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Green Silver Nanoparticles induced apoptotic pathways and oxidative stress in cells of *Culex pipiens* larvae

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Abstract:
Background*: Nanobiotechnology had emerged with leading contributions in applications of cell biology and antioxidants. Meanwhile, Culex pipiens are the most widely distributed mosquitoes among Egyptian governorates where they represent significant vectors for filariasis, St. Louis encephalitis, West Nile virus, western equine encephalitis, Japanese encephalitis and Rift Valley fever. The main aim of this study is to investigate the effect of green silver nanoparticles, GSNPs, on the activities of some oxidative stress enzymes and accordingly on apoptotic pathways in cells of Culex pipiens larvae in purpose of controlling their prevalence.*
Materials and Methods*: In this prospective four groups were regarded, 100 of third instar of Culex pipiens larvae each. One was the control group and the remaining were test groups representing Lc10, Lc50 and Lc90 of GSNPs. Measurements of the activities of Superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) were recorded as well as the levels of nitric oxide and Caspase 3 which were also detected in tissue homogenates of control group and test groups .*
Results*: Results revealed decreased activities of oxidative stress enzymes in tissue homogenates of Culex pipiens larvae upon increasing GSNPs concentrations. Activities of catalase enzyme recorded 1.09, 0.96 and 0.87 U/g for LC10, LC50 and LC90 respectively compared to 2.11 U/g for control samples. Similarly, the activities of glutathione reductase recorded 15.83mg/g. tissue for control samples and 14.7, 13.4 and 11.6 mg/g. tissue for LC10, LC50 and LC90 respectively. Furthermore, superoxide dismutase activity recorded 373.7 U/gm tissue in control samples and 357.8, 322.5 and 301.2 U/gm tissue for LC 10, LC50 and LC90 of GSNPs respectively. On the other hand, nitric oxide levels were significantly increased upon increasing GSNPs concentrations which recorded 8.24, 43.37 and 47.0 umol/L for LC10, LC50 and LC90 respectively while it recorded 4.65 umol/L in control samples. Additionally, Caspase3 protein which is encoded by caspase3 gene showed a significant increase upon increasing the concentrations of GSNPs as well, ($p<0.05$).*
Conclusion: *This study signifies the toxic effect of green silver nanoparticles, GSNPs on cells of Culex pipiens larvae leading to induction of prominent apoptotic pathways and eventually their death.*
Key Word*: Apoptotic pathways, Caspase3, Culex pipiens larvae apoptosis, Green Silver Nanoparticles toxicity, Oxidative stress enzymes*

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I. Introduction

Nanobiotechnology has recently emerged and become an integral part of modern disease prevention; diagnosis and treatment [1] Nanomaterials provide solutions to technological and environmental challenges in many areas including catalysis, biology and biomedical science [2]. Nanotechnology exhibits a variety of promising approaches in the area of material sciences on a molecular level, and silver nanoparticles (SNPs) are of leading interest in the current researches [3]. Previous studies revealed that physical, optical and catalytic properties of silver nanoparticles (SNPs) are strongly influenced by their size, distribution, morphologic shape and surface properties [4]. Therefore, in nano-biotechnological research, SNPs have received significant attention [5]. For medical applications, NPs synthesis must be biocompatible and either low-toxicity protocols must be used. Hence, SNPs syntheses through green chemistry (such as the use of biologic sources) have shown great promise recently [1]. During the past decade, it has been described that many biological systems, including plants, algae, bacteria, yeast and fungi can transform inorganic metal ions into metal nanoparticles by means of the reductive capacities of the proteins and metabolites present in these organisms [6]. Biosynthesized silver nanoparticles or- as so called- Green Silver Nanoparticles (GSNPs) are a class of eco-friendly, cost-effective

and biocompatible agents that have attracted attention for their possible biomedical applications [1]. Many biomedical applications of SNPs are recently known including antibacterial, antifungal, antiviral, in medical devices such as wound dressings, catheters and bone cement, in treatment of tumors, water purification, biosensors, catalytic activity enhancement and bioimaging [7]. Moreover, GSNPs have emerged with leading contributions in applications of cell biology [8] and antioxidants [9] .Also, SNPs showed molluscicidal effect on *Biomphalaria alexandrina* snails, the intermediate host of *Schistosoma mansoni*, [10].

