the treated tomato juice, no significant body weight difference was recorded among the tested groups, neither the fungicide treatments nor the two control treatments. Body weight ranged from 114.0 to 117.3 g. After the second week, the mean body weight in all treatments increased, no significant body weight difference was recorded between any of the tested groups. Body weight ranged from 118.3 to 121.75 g. After the third week, significant differences were recorded. The body weight of the different treatments groups showed three categories. First category contained Adwicopper and Flowcopper (120 g for each). The second category contained (CuNPs) and Control– recording 125.0 and 124.5 g. respectively. The third category contained Control + with 129.5 g.

Table 4: Rat body weight (in gm.) during six weeks of feeding on fodder containing 10% tomato juice from plants treated with copper fungicides, compared with rats fed juice from non-treated plants.

Treatments	After 1 week	After 2 weeks	After 3 weeks	After 4 weeks	After 5 weeks	After 6 weeks
Copper (NPs)	117.3±2.2[a]	121.75±1[a]	125.0±1.6[b]	130.3±1.9[b]	115.3±2.3[c]	113.5±2.5[c]
Adwicopper	114.0±3.1[a]	118.3±1[a]	120.0±0.7[a]	123.3±1.2[a]	80.3±3.6[a]	75.3±3.3[a]
Flacopper	115.5±3.1[a]	119.3±1.4[a]	120.0±3.1[a]	123.8±2.6[a]	97.3±1[b]	93.3±4.1[b]
Control +	116.5±3.5[a]	121.5±4.8[a]	129.5±3.8[c]	136.0±2.3[c]	142±1.8[d]	147.3±1.7[d]
Control -	115.7±3.4[a]	118.3±3.2[a]	124.5±3.8[b]	129.5±3.3[b]	139.3±2.5[d]	142.0±1.9[d]

* Data representing body weight mean (in grams) ± SEM (standard error of the mean) and category letter

** Treatments with same letter showed no significant difference

After the fourth week, body weight in all treatments increased and could be significantly classified into three significantly different categories. The first category contained Adwicopper and Flacopper with the lowest body weight (123.3 and 123.8 g). The second category contained (CuNPs) and control- with 130.3 and 129.5 g respectively. The third category contained Control+ with 136.0.

After fifth week, the rat body weight could be significantly classified into four categories. The first contained Adwicopper with the lowest body weight (80.3 g). The second category contained Flacopper with 97.3 g. The third category contained (CuNPs) with115.3 g. The fourth category contained Control+ with 142.0 g. and Control-with body weight of139.3 g.

These results were more obvious after six weeks. The rat body weight could be classified into the same four categories too. The first category contained Adwicopper with the lowest body weight (75.3 g). The second category contained Flacopper with 93.3 g. The third category contained (CuNPs) with 113.5 g. The two control treatments (control+ and control-) represented the fourth category with the highest body weights, 147.3 and 142.0 g respectively.

Drawing a body weight (development) curve, for the experiment period (Fig. 4) showed that all the treatments resulted in gradual body weight increase till the fourth week. During this period control+ recorded the highest body increase (136.0g), followed by control- (129.5 g) and (CuNPs) (130.3g). After the fifth week, all the copper treatments resulted in drastically lost weight, in contrast to the two control treatments, in which the rats gradually gained weight along the six weeks. Adwicopper was the worst one, followed by Flacopper recording 75.3g compared with 142.0g recorded for control- and 147.3 g recorded for control+.

