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Biological effects of synthesized silver nanoparticles using *Dodonaea viscosa* leaf extract against *Aedes aegypti* (Diptera: Culicidae)

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Abstract
Nowadays Metallic nanoparticles is a hot topic used for cleaning waste water as well has the applications in the field of Medical sciences. In the present study, fresh leaves of *Dodonaea viscosa* have been used for synthesis of silver nanoparticles and its Larvicidal activity was evaluated against *Aedes aegypti* larvae. The biosynthesized silver nanoparticles **(**AgNPs) were characterized using Scanning Electron Microscopy (SEM) and Fourier Transformed Infrared spectroscopy (FTIR). Randomly amplified polymorphic DNA technique was used for the genetic toxicity of the dengue mosquito larvaes. Results revealed that LC_{50} of AgNPs was more effective as compared to that of *D. viscosa* extract by 1.38 fold. Molecular assessment using three set of primers did not showed any mutation in the DNA. However the growth of larvae was greatly affected by AgNPs treatment as compared to control one. The retardation in the larval growth might be due the under expression and/or mutational effects of the AgNPs nanoparticles. In addition, the AgNPs resulted deformation in larvae stage of the *A. Aegypti* and also disturbed the metamorphosis of larvae to pupa and pupa to adults. Therefore, our results revealed a promising effects of *D. viscosa* AgNPs and could be used as ecofriendly pesticide for the control of dengue fever vector.

Keywords: Silver nanoparticles, *Dodonaea viscosa*, larvicidal, *Aedes aegypti*, dengue fever

Introduction
Dengue fever is a vector borne disease caused by dengue virus, transmitted by mosquitoes *Aedes aegypti* (L.) to humans. Dengue fever has infected 5% of the population of the total world population. The disease is classified as emerging or re-emerging. It is endemic to most countries outside the European Region. It is increased fourfold between 1970 and 1995; in the American Territory, there were 1.3 million cases in 1998, of which 3,600 were deaths [1].
Disease control methods in the past have focused on using conventional chemical pesticides [2]. Frequent and intensive use came to be associated with harmful effects on non-target organisms, such as mosquito predators or through the food chain, known as bio-magnification, causing serious damage to the environment and chronic health problems [3].
A number of studies have reported the importance of applications of non-conventional pesticides such as biocides, insect growth regulators, plant extracts as alternative methods in mosquito control [4, 5]. The application of conventional chemical pesticides caused a problem, because kills natural enemies resulting unbalancing the equilibrium in the environment and the emergence of insecticide-resistant mosquito strains [6]. In recent studies nanotechnology has been used to produce nanoparticles using some plant extracts as biochemical agents to convert metal ions into nanoparticles [7-11]
In the present study, *Dodonaea viscosa* extract was used in combination of AgNPs to evaluate the Larvicidal and genotoxic effects on larvae of *Aedes aegypti* to innovate a new and environmentally friendly method against dengue fever.

Materials and methods
Insect rearing and plant extraction
The eggs of *A. aegypti* were obtained from the department of biological sciences, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia. The eggs were put for rearing in standard conditions to get larval colonies [12].
Dodonaea viscosa plants leaves were collected from Al Baha area located in the southwestern part of Saudi Arabia. The leaves of these plants were washed carefully by tap water and then

dried at room temperature. The dried leaves of the plant material was powdered mechanically using commercial electrical stainless steel blender. 30 g of the powder was added to 90 ml of ethanol and incubated for 72 h. The extract was filtered and transfer to Soxhlet apparatus (boiling point range 60-80 ºC) to evaporate the ethanol from it. The crude plant extract was stored at 22 ºC for further use.

Larvicidal assay of plant extract and silver nanoparticles (AgNPs)

Standards solutions were prepared for experiments by taking 1 gm of the plant debris that was dissolve in 99.5 mL of distilled water and 0.5 Triton X-100. This was considered as 1% stock solution. From this stock solution, a series of concentrations was prepared and tested for their effectiveness against *A. aegypti* mosquito larvae.

The silver nanoparticles were synthesized using 1 ml of the extract and 1ml AgNO3 in addition to 0.5 Triton x-100 and 97.5 distilled water. The extract along with silver nitrate was left on magnetic stirrer at room temperate until the color changes.