In the mean time, mosquitoes are considered the most serious insect pests of medical importance. *Culex pipiens* (Linnaeus) complex, is the common and widely distributed mosquitoes among Egyptian governorates [11]. Considerably, these are important vectors for filariasis [12], St. Louis encephalitis [13], West Nile virus [14], western equine encephalitis, Japanese encephalitis, and Rift Valley fever [15, 16, 17]. Now that, *Culex pipiens* is the main target in control programs for reducing the prevalence of these diseases. In Egypt, the intensive use of insecticides often leads to the emergence of their resistance. Insecticide resistance displays major problems in the control of medical and agricultural arthropod pests [18]. The appearance of such problems has been accompanied by forcing interests to the use of novel pesticides with a new mode of action especially upon dealing with undesired biological entities [19].

It is notable that GSNPs can cause cytotoxicity of cells by altering their morphology and reducing their viability along with oxidative stress [20, 6]. Recently, GSNPs imply prominent effect in controlling the prevalence of *Culex pipiens* larvae via increasing the lipid peroxidation levels in their tissues [21]. Accordingly, the main aim of the present work is to investigate the effect of Green Silver nanoparticles (GSNPs) on cells of mosquitoes larvae (*Culex pipiens*), regarding alteration in levels of oxidative stress enzymes and consequently altering their apoptotic pathways regarding caspase3 protein under laboratory condition in Egypt.

II. Material And Methods

This prospective comparative study was carried out on larvae belong to the species *Culex pipiens*, which were obtained from Research Institute of Medical Entomology, Giza, Egypt. They were maintained in the water of their biotopes under laboratory conditions (75% relative humidity and 25°C).All 3rd instars larvae selected for larvicidal activity came from the same generation and from the same breading sites. The methodology of the toxicity tests was based on the WHO standardized sensitivity tests **[22, 23].**

Study Design: Prospective open label observational study

Study Location: The study was carried out in Rodents Research Dept. (RRD), Research Institute of Medical Entomology, (RIME), Giza, Egypt.

Study Duration: September 2020 to August 2021.

Sample size: 400 third instar *Culex pipiens* larvae.

Sample size calculation: The sample size was estimated on the basis of a single proportion design. The target population from which we randomly selected our sample was considered 100,000. We assumed that the confidence interval of 10% and confidence level of 95%. The sample size actually obtained for this study was 100 third instar *Culex pipiens* larvae for each group. We planned to include 400 third instar *Culex pipiens* larvae (Group I- Control, Group II- Cases of 100 third instar *Culex pipiens* larvae for each group) with 4% drop out rate.

Materials:

Green silver nanoparticles: Ginger extract was prepared by cutting 5.0 g of rhizome into small pieces, which were refluxed with 100 ml of 70% ethanol at70°C for 2 hours. After cooling, the obtained extract was filtered using Whatman filter paper and then centrifuged. The supernatant was collected and stored at 4°C.For the biosynthesis of SNPs, 1 ml of ginger extract was added to 20 ml of AgNO3 solution (1 m mol/L) in a round-bottom flask. The mixture was heated at 85°C and color change of the solution was recorded within 20 minutes. This prepared GSNPs were characterized by UV–visible spectroscopy on a UV-2550 spectrophotometer in the range of 200–800 nm **[24].**

Procedure methodology

I- Preparation of larval tissue homogenates:

