Research Article

Biocompatible green synthesized silver nanoparticles impact on insecticides resistant developing enzymes of dengue transmitted mosquito vector

Ezhumalai Parthiban[1] · **Maduariveeran Ramachandran**[1] · **Manickam Jayakumar**[1] · **Ravichandran Ramanibai**[1]

Abstract
The growing interest in nanomaterial-based products in various fields urged to study the influence of different nano-products on the ecosystem and on various insect pests. In this study, we evaluated insecticides resistant influenced important biochemical constituents of *A. aegypti* upon the exposure of green synthesized *A. reticulata* silver nanoparticle (Ar–AgNPs) for 24 h at their lethal threshold concentration. The AgNPs exposed and control mosquito larval homogenates of the protein content, acetylcholinesterase, specific detoxification enzymes α- and β-carboxylesterase and other anti-oxidant or effective insecticides resistant developed enzyme named glutathione *S*-transferase (GST) were studied. The larvicidal activity of AgNPs at their lethal threshold concentration (4.5 µg/ml) were monitored for 12 and 24 h and prompted time dependent mortality rate were 23.3 ± 0.57 and 46.6 ± 0.57% respectively. Further, the biochemical constituents of protein content of AgNPs exposed larval homogenates were quantitatively higher compared to the control, but, in the qualitative analysis of separated protein in the electrophoresed gel revealed, the expression protein was down regulated. The important esterase enzymes of acetylcholinesterase, α-and β-carboxylesterase and GST enzymes were significantly reduced upon the exposure of AgNPs compared to the control larval body homogenates respectively. In the qualitative analysis, isoenzyme of β-carboxylesterase expression level was also down regulated. Followed by these investigations, it is clearly showed that *A. reticulata* mediated synthesized AgNPs have the potent deleterious impact on fourth instar larvae of *A. aegypti* physiological system by inhibiting important esterase enzyme groups.

Keywords *Annona reticulata* · *Aedes aegypti* · Larvicidal activity · Eco-friendly · Esterase

1 Introduction

Arthropod insect order, *Aedes aegypti* is a deadly disease transmitting vector originated from Africa but now it is globally distributed especially in tropical and subtropical regions of India. Global distribution of this vector was assisted by mass human migrations as well as lack of awareness among the population leads to transmitting of the deadly diseases such as Zika, dengue, Chikungunya and yellow fever between the human populations [1]. Application of synthetic insecticides for controlling of mosquito larvae leads to adverse impacts on other some non-target aquatic organisms in the environment. Moreover, such chemical insecticides cause resistance development species and also its production cost of these insecticides are very high [2]. To overcome these issues, there is an alternate eco-friendly method are in necessity to control mosquito population at their community level with specific features such as low or lack of toxic effects on non-target organisms and their ecosystem, etc. There are few alternate methods have been used preferentially such as botanicals, entomopathogenic fungus and aquatic predator etc. [3–6]. At present, nanotechnology have been widely used in all fields, especially, the silver nanoparticles

✉ Ravichandran Ramanibai, rramani8@hotmail.com | [1]Department of Zoology, University of Madras, Guindy Campus, Chennai 600025, India.

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are used in various field research, including pharmaceutical industries to develop a best anti-microbial drugs, agriculture, bio labeling, parasitology and pest management [7, 8]. Silver naoparticles have been synthesized using different types of reducing agent, of which, the plant-mediated biosynthesis of silver nanoparticles is advantageous over chemical and physical methods, since it is a cheap, single-stepped and did not require high energy, temperature, pressure [9, 10]. A number of plants-synthesized nanoparticles have been reported as effective adulticides, pupicides, larvicides, ovicides and ovideterrents effects against various mosquito species [2]. In the current scenario, plenty of research has been carried out using botanicals as reducing agent for the green synthesis of silver nanoparticles that showed a reliable insecticidal property against various insect pests. The investigation on mode of action of these botanicals based synthesized nanoparticles on experimented insect pests is very limited. While using of these biocompatible silver nanoparticles with insecticidal property will produce changes on the important biomarker enzymes in the insecticidal resistant insect pests. The underlying mechanisms of these kinds compounds and how physiological alterations occur in the insect pests upon exposing to botanicals is necessary to explore the biomarker enzyme expression in the insect pests and its provide a fundamental understanding about the mode of action of insecticide resistant to various insect pests. There are few major biomarker enzymes were evaluated to study the effects of botanical biocides in the insect resistant developed enzymes, namely, esterases, phosphates, glutathione-*S*-transferase, monooxygenase etc. [11]. These enzymes are playing a vital role in insect pests; especially, the esterase family is helped in digestion, reproduction and metabolism of juvenile hormone, metamorphosis and detoxification of toxic substances [12, 13]. Among the various enzymes in esterase families, one of the enzyme called acetylcholinesterase is present at nerve synapses and it helps to transmission of nerve impulse by hydrolysis of acetylcholine [14]. Carboxylesterase, is another important enzyme that plays a vital role in degradation of neurotransmitter, metabolism of specific hormones and pheromones, consequently involved in insect development and behavior, detoxification of toxic substances etc. [15–17]. On the other hand, glutathione *S*-transferase enzymes are highly involved in the insecticide resistance development in various insect pests by detoxification of diverse allelochemicals exposure to the diverse insect pests [18].

