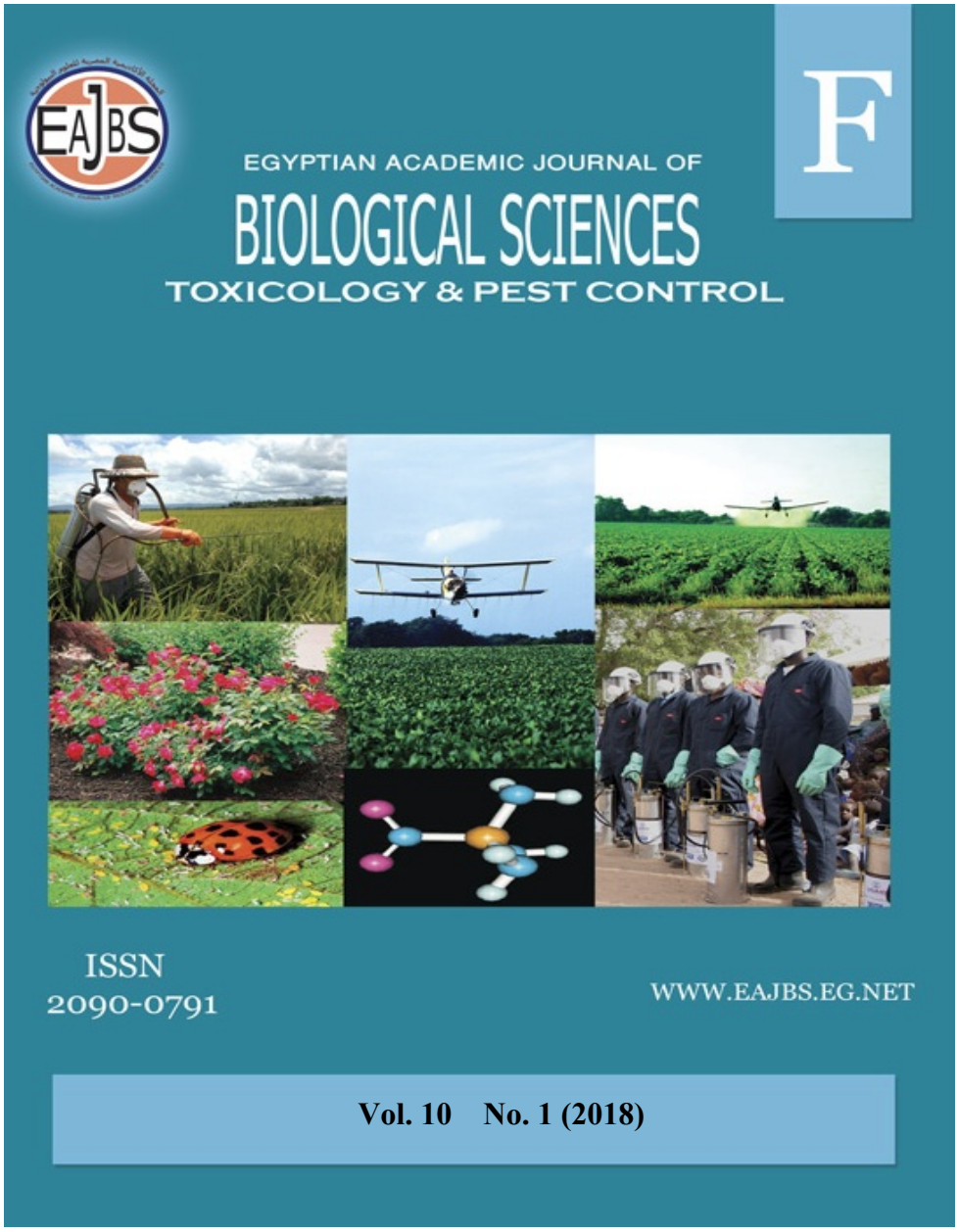

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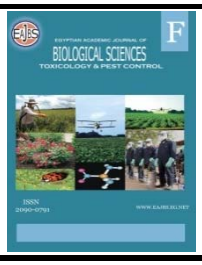