In this step, the selected larvae were incubated with sub-lethal doses of green silver nanoparticles according to the predetermined concentrations of Lc90, Lc50 and Lc10 which were obtained from the established regression log concentrate response lines after 24 hours of incubation. The treatment doses were 0.576 ppm, 1.719 ppm and 5.129 ppm for LC**10**, LC**50,** and LC**90** respectively **[21].** LC90, LC50 and LC10 of GSNPs concentrations were prepared using dechlorinated water as diluent. Twenty five 3rd instar larvae were put into a small beaker (500 ml) containing the test solution of each concentration. Four replicates were performed for each concentration. In control experiments, larvae were placed into plain dechlorinated water. Larval mortalities were determined 24 hours post treatment. Larva was considered dead if it did not move when prodded with a fine wooden dowel **[25].**

Subsequently, alive larvae were homogenized using UP 200H ultrasonic processor, one gram tissue in 5ml phosphate buffer solution (PH 7.4). The obtained suspension will be centrifuged at 4000 rpm for 45 minutes at room temperature. The pellet will be discarded while the aliquots of supernatants will be involved in determining the activities of several cellular parameters [21].

II- Detection of antioxidant status in tissues of *Culex pipiens* larvae treated with silver nanoparticles.

In this experiment, antioxidant enzymes will be detected spectrophotometrically as indicators for the assessment of oxidative stress in the larval tissue homogenates previously prepared in step I. Superoxide dismutase (SOD) is determined as described in **Peltola *et al*.** [26].While the activity of catalase (CAT) was assessed using **Beers and Sizer** [27] method, which is essential for removing the hydrogen peroxide produced by SOD. Glutathione reductase (GR) is evaluated indirectly based on the oxidation of NADPH to NADP+ regarding **Paglia and Valentine** method of detection [28]. Finally, nitric oxide in the tissue homogenates will also be examined following **Green *et al.*** method [29].

Effect of silver nanoparticles on Caspase 3 enzyme in tissue homogenates of *Culex pipiens* larvae:

In this experiment the aliquots of supernatants of larval tissue homogenates which were prepared as described in step I**,** were used in determining the concentration of Caspase 3 protein as a determining factor of apoptotic pathway induction following the instruction manual of Cloud- clone Corp. kit- USA and using ELISA reader (STAT FAX, 2100- Awareness Technology INC.) Japan.

Statistical analysis

Statistical analyses were performed using the Statistical Package for Social Science (SPSS) version 25 (SPSS Inc., Chicago, IL, USA.). Data were displayed as means ± SD. Differences in the various parameters in more than two groups were evaluated by a one-way analysis of variance (ANOVA). Differences between groups were considered significant at $p < 0.05$ [30]**.**

III. Result

Decreased activities of oxidative stress enzymes in tissue homogenates of *Culex pipiens* larvae were observed upon increasing GSNPs concentrations (table 1). Activities of catalase enzyme recorded 1.09, 0.96 and 0.87 U/g for LC10, LC50 and LC90 respectively compared to 2.11 U/g of control samples (Figure 1 and table 1). Similarly, the activity of glutathione reductase recorded 15.83mg/g. tissue for control samples and 14.7, 13.4 and 11.6 mg/g. tissue for LC10, LC50 and LC90 respectively (figure 2 and table 1). On the same track, superoxide dismutase activity has decreased significantly upon increasing GSNPs concentrations (Figure 3 & table 1) to record 373.7 U/gm tissue in control samples and 357.8, 322.5 and 301.2 U/gm tissue for LC 10, LC50 and LC90 of GSNPs respectively. On the contrary, nitric oxide levels have significantly increased upon increasing GSNPs concentrations that recorded 8.24, 43.37 and 47.0 umol/L for LC10, LC50 and LC90 respectively while it recorded 4.65 umol/L in control samples (figure 4and table1). The activities of caspase3 have increased significantly upon increasing the GSNPs concentrations to record 3.41, 5.15 and 9.17 ng/ml for LC10, LC50 and LC90 respectively compared to1.65 ng/ml for control samples as implied in table 1 and figure 5).

Table no 1: Levels of catalase, glutathione reductase, superoxide dismutase, nitric oxide and caspase3 in control and GSNPs treated groups of tissue homogenates of *Culex pipiens* larvae.