Annona reticulata (Annonaceae) has been used to treat various ailments, diseases [19]. It is widely distributed in tropical and subtropical regions of countries [20]. It is reported to have high anti-bacterial, insecticidal, anti-proliferative and primarily mosquito larvicidal activity with lack of these enzymes assessments in *A. aegypti* mosquito larvae using *A. reticulata* based metabolites [21–24]. Based on this background information, we selected this plant for the silver nanoparticles synthesis, assayed the larvicidal and toxicity to non-target aquatic organisms previously. Moreover, this is the first report of biochemical assessment in the *A. aegypti* larvae upon exposed to the *A. reticulata* silver nanoparticles. Therefore, we carried out the experiment to find out the influence of *A. reticulata* mediated synthesized silver nanoparticles on the biochemical constituents of mosquito vector larvae and which could provide better understanding of the mode of action of the AgNPs on mosquito larvae.

2 Materials and methods

2.1 Fine chemicals and reagents

Acetylthiocholine iodide and fast blue-B salt were purchased from Hi-Media (Mumbai, India), and α- and β-naphthol, reduced glutathione *S*-transferase, 1-chloro-2,4-dinitrobenzene (CDNB) were purchased from SRL (Mohali, India). β-naphthyl acetate, DTNB reagent (5-5-dithiobis 2-nitro benzoic acid) were purchased from Sigma (Mumbai, India). Other fine chemicals and reagents were used of the highest analytical grade reagents.

2.2 Plant collection, extraction, synthesis and characterization of silver nanoparticles

For the investigation of this work, the active plant was collected; authenticated [25] and processing of extraction, synthesis and characterization of silver nanoparticles were detailed described in our previous investigation [26]. For the purpose of this work, the previously synthesized silver nanoparticles were used in this study.

2.3 Collection and rearing of mosquito larvae

The *A. aegypti* eggs were collected from the laboratory reared mosquitoes. Eggs were allowed to hatch out and reared until it reaches the fourth instar larvae with the regular fed with a mixture of powdered dog biscuit and brewer's yeast at 3:1 ratio and used for all the experimental studies [26].

2.4 Larvicidal bioassay

The larvicidal bioassay using silver nanoparticles was performed according to methods followed in WHO protocol with insignificant modification [27]. The larvae were exposed to AgNPs at their lethal threshold concentration

(4.5 µg/ml) in 50 ml of tap water. This experiment was performed with five replicates. Control containers maintained with equal volume of tap water instead of AgNPs. The percentage of mortality of larvae was recorded every 12 h exposure period.

2.5 Preparation of larval whole body homogenates

The control and AgNPs exposed (LC_{50}) larvae were separately taken and washed it with double distilled water and removed the water content from body surface by blotting with tissue paper. The larvae of ten individuals were homogenized in eppendorf tubes (held in crushed ice) using a Teflon hand homogenizer containing 0.5 ml of ice cold 20 mM phosphate buffer (pH 7.2). The whole body homogenates were centrifuged at 10,000×*g* in 4 °C for 15 min and the clear supernatants were used for the all the quantitative and certain (protein and α-carboxylesterase) qualitative analysis. The homogenates were stored at − 20 °C until use.