The fourth instars larvae of the *A. aegypti* mosquito was selected. The tests were carried out in petri plate containing 100 ml of tap water. In all sensitivity tests, five replicates were used for the LC_{50} concentration of *D. viscosa* plant extracts and AgNPs. Twenty larvae were used per replication that was monitored daily**.**

Characterization of AgNPs by SEM and FTIR

The plant extract was centrifuged at 4000 rpm for 30 min and supernatant was dried completely in the oven at 40°C. The fraction of the dried precipitate placed on a conductive carbon adhesive connector, for the analysis of its phenotype and elements. This analysis was conducted through scanning electron microscope and the X-ray spectrometer with dispersed energy. (JEOL, JSM7600F, Field Emission Scanning Electron Microscope).

A spectral study of plant extracts was carried out before and after the reduction process for the infrared region using an infrared spectroscopy. The spectroscopy was done by placing a small fraction of the plant extract after drying.

Larvicidal bioassay of AgNPs

The larvicidal activity was monitored of AgNPs prepared from them under laboratory. The fourth instars larvae of the *Ae. aegypti* mosquito was selected. The tests were carried out in small white plastic containers (diameter 11 cm and depth 4 cm) containing 100 ml of water. In all sensitivity tests, five replicates were used for each concentration and for all extracts. Where each single contains 20 larvae in addition to five replicates for control. Taking into account the supply of larvae with food to avoid starvation factor. The ratios of death obtained by Abbott equation were corrected [13]. The resulting dead stages are collected and examine for the identification of abnormalities that may have evolved in the form of a virtual microscope with a digital camera connected to a computer (Stereo Dissection microscope (Leica EZ4D S/NO: 5649900 made in Singapore).

The DNA of larvae and RAPD analysis

The DNA was extracted according procedure of thermo-scientific kit [14]. RAPD amplification was done using different primers [15]. The amplification products were analyzed by electrophoresis. The PCR amplified products, 100 bp DNA ladders as standard marker were subjected to electrophoresis in 1.5% agarose gel in TAE buffer and stained with ethidium bromide. Molecular size of the marker was 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100 bp. The amplified pattern was visualized on a UV transilluminator. Polymorphism was evidenced as the presence and/or absence of DNA fragments between the samples. The RAPD profiles of the treated insects were evaluated on the same 1.5% agarose gel run for 30 min at 120 volts.

Statistical analysis

SAS program was used to compare mortality rates and their relationship to the concentrations used in the experiments. The laboratory toxicity results were analyzed according to [16] the method using a statistical program that analyzes the results of the LDP line.

Result and discussion

The results (Table 1) showed that the percentage of larval mortality of the 4th instar larvae of *Ae. aegypti* mosquito was treated with a series of concentrations of the methanolic extract of the *D. viscosa* plant ranged from 100 - 900 ppm. Which were directly proportional to the death rates of the 4th instar larvae of *A. aegypti* mosquito, which ranged from 28.125 - 94.792%. On the other hand, the results of the laboratory toxicity analysis of the tested compound the difference in the level of mosquito sensitivity for these preparations. By studying the LC-p line and the values of the lethal concentrations of 50% and 90% of the larvae exposed to different concentrations of extract. The lethal concentration of 50% of the treated larvae was 214.3288 ppm while the lethal concentration of 90% of the treated larvae was 932.3018 ppm. The effective concentrations of AgNPs from the methanolic extract of the *D.viscosa* plant ranged from 50 to 250 ppm and the mortality percentages for larvae treated with these concentrations were between 7.292 and 79.167% (table 2). In the case of the methanolic extract of D.*viscose* with AgNPs, the concentration values for killing 50% and 90% of the larvae within 24 hours of treatment were 154.7695 and 376.2513 ppm, respectively. According to the lethal concentration LC_{50}, the result shows differentiation in the senetivity on mosquitoes. The AgNPs (154.77ppm) has more effect to mosquito larvae than the extracts alone (214.328ppm) by about 1.385 fold (Fig. 1).