	Catalase (U/g)±SE	**Glutathione reductase(mg/g. tissue) ±SE**	**Superoxide dismutase (U/gm) ±SE**	**Nitric oxide (umol/L) ±SE**	**Caspase3 (ng/ml) ±SE**
Control	2.11±.26476**	15.83±.32285**	373.7±2.69176**	4.65±.33441**	1.65±.12547**
LC10	1.09±.16463**	14.7±.34705**	357.8±2.13333**	8.24±.35408**	3.41±.18705**
LC50	0.96±.07485**	13.4±.47093**	322.5±2.60021**	43.37±2.27909**	5.15±.21305**
LC90	0.87±.02171**	11.6±.49889**	301.2±7.38083**	47.0±2.00000**	9.17±.19382**

Values are represented as mean± SE, ** is highly significant at P< 0.001, * is significant at P<0.005, NS is non significant at P>0.005.

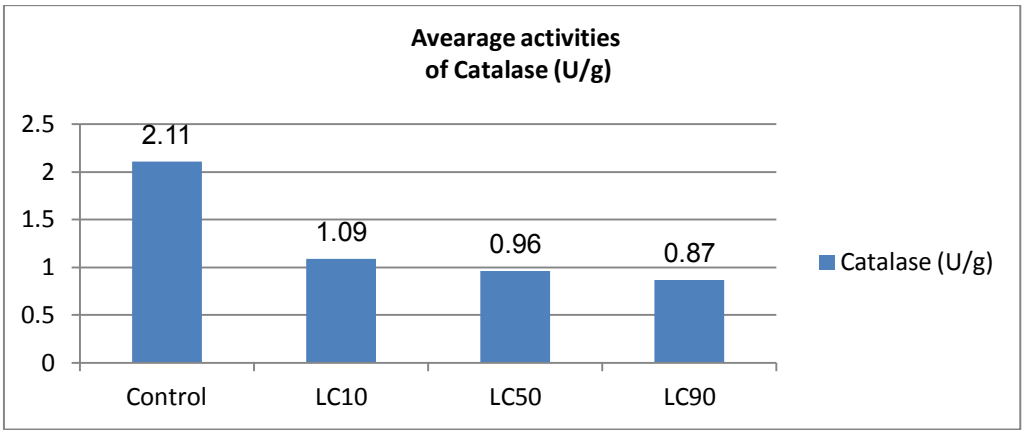

Figure 1: Decreasing activities of catalase enzyme among different concentrations of GSNPs in tissue homogenates of *Culex pipiens* larvae.

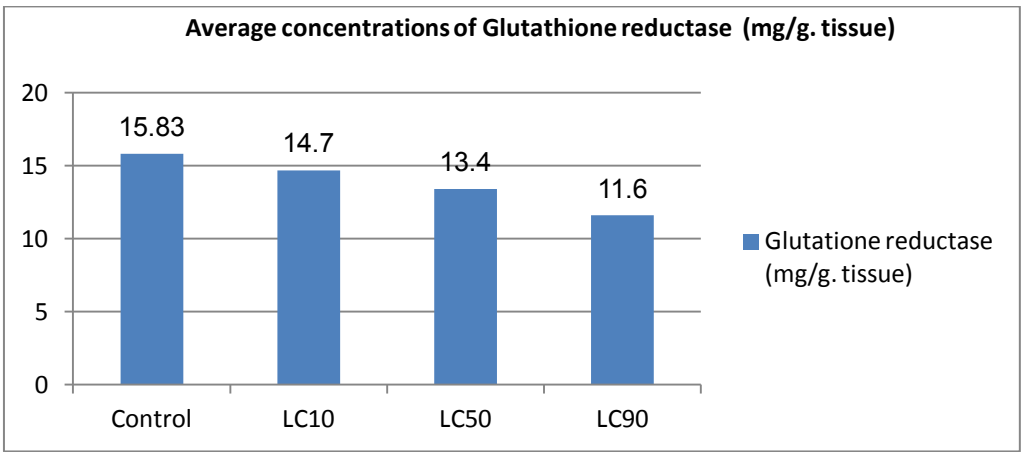

Figure 2: Decreasing concentrations of glutathione reductase enzyme among different concentrations of GSNPs in tissue homogenates of *Culex pipiens* larvae.