Effects of Two Neonicotinoids Insecticides on Some Anti-Oxidant Enzymes and Hematological Parameters in Egyptian Frogs, *Bufo regularis.*

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ABSTRACT

The effect of two neonicotinoid pesticides, Actra and Acetamore, on the Egyptian frog (*Bufo regularis*) was evaluated. Some parameters were selected to reflect and measure the stress caused by exposing frogs to 1/6 of the recommended field concentration. Stress parameters included malondialdehyde (MDA), the antioxidant enzyme, reduced glutathione (GSH) and superoxide dismutase (SOD) besides some hematological parameters. Results showed that activities of the enzymes GSH and SOD decreased significantly after treatments with both pesticides. GSH content decreased by 29.47% and 47.37% compared to control frogs, while SOD activity decreased by 11.37% and 18.88% after exposure to Actra and Acetamore pesticides, respectively. The exposure for both pesticides promotes significant increase in MDA content and also caused a significant reduction for serum albumin fraction and total proteins. The number of total red blood cells (RBCs) and hematocrit values showed a non-significant decrease, whereas the number of white blood cells (WBCs) increased significantly by 82.54% and 127.5%, after the exposure to Actra and Acetamore, respectively. Hemoglobin content and the blood indices such as Mean corpuscular volume (MCV), Mean Corpuscular Hemoglobin (MCH) and Mean Corpuscular Hemoglobin Concentration (MCHC) declined significantly only in Acetamore treated frogs. Changes caused by Acetamore exposure were much more profound for nearly all studied parameters in comparison to changes caused by Actra. Alteration took place in the studied parameters could have adverse influence on major physiological functions in frogs, which could result in more vulnerable adults.
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INTRODUCTION

Amphibians are subjected to a diverse set of environmental stressors. Amphibians are distinctive in many ways, with significant role in many ecosystem services, and they have an important ecological role. Contaminants have the potential

to cause lethal effects in amphibians that might reduce their survival rate. Exposure to sub-lethal concentrations of many contaminants can cause immunity suppression, malformations, compromised reproduction, and reduction in growth and development (Taylor *et al.,* 2005; Gahl *et al.,* 2011; Groner and Relyea, 2011).

Amphibians have a number of properties that make them useful bioindicator species, in particular their biphasic life and semi-permeable skin (Venturino *et al.,* (2003). Frogs also serve as good indicator species gauging much of the ecological changes within their environment because of their remarkable abundance in various ecosystem niches. (Wilson and McCranie, 2003).

Recently, decreased abundance of amphibian species has become a global phenomenon, and agricultural contaminants including pesticides, are thought to contribute to this decline (Houlahan and Findlay 2003; Mann *et al.,* 2009). Up to 90% of pesticides never reach their intended targets (Sparling *et al.,* 2001) and amphibians are one of the non-target groups mostly affected (Berrill *et al.,* 1994; Sparling *et al.,* 2001). Egypt has a relatively small (nine species) amphibian fauna whose current status and needs for conservation are not well observed. Egyptian amphibians are facing potential threats that affect their density, abundance and vitality including the overuse of pesticides (Ibrahim, 2013).

The most common route for amphibians exposure to pesticides is agricultural runoffs. Pesticides are picked up into frogs by passive diffusion through the gills, skin, or gastrointestinal tract via the dietary route. The routes via gills and skin are involved in the uptake by tadpoles, while the skin would be a main route for the adult frogs. Skin absorption by dipping their partial or whole body in water containing a pesticide, is also a key route in the uptake process especially in the adult frogs (Katagi and Ose 2014).

Neonicotinoids insecticides offered great promise for long-term plant protection. These pesticides mimics the nicotine receptor of the nervous system (Tomizawa and Casida, 2005). They are utilized in veterinary medicine, urban landscaping, and crop protection.

