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Oxidative stress and X-ray metal assessment in ovarian tissues of the beetle, *Calosoma olivieri* (Coleoptera, Carabidae) collected from Kafr El Zayat, Egypt

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ABSTRACT

X-ray analysis was applied to estimate the percentages of heavy metals in ovarian tissues of the beetle *Calosoma olivieri*. This research was performed to assess the influence of heavy metals on biochemicals in ovarian tissues in the ground beetle *Calosoma olivieri.* A total of 320 beetles with an average body weight of 1.96 g were obtained from two locations the garden of the Faculty of Science, Alexandria University, Alexandria, Egypt, which was previously classified as an uncontaminated zone, and a site surrounding the KZ factory of pesticides and chemicals. The results revealed a high activity level of MDA, Low activity level of glutathione-S – transferase GST, Glutathione Peroxidase- GPx, Reduced glutathione GSH and glutathione reductase GR, Ascorbate-Peroxidase APOX, Superoxide dismutase SOD, Catalase activity CAT, on the other side high concentration of Protein carbonyl. Our data showed the detection of heavy metals in soil samples collected from the polluted group more than those of control site samples. The results showed that *C. olivieri* is a valuable indicator of heavy metal pollution. Moreover, the metrics under examination are valuable markers for evaluating the deleterious consequences of environmental pollutants.

KEYWORDS: Heavy metals, Insects, Soil pollution, Toxicity, Oxidative stress, X-ray analysis.

INTRODUCTION:

Heavy metals are well-known environmental pollutants owing to their toxicity, presence in the atmosphere, and ability to accumulate in the human body via bioaccumulation. Toxic heavy metal poisoning of terrestrial and aquatic ecosystems is a serious environmental issue that impacts public health. The majority of heavy metals are found in nature, while some come from human activity (1). The high atomic mass and toxicity of heavy metals to living things are their defining characteristics. The majority of heavy metals can be fatal to humans and pollute the ecosystem and atmosphere. The food chain can expose humans and other living things to heavy metals, which can become extremely dangerous when they combine with other environmental factors like water, soil, and air (2).

A significant fraction of the heavy metals with high atomic weight and density are found in the periodic table. The majority of them are found in the

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biosphere, which includes rocks, soils, and water. They are also released into the environment by manmade resources, primarily those used in commerce and industry. Heavy metal toxicity has long been understood, according to Qidong (3).

Heavy metals have several undesirable repercussions on the environment; for example, the conversion of mercury into methylmercury in the presence of water creates sediments with high toxicity (4). Chromium is used extensively in industry and can be carcinogenic (5). However, some heavy metals are involved in the control of certain physiologic bodily functions. Naturally found vital heavy metals penetrate the body via food, air, and water, where they regulate numerous biological activities (6). Most of the toxic heavy metals including lead, thallium, cadmium, and antimony, are common in industrial operations and are substantial polluters of the environment.

Environmental problems have always received immense attention from scientists. Toxicant pollution is a critical environmental concern that has posed serious threats to human health and agricultural production. Heavy metals and pesticides are top of the list of environmental toxicants endangering nature (7).

Heavy metals arise from many sources, such as industry, mining, and agriculture. In terms of the sources in the agricultural sector, these can be categorized into fertilization, pesticides, livestock manure, and wastewater (8). Recently, the risk of heavy metals pollution in the environment has been increasing rapidly and creating turmoil, especially in the agricultural sector, by accumulating in the soil and plant uptake (9). The heavy metals contamination problem has become urgent and needs radical and practical solutions to reduce the hazards as much as possible.

Today, an ever-rapidly increasing human population also increases their consumption needs. To make life more standardized, and to increase production and productivity, the application of widespread and unconscious use of chemicals causes direct environmental damage, such as air, soil, and water pollution, degrading plant and animal health and existence (10). Among these chemicals, pesticides are used extensively in the fields of agriculture, public health, environmental health, and veterinary medicine. Pesticides are chemical substances used to prevent, control, or reduce harmful biological organisms such as insects, plant pathogens, weeds, molluscs, birds, mammals, fish, nematodes, and microorganisms that are harmful to human and animal food sources and also to the ecosystems that inhabit them (11).