2.6 Quantitative analysis of biochemical constituents of *A. aegypti*

2.6.1 Determination total of protein concentrations

The total protein content of control and experimental larval homogenates were estimated according to method Lowry et al. [28] using bovine serum albumin (BSA) as standard.

2.6.2 Acetylcholinesterase assay

The acetylcholine esterase activity was determined in the larval homogenate by the Ellman et al. [29] using acetylcholine iodide as a substrate. Fifty micro liter of larval homogenate was dissolved in 850 µl of 100 mM sodium phosphate buffer (pH 7.5). To each reaction mixture, 50 µl of 10 mM DTNB and 50 µl of 12.5 mM acetylcholine iodide were added and incubated at RT for 5 min. The optical density of the sample was read at 405 nm using Thermo Scientific Multiskan EX (200–240 V) spectrophotometer against suitable blank.

2.6.3 Carboxylesterase assays

The determination of α- and β-carboxylesterase activities in the larval homogenate was measured by method of Van Asperen [30]. For the assays, 30 µl of the homogenate was added with 1 ml of sodium phosphate buffer (100 mM, pH 7.0) containing 250 µM of α-naphthyl and β-naphthyl acetate and incubated for 30 min at RT. For each reaction mixture, 400 µl of 0.3% fast blue B in 3.3% SDS was added to stop the enzymatic reaction and it was allowed for color development for 15 min at RT. The optical density (Thermo Scientific Multiskan EX-200-240V) of the sample was read at 430 nm for α-carboxylesterase and 588 nm for β-carboxylesterase against a suitable reagent blank. The carboxylesterase activity was calculated with standard blot curve made using α- and β-naphthol as standard.

2.6.4 Glutathione *S*-transferase assay

The activity of this enzyme level was measured according to method of Devonshire et al. [31]. Briefly, 30 µl of homogenate were mixed with 200 µl of 10 mM of reduced glutathione and 63 mM of CDNB mixture solution. It was then incubated for 5 min at RT. The optical density was read at 340 nm against a suitable reagent blank.

2.7 Qualitative analysis of biochemical constituents of *A. aegypti*

The separation of proteins in the homogenates was analysed with native-PAGE (8%). Briefly, both control and experimental larval (40 µl) homogenates were electrophoresed at a constant current of 80 V at 10 °C on a slab gel system. After electrophoresis, the gels were stained with coomassie brilliant blue (CBB) R-250 for detection of protein bands and incubated with appropriate substrates for the detection of protein bands for esterase activity.

2.7.1 Detection of β-carboxylesterase activity

The β-carboxylesterase activity in the homogenate was detected according to the method of Kirkeby and Moe [32]. The gel was first incubated with 20 mM phosphate buffer, pH 7.0 for 15 min at RT. After decanting the buffer, the gel was then re-incubated with freshly prepared β-naphthyl acetate with fast blue B in 20 mM of phosphate buffer, pH 7.0 for 30 min at RT. Then the gels were washed with distilled water and stored at 7% glacial acetic acid.

2.8 Statistical analysis

For biochemical analysis, each experiment was conducted with three replicates and the significance level between control and experimental larvae were calculated by Student's t test using the Statplus (V.5.00) software. Other statistical analysis was performed by using excel.

3 Results

3.1 Larvicidal effects

The larvicidal bio assays were performed to determine their threshold time for lethal effect of Ar–AgNPs against fourth instar larvae of *A. aegypti* by exposing them to specific concentrations of this AgNPs. As shown in Fig. 1, the AgNPs tested at a concentration of 4.5 µg/ml did not elicit mortality of the larvae up to 6 h, but ~ 23% larvae were found dead at 12 h, and 46% larvae were found dead at 24, whereas, remaining larvae were found to be killed at 48 h exposure time. A mortality of 20–46% of the test organisms at the 24 h time point upon their exposure to a specific concentration of AgNPs was considered as the threshold time for its lethal effects. According to the exposure time of 24 h for fourth instar larvae to the particular test concentrations of the AgNPs were chosen to investigate the impact of AgNPs on the biochemical constituent's of fourth instar larvae of *A. aegypti*.