The neonicotinoids insecticides, has become one of the fastest growing chemical class of insecticides on the global market (Jeschke *et al.,* 2011; Casida and Durkin 2013). Neonicotinoids have proven less toxic than other classes of pesticides. They are less toxic to birds and mammals than older classes of insecticides and thus have been promoted as being safer for wildlife. On the other hand, they remain toxic within the plant for longer than other insecticides. One thing that has made neonicotinoids popular in pest control is their water solubility, which allows them to be applied to soil and be taken up by plants. Evidence suggests that systemic insecticides may remain in plant for months or even more than a year (e.g., Maus *et al.,* 2005). Furthermore, relatively rapid degradation of neonicotinoids in both soil (half-life = 39 days) and water (half-life < 3 hours) is another reason that makes this class of insecticide particularly promising (Kollman and Segawa, 1995; Moza, 1998). Some neonicotinoids can persist for extended periods in soil. For example, Acetamiprid (Acetamore) and thiamethoxam (Actra) half-life in soil are (1-8 days) and (25-100 days) (aerobic soil metabolism) (EPA 2002 and Syngenta Group 2005, respectively). Also clothianidin, a primary metabolite of thiamethoxam, has a soil half-life of 148 to 1,155 days depending upon soil types (EPA 2003).

Direct or indirect exposure to pesticide may lead to generation of reactive oxygen species (ROS). Oxidative stress is a general response to toxicity induced by many contaminants. Therefore, its assessment is included in bio-monitoring programs as a nonspecific biochemical marker (Livingstone, 2001). The enzymatic and non-

enzymatic antioxidant defense systems protect cells against the cytotoxic effects of ROS under normal conditions. Actually, it has been shown that numerous pesticides can impair the antioxidant defense system in tadpoles. However, the sensitivity of species to these chemicals may be species-specific (Juliane Silberschmidt Freitas *et al.,* 2017).

Blood parameters respond to low doses of pollutants and are considered to be pathophysiological indicators of the whole body. Therefore, hematological parameters have become promising biomarkers for measuring pollution effects and considered important in diagnosing the structural and functional status of animals exposed to contaminants (Maheswaran *et al.,* 2008).

It is widely accepted that pesticides can reach amphibians' environment, causing several impacts. Nevertheless, a limited number of studies have been performed to assess the potential negative effects that pesticides inflict on frogs. As reported by Venturino *et al.,* (2003), toxicological research on amphibians has been rather scarce compared with that on other vertebrates with only 2.7% of the literature addressing aspects of ecotoxicology over the last 25 years (up to 1998). Also some of the recent studies mentioned that only a few reports have investigated antioxidant responses in amphibians (Jones *et al.,* 2010).

At local level, the impacts of agricultural pesticides on Egyptian amphibians have not been well explored and sub-lethal effects have not been widely studied and no research have been focused on antioxidant responses in amphibians after exposure to neonicotinoids pesticides. Therefore, our objective was to investigate how some enzymatic and non-enzymatic antioxidant parameters of the frog (*Bufo regularis)* would respond to exposure of a sub lethal concentration of two neonicotinoids pesticides, as well as to observe the changes in some hematological parameters.

MATERIALS AND METHODS

Chemicals:

Actara 20% with thiamethoxam as active ingredient (chemical name: 3-[(2-chloro-5-thiazolyl)methyl]tetrahydro-5-methyl-N-nitro-4H-1,3,5-oxadiazin-4-imine, Syngenta Company) and Acetamore 25% with acetamiprid as active ingredient (chemical name: N-[(6-chloro-3-pyridyl)methyl]-N'-cyano-N-methyl-acetamidine) were used in this study. All chemicals used in this study were purchased from Sigma-Aldrich except for the pesticides, which were obtained from a local market. Other chemicals were obtained from highest commercial grade available. Kits for determination of albumin and total protein were purchased from DIACHEM Ltd Budapest, Hungary, while kits for measuring SOD, GSH, and MDA were purchased from BioVision Inc., Milpitas, USA.