These chemicals, which are useful when applied at the required dosages and durations, may affect the non-target organisms as a result of their careless and intensive use while polluting the soil and aquatic ecosystem (3). Pesticides are transported by surface streams, evaporated into the atmosphere, go through the absorption/desorption process, infiltrated from the soil or by taking plants into the plant, or are degraded by the chemical, as microbial and photodegradation directly from one ecosystem to another. The transport of pesticides into aquatic ecosystems is direct, via spray application to the water sources or by being mixed into the groundwater. This phenomenon threatens the life of the environment (12).

The structures of these biological membranes and toxic compounds are the most important factors controlling access to the organism. For example, small molecules and fat-soluble substances are taken by passive diffusion that does not require energy use by the organism (13).

The insect is utilized as an efficient bioindicator of heavy metal pollution because of their diverse richness of species, easy handling, and traps are good enough for effective statistical analysis. They are generally collected for their role as predators and significant for biological control such as spiders and

beetles (14). Heavy metals have a negative influence on insects impacting their fecundity, weight, mortality, and developmental stages. Insects can be impacted directly by various means as associated with polluted soil and air deposition. Parasites and predators are also affected if they consume insects that have a greater number of heavy metals (15).

Ground beetles as efficient bioindicators are commonly used by researchers because they show a response to ecological variation as a consequence of anthropogenic activities including overgrazing, and soil and land pollution (16). Concerning their cosmopolitan distribution in land, Carabid beetles are commonly utilized to assess the heavy metal pollution in soil. Previously, it was discovered that the significant bioaccumulation factor range of mercury and arsenic in research on *Carabus lefebvri* indicating that beetles were favorable for assessing mercury and arsenic in the environment (17). So, in this study, we used *Calosoma olivieri* as a bioindicator for assessing the effect of heavy metals exposure on the biochemical functions in ovarian tissues.

Materials and Methods

Sampling procedure & Study areas

A total of 320 beetles with an average body weight of 1.96 g were obtained from two locations the garden of the Faculty of Science, Alexandria University, Alexandria, Egypt, which was previously classified as an uncontaminated zone, and a site surrounding the KZ factory of pesticides and chemicals (18) The beetles were immediately transported to the lab and then characterized as *Calosoma olivieri*, Family: Carabidae. Preliminary collections of coleopteran insects inhabiting the selected sites showed that *C. olivieri* is a dominant adult species in the studied sites. The specimen was identified at the Department of Entomology, Faculty of Agriculture, Alexandria University as *C. olivieri*. The studied species belong to the family Carabidae.

Two sites were chosen for sampling the studied insect. These sites were (A) the garden of Faculty of Science El Shatby, Alexandria University, Alexandria, Egypt, which is considered a reference site (14) and (B) an urban area with high population density, The KZ Company for pesticides and chemicals is located in Kafr EL-Zayat, Al Gharbia governorate.

Live specimens of *C. olivieri* were collected randomly from different sampling areas in July 2022. The sampling areas in Kafr El-Zayat (the polluted site) were selected around the chemicals and fertilizers factories. The mean air temperature ranged from 27 to 35 °C and the mean relative humidity was 76 %. 320 insects were collected from each site. The specimens were sexed. 160 females were transported to the lab. Beetles were anesthetized with ethanol (95 %) and then dissected under a dissecting microscope in a drop of Ringer's physiological solution. The abdominal cavity was opened and the ovaries were removed. Maintenance of the insects was done in compliance with ethical guidelines for the protection and use of laboratory animals. Synchronously with the beetle's collection, soil samples at a depth of 25 cm below the surface were gathered from the mentioned sites.

Fig (1): Map of the polluted location

Determination of heavy metals in soil samples and ovarian tissues of *C. olivieri*.

Metal percentages in the soil and the ovarian tissues were determined by using energy-dispersive X-ray microanalysis (EDX). This analysis was applied using a JEOL (JSM-5300) scanning microscope at Electron Microscope Unit (E.M.), Faculty of Science, Alexandria University, Egypt. ovarian tissues were immediately fixed in the fixative solution at 4 ◦C for 3 h, then post-fixed with 2% osmium tetroxide (OsO4) for 2 h at 4 ºC. Fixed samples were washed in 0.1 M phosphate buffer and dehydrated through a series of ethanol concentrations, mounted on an aluminum stub, and coated with gold-palladium in a sputter coating unit (JFC-1100 E). Specimens were then visualized using SEM. For the accuracy of the analytical results, eight samples of soil and testicular tissues were analyzed from each site. Peaks were allocated automatically by the SEM–EDX software for identification. Measuring line intensities for each element in the sample and the same elements in calibration standards of known composition occurred. A stationary spot (X500) was analyzed at random for 110 sec.