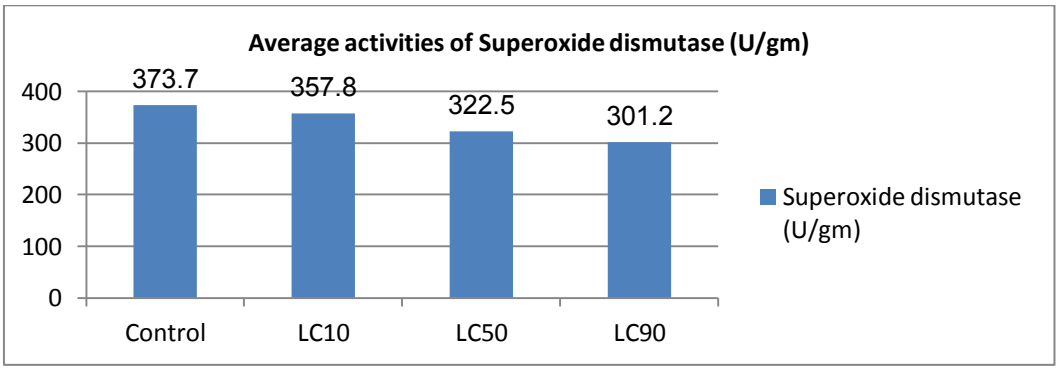

Figure 3: Decreasing activities of superoxide dismutase among different concentrations of GSNPs in tissue homogenates of *Culex pipiens* larvae.

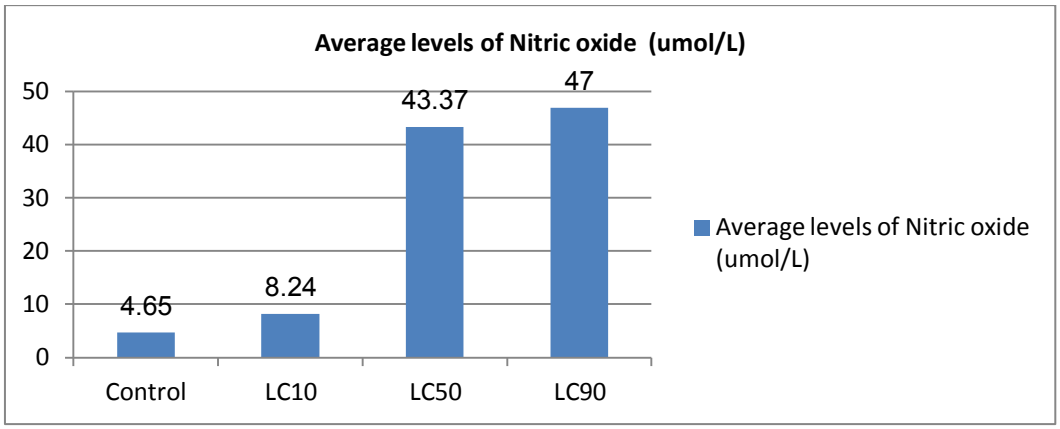

Figure 4: Increasing levels of nitric oxide among different concentrations of GSNPs in tissue homogenates of *Culex pipiens* larvae