Experiment Design:

Adult toads, *Bufo regularis* of both sexes were collected during night time by hand net from their spawning ponds in unpolluted areas far away from agricultural areas. Toads of relatively similar sizes were selected (35±10 g) and transferred to glass container (51 × 60 × 33 cm3) supplemented with mud, sand, and a pool of 2 L of dechlorinated tap water, to provide a natural habitat to the frogs to provide the option of both aqueous and dry environment (Allran and Karasov, 2001). Water was changed every 3 days and the container was cleaned thoroughly. The animals were fed every two days with earth worms.

Frogs were maintained under lab conditions (12/12 hrs. light/dark cycle and at 29/32°C) for a period of seven days for acclimatization to the laboratory condition and

then used for experimentation in the eighth day. Dechlorinated tap water was used for acclimatization, control tests and for the preparation of pesticides concentrations.
To study the effect of the two neonicotenoid insecticides, fifteen frogs were placed in each glass container and sorted out into three groups; one set for control where toads exposed only to dechorinated tap water. The second and third groups were exposed to 1/6 of the Egyptian recommended field concentration of Actra (0.03 g/l) and Acetamore (0.04 g/l). Fresh solutions of pesticides were prepared each 3 days, which mean that animals were exposed four times to the pesticides concentrations within twelve days. Animals were sacrificed by pitching, and blood was collected in heparinized polyethylene tubes to assess hemoglobin, white blood cells, and red blood cells in both experimental and control frogs. Alongside, liver samples were also collected and kept at 80°C until analysis of biochemical parameters. Finally, all experiments were carried out in accordance with the Egyptian laws and university guidelines for the care of experimental animals.

Biochemical Analyses:

Serum total protein level was measured according to the method of Armstrong & Carr (1964), in which cupric ions in an alkaline solution react with the peptide bonds of protein and polypeptides containing at least two peptide bonds to produce a violet colour complex. The absorbance of the complex at 546 nm is directly proportional to the concentration of protein in the sample. Protein content of the liver tissue homogenate (2.5%) prepared in distilled water was measured according to the method of Lowry *et al.,* (1951) using Bovine serum albumin as standard.

Serum albumin level was measured according to the method of Doumas *et al.,* (1971). Albumin binds with bromocresol green to produce a blue-green complex at pH4.2. The change in absorbance at 628 nm correlates with the concentration of albumin.

The GSH content of the liver tissues as non-protein sulphydryls was estimated according to the methods described by Sedlak & Lindsay (1968). 10% TCA was added to the homogenate and the mixture was centrifuged. 1.0 ml of supernatant was treated with 0.5 ml of Ellimans reagent (19.8 mg of 5, 5-dithiobisnitro benzoic acid (DTNB) in 100 ml of 0.1% sodium nitrate) and 3.0 ml of phosphate buffer (0.2, pH 8.0). The absorbance was read at 412 nm.

Superoxide Dismutase activity was determined by its ability to inhibit the auto oxidation of epinephrine determined by the increase in absorbance at 480 nm as described by Sun & Zigma (1978). The reaction mixture (3 ml) contained 2.95 ml 0.05 M sodium carbamate buffer pH 10.2, 0.02 ml of liver tissue homogenate and 0.03 ml of epinephrine in 0.005N HCl was used to initiate the reaction. The reference cuvette contained 2.95 ml buffer, 0.03 ml of substrate (epinephrine), and 0.02 ml of water. Enzyme activity was calculated by measuring the change in absorbance at 480 nm for 5 minutes.

The activities of the antioxidant enzymes were measured simultaneously in triplicate for each sample, using a Shimadzu UV-160 spectrophotometer with a temperature-controlled cuvette holder.

Malondialdehyde (MDA) an index of lipid peroxidation was determined using the method of Buege & Aust (1978). 1.0 ml of the supernatant of liver tissue was added to 2 ml of (1:1:1ratio) tricarboxylic acid-thiobarbituric acid-hydrochloric acid reagent TCA-TBA-HCl reagent (thiobarbituric acid 0.37%, 0.24N HCl and 15% TCA) boiled at 100ºC for 15 minutes and allowed to cool. Flocculent materials were removed by centrifuging at 3000 rpm for 10 minutes. The supernatant was removed and the absorbance read at 532 nm against a blank. MDA was calculated using the molar extinction coefficient for MDA-TBA complex of 1.56 × 105 M-1 CM-1.