Biochemical analyses

Ovarian tissues were obtained by dissecting the beetles on ice, and the tissues were then washed three times with sterilized saline solution before blotting with filter paper. This process was done to assess the performance of biochemical parameters, such as antioxidant enzymes, protein content, and other enzymes implicated in the ovarian tissues of the polluted group and the control group. Subsequently, the tissues were weighed and homogenized at a ratio of 1:5 of tissues to buffer in potassium phosphate solution (67 mM, pH 7). Subsequently, the mixtures were centrifuged at 10,000 ×g and 4 °C for 30 minutes.

The measurement of SOD activity followed Misra (19) guidelines. The technique relies on tracking the rate at which adrenaline is being autooxidized. The reaction mixture was evaluated for absorbance at 480 nm. It contained the supernatant, sodium carbonate buffer (200 mM; pH 10.0), EDTA (10 mM), and newly made epinephrine

(15 mM). SOD activity is shown in mU/mg protein on the chart. The amount of enzyme that inhibited 50% of the adrenaline autooxidation control reaction per minute was the definition of an activity unit. The Cayman Chemical Company (Michigan, USA) kits were used to assess the catalase activity (CAT). Glutathione peroxidase (GPx) activity was measured using the methodology previously reported by Cichoski (20). the catalase activity (CAT) and glutathione S-transferase (GST) activities were evaluated using the kits procured from Cayman Chemical Company (Michigan, USA).

The kits obtained from Cayman Chemical Company (Michigan, USA) were utilized to assess the quantity of malondialdehyde (MDA). The reduced glutathione (GSH) test was conducted using the previously published Beutler (21) procedure. The methodology involved reducing 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) with GSH to yield a yellow molecule, which was then detected using a spectrophotometer at 412 nm.

Glutathione reductase (GR) activity was determined as described in the GR assay kit developed by ZeptoMetrix Corp. (Buffalo, USA). Briefly, soluble protein extracts (35 µL) were mixed with the kit working solution (935 µL), and GR activity in the reactive mixture was monitored spectrophotometrically at 340 nm for 1 min. Results were expressed as Ug-1fw (U: 1 unit of GR that forms 1 µM of NADP+ after NADPH reduction at 25◦C) (22).

The activity of ascorbate peroxidase (APOX) was measured following Asada (23). The control samples were boiled. After adding H_2O_2, the absorbance changed and was measured at 290 nm. Carbonyl content of proteins (Levine et al., 1990). Tissues were separated and mixed thoroughly in 5 milliliters of PBS containing additives (Triton X-100 and CaCl2), then centrifuged at 2000 rpm for ten minutes at 4 degrees Celsius. Next, 800 µL of 30% trichloroacetic acid (TCA) was combined with 800 µL of supernatant aliquots. The samples were centrifuged (5000×g; 10 min, 4 °C) after being incubated for 30 minutes at room temperature. Lipid peroxide assay was carried out utilizing supernatant, and protein carbonyl (PC) assay was carried out using precipitated pellets. The pellet, after washing four times with an ethanol/ethyl acetate (1:1) mixture, was redissolved in 3.5 mL of PBS. Next, the mixture was centrifuged (2000×g for 1 min) and the absorbance of the supernatant was measured at 366 nm.

Statistical analysis

All determinations were carried out from 3 to 6 independent experiments, and the data were analyzed employing a Student's t-test in Statistical Product and Service Solutions (SPSS), version 25, produced by IBM Software Inc., Chicago, IL, USA. The values are presented as mean ± SD, and the significant differences were considered at $P \leq 0.05$.

RESULTS

Heavy metals percentages in soil samples and ovarian tissues of *C. Olivieri*

The beetles were dissected and the ovarian tissues of *C. Olivier* were obtained. The ovarian tissues of polluted and the control group and soil samples were probed using SEM and EDX to determine the heavy metals content as illustrated in (Fig 2 A-B) & Fig 3, Fig 4. It is manifest from the results that the development of peaks related to carbon (C), oxygen (O), sodium (Na), phosphorus (P), sulfur (S), Nitrogen (N), and Magnesium (Mg) in the ovary of the control group. Considering the EDX analysis of ovarian tissues of a polluted group, similar elements could be detected. Furthermore, other elements emerged in the treated group, including Cobalt (Co) and Chromium (Cr). This performance could be derived from the physiological alterations in the beetles as a consequence of heavy metals accumulation.