Furthermore, mortality in the pupal stage and the incomplete emergence of adults were observed. The results revealed that the treated larvae with medicinal plant extract of D.viscose showed deformed abnormalities in developmental stages of *A. aegypti* larval and pupal stages after treatment. Shrinks of the larva body parts. Cell explosion and pigmentation. Clear larva neck prolongation andenetration of nanoparticles on the effected larvae body. There is other morphological affect for the treatment of the plant extract as intermediate stages including larval siphon, pupal trumpets, un melanized pupa (Albino pupa) and failure of adults to emerge from the pupa's skins (Fig. 2)

Analysis of nanoparticle particles.

This study involved the diagnosis of nanoparticle particles, biologically prepared from plant extract (D. *viscosa*), which reduced the silver nitrate (AgNO3) to nanoparticles. After adding silver nitrate to the selected plant extract by 1:1 and using water as a solvent in preparation of the extract, Which is a clear indicator of the formation of nanoparticles as soon as

the color change Figure 3 while no change was observed for plant extracts that did not add silver nitrate. When we diagnosed nanoparticles with the UV / Vis / NIR Spectrophotometer, There was a clear increase in the absorption peak of the particles prepared from the *D. viscosa* plant extract at 216 nm wavelength in the extract after adding the nitrates (Fig. 4).indicating that silver nitrates are reduction into nanoparticles. When comparing the analysis of the Fourier Transform Infrared Spectrometer (FTIR) absorption spectrophotometer to the extract of the D. *viscosa* plant before and after the addition of nitrate, the results (Fig. 5) shows the displacement of the absorbent peaks after mixing the extract with the silver nitrate from 3339.78 to 3354.00 cm. Scanning electron microscopy (SEM) image in Figure 6 and shows different size between plant extract for D.viscosa only and with silver AgNPs

Genotoxic effect of *D. viscosa* plant extract against *A. aegypti* larvae.

The extracted of DNA samples of 4th instar *A. aegypti* larvae exposed to the ethanol extract of *D. viscosa* at LC_{50} concentration for plant extracts were further evaluated for their DNA changes in comparison with untreated control larvae. DNA samples from mosquito larvae were taken and placed in the gel electrophoresis and detected by the Ultraviolet (Fig. 7) shows two replicant of the DNA extracted for the 4th instar larvae of *A. aegypti* taken from the first day of experiment until day 8 (the last day of larval stage before pupation) comparing with the control.

We were also able to perform the RAPD-PCR on genomic DNA extracted from a single 4th instar mosquito larvae.the result shows that the plant extract doesn't effect in the DNA structured damage (Apoptosis) and in the programmed cell death (PCD). Results (Fig. 8) shows the plant extract *D. viscose* for each 3 primers that used in this study (primer MA-09, primer MA- 12, primer MA-26) by using 10- kb ladder (molecular size markers).

In general, the study agreed with many scientific studies conducted in most countries to confirm the effectiveness of many plant extracts against different types of mosquitoes [17-20]. Also, [21] confirmed that the use of sub-lethal concentrations of Lemna minor extract against *Cx. pipiens* larvae leads to malformation in all mosquito larvae, especially larval and pupal stages, where they were more sensitive to this extract that confirm this study

In addition to the color factor, it was confirmed that the reaction and the formation of particles using the UV device and the scanning electron microscope and the addition of X-ray spectroscopy for the study of the shape and analysis of elements and the infrared device (FTIR), all confirmed the composition of particles and composition of peaks absorption in the areas of visible and ultraviolet The results of this study are consistent with many previous studies in this field [22-24].

As for the results of the combined effect of mixing the silver nitrate with the extracted plant, the silver particles played a significant developmental role in raising the efficiency of the extract of D. *viscosa* by 1.385 fold. This may be due to the fact that small particle size allows the permeability of active substances through the wall of the body as well as the insert of oral substances, so that the active substances have more than an effect on the larval body [25] effective substances so increase the exposure area [26] the reason for the small particle size and its relation to the different sites of the body of larvae makes them unable to remove them by the contact effect continuous genocidal addition to the gastrointestinal effect which accelerates the occurrence of loss of treated larvae [27].