Hematological Parameters:

At the end of the treatment, heart blood samples were collected from ether anaesthetized frogs in EDTA tubes. Blood samples were diluted in a rate of 1: 100 with Shaw's solution (0.4 ml formaline , 10 mg crystal violet, 3.8 g sodium citrate add to 100 ml distilled water). Red blood cells (RBCs) count and total leukocyte count (TLC) were carried out immediately after sampling using Neubauer's improved haemocytometer (Gatten and Brooks 1969).

The hematocrit (Ht) was determined by the micro hematocrit method and hemoglobin concentration (Hb) by modified cyanmethemoglobin method (Das and Mahapatra 2014 (while differential leukocyte counts (DLC) were performed in blood smears after staining with Giemsa stain according to Heatley and Johnson (2009).

Statistical Analysis:

Results are given as mean values ± standard deviation (SD) for five animals. The obtained data were analyzed by using one way ANOVA. Differences between treatments and the control were tested for significance by using Least Significant Difference (LSD) Test at $P<0.05$ level of significance.

RESULTS AND DISCUSSION

Biochemical Parameters:

This study aimed mainly to assess the effects of Actra and Acetamore pesticides on some biomarkers in frogs, *Bufo regularis*, after exposure to 1/6 of the recommended field concentration of both pesticides. Biomarkers selected for stress monitoring in this study include malondialdehyde (MDA) and some antioxidant enzymes (Table 1). Liver, is a highly metabolically active organ, with a high activity of antioxidants and associated enzymes, so that it is the main organ responsible for detoxification of xenobiotics (Perez-Campo *et al.,* 1993, Czarniewska *et al.,* 1995).

Subjected to one way ANOVA our results showed a significant decrease of GSH content and SOD activity in frogs liver after 4 times of exposure to Actra and Acetamore pesticides compared to the control (Table 1). The GSH content has been reduced by 29.47 and 47.37 % in the frogs treated with Actra and Acetamore pesticides respectively. While the reduction percentage in case of SOD activity was 11.37 and 18.88%, respectively.

According to Van der Oost *et al.,* (2003), the activity of antioxidant enzymes may be increased or inhibited depending on the type and concentration of the stressor. The present study documented a considerable impairment of antioxidants enzyme of *B. regularis* exposed to a commercial formulation of the two tested neonecotinoids. These results are attributed to oxidative stress induced by both insecticides (Qiao *et al.,* 2005). Oxidative stress is a general response to toxicity induced by many contaminants that disturb the balance between oxidants and antioxidants. This imbalance is due to the depletion of antioxidants like GSH or excessive accumulation of reactive oxygen species (ROS) that depletes SOD, or both (Scandalios, 2005).

Regarding the reduction of SOD activity recording in this study, Dimitrova *et al.,* (1994) suggest that the superoxide radicals by themselves or after their transformation to H2O2 can cause an oxidation of the cysteine in the enzyme and decrease SOD activity.

GSH is perhaps the most important ROS scavenger participating in the control of the intracellular redox status (Finkel and Holbrook, 2000). The considerable decline in GSH content occurred under the current experimental condition may be due to its

utilization to challenge the prevailing oxidative stress under the influence of ROS generated from Actra and Acetamore stress.

The considerable impairment of antioxidants enzyme in *B. regularis* reported in the this study is in line with previous studies on *S. fuscovarius* tadpoles exposed to the commercial formulation of Fipronil, a Phenylpyrazole insecticides (Margarido *et al.,* 2013), and frog *Rana esculenta L* exposed to Paraquat, a bipyridinium herbicide for 6 days (Czarniewska *et al.,* 2003), *P. nattereri* tadpoles exposed to Fipronil for short periods (Gripp *et al.,* 2017). In addition, Hai *et al.,* (1997) reported that alterations in the cellular antioxidant defense have been associated with organophosphorus insecticides exposures. Previous studies concluded that the inhibition of these antioxidant enzymes should decrease the ability of frogs to metabolize environmental xenobiotics, turning the animals more vulnerable for intoxication.