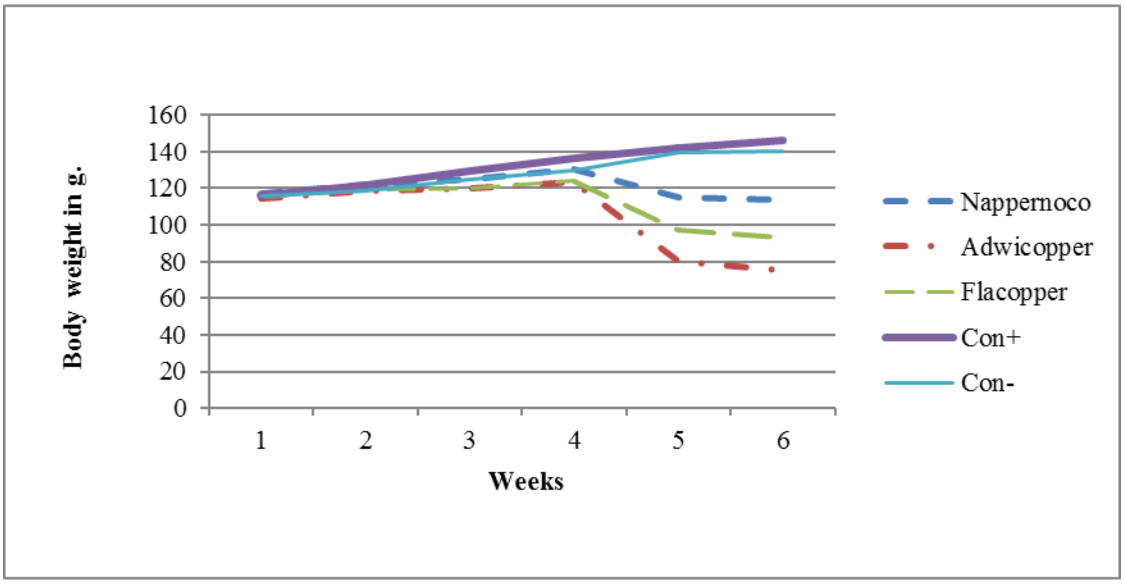


Fig 4: Rat body weight (in gm.) curve during six weeks feeding on fodder containing 10% tomato juice from plants treated with different copper fungicides, compared with rats fed juice from non-treated plants.

Effect on organ weight:

Feeding rats for six weeks, on fodder mixed 10% tomato juice from plants treated with copper fungicides, had in different effects on the different organs. Liver weight with (CuNPs) and Flowcopper was significantly reduced to 2.625 and 2.625 g respectively, compared with 3.33 and 3.04 g liver weight in the control treatments, control+ and control-, respectively. Adwicopper resulted in no significant difference compared with the two control treatments.

Calculating the relative liver weight to the body weight indicated that Adwicopper resulted in the highest relative liver weight (2.94%), followed by Flacopper (2.69%) and 2.32, 2.27and 2.16% in (CuNPs), control+ and control- respectively.

Adwicopper and Flacopper resulted in significant decreases of kidney weight, 0.585 and 0.625 g respectively, compared with 0.745, 0.815 and 0.795 g for (CuNPs), control+ and control- respectively.

According to the relative kidney weight to body weight, the treatments could be classified into two groups; the first group containing Adwicopper and the two control treatments (control+ and control-) with 0.58, 0.56 and 0.57% respectively; the second group containing (CuNPs) and Flowcopper with 0.66 and 0.67% respectively.

As for brain weight, however, no significant difference was found among the different treatments. (CuNPs) showed the highest relative brain weight with 1.16%, followed by Flawcopper with 0.96% and Adwicopper with 0.90%, then control+ and control- with 0.72 and 0.74% respectively.

Table 5: Effect of feeding rats for six weeks on fodder mixed with 10% tomato juice from plants had been treated with copper fungicides on the weight of different organs.

