

Carbon and silver nanoparticles in the fight against the filariasis vector *Culex quinquefasciatus*: genotoxicity and impact on behavioral traits of non-target aquatic organisms

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Received: 2 October 2015 / Accepted: 17 November 2015 / Published online: 28 November 2015
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Abstract Mosquito-borne diseases represent a deadly threat for millions of people worldwide. The *Culex* genus, with special reference to *Culex quinquefasciatus*, comprises the most common vectors of filariasis across urban and semi-urban areas of Asia. In recent years, important efforts have been conducted to propose green-synthesized nanoparticles as a valuable alternative to synthetic insecticides. However, the mosquitocidal potential of carbon nanoparticles has been scarcely investigated. In this study, the larvicidal and pupicidal activity of carbon nanoparticle (CNP) and silver nanoparticle (AgNP) was tested against *Cx. quinquefasciatus*. UV–Vis spectrophotometry, Fourier transform infrared (FTIR) spectroscopy, X-ray diffraction (XRD) analysis, scanning electron microscopy (SEM), transmission electron microscopy (TEM), energy-dispersive X-ray (EDX) spectroscopy,

and Raman analysis confirmed the rapid and cheap synthesis of carbon and silver nanoparticles. In laboratory assays, LC₅₀ (lethal concentration that kills 50 % of the exposed organisms) values ranged from 8.752 ppm (first-instar larvae) to 18.676 ppm (pupae) for silver nanoparticles and from 6.373 ppm (first-instar larvae) to 14.849 ppm (pupae) for carbon nanoparticles. The predation efficiency of the water bug *Lethocerus indicus* after a single treatment with low doses of silver and carbon nanoparticles was not reduced. Moderate evidence of genotoxic effects induced by exposure to carbon nanoparticles was found on non-target goldfish, *Carassius auratus*. Lastly, the plant extract used for silver nanosynthesis was tested for 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging activity.

Electronic supplementary material The online version of this article (doi:10.1007/s00436-015-4837-9) contains supplementary material, which is available to authorized users.

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Overall, our results pointed out that AgNP and CNP can be a candidate for effective tools to reduce larval and pupal populations of filariasis vectors, with reduced genotoxicity and impact on behavioral traits of other aquatic organisms sharing the same ecological niche of *Cx. quinquefasciatus*.

Keywords Antioxidant activity · Biological control · *Carassius auratus* · *Lethocerus indicus* · Nanotechnology · Predation efficiency

Introduction

Mosquitoes transmit a number of important diseases, such as malaria, filariasis, chikungunya, dengue, and Japanese encephalitis, causing millions of deaths every year (Mehlhorn et al. 2012; Benelli 2015a). Lymphatic filariasis, commonly known as elephantiasis, is a neglected tropical disease. More than 1.4 billion people in 73 countries are living in areas, where lymphatic filariasis is transmitted, and are at risk of being infected. Globally, an estimated 25 million men suffer with genital disease and over 15 million people are afflicted with lymphedema (WHO 2014). In this scenario, vector control of mosquito-borne diseases is a priority, particularly in situations of epidemic outbreaks. Recently, vector control using botanical products arising as a valuable alternative, due to their reduced toxicity towards vertebrates and high biodegradability (e.g., Mehlhorn et al. 2005; Amer and Mehlhorn 2006a, b, c, d; Hafëez et al. 2011; Ravikumar et al. 2011a, b; Benelli 2015b; Benelli et al. 2015).

The development of reliable green process for the synthesis of nanoparticles is an important aspect of current nanotechnology research. Nanotechnology research opens newer avenues for a wide array of applications in the fields of biomedical, sensors, antimicrobials, catalysts, electronics, optical fibers, agricultural, and biolabeling (Salam et al. 2012). Nanoparticles play an indispensable role in drug delivery, diagnostics, imaging, sensing, gene delivery, artificial implants, and tissue engineering (Morones et al. 2005). The synthesis of nanoparticles (NPs) using chemical and physical methods requires high pressure, energy, temperature, and toxic chemicals (Goodsell 2004). Microbes and plants are currently used for nanoparticle synthesis. The use of plants for the fabrication of NP is a rapid, low-cost, and eco-friendly method for biosynthesis process (Huang et al. 2007). A growing number of plant extracts and metabolites have been successfully used for efficient and rapid extracellular synthesis of mosquitocidal NP (Veerakumar et al. 2014; Murugan et al. 2015a, b, c; see Benelli 2016 for a review).