MDA is one of the end products of lipid peroxidation. Imbalance of free radicals within the body can affect processes like lipid peroxidation that are vital to the survival of an animal (Akhgari *et al.,* 2003). Our results showed that exposure to Actra and Acetamore pesticides promotes MDA content significantly in the liver tissues of frogs compared to the control (Table 1), with no significant difference observed between both pesticides. The level of MDA in Acetamore treated group (0.16 U/mg protein) was almost three time the MDA level (0.05 U/mg protein) in control group.

This elevation is probably resulted from the degeneration of polyunsaturated fatty acids of cell membranes after treatment with both pesticides that promoted free radicals elevation.

Our results are in accordance with Özkol *et al.,* (2011) who concluded that exposure of frogs (*Rana ridibunda Pallas*) to Omethoate organophosphate insecticide for 24, 48, 72 or 96 hrs., was characterized by significant increase of MDA levels in lung and stomach tissues. It has been also reported that organophosphorus pesticides increase the lipid peroxidation level in vertebrate (Bagchi *et al.,* 1995; Gultekin *et al.,* 2000). Kanter and Celik (2011) have also reported an elevation of MDA levels in liver and other tissues of frogs (*Rana ridibunda*) following exposure to fenthion (organophosphorus insecticide). It has been also reported that organophosphorus pesticides increase the lipid peroxidation level in vertebrate (Bagchi *et al.,* 1995; Gultekin *et al.,* 2000).

Proteins are one of the earliest indicators of pesticides poisoning. The results of this study demonstrated a significant decrease ($P<0.005$) in the mean levels of serum total protein (24.5% and 36.6%) and albumin (30.7% and 37.6%) after exposure to Actra and Acetamore respectively (Table 1). Moreover, differences in the reduction rate between animals treated with Actra and Acetamore pesticides were not significant.

Our finding regarding proteins are in agreement with that obtained by other investigators who reported lower levels of total proteins in animals exposed to pesticides. Mahananda and Mohanty (2012) documented a reduction in protein content by 42.85% and 48.57% in liver tissue of frogs (*Bufo melanostictus*) exposed for 96 h, to 25 and 50 ppm of malathion, an organphosphorus insecticides respectively. Pradhan and Pradhan (2016) also reported a reduction of total protein content in both liver and brain of Indian toad after treatment with Sevin, a carbamate insecticide. Gopal *et al.,* (1997) observed a decreasing level of serum protein, albumin and globulin level in the fish, *Cyprinus carpio* exposed to some essential and toxic heavy metals.

Most of the previous studies have attributed the reduction in protein to less incorporation of amino acids in the translation process that mainly happens in hepatic tissue where about 25% of albumin is produced in liver (Park *et al.,* 2014). Albumin is considered the most abundant circulating protein in plasma accounting for

approximately 60% of total plasma protein (Roche *et al.,* 2008). The presence of hepatic oxidative stress in both insecticides treated groups illustrates the hepatic failure to produce albumin and hence its reduction in peripheral blood. Another explanation is that the albumin decrement and subsequently total protein are considered physiological adaptation to compensate for pesticide stress. To overcome the stress, exposed animals use more energy, which leads to stimulation of protein catabolism (Sancho *et al.,* 1998).

Table (1) Effect of two neonicotinoids insecticides on some toxicological parameters in *Bufo regularis*

Pesticides	Total protein (g/dl)	Albumin (g/dl)	GSH (U/mg protein)	SOD (U/mg protein)	MDA (U/mg protein)
Actara	3.42±0.082 [b]	2.1±0.064 [b]	0.67±0.036 [b]	4.13±0.058 [b]	0.13±0.010 [a]
Acetmore	2.87±0.065 [c]	1.89±0.061 [c]	0.5±0.014 [c]	3.78±0.080 [c]	0.16±0.009 [a]
Control	4.53±0.066 [a]	3.03±0.058 [a]	0.95±0.014 [a]	4.66±0.0127 [a]	0.05±0.002 [b]