Biochemical parameters in ovarian tissues of *C. Olivieri*

Figure (4- 12) presents the assay of biochemical factors of the ovarian tissues in beetles obtained polluted group and the control group. It can be recognized from the data that the exposure of beetles to heavy metals provokes substantial malfunctions in comparison to the control group. Specifically, the MDA level in the ovarian homogenate of the polluted group was considerably augmented compared to the control group. In contrast, the protein content was significantly diminished in the ovaries of the polluted group in comparison with the control group. Besides, GPx, GST, SOD, and CAT enzymes, which regulate the overabundance of ROS, were noticeably decreased in the ovarian tissues of the polluted group compared to the control group. Moreover, the level of GSH was remarkably decreased in the polluted group in comparison to the control group beetles and the statistical analysis exhibited a significant difference between the two groups ($P \leq 0.05$).

In general, The APOX activity was significantly higher in beetles from the polluted group compared to those from the control group. figure (11). Also, the concentration of protein carbonyls was higher in ovarian cells of beetles collected from polluted sites. As shown in figure (12).

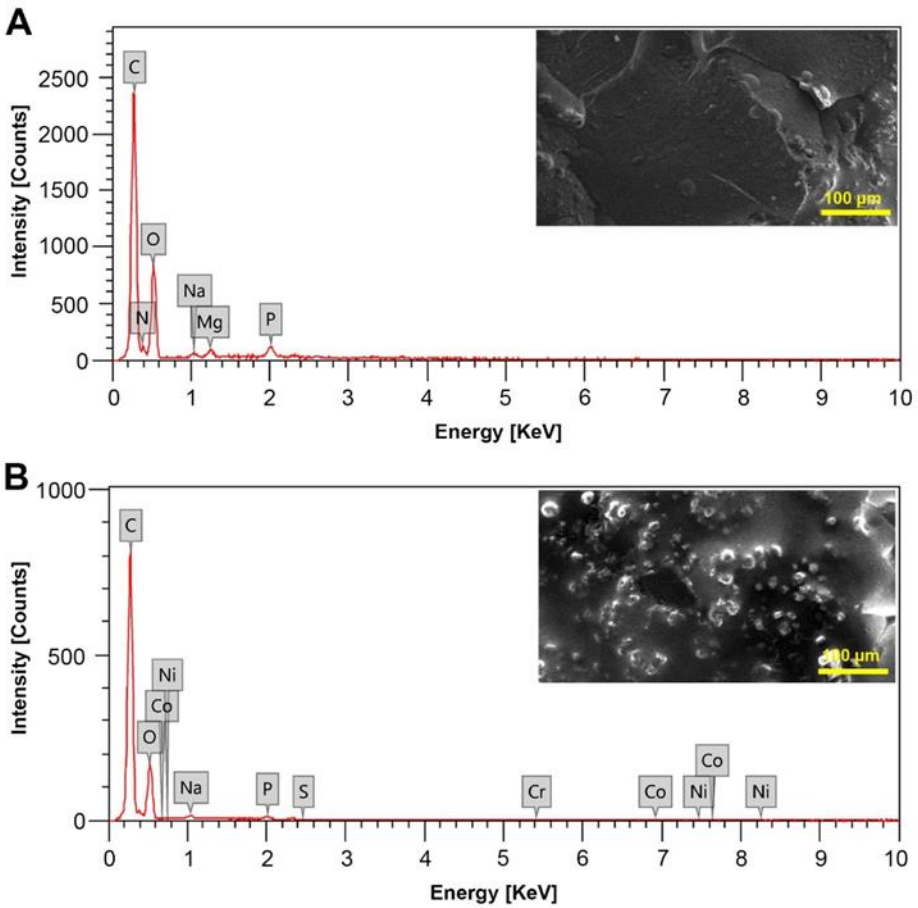

Fig (2): SEM images of (A) ovarian tissues harvested from the control group and (B) ovarian tissues harvested from the polluted group demonstrate the agglomeration of the heavy metals in the ovarian tissues of the polluted group in comparison with the control group.