3.2 Effects of silver nanoparticles on general protein metabolisms

The AgNPs exposed alive and control larvae were used to examine the all the biomarker enzymes after determination of their protein contents from the control and AgNPs exposed larvae. The protein contents of the control and exposed larvae were noticed statistically significant ($p \leq 0.05$), compared to control. The protein level of the exposed larvae was significantly increased were 2.7 and 2.025 mg/ml in the AgNPs exposed and control larval homogenates of the *A. aegypti* respectively (Fig. 2a). Interestingly, in the qualitative analysis of AgNPs exposed larval protein expression level in the electrophoresed gel was down regulated (Fig. 2b).

3.3 Acetylcholinesterase activity

The acetylcholine esterase level of the of AgNPs exposed larvae for 24 h at their lethal threshold concentration was abruptly reduced while normal physiological level maintained to the control larval homogenates was depicted in Fig. 3. The noticed level of acetylcholine esterase was highly statistically significant to AgNPs exposed larvae were ($p \leq 0.01$).

3.4 Isozyme of carboxylesterase activity

As depicted in Fig. 4a, b, both α and β-carboxylesterase level of the AgNPs exposed larval homogenate were decreased steadily when compare to control larvae. The reduced level of these both enzymes is statistically significant ($p \leq 0.05$) due to the low level of α- and β-naphthol released, when the respective carboxylesterase enzyme was inhibited by AgNPs exposed larvae. Similarly, in the qualitative analysis, the important allelochemicals detoxify isozyme of β-carboxylesterase expression level in the gel electrophoresis was in down regulated by AgNPs exposed (Fig. 5).

3.5 Glutathione *S*-transferase activity

The glutathione *S*-transferase enzyme level to the control and silver nanoparticles exposed larval homogenates were observed after 24 h at their lethal concentration. The enzyme level of AgNPs exposed larvae was immensely declined with statistically significant ($p \leq 0.001$) compared to the control larval homogenates as depicted in Fig. 6.

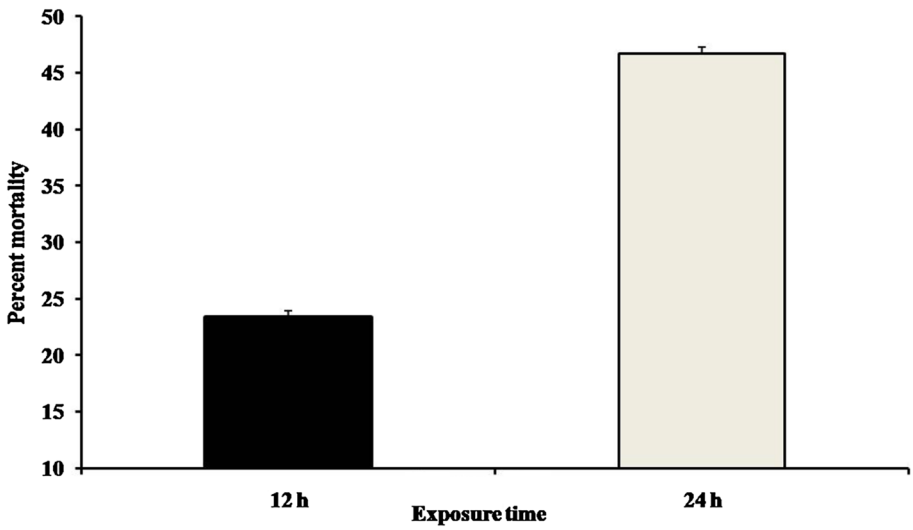

Fig. 1 The larvicidal activity of *A. reticulata* AgNPs against fourth instar larvae of *A. aegypti* at their lethal threshold concentration (4.5 µg/ml). Each bar represents as mean of five determinations and the vertical line represents ± SD