Results are expressed as mean ± SD for 5 animals.
Values bearing different superscripts in a column differed significantly at $P < 0.05$

Hematological Parameters:

Intrinsic and extrinsic factors contribute to the wide variability in normal hematological parameters in amphibians. Reference values are scarce, and normal hematology of many species is poorly understood (Allender and Fry, 2008). Despite the limitations, it is possible to obtain hematologic data which is useful in assessing an animal's health. In this article, effects of two neonicotinoids pesticides on some hematological parameters are described (Table 2).

Table (2) shows the values of the total RBCs and WBCs at the end of the experiment. In Actra and Acetamore treated frogs the numbers of RBCs were reduced non significantly by 5.5% and 11.04%, respectively, in comparison to control frogs. Hemoglobin content has been decreased nonsignificantly in Actra treated group (15.11%) and significantly in Acetmore (36.38%) treated group.

The blood indices such as MCV, MCH and MCHC have significantly declined in Acetmore treated group than the control one. While these indices did not change significantly in Actra treated group (Table 2).

Table 2: Effects of two neonecotinoids pesticides on some hematological parameters of *Bufo regularis*.

Pesticides	Hemoglobin (g/100 cc)	RBCs (10^6/mm^3)	HCT (%)	WBCs (10^3/mm^3)	MCV (Femto litre)	MCH (pg)	MCHC (g/dl)
Actara	**8.54 ± 01.65[ab] (15.11%)**	**3.08±0.44 [a] (5.5%)**	**26.42±4.11 [a]**	**15.26±5.25 [a] (82.54%)**	**85.85±6.52[a] [b]**	**27.61±1.74 [a]**	**32.38±3.74 [a]**
Acetmamore	**6.40±02.30[b] (36.38%)**	**2.90±0.54 [a] (11.04%)**	**23.52±6.61 [a]**	**19.02±5.91 [a] (127.5%)**	**70.58±20.82 [b]**	**20.91±4.66 [b]**	**26.64±2.83 [b]**
Control	**10.06±02.71 [a]**	**3.26±0.06 [a]**	**30.76±8.88 [a]**	**8.36±2.44[b]**	**92.80±10.05 [a]**	**30.49±3.54 [a]**	**32.86±1.60 [a]**

Data expressed as mean ± SD for 5 animals.
In parentheses; percentage change against control
Values bearing different superscripts in a column differed significantly at $P < 0.05$

The use of hematological parameters for health assessment of amphibians has been reported in some species, such as the common toad (*Bufo arenarum*) in agricultural areas in Argentina (Cabagna *et al.,* 2005), the marsh frog (*Rana ridibunda*) in an industrial area in Bulgaria (Zhelev *et al.,* 2006), and the northern leopard frog (*Lithobates pipiens*) in pesticide contaminated areas in Canada (Shutler and Marcogliese, 2011).

Our results are suggestive for microcytic hypochromic anemia in Acetmore treated group. This anemia could be attributed to impaired erythropoiesis due to the direct effect of pesticides on hematopoietic centers like bone morrow, liver, and kidney or due to interferance with iron absorption from gastrointestinal tracts (Ford, 2013). Current results are in agreement with several previous reports such as Pradhan and Pradhan (2016) who observed that the haemoglobin, WBC and RBC of *Rana cyanophlictis* were reduced drastically after 4, 48, 72 and 96 hrs. of exposure to 25 and 50 ppm of Sevin insecticide . Also Malathion has a strong potential to reduce hemoglobin, WBC, and RBC in *Bufo melanostictus* (Mahananda and Mohanty 2012).

The number of WBCs has significantly increased by 82.54% and 127.5% in Actara and Acetmore groups, respectively, than control one. The increase in the total WBCs count may play a part in the immunological defense system during exposure to toxicant (Dick and Dixon, 1985).