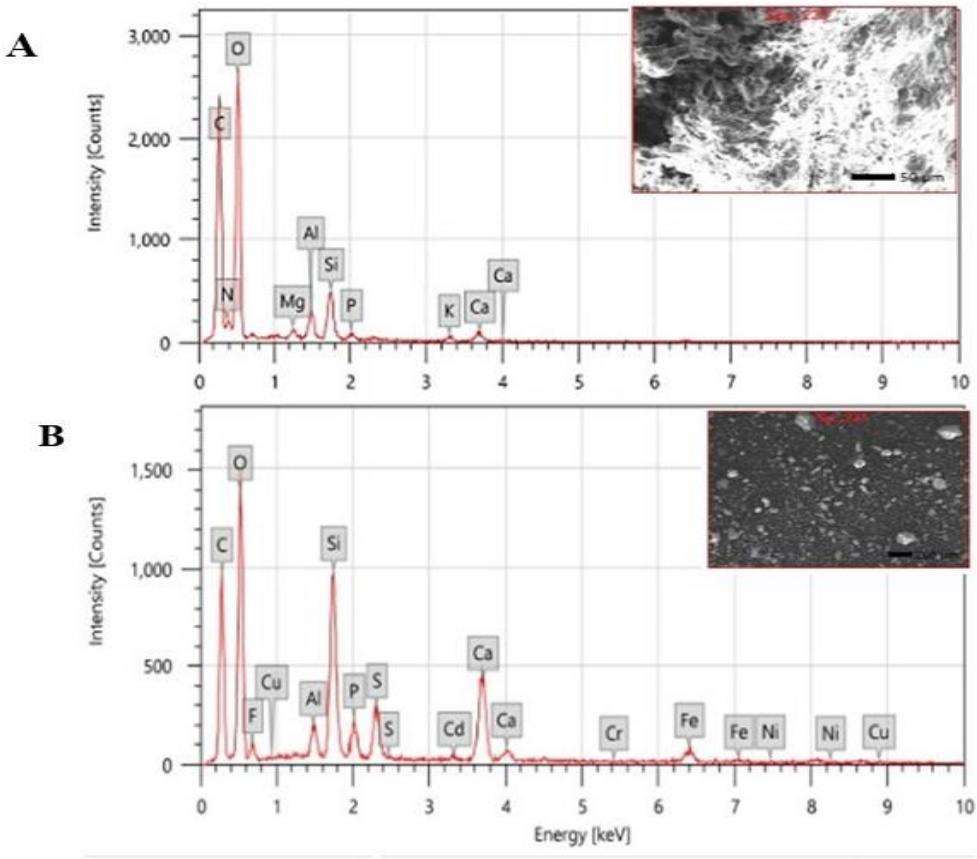

Fig (3): SEM images of (A) soil samples collected from the control group and (B) soil samples collected from the polluted group.

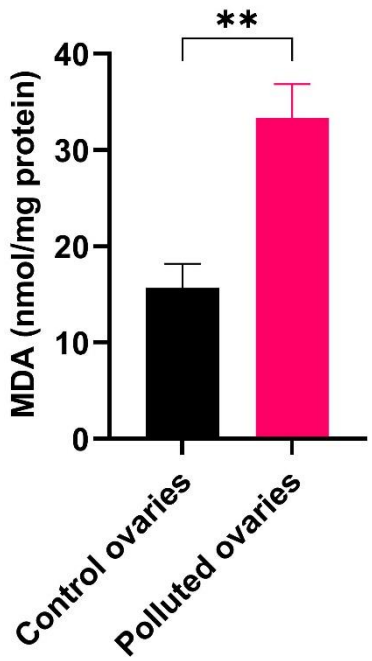

Fig (4): Assessment of MDA level in the ovarian tissues of *C. Olivieri* collected from the clean site (site A) and the polluted site with industrial pesticides (site B). Values are presented as mean ± SD and **p < 0.01.

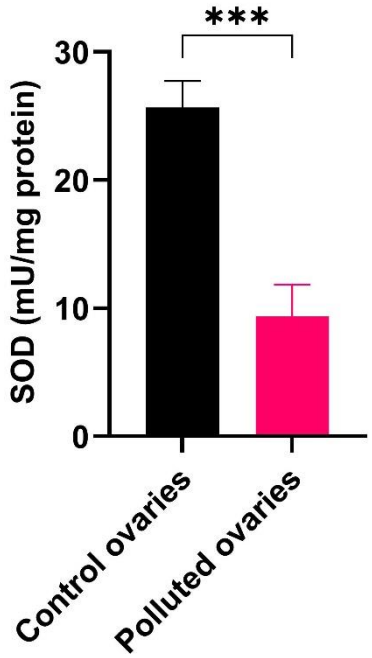

Fig (5): Evaluation of SOD in the ovarian tissues of *C. Olivieri* collected from the clean site (site A) and the polluted site with industrial pesticides (site B). Values are presented as mean ± SD and ***p < 0.001.