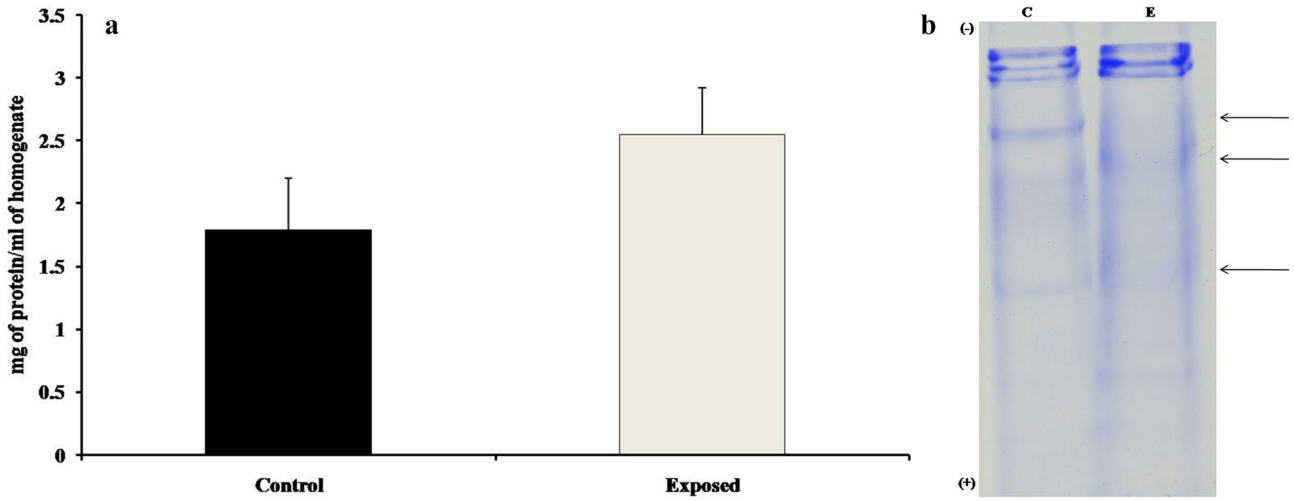

Fig. 2 **a** The bar diagram represent the protein concentration of fourth instar larvae of *A. aegypti* for 24 h exposed to *A. reticulata* AgNPs at their lethal threshold concentration. Each bar represents as mean ± SD of three replicates, **b** separation of proteins in the whole body homogenates of fourth instar larvae of *A. aegypti* by discontinuous polyacrylamide gel electrophoresis under non-denaturing conditions (native PAGE). An aliquot (40 µl homogenate) from control (C) and AgNPs exposed (E) larvae were loaded into each well. The gels were stained with Coomassie brilliant blue R-250 (CBB-R20). Arrows indicates perceptible changes in protein banding pattern between control and experimental larvae

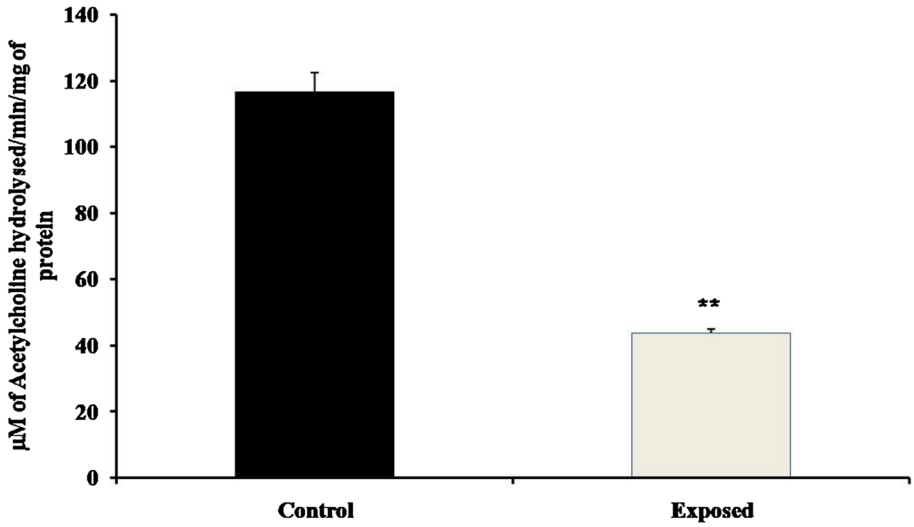

Fig. 3 Acetylcholinesterase activities in the whole body homogenates of the fourth instar larvae of *A. aegypti*. The bar diagram represent the levels of acetylcholine hydrolyzed in the fourth instar larvae exposed (24 h) to *A. reticulata* AgNPs. Each vertical bar represents as mean ± SD of three replicates. Star sign indicates statistically significant difference between control and experimental larvae ($p \leq 0.01$)

4 Discussion

In recent years, nanotechnology has been used in all fields, including the pesticide formulations for the effective control of diverse insect pests. A green synthesized silver nanoparticles are more widely used rather than other metal nanoparticles was due to low toxic, an eco-friendly and low cost. The green synthesized silver nanoparticles cause efficient mortality compared to plant extract due to small spherical shaped particles obtained through green synthesis of AgNPs is able to passing through the cellular membrane barriers without any obstructions [33, 34]. The reason of this green synthesis mechanism was due to by the effective functional group of plant compound was embedded with Ag ions contains solution during the reduction process cause formed small sizes of nanoparticles, therefore it has easily passed through the cellular barrier and cause a damages in inner cellular organelles or disturbs their normal physiological functions leads to changes in the entire organ system, sub sequentially, the insect were met to dead as reported by few investigators [35, 36].