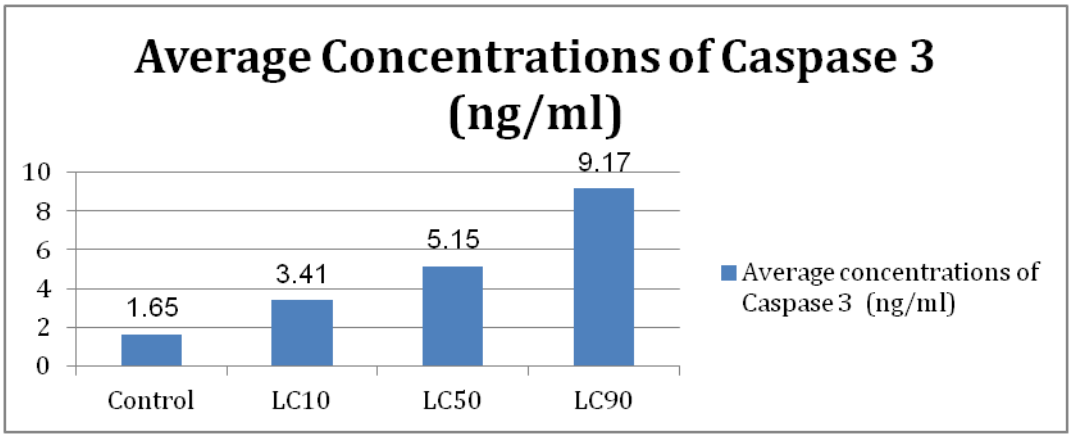

Figure 5: Increasing concentrations of caspase 3 among different concentrations of GSNPs in tissue homogenates of *Culex pipiens* larvae.

IV. Discussion

Green nanotechnology is that chemical philosophy which enhances the design of nano products that mitigates the use and generation of hazardous substances [31]. Nowadays, biosynthesized metallic nanoparticles or so called green nanoparticles have drawn great attention as they are simple and viable alternatives when compared to chemical and physical methods [32]. Owing to their nature of being green, they are eco-friendly and low-priced processes that can provide biocompatible nanostructures with potential applications in fields of biotechnology [32]. However, the use of plant extract in synthesizing silver nanoparticles is most favorable as they are easily accessible and contain various metabolites, which may help in the reduction of silver ions, and improves the rate of synthesis [33]. Green Silver nanoparticles, GSNP were recently regarded as suitable carriers of various therapeutic molecules, including anti-inflammatory [34], antimicrobial [35], anticancer [36]. Mousavi *et al* [37] have indicated that Silver nanoparticles synthesized using *Piper longum* fruit had showed antibacterial and cytotoxic effects. They also reported that GSNPs synthesized by using the leaf extracts of *Artemisia turcomanica* induced cytotoxic and apoptotic effects on gastric cancerous cells [37]. Recently, Ragheb *et al* have showed that Green Silver Nanoparticles (GSNPs)synthesized using ginger extract had larvicidal potency against *Culex pipiens* larvae [21]. Hence, this study was concerned with studying the cytotoxic effect of GNSPs synthesized using ginger extract in initiating oxidative stress in *Culex pipiens* larval tissues which may proceed to apoptotic pathways. Initially, many studies had showed that the cytotoxic characteristics of GSNPs can be attributed due to presence of organic species such as phenolics and flavonoids present in the extract [38]. An earlier study which was concerned with the effect of GSNPs on *Culex pipiens* larvae had showed that LC50 and LC90 of GSNPs induced sever toxicity to larval tissues which were indicated as a function of elevated lipid peroxidases levels [21], this effect was previously described by Chauhan *et al* who showed that SNPs affect the integrity of lipid bilayer and permeability of the cell membrane which is responsible for the proper regulation of transport through the plasma [39]. Moreover, SNPs and/or silver ions inside the living cells can interact with cellular structures (e.g., ribosomes) and biomolecules such as proteins, lipids, and DNA, which have a damaging effect on these cells [40]. Results in this study have implied gradual decrease in the activities of antioxidant