Carbon NPs (CNP) are of great interest for both fundamental studies and practical applications. CNPs have been widely used in super capacitors (Laforguem et al. 2003), high-performance electrode materials in batteries (Dominko et al. 2003), and photoluminescent materials (Li et al. 2011).

Specifically, CNPs with large surface areas can be readily functionalized, allowing an efficient binding of biomolecules. Compared with traditional quantum dots and organic dyes, photoluminescent carbon nanomaterials have advantages due to their chemical inertness and lower toxicity (Dominko et al. 2003). They have been also proposed as possible candidates to develop newer and safer mosquitocidals (see also Saxena et al. 2013), even if field evidences on a wider scale are lacking (Benelli 2016).

Furthermore, mosquito larval population can be controlled by a number of aquatic predators, including odonate young instars, water bugs, tadpoles, fishes, crabs, and copepods (Bowatte et al. 2013; Kalimuthu et al. 2014; Murugan et al. 2011, 2015a, b, c, d). Predation is reported as one of the most limiting factors causing a high level of mortality to immature stages of mosquitoes (Service 1973, 1977). *Lethocerus indicus* is a giant water bug in the family Belostomatidae; these large aquatic insects have the largest body size among Heteroptera and are reported as common inhabitants and major predators in many aquatic ecosystems (Perez-Goodwyn 2006).

As many chemicals with genotoxic potential are emitted to surface water, genotoxicity tests are gaining importance which led to the development of several techniques to detect directly DNA damage and to identify such pollutants. The relevance of detecting the genotoxic risks associated with water pollution was firstly perceived in the late 1970s. Since that time, several tests have been developed for evaluating DNA alterations in aquatic animals. The possibility of using changes in DNA integrity to the genetic material as markers of exposure and effect of genotoxicants has been investigated (McCarthy and Shugart 1990). The presence of DNA adducts has been taken as evidence of exposure to specific genotoxicants (Shugart 1999). These tests rely on the premise that any changes to DNA may have long-lasting and profound consequences (Lam and Gray 2003).

In this research, the larvicidal and pupicidal activity of green-synthesized silver nanoparticle (AgNP) and CNP was tested for acute toxicity against *Culex quinquefasciatus*. UV–Vis spectrophotometry, Fourier transform infrared (FTIR) spectroscopy, Raman spectroscopy, X-ray diffraction (XRD), scanning electron microscopy (SEM), transmission electron microscopy (TEM), and energy-dispersive X-ray (EDX) spectroscopy confirmed the rapid and cheap synthesis of AgNP and CNP. The predation efficiency of the water bug *L. indicus* against *Cx. quinquefasciatus* larvae, after a single treatment with low doses of AgNP and CNP, was evaluated. The possible genotoxic effects of CNP on non-target goldfish, *Carassius auratus*, were also investigated. Finally, the plant extract used for silver nanosynthesis was also tested for 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging activity.

Materials and methods

Collection of *Moringa oleifera* seeds and preparation of seed extracts

M. oleifera belongs to the family Moringaceae and is commonly known as “miracle tree.” It is a multipurpose, rapidly growing crop indigenous to North West India (Cidamis et al. 2003). It is commonly planted in Africa as a living fence tree (Von Maydell 1986). These trees have a medicinal value for rural human communities, due to their antibiotic, anti-inflammatory, and analgesic properties (Vinoth et al. 2012). The leaves have been reported to be a rich source of carotene, protein, vitamin C, calcium, and potassium and act as a good source of natural antioxidants (Dillard and German 2000; Siddhuraju and Becker 2003). The seeds possess antimicrobial (Ali et al. 2004; Chuang et al. 2007), antitumor (Guevara et al. 1999; Bharali et al. 2003), anti-inflammatory, antispasmodic, and diuretic (Cárceles et al. 1992) properties. The use of traditional medicine is widespread, and plants still present a large source of natural antioxidants that might lead to the development of novel drugs (Perry et al. 1998).