Treatments	Liver		Kidney		Brain	
	Organ Weight in g	RBW %	Organ Weight in g	RBW %	Organ Weight in g	RBW %
Copper (NPs)	2.625±0.05[bc]	2.32	0.745±0.03[a]	0.66	1.070±0.1[a]	1.16
Adwicopper	2.965±0.11[ab]	2.94	0.585±0.01[b]	0.58	0.900±0.0[a]	0.90
Flacopper	2.505±0.05[c]	2.69	0.625±0.02[b]	0.67	0.900±0.1[a]	0.96
Control +	3.330±0.24[a]	2.27	0.815±0.02[a]	0.56	1.055±0.1[a]	0.72
Control -	3.040±0.14[a]	2.16	0.795±0.01[a]	0.57	1.045±0.1[a]	0.74

* Data representing body weight mean (in grams) ± SEM (standard error of the mean)

and category letter

** Treatments with same letter showed no significant difference

***RBW% = relative organ weight to body weight (%)

DISCUSSION

Sulfur and copper are protective fungicides. They are the only non-organic fungicides allowed under organic agriculture guidelines (**Letourneau and van Bruggen, 2006).**

During the last few decades, nanotechnology evolved very rapidly, and nanoparticles were involved in most of our life branches (**Filipponi and Sutherland, 2013).** In this study the fungicidal effect of CuNPs. was compared with two recommended conventional copper fungicides in *vitro* and in *vivo*, in addition, to observe their toxic effects on experimental animals.

In *vitro*, all three tested copper fungicides showed remarkable fungicidal effects on mycelium growth. CuNPs showed in the least fungicidal efficiency, compared with the two conventional copper fungicides, Adwicopper and Flacopper; however, there was a remarkable higher efficacy of the copper Nps (copper sulfate pentahydrate NPs) compared with the bulk copper (copper sulfate pentahydrate). These data were confirmed in *vivo*. Calculating the disease severity and fungicidal efficiency for each fungicide showed that Adwicopper resulted in the least disease severity and the highest fungicidal efficiency followed by Flacopper, then CuNPs, which was the least effective one. Bulk copper sulfate was not included, the in *vivo* study due to relatively low efficiency in *vitro.* Since tomato fruits are harvested many times periodically during the growth time, it was very important to calculate the area under disease progress curve during the whole disease period. The area under disease progress curve revealed that no significant difference was found between Adwicopper and Flacopper. However, there was significant difference between Adwicopper and Copper NPs, but not difference between Flacopper and CuNPs. This data is in accordance with **Kanhed, *et al*., (2014), Ouda, (2014), Ponmurugana, *et al*., (2016)** and **Sidhu, *et al*., (2017)** they found that nano copper showed significant fungicidal effects against different plant pathogenic fungi. On the other hand, these data do not support the work done by **(Thejakumar and Devappa 2016)** they found that copper hydroxide was more efficient than copper oxychloride. Cu^{2+} and Cu^{+} ions are soluble in water and provide antifungal and antibacterial effects (biostatic elements) at low concentration levels, wherefore their contribution to the production of fungicides is, up to the present day, irreplaceable **(Rusjan, 2012).** Products such as copper belong to Group Y and have multisite activity. They interfere with several of fungus vital life functions. Copper oxychloride is a protectant fungicide and bactericide, which prevents infection on plants. Its mode of action is by interfering with the enzyme system of spores and mycelium, a process which is usually irreversible (**Burrows, *et al.* 2017**). On the other hand, Cu as either CuNPs or $CuSO_4$ causes oxidative stress and cell apoptosis programmed death of cell. In addition, Cu as either CuNPs or $CuSO_4$ impaired mitochondrial membranes **(Wang, *et.al.* 2015**).