The RAPD method has recently been applied to detect genetic instability in tumors and successfully detected genomic DNA alteration induced by several DNA damaging agents, such as benzopyrene, heavy metal and UV radiation. [15]. In the present study, the fourth instar larvae of Ae.aegypti exposed to ethanolic extract of D.viscosa was used to screen genome-wide DNA alteration by using 10-base primers (primer MA-09, primer MA-12, primer MA-26) all at an annealing temperature of 32 °C in the random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR). We use the random amplified polymorphic DNA (RAPD) technique, which is one of the most dependable means of discovering DNA damages when the extension stops at the damage site. The result shows that these plant extract dose not effect in the DNA damages and the reproducibility of RAPD bands scored in replicate data.

Table 1: Susceptibility of *Ae. aegypti* after treated with different concentrations of *D. viscose* extract using dipping technique.

Concentration (ppm)	Larval Mortality (%)* Mean** ± SE
100	28.125^{e}±0.57
300	58.333^{d}±1.15
500	72.917^{c}±1.15
700	83.333^{b}±1.73
900	94.792^{a}±1.73
LC_{50} L. limit- U. limit)	214.3288 (176. 4813– 250. 9126)
LC_{90} L. limit- U. limit)	932.3018 (761.9124– 1222.9745)
Slope	2.0073
Calculated$(Chi)^2$	5.0195

* Five replicates; 20 mosquito larvae each.

**Means followed by the same letter in the same column are not significant differences according to LSD at (0.05).

Tabulated Chi-square at 0.05 probability level = 7.81> Chi-square calculated from the data (5.0195), the line is good fit and the data are significantly homogenous.

Table 2: Susceptibility of *Aedes aegypti* after treated with different concentrations of *D. viscosa* extract silver nanoparticles (AgNPs) using dipping technique.

Concentration (ppm)	Larval Mortality (%)* Mean** ± SE
50	7.292^{d}±1.73
100	$25±2^{c}.89$
150	40.625^{b}±5.00
200	66.667^{a}±4.04
250	79.167^{a}±6.66
LC_{50} L. limit- U. limit)	154.7695 (142.1023– 169.0539)
LC_{90} L. limit- U. limit)	376.2513 (319.4636 – 472.4089)
Slope	3.32
Calculated$(Chi)^2$	4.2884

* Five replicates; 20 mosquito larvae each.

**Means followed by the same letter in the same column are not significant differences according to LSD at (0.05).

Tabulated Chi-square at 0.05 probability level = 7.81> Chi-square calculated from the data (4.2884), the line is good fit and the data are significantly homogenous.

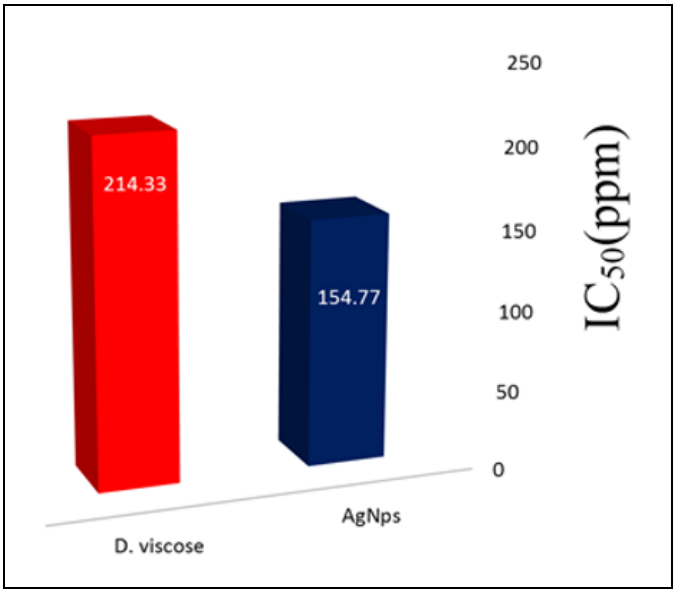

Fig 1: Toxicity values of *D. viscosa* against *Aedes aegypti* only and with silver nanoparticles (AgNPs).

Fig 2: The Biological effects of *D.viscosa* plant extracts and silver Nanoparticles on larvae *Ae. aegypti* (A) Control, B) Segment Body Contraction, C) intermediate stage (between larva and pupa) D) Pigmentation.