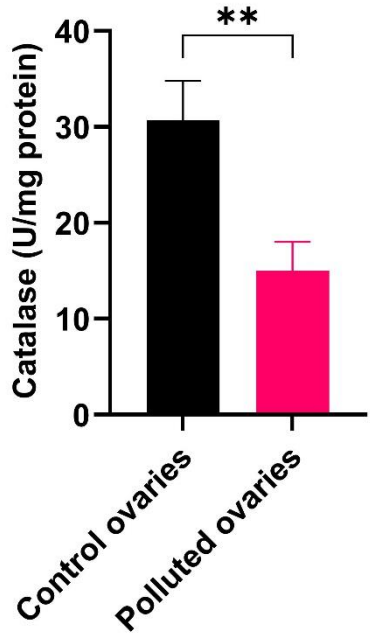

Fig (6): Assessment of catalase in the ovarian tissues of *C. Olivieri* collected from the clean site (site A) and the polluted site with industrial pesticides (site B). Values are presented as mean ± SD and **p < 0.01.

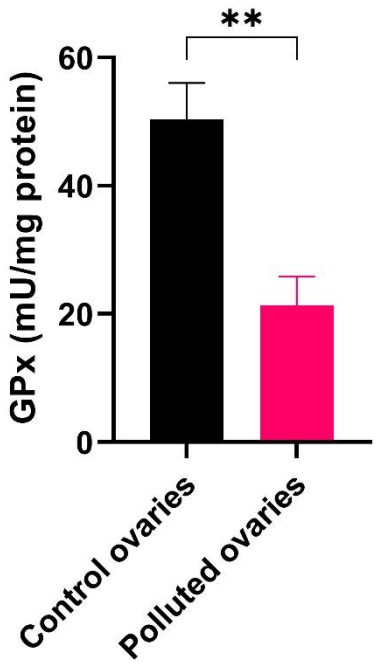

Fig (7): Evaluation of GPx in the ovarian tissues of *C. Olivieri* collected from the clean site (site A) and the polluted site with industrial pesticides (site B). Values are presented as mean ± SD and **p < 0.01.

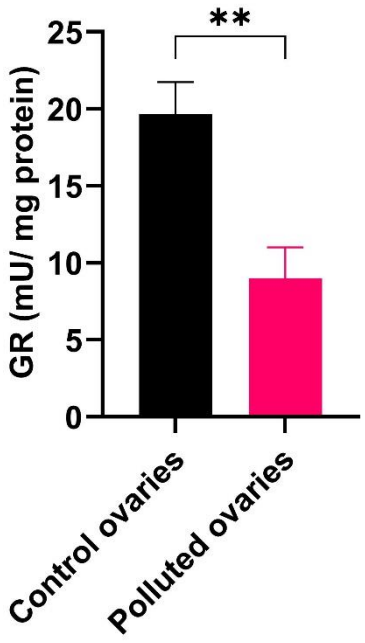

Fig (8): Estimation of GR in the ovarian tissues of *C. Olivier* collected from the clean site (site A) and the polluted site with industrial pesticides (site B). Values are presented as mean ± SD and **p < 0.01.

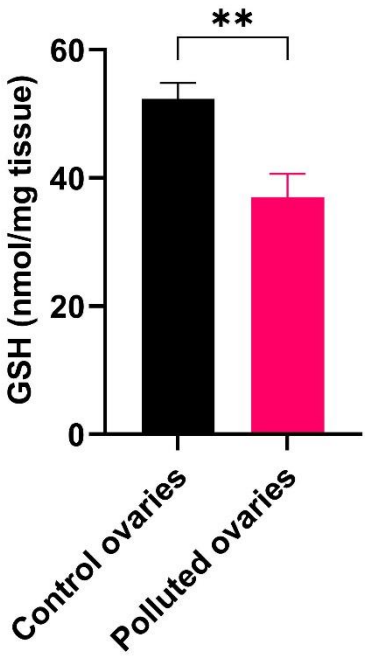

Fig (9): Estimation of GSH in the ovarian tissues of *C. Olivieri* collected from the clean site (site A) and the polluted site with industrial pesticides (site B). Values are presented as mean ± SD and **p < 0.01.