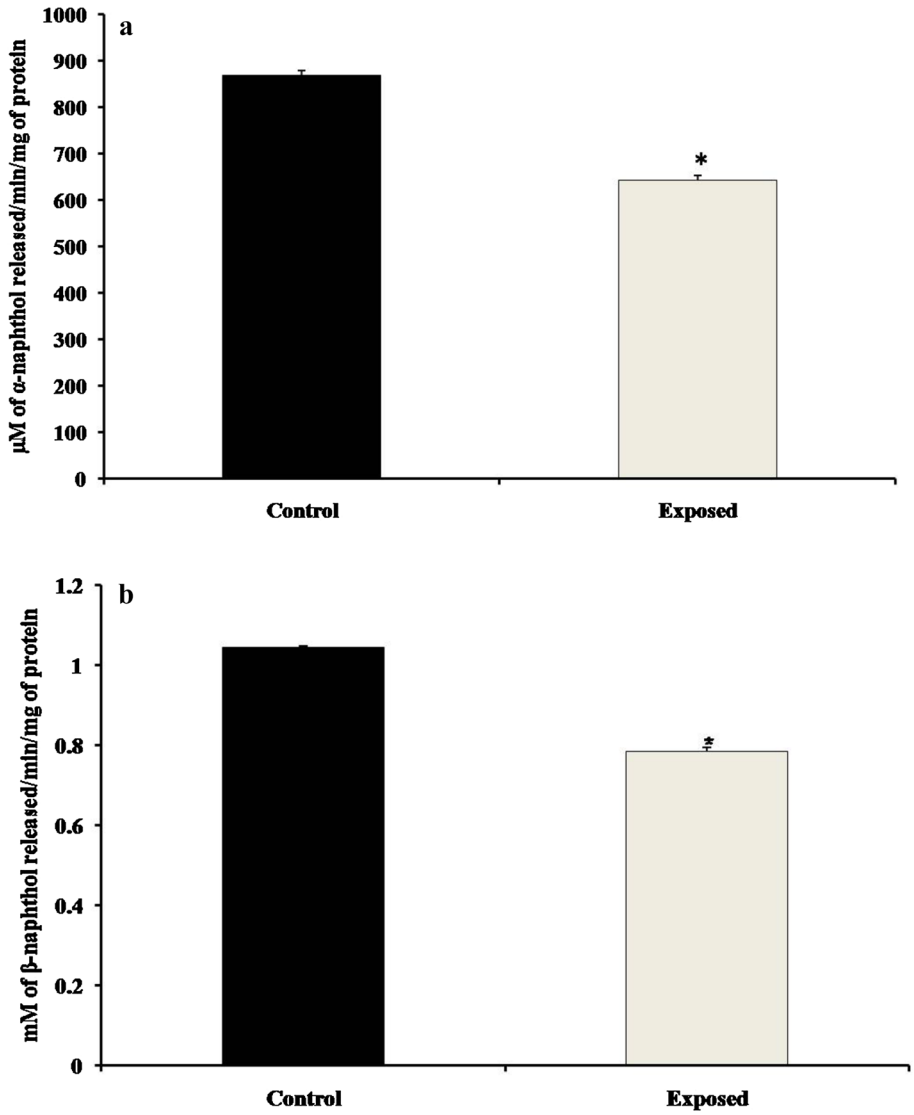

Fig. 4 **a** α-carboxylesterase activity in the whole body homogenates of the fourth instar larvae of *A. aegypti*. The bar diagram represent the levels of α- naphthol released in the fourth instar larvae exposed (24 h) to *A. reticulata* AgNPs. Each bar represents as mean ± SD of three replicates. Star sign indicates statistically significant difference between control and experimental larvae ($p \leq 0.05$), **b** β-carboxylesterase activity in the whole body homogenates of the fourth instar larvae of *A. aegypti*. The bar diagram represent the levels of α-naphthol released in the fourth instar larvae exposed (24 h) to *A. reticulata* AgNPs. Each bar represents as mean ± SD of three replicates. Star sign indicates statistically significant difference between control and experimental larvae ($p \leq 0.05$)