enzymes in tissue homogenates of *Culex pipiens* larvae corresponding to gradual increase of GSNPs concentrations. These findings could be explained through the fact that, the cytotoxicity of green synthesized SNPs is associated to the involvement of the level of cellular reactive oxygen species (ROS) and mitochondrial membrane disruption [41]. As they disturb DNA replication as well as translation process in ribosomes and protein activities [39]. It was also thought that induction of high levels of reactive oxygen species (ROS) is a general mechanism of nanoparticles mediated cytotoxicity where, reactive oxygen species (ROS) are capable of causing cell death and conversely, that of antioxidant agents can prevent cell death [42]. In addition, nanoparticles can cause a dose-dependent increase in oxidation and DNA damage [43]. In general, the cellular damaging mechanism of SNPs is associated with the generation of high level of ROS and free radical species (e.g., hydrogen peroxide, superoxide anion, hydroxyl radical, hypochlorous acid, and singlet oxygen) [44] which inhibit respiration and growth of cells [45]. Results in this study revealed significant decrease in the activity of superoxide dismutase enzyme (SOD) and catalase enzyme (CAT) upon increasing GSNPs concentrations. These findings were in accordance with Valerio-Garcia *et al* [46]and Huang *et al* [47] who reported marked inhibition of SOD and CAT activities in different aquatic organisms upon exposure to SNPs . Also, Liu *et al* have showed that SNPs considerably inhibited the enzymatic activity of CAT while they did not affect the SOD activity[48]. It is currently accepted that the alteration of the enzyme activity might be due to either regulation of genes or to direct surface interaction of the enzymes with SNPs [49]. Results in this study also revealed decrease in glutathione reductase enzyme upon increasing GSNPS concentrations. Many studies showed that an in vitro exposure to SNPs cause reduction of glutathione (GSH) level, elevated ROS levels, increased expression of ROS responsive genes [42] . A decreased enzymatic activity of glutathione reductase (GR) enzyme with increasing concentrations of SNPs has not been only reported in human liver cell line, but also in rainbow trout hepatocytes and erythrocytes [50, 51]. Moreover, Zayed *et al* have also reported the decrease of SOD, CAT and glutathione reductase (GR) in Schtosomiasis snails treated with SNPs [52]. These findings could be explained owing to the ability of GR to regenerate glutathione (GSH) from its oxidized form, glutathione disulphide. GSH is one of the major endogenous antioxidant scavengers that is able to bind and reduce ROS. Thus, maintaining a sufficient GSH pool is critical to ensure a significant defense system for cell survival. It has been indicated that GSH can interact with metal ions, including Ag+. Hence, it could bind directly to Ag+ as a response against oxidative stress [51]. Results in this study demonstrated an increase in the levels of nitric oxide in tissue homogenates of tissues of *Culex pipiens* larvae upon increasing GSNPs concentrations. These results were in accordance with Barcińska *et al.*, who described the increase of nitric oxide levels of human pancreatic ductal adenocarcinoma cells treated with silver nanoparticles [53]. Meanwhile, Chakraborty *et al.* proved a significant increase of nitric oxide synthase protein level and nitric oxide level in murine fibrosarcoma cells after treatment with SNPs [54]. Silver nanoparticles, significantly increased the level of measured oxidative stress parameters of nitric oxide (NO) and lipid peroxidation (malondialdehyde, MDA) in tumor cells [53]. Also, Jadeski and Lala described that nitric oxide synthase expression in human pancreatic cancer cells is positively correlated with apoptosis [55].