The difference in the different tested fungicides efficiency may due to the chemical and physical properties of the tested fungicides. Efficiency of copper formulation is related to particle size and particle retention on the plant surface. Copper oxychloride particle size is 1.8 - 3.1 µm, copper hydroxide 2.5 - 3.1 µm, Copper sulphate 0.7 - 3.0 µm and our tested copper Nps ranged from 9.61 to 22.6 nm. The smaller the particle size the greater the surface area. Therefore, more particles are available to react. In addition, the smaller the particle size, the greater the surface area; this results in better adhesion on plant surfaces, and particles have difficulty dislodging (**Anonymous, 2016**). However, in this study, the tested fungicides reacted adversely, which may be due to the fact that, soluble coppers crude faster and require more frequent applications as they are less persistent on plant. The tested copper fungicides, classified according to their solubility as flow; copper oxychlorides - copper hydroxide then copper sulfate. The most soluble coppers crude faster and require more frequent applications as they are less persistent. The least soluble coppers are more persistent and release a supply of copper ions for a longer period. These least soluble formulations require less frequenter – application (**Anonymous, 2016).** (**Menkissoglu and Lindow.1994),** speculated that it is likely that the free copper ion is the only form of copper that is toxic to the organism. Since nano particles are very reactive, they react with the organic matter in the media or on the leaf surfaces resulting in complexes forms of copper. Such complexes forms of copper are not toxic to the organism and only the remaining free ionic copper will react against the organism, resulting in reducing the efficacy of the copper fungicide.

On the other hand, copper is an essential element for the normal healthy growth and reproduction of all higher plants and animals, especially for of hemoglobin in the blood, the formation of collagen and protective coverings for nerves. In combination with other metallic elements, along with fatty and amino acids as well as vitamins, Cu is necessary for normal metabolic processes. The human body is unable to produce metals; therefore the human diet must supply regular amounts of bioavailable Cu **(Rusjan, 2012).**

Copper toxicity is a central factor in many of today's modern disease epidemics including: cancer, Alzheimer's, Parkinson's, schizophrenia, OCD, ADD, rheumatoid arthritis, cardiovascular disease. Copper toxicity is even a major player in women's health issues such as estrogen dominance, Candida overgrowth, and PMS. While copper toxicity is a major cause for concern, it is something that can be effectively dealt with by powerful nutritional therapies (**Mcevoy, *et al.* 2014**). On other hand, all aspects of our life will benefit from the revolution in nanotechnology. This revolution will necessitate large-scale production of nano size particles/structures, new formulations and novel surface properties to meet demands of novel functions. Meanwhile, the rapid development of nanotechnology is likely to become a new source of human or environmental hazards through inhalation, ingestion, skin uptake, or injection of engineered nanomaterials in the workplace or the use of consumer products **(Zhao, *et al.* 2012).** Cu2+ and Cu+ ions are soluble in water and provide antifungal and antibacterial effects (biostatic elements) at low concentration levels, wherefore their contribution to the production of fungicides is, up to the present day, irreplaceable. On the other hand, high concentrations of copper salts affect physiological and biochemical processes in higher organisms. Copper takes part in numerous physiological processes and is an essential cofactor for many metalloproteins. However, copper excess leads to problems in cell function and metabolism, as copper surplus inhibits plant growth and impairs important cellular processes (*i.e.*, photosynthetic electron transport).

In this study, rats fed on fodder mixed with the juice of tomatoes harvested from plants treated with any of the three tested fungicides resulted in a significant elevation in the level of the liver enzyme alanine aminotransferase (ALT) compared with the two control treatments (control + control -). The highest ALT level was found in case of Adwicopper, followed by Flacopper then copper nanoparticles. Only rats fed on fodder mixed with the juice of tomatoes harvested from plants treated with Adwicopper showed significantly elevated aspartate aminotransferase (AST) levels. Aspartate aminotransferase and alanine aminotransferase are enzymes found mainly in the liver, but also found in red blood cells, heart cells, muscle tissue and other organs, such as the pancreas and kidneys. AST or ALT levels are a valuable aid primarily in the diagnosis of liver disease; it can be used in combination with other enzymes to monitor the course of various liver disorders. When body tissue or an organ such as the liver or heart is diseased or damaged, additional AST and ALT are released into the bloodstream, causing levels of the enzyme to rise. Therefore, the amount of AST and ALT in the blood is directly related to the extent of the tissue damage (**Huang, *et al.* 2006).**