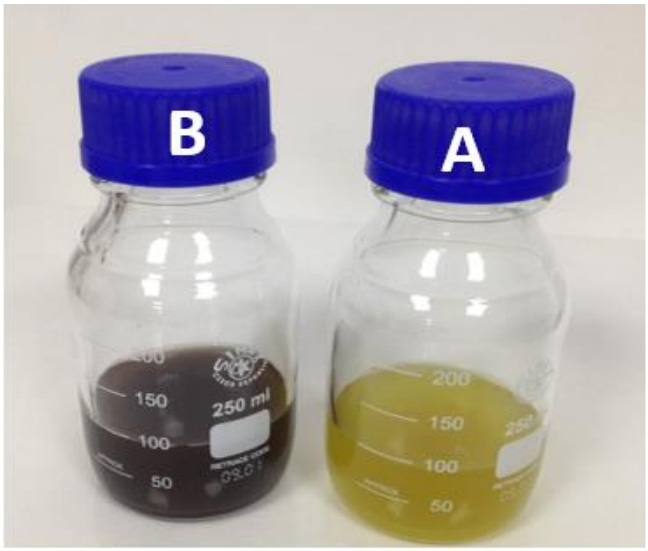

Fig 3: Biosynthesis and silver nanoparticles for *D. viscosa* plant extracts

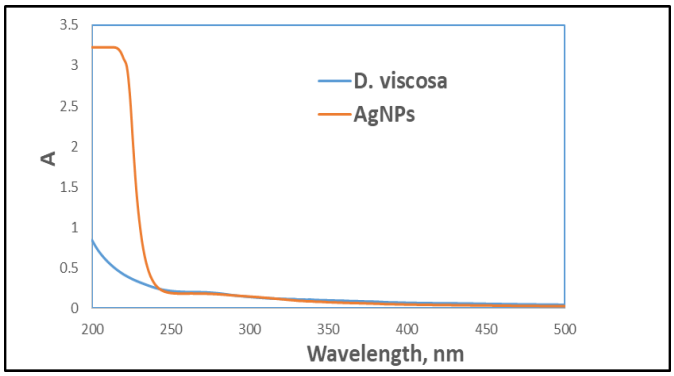

Fig 4: The ultraviolet radiation spectrometer for *D.viscosa* plant extract before and after the silver nitrate.

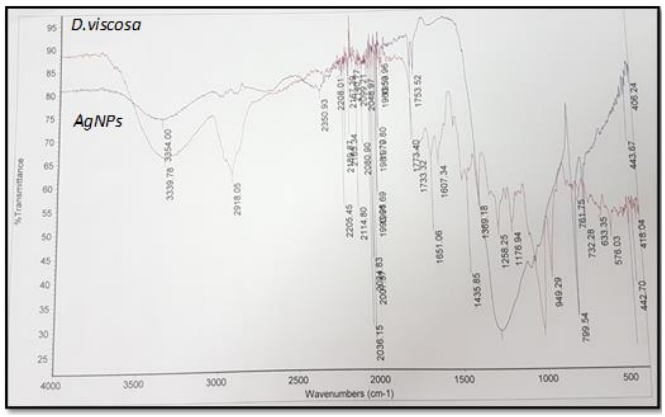

Fig 5: Analysis of the FTIR for *D. viscosa* plant extract before and after adding silver nitrates.