In this study, seeds of *M. oleifera* were collected from the garden of the Bharathiar University (Coimbatore, India) and identified at the Department of Botany of Bharathiar University, Coimbatore, India. Voucher specimens were stored in our laboratories. *M. oleifera* seeds were washed with tap water and shade dried at room temperature. The dried seeds were powdered using an electrical blender; 500 g of the powder was macerated in 1.5 l of ethanol for 72 h. The crude plant extract was concentrated at reduced temperature using a rotary evaporator and stored at 22 °C. One gram of the seed residue was dissolved in 100 ml of acetone (fixative agent to separate the aqueous impurities altering the chemical composition of plant crude extract) and considered as 1 % stock solution. From this stock solution, experimental concentrations were prepared.

Synthesis and characterization of silver nanoparticles

The *M. oleifera* aqueous seed extract was prepared by adding 20 g of seeds washed with distilled water and was shade dried at room temperature (28 ± 2 °C) for 2 days. The air-dried materials were powdered separately using a commercial electrical blender. The plant seed material (5 g) in a 300-ml Erlenmeyer flask filled with 100 ml of sterilized double-distilled water was finely ground, and the mixture was boiled for 5 min, before finally decanting it. The extract was filtered using a Whatman N. 1 filter paper, stored at -4 °C, and tested within 5 days. The filtrate was treated with aqueous 1 mM silver nitrate in an Erlenmeyer flask and incubated at room temperature. A color change indicated the formation of AgNP, since aqueous silver ions were reduced by the *M. oleifera* seed extract generating

stable AgNP in water (Sujitha et al. 2015). Silver nitrate (AgNO_3) was purchased from the Precision Scientific Co. (Coimbatore, India).

As recently reported by Sujitha et al. (2015), the green synthesis of AgNP was confirmed by sampling the reaction mixture at regular intervals and the absorption maxima were scanned by UV–Vis spectrophotometry, at the wavelength of 200–800 nm in a UV-3600 Shimadzu spectrophotometer (1 nm resolution). Furthermore, the reaction mixture was subjected to centrifugation at 15,000 rpm for 20 min and the resulting pellet was dissolved in deionized water and filtered through a Millipore filter (0.45 μm). An aliquot of this filtrate containing AgNP was used for SEM, TEM, FTIR spectroscopy, XRD analysis, and EDX spectroscopy. In detail, the structure and composition of freeze-dried purified AgNP was analyzed using a 10-kV ultra high-resolution scanning electron microscope with 25 μl of the sample that was sputter coated on a copper stub and the morphology of AgNP was investigated using a FEI Quanta 200 SEM and HT7700 120 kV High-Contrast/High-Resolution Digital TEM. The surface groups of the AgNP were qualitatively confirmed by FTIR spectroscopy (Stuart 2002), with spectra recorded using a Perkin-Elmer Spectrum 2000 FTIR spectrophotometer. EDX assays confirmed the presence of metals in analyzed samples (Sujitha et al. 2015).

Synthesis and characterization of carbon nanoparticles

Citric acid and urea were purchased from HiMedia Chemicals; 1.0 M of citric acid and 1.0 M of urea were dissolved in 40 ml of DDW and transferred into a Teflon-lined autoclave. The reaction temperature was maintained at 180 °C for 3 h. Then, the autoclave was cooled naturally and a dark brownish solution, containing carbon quantum dots, was collected. The resultant solution was centrifuged at 10,000 rpm to remove the larger particles. The collected supernatants were subjected to nanocharacterization, following the methods described above for AgNP (Qu et al. 2014).