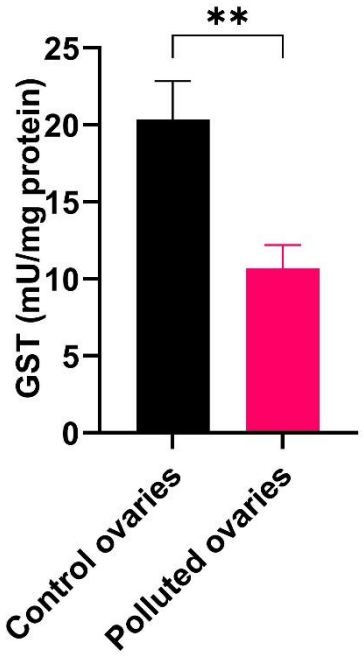

Fig (10): Estimation of GST in the ovarian tissues of *C. Olivieri* collected from the clean site (site A) and the polluted site with industrial pesticides (site B). Values are presented as mean ± SD and **p < 0.01.

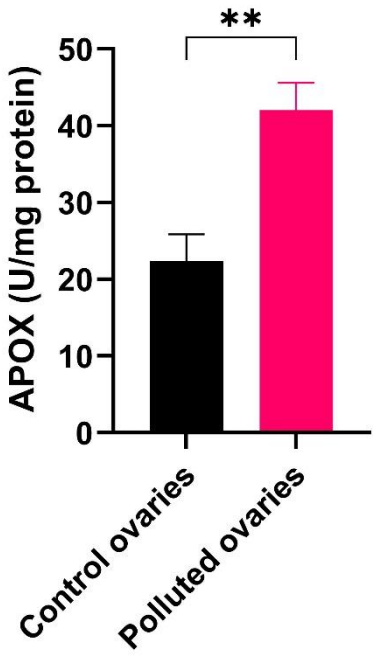

Fig (11): Assessment of APOX in the ovarian tissues of *C. Olivieri* collected from the clean site (site A) and the polluted site with industrial pesticides (site B). Values are presented as mean ± SD and **p < 0.01.

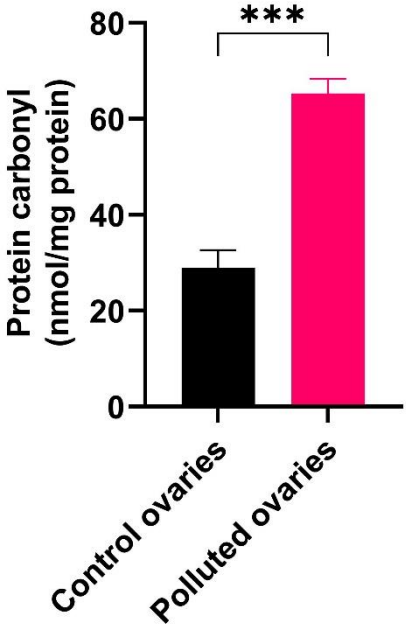

Fig (12): Evaluation of protein carbonyl in the ovarian tissues of *C. Olivieri* collected from the clean site (site A) and the polluted site with industrial pesticides (site B). Values are presented as mean ± SD and ***p < 0.001.

Discussion

Heavy metals are naturally occurring elements that have a high atomic weight and a density at least 5 times greater than that of water. Their multiple industrial, domestic, agricultural, medical, and technological applications have led to their wide distribution in the environment, raising concerns over their potential effects on human health and the environment (24). Their toxicity depends on several factors including the dose, route of exposure, and chemical species, as well as the age, gender, genetics, and nutritional status of exposed individuals. Because of their high degree of toxicity, arsenic, cadmium, chromium, lead, and mercury rank among the priority metals that are of public health significance. These metallic elements are considered systemic toxicants that are known to induce multiple organ damage, even at lower levels of exposure. They are also classified as human carcinogens (known or probable) according to the U.S. Environmental Protection Agency, and the International Agency for Research on Cancer (25).

The first symptom of increased industrial activity of humans is usually an increase in pollution of biotopes, including soil and water. The consequence might be several negative changes in organisms living in these degraded ecosystems. In this research, a significantly higher concentration of certain elements (especially P, S, and Na) has been observed in beetles of a polluted site, close to a chemical industry, when compared to a reference group. The observed increase in the concentration of P, S, and Na was likely caused by the chemicals factory, as it is the main source of pollution in the Kafr El-Zayat area. Moreover, the relationships observed by us are in agreement with what other authors have been postulating. (2). detected high concentrations of Cd, Cu, Zn, and Pb in the soil adjacent to the point of discharge of untreated industrial effluents. Li (26) reported that the metals were more concentrated in the soil of industrial cities compared to that of coastal cities.