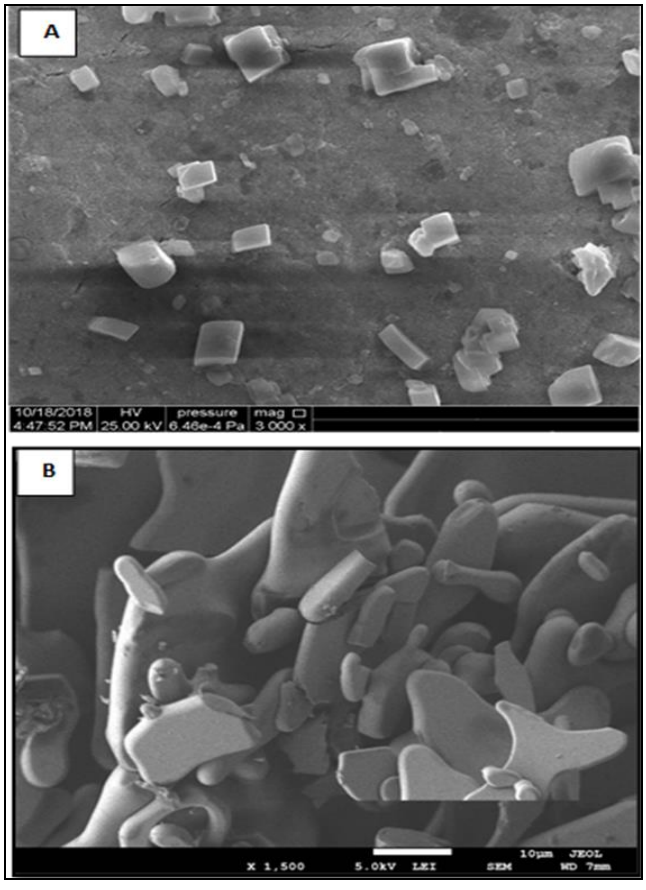

Fig 6: (A) Analysis of the scanning electron microscopy device for the plant only (B) silver particles prepared from the *D.viscosa* plant extract.

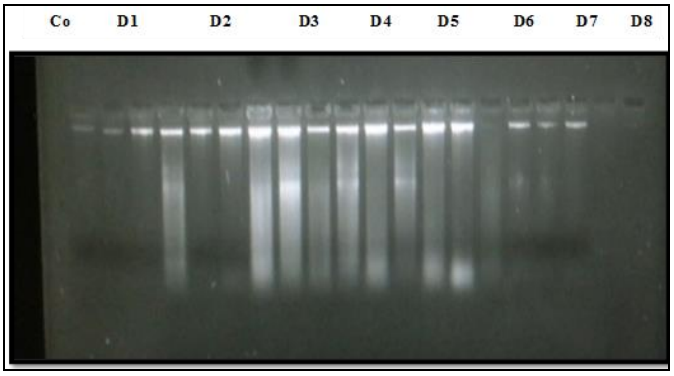

Fig 7: Shows the DNA extracted in the gel with the different plant extracts.

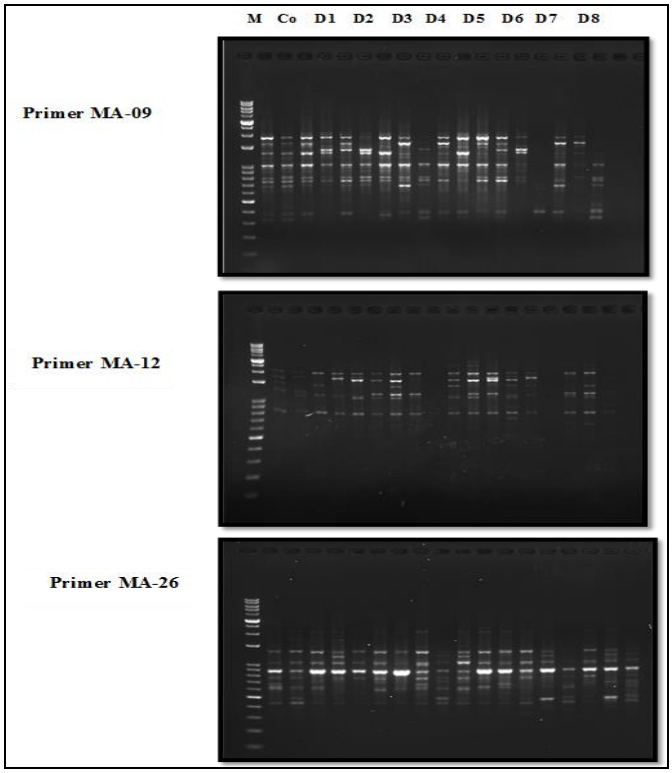

Fig 8: Fragment amplified using Primer MA-09, Primer MA-12 and Primer MA-26 on DNA extracted from *Dodonaea viscosa*.

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