Protein plays a major role in the insects during metamorphosis such as cuticle formations and chitin synthesis, etc., while insects exposed with botanical based pesticide, it could be leading to changes in the whole body level of protein by down regulation or up regulation. In our investigations, the AgNPs exposed to fourth instar larvae of *A. aegypti* protein level was steadily increased compared to the control larvae by quantitatively, but contrast, in the qualitative analysis, the protein expression pattern in the electrophoresed gel was observed in down regulated by inhibiting the proteins involved in the detoxification process apart from the other tissue protein was raised in the quantitative analysis. The similar phenomenon was also observed by few investigators, that the level of protein was increased to the botanical biocides exposing insects compared to control, besides other remaining biochemical constituents was down regulated. It's due to the increased synthesis of rest of proteins from the fat body, haemolymph and other related tissues of the insect pests, when it was participated in the detoxification process, while toxicants intruded into the insect body [37–40]. Therefore, the evident reports from previous studies would be the reasoning of higher level of protein synthesis in the whole body homogenates of *A. aegypti* larvae upon exposed to AgNPs, while other rest of important specific detoxifying enzyme level was inhibited in the tested mosquito larvae.

Acetylcholinesterase is the major neurotransmitter enzyme to hydrolyze the substrate of acetylcholine into acetate as well as choline. This acetylcholine is released from pre synaptic neuron into a synaptic cleft bind with the receptor for passing the sodium ions through this channel. Acetylcholinesterase enzyme is released to break the acetylcholine, which is present in the synaptic cleft and send back the choline to the presynaptic terminal to take part in regular cycles for synthesis of acetylcholine along

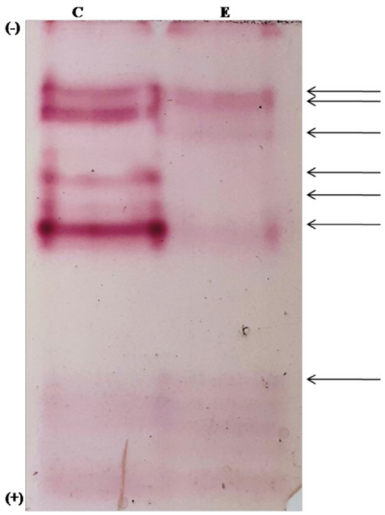

Fig. 5 Detection of β-carboxylesterase in the whole body homogenates of control (C) and AgNPs exposed (E) fourth instar larvae of *A. aegypti* under non-denaturing condition (Native-Page). An aliquot of 40 µl of homogenate was loaded into each well. Arrows indicates perceptible differences in banding pattern between control and experimental larvae

with Acetyl CoA [41]. In our studies, the synthesized AgNPs exposed larvae acetylcholine esterase activity was inhibited revealed by releasing of low quantity of acetylcholine compared to control larva in the experimental result. The inhibition of this esterase activity was due to by the green synthesized silver nanoparticles directly to bind with the acetylcholinesterase receptor binding site and its block the releasing of acetylcholinesterase enzymes for the breaking acetylcholine substrate. Resulting, the inhibited acetylcholinesterase enzyme was not able to hydrolyze the continuous releasing of acetylcholine substrate and its leads to an accumulation of acetylcholine at the postsynaptic terminal junction. This continuous accumulation of acetylcholine at postsynaptic terminal, resulting continuous opening of sodium channel as well as assist non-stop passing of sodium ions made a permanent stimulation state in the tested mosquito nerve system. Therefore, the lack of coordination at the point of neuromuscular system named ataxia was occurring and it's eventually larvae were met with dead [42, 43].