Cholesterol level was significantly elevated in blood of rats fed on fodder mixed with the juice of tomatoes harvested from plants treated with any of three tested copper fungicides; with significant difference among them. Adwicopper recorded the highest cholesterol levels, followed by copper nanoparticles, then Flacopper. All the three copper fungicides (Copper nanoparticles, Adwicopper and Flacopper) resulted in significant elevated level of triglisroid; without significant difference among them, but with significant differences between any of them and the two control treatments. At the same time, feeding rats on any of the tested copper fungicides resulted in significant elevation of LDL levels. Adwicopper, resulted in the highest LDL level, followed by copper nanoparticles then Flacopper. (**Flower *et al.*, 2011**) reported the suppression of statins effectiveness by copper and zinc in yeast and human cells. Statin inhibit HMG-CoA reductase, which is the rate-limiting enzyme in the synthesis of cholesterol and used to treat high cholesterol and other sclerosis. In addition, these drugs act by decreasing liver cholesterol synthesis resulting in up-regulation of LDL receptors increased clearance of LDL from plasma, and diminution of plasma LDL levels (**Kostner, *et al.*, 1989**). Thus, suppression of statin effectiveness by copper leads to elevation of cholesterol, triglisroid and LDL levels in blood. The tested fungicides (Copper nanoparticles, Adwicopper and Flacopper) resulted in significant elevations of creatinine level. This data is in harmony with that found by (Gooneratne and Howell, 1980) who reported a sudden rise in the level of plasma creatine kinase in sheep after repeated dosing with an aqueous solution of copper sulphate. Copper nanoparticles, Adwicopper and Flacopper resulted in significant elevation of urea levels in blood. Copper nanoparticles and Adwicopper resulted in the highest urea levels in rat blood, followed by Flacopper.

(**Babaknejad *et al.*, 2015**) found that, Cu injection in rats induced nephrotoxicity with accompanying renal dysfunction; they suggested that, copper mediates in oxidative-induced renal dysfunction.

The body weight development curve during the six weeks of feeding the rats on fodder containing tomato juice from plants which had been treated with copper fungicides, showed that all the treatments resulted in gradual body weight increases till the fourth week. During this period control + recorded the highest body increase, followed by control- and CuNPs. After the fifth week, all the copper treatments resulted in drastically significant weight loss, in contrast to the two control treatments, in which the rats gradually gained weight throughout the six weeks. Adwicopper was the most toxic; followed by Flacopper, then CuNPs. Copper takes part in numerous physiological processes and is an essential cofactor for many metalloproteins. However, copper excess leads to problems in cell function and metabolism, as copper surplus impairs important cellular processes *i.e.,* electron transport (**Rusjan, (2012**). During the first 4 weeks of feeding, copper reacted friendly in the rats physiology resulting in normal growth; after the fourth week, the copper accumulated in the rats body and exceeded the normal levels, leading to disturbance of the cell physiology, resulting in weight loss. **(Sajid, *et al.* 2014)** reported that, NPs may behave differently when entering hepatic circulation. They can be hepatotoxic, or sludge in the biliary tree or the pancreatic ducts and may cause obstruction or gradual fibrosis. They may change the permeability of the gastrointestinal lining and can result in ulcers, weakening of the epithelium, cause metaplasia or dysplasia of the epithelium, malabsorption of nutrients, or in severe cases may lead to chronic bleeding. These data were estimation of the different organ weight confirmed the last data. Since, Copper (NPs) and Flowcopper resulted in significant decreases of liver weight, while Adwicopper resulted in no significant difference. At the same time, Adwicopper and Flacopper resulted in significant decreases of kidney weight.

It can be concluded that, although copper nanoparticles showed better efficacy in *vitro* than the bulk copper sulphate, there was no real efficiency difference compared with the two other fungicides, Adwicopper and Flacopper. This data was confirmed in *vivo.* Toxicological, however, all the three tested copper fungicides showed significantly different toxic effects, Adwecopper was the most toxic one.

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