The leukocytosis recorded in this study could be attributed to the upsurge in leukocytes mobilization stimulated by the severe stress caused by insecticide application (Celick *et al.,* 2009). This would also indicate the more profound impact of Acetmore in comparison to that of Actra. Similarly, the immune system activation indicated by leukocytosis could explain the stressful condition and/or the need for removal of cellular debris (Garg *et al.,* 1989) resulted from both insecticides as well as the possibility of inflammation in liver tissues (Yousef *et al.,* 2003) triggered by higher oxidative biomarkers observed in this study.

Conclusion:

Efforts to elucidate the effects of pesticides on frogs toxicity have not been well pursued in Egypt. *Bufo regularis* were chosen to study the effect of sub lethal exposure to two neonicotinoids pesticides. Short exposure to sublethal concentration of commercial formulation of Actra and Acetamore (1/6 of field recommended concentration for 4 times within 12 days), has negatively affected SOD, GSH, MDA, and hematological parameters. The effect of Acetamore pesticide was much more profound for nearly all studied parameters. The alteration that took place in the studied parameters would have an adverse influence on major physiological events in the studied frogs, which could result in more vulnerable adults, promoting adverse consequences at population level.

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ARABIC SUMMERY

تأثير مبيدين من مجموعة النيونيكوتينويدات علي بعض الانزيمات المضادة للأكسدة ومؤشرات الدم في الضفادع المصرية

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تمت دراسة تأثير اثنين من المبيدات التي تنتمي الي مجموعة النيونيكوتينويدNeonicotinoids وهما مبيد اكترا ومبيد اسيتامور علي بعض الانزيمات المضادة للاكسدة وبعض مؤشرات الدم فى الضفادع المصرية .تم جمع الضفادع من مناطق بعيدة عن مصادر التلوث وابقاؤها تحت الظروف المعملية لفترة حتي يتم تأقلمها .تمت معاملة الضفادع بتركيزات تحت مميتة من المبيدين تساوى 6/1من الجرعات الموصي بها حقليا، كلا علي حدة .تمت دراسة تأثير التعرض لهذين المبيدين علي بعض المؤشرات التي تشمل مالونداياالدهيد(MDA)، جلوتاثيون المختزل (GSH)، سوبر اكسيد ديسميوتيز (SOD)وبعض مؤشرات اخري الى جانب عدد من المؤشرات الخاصة بالدم .اظهرت النتائج ان التعرض لتركيزات المبيدات المختبرة قد ادي الي الخفض المعنوي لانزيم جلوتاثيون المختزل (GSH)ونشاط انزيم سوبر اكسيد ديسميوتيز (SOD)وكان الخفض مقارنة بالكنترول بنسبه 29.47%و 47.37%في حاله انزيم جلوتاثيون المختزل، و بنسبه 11.37%و 18.88% فى حالة انزيم سوبر اكسيد ديسميوتيز بعد التعرض لمبيدى اكترا واسيتامور بالترتيب. كما ادى التعرض لهذه المبيدات الى زيادة معنويه في مستوى مالوندالدهيد بينما انخفض معنويا محتوى الألبومين والبروتين الكلى في الدم .

بالنسبة لمؤشرات الدم فقد حدث انخفاض غير معنوى في عدد كرات الدم الحمراء(RBCs) وقيم الهيماتوكريت (hematocrit)،بينما ارتفع عدد كرات الدم البيضاء(WBCs) معنويا بنسبه 82.54%و 127.5% بعد التعرض لمبيدى اكترا واسيتامور بالترتيب .كما انخفض محتوى الهيموجلوبين وبعض المقاييس الاخرى مثل (MCV)و (MCH)و (MCHC)معنويا فقط في حالة المعاملة بمبيد اسيتامور. اظهرت النتائج ان تأثير مبيد اسيتامور كان اشد من تأثير مبيد اكترا على جميع المؤشرات التي تم دراستها .التغيرات التي حدثت على المؤشرات المدروسة يمكن أن يكون لها تأثير سلبي على الوظائف الفسيولوجية الرئيسية في الضفادع، والتي يمكن ان تجعل افراد العشيرة أكثر عرضة للخطر.