Natural ecosystems are adversely affected by manmade interventions. Among living organisms, insects are regarded as susceptible to environmental disruption as delicate bodies confirm the presence or absence of a polluted environment thus found as suitable indicators of the aquatic and terrestrial ecosystem (17). Insects are considered indicators of environmental pollution because different taxa of different localities provide robust information, a comparison of various communities, and quantitative data associated with indicators, etc. Most of them present the quick reliable influence of heavy metal accumulation as pronounced disruptions were observed at molecular and biochemical levels and hence considered as best opted indicators of environmental pollution (27).

This research done on *C. Olivieri*, a species common around Kafr El-Zayat, brings further interesting results, which show how this population can survive in a transformed ecosystem. The first effect of exposition to chemical elements, including metals, is usually increased accumulation in the body (predominantly in the ovary) of exposed beetles. However, it is possible that a significant increase in element accumulation will not occur, as some animals are capable of regulating the intake of elements with food (avoidance strategy) or increasing excretion, (9). Our results show that *C. Olivieri* does not utilize these strategies, especially for non-essential elements, because an increased concentration of these elements is observed in the animals' ovaries.

In our study, oxidative stress was evaluated by determining both the activity of antioxidant enzymes and the oxidative damage of macromolecules (DNA, proteins, and lipids) in the ovary of *C. Olivier*i. There was a significant decline in the activity of GST, SOD, CAT, and APOX in the ovarian tissues of beetles gathered from the polluted site, compared to those from the reference site. This result may be explained as follows: high concentrations of heavy

metals in midgut tissues can a) trigger ROS production and damage to plasmatic membranes, b) bind to proteins and phospholipids of membranes, c) inhibit Na- and K-dependent ATPases, d) inhibit the trans-membrane amino-acid transport, e) inhibit enzyme activity, increase lipid peroxidation and oxidative DNA damage. This can finally lead to cell death. Such scenarios were presented by El Gendy (15). In insects treated with Cd, it was hypothesized that CAT serves the most crucial role in scavenging ROS and reducing their harmful effects (28).

Our data revealed a significant decrease in GR activity in the control group compared with the polluted group, which comes in agreement with Augustyniak (29) who noticed that Glutathione reductase (GR) activity was generally lower in males compared to females and also 5th instar of *A. thalassinus*. If some part of the glutathione is bound to metals, GR activity in insect tissues can be expected to decline in proportion to the glutathione deficiency.

Alternatively, it may be due to the influence of ROS on enzymes (30). Our results are following those of Yousef (31) who reported a decline in antioxidant enzyme activity in insects exposed to different environmental stress caused by xenobiotics, particularly heavy metals. In contrast to our results, El-Samad (32) verified the maximum activities of GST and APOX enzymes in spiders gathered from highly polluted sites. Also, Haihua (33) showed that Cd-induced a significant increase in SOD and CAT in *Oxya chinensis*.

Ascorbate is used as a reducing agent by APOX to catalyze the reduction of H_2O_2. As a result, the amount of decreased ascorbate present may have an impact on the enzyme's activity. Under ideal circumstances, the production of decreased ascorbate is sustained; but, under stressful circumstances, the process may be interrupted (34) Our findings support the findings of Yousef (31), who reported that antioxidant enzyme activity in *Aiolopus thalassinus* nymphs in their fifth instar are altered by heavy metal pollution.

It is most probably an effect of compensation based on increased synthesis of antioxidative enzymes, which limits oxidative stress through early scavenging of ROS, as well as preventing further damage to molecules and cell structures. Such a strategy, for reasons not fully understood, is not very common, especially if chronic exposure to xenobiotics is considered. It seems that during short-term exposure, an organism can increase energy allocation towards antioxidative enzyme synthesis (within phenotypic plasticity). In a longer perspective, when multigenerational exposure to toxins is considered, epigenetic phenomena might take place, which could lead to muting the expression of certain genes (14).

CONCLUSION

The results showed that C. olivieri is a valuable indicator of heavy metal pollution. Moreover, the metrics under examination are valuable markers for evaluating the deleterious consequences of environmental pollutants. The investigation revealed anomalies related to the overproduction of ROS caused by exposure to heavy metals.

Conflict of interest

All authors declared that there were no conflicts of interest.

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