The α- and β-Carboxylesterase is the important detoxification enzyme in the insect physiological system to defend itself against various allelochemicals. The activity of these both α-and β-carboxylesterase enzyme level were reduced in our studies due to the inhibition of this enzyme activity upon exposed to AgNPs against *A. aegypti* fourth instar larvae by quantitatively. At the same time, one of this isoenzyme of β-Carboxylesterase activity were detected in the gel electrophoresis by reveling these responses upon exposed to AgNPs shows many protein patterns of these enzyme levels was down regulated by disappearing or faint of a specific enzyme level in the qualitative analysis. This indicated that *A. reticulata* mediated synthesized AgNPs have the ability to inhibit specific enzyme activity of the tested organism without developing any resistance. Regrettably, and to our knowledge, there is only a few reports are investigated using green synthesized silver nanoparticles on isoenzyme of carboxylesterase activity in the insect especially mosquitoes. Accordingly, Kamaraj et al. [44] who reported the evaluation of various esterase enzyme activities including carboxylesterase in the *Helicoverpa armigera* insects using *Trichoderma viride* formulated titanium dioxide nanoparticle. Upon exposing this nano formulation, they observed, the reduced level of carboxylesterase activity and other enzyme called glutathione *S*-transferase level was elevated. Therefore, in our study strongly evidenced to develop new insecticides towards diverse insect pests, including mosquito with reference support of α-and β-carboxylesterase enzyme level

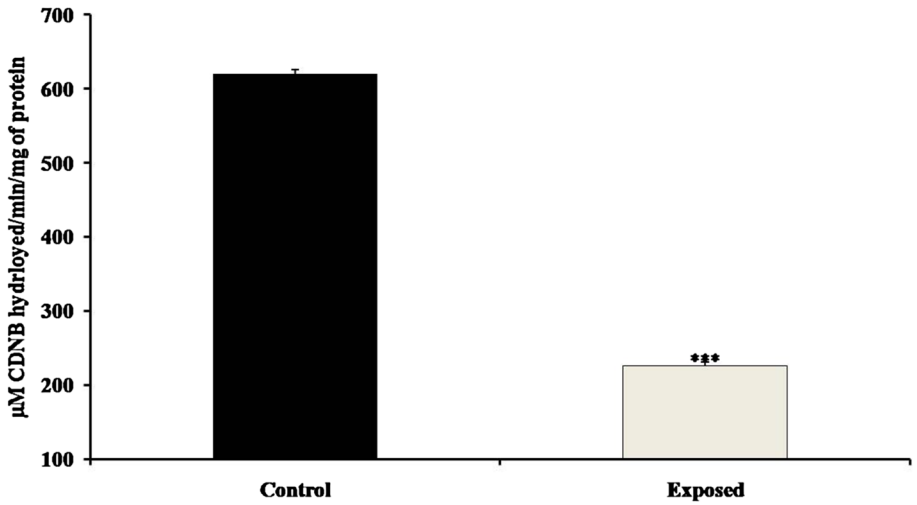

Fig. 6 Glutathione *S*-transferase inhibitions in fourth instar larvae of *A. aegypti* after exposed to *A. reticulata* AgNPs. Each bar represents as mean ± SD of three replicates. Star sign indicates statistically significant difference between control and experimental larvae ($p \leq 0.001$)

reduced upon exposed to the *Annona reticulata* mediated synthesized AgNPs.

In another one esterase enzyme called glutathione *S*-transferase are important key biomarker enzyme to detect whether it was developed a resistance or susceptibility upon exposed to the particular botanicals based insecticides. Moreover, this glutathione *S*-transferase (GST) enzyme exist in highly in various insect pests especially in mosquitoes. Therefore, it was confirmed that this enzyme plays a major significant role in the detoxifying process. When plant based insecticides are involved in inhibition or stimulation of these enzymes in the insects may result in metabolic disparity, impairment of growth and induction of mortality [45]. In our study, the GST enzyme level was significantly ($p \leq 0.001$) reduced and its indicated that the AgNPs might be involved in the redox reaction and causes oxidative stress damage in the larvae tissues upon exposed to AgNPs [46–48].

5 Conclusion

We bring out the possible mechanism in the mosquito larvae upon exposed to synthesized silver nanoparticles have the potential ability to inhibit the specific major enzyme activity like esterase family rather than whole body homogenates of the protein content. This biological and eco-friendly synthesized AgNPs were only a toxic to the target mosquito larvae, but not for non-target aquatic organism which was reported in our earlier work. Therefore, it could be a considered as alternate insecticides agents for the control of mosquito populations in the current scenario.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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