In mosquitoes, apoptosis occurs as a result of infections in the mid gut, this suggests that apoptosis plays a role in mosquito innate immunity [56]. Recently it has been shown that exposing a lab-derived strain of *Culex pipiens* to infection with West Nile virus caused extensive cell death in the midgut epithelial cells of these mosquitoes [57]. In spite of only few studies have been conducted in insects, the available data suggest that mid gut epithelial tissue of the insects challenged with either pathogenic or nonpathogenic agents and is able to trigger an immune response to reduce the cellular and tissue damage [58].Apoptosis is mediated via a major family of evolutionarily conserved cysteine dependent aspartate-specific proteases, called caspases [59]. Apoptotic caspases are generally divided into "initiators" and "executioners" [60]. Apoptosis can be activated via two distinct pathways: the extrinsic and intrinsic pathways. The intrinsic pathway can be induced when the cell is under stress, which leads to the depolarization of the mitochondrial membrane and the subsequent release of pro-apoptotic molecules at the origin of initiator caspase-9 activation where, these activated initiator caspases initiate the executioner caspases-3, -6 and -7, with caspases-3 and -7 having redundant roles [61]. Increased level of ROS lead to an apoptosis-like response, lipid peroxidation, depletion of antioxidant enzyme such as glutathione, and DNA damage [62].Considerably, caspase-3 is a frequently activated death protease, catalyzing the specific cleavage of many key cellular proteins. It is important or essential in other apoptotic scenarios in a remarkable tissue-, cell type- or death stimulus-specific manner. It is also required for some typical hallmarks of apoptosis, and is indispensable for apoptotic chromatin condensation and DNA fragmentation in all cell types examined. Thus, caspase-3 is essential for processes associated with the dismantling of the cell and the formation of apoptotic bodies, but it may also function before or at the stage when commitment to loss of cell viability is made [63]. At the mean time SNPs have been shown to induce the apoptotic pathway in vitro through free oxygen radical generation and also showed anti tumor, anti proliferative, and anti angiogenic effect in vitro [64, 65, 66].

Results in this study displayed significant increase in the levels of caspase3 marker in tissue homogenates of *Culex pipiens* larvae upon increasing the concentration of GSNPs. A finding that indicate the

damaging effect that GSNPS induced in the tissues of *Culex pipiens* larvae that lead to decreasing the levels of the protecting ROS enzymes, increasing the level of nitric oxide and eventually increasing the apoptotic markers and inducing intrinsic apoptotic pathways. Further studies implied that SNPs can induce cell death and oxidative stress in skin carcinoma cells and in human fibrosarcoma [67]. Furthermore, silver nanoparticles exert their antifungal effect through apoptosis via the accumulation of reactive oxygen species induction .Adding to this, hydroxyl radicals are considered an important component of cell death. Cells exposed to silver nanoparticles showed increased reactive oxygen species and hydroxyl radical production [68]. Recent experiments, observed high expression levels of the pro-apoptotic proteins such as P53, caspase 3 and Bax levels in GSNPS treated cancer cells. The activation of these proteins leads to mitochondrial permeability. These findings suggest that GSNPs significantly induced mitochondria mediated caspase dependant apoptosis. [33]. It was also recently mentioned that higher concentrations of GSNPs reduced liver cells viability and proliferation through induction of apoptosis with caspase-3 activation with dose-dependent manner [69]. The studies of molecular mechanisms revealed that the major mode of cell death induced by SNPs was cell apoptosis. The mechanisms indicated that SNPs promote caspase-3-mediated apoptosis by the involvement of ROS generation [70]. Silver nanoparticles induce apoptosis via caspase dependent pathway. It was described that the possible molecular mechanisms of triggering the apoptosis was identified through the activation of the caspase cascades in tumor cells treated with SNPs [69]. Finally, all the findings in this study were in agreement with the statement that mentioned that SNPs cytotoxicity occurred due to their chemical transformation of neutral silver (Ag°) to Ag+, Ag-O-, Ag-S-, which lead to ROS production by chain effect, where an increase in the intracellular ROS levels was observed. Hence, GSNPs are capable of inducing cytosolic oxidative stress and thereby promote cell death.

V. Conclusion

In conclusion, green silver nanoparticles (GSNPs) exhibit cytotoxic and deteriorating effects on cells of *Culex pipiens* larvae regarding their ability to decrease the activities of enzymes of cell damage and oxidative stress while increasing the levels of nitric oxide and provoking the expression of caspase3 gene that is involved in the execution of apoptotic pathways in damaged cells. Hence, GSNPs might be a promising candidate for *Culex pipiens* larval control concerning their prevalence.

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Mona Ragheb, et. al. "Green Silver Nanoparticles induced apoptotic pathways and oxidative stress in cells of Culex pipiens larvae." *IOSR Journal of Biotechnology and Biochemistry (IOSR-JBB)*, 7(6), (2021): pp. 31-39.