DPPH radical scavenging activity

The scavenging activity of *M. oleifera* seed extract against DPPH radical was assessed according to the method of Blois (1958) with minor modifications; 1 ml of *M. oleifera* seed extracts ($0.01 \text{ mg dw ml}^{-1}$) was mixed with 4 ml of 0.005 mg ml^{-1} DPPH methanol solution. The reaction mixture was vortexed and left in the dark at room temperature for 30 min. The absorbance of the mixture was measured at 517 nm. Ascorbic acid and butylated hydroxytoluene (BHT) were

used as references. The ability to scavenge DPPH radical was calculated using the following equation:

$$\text{DPPH radical scavenging activity (\%)} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100$$

where $\text{Abs}_{\text{control}}$ is the absorbance of DPPH radical in methanol and $\text{Abs}_{\text{sample}}$ is the absorbance of DPPH radical solution mixed with sample extract/standard. All determinations were performed in triplicate ($n=3$).

ABTS radical scavenging assay

Here, we followed the method by Arnao et al. (2001) with minor modifications; stock solutions included 7 mM ABTS solution and 2.4 mM potassium per sulfate solution. Then, the experimental solution was prepared by mixing the two stock solutions (1:1, v/v) and allowing them to react for 14 h at room temperature in the dark. The solution was then diluted by mixing 1 ml ABTS solution with 60 ml methanol to obtain an absorbance of 0.706 ± 0.01 units at 734 nm using a spectrophotometer. Fresh ABTS solution was prepared for each assay. Seed extracts (1 ml) were allowed to react with 1 ml of the ABTS solution, and the absorbance was taken at 734 nm after 7 min using a spectrophotometer. The ABTS scavenging capacity of the extract was compared with that of BHT and ascorbic acid, and the percentage inhibition was calculated as follows:

$$\text{ABTS radical scavenging activity (\%)} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100$$

where $\text{Abs}_{\text{control}}$ is the absorbance of ABTS radical in methanol and $\text{Abs}_{\text{sample}}$ is the absorbance of ABTS radical solution mixed with the sample extract/standard. All determinations were performed in triplicate ($n=3$).

Cx. quinquefasciatus rearing

The mosquitoes tested in this study were laboratory reared and pathogen free. The rearing was originally established as described by Murugan et al. (2015d). Eggs of *Cx. quinquefasciatus* were provided by the National Centre for Disease Control (NCDC) field station of Mettupalayam (Tamil Nadu, India). Batches of 100–110 eggs were transferred to 18 cm (*L*) \times 13 cm (*W*) \times 4 cm (*D*) enamel trays containing 500 ml of water where they were allowed to hatch at laboratory conditions [27 ± 2 °C; 7–85 % R.H.; 14:10 (L:D) photoperiod]. Larvae were fed daily with 5 g of ground dog biscuits (Pedigree, USA) and brewer's yeast (Sigma-Aldrich, Germany) in a 3:1 ratio. First- to fourth-instar larvae and recently pupated individuals (i.e., pupation occurred within 24 h) were collected and used in the experiments (Murugan et al. 2015d).

Larvicidal and pupicidal experiments against *Cx. quinquefasciatus*

Twenty-five *Cx. quinquefasciatus* larvae (first, second, third, or fourth instar) or pupae were placed for 24 h in a glass beaker filled with 250 ml of dechlorinated water plus the desired concentration of the *M. oleifera* seed extract, AgNP or CNP. Larval food (0.5 mg) was provided for each tested concentration. Each concentration was replicated five times against all instars. Control mosquitoes were exposed for 24 h to the corresponding concentration of the solvent. Percentage mortality was calculated as follows:

$$\text{Percentage mortality} = \frac{\text{Number of dead individuals}}{\text{Number of treated individuals}} \times 100$$

Impact of nanoparticles on water bug predation

L. indicus were collected from rural ponds in Coimbatore (Tamil Nadu, India) and maintained in laboratory conditions. Water bugs are cannibalistic among the five stages, here only second-instar nymphs were used for the trials. In this experiment, the predation efficiency of second-instar nymphs of *L. indicus* was assessed against second- and third-instar larvae of *Cx. quinquefasciatus*. Five hundred mosquitoes were introduced, with two water bugs, into a glass arena (5 l) containing dechlorinated water. Mosquito larvae were replaced daily with new ones. Both for second- and third-instar mosquito larvae, five replicates were conducted. The control was 5 l of dechlorinated water without water bugs. All experimental arenas were checked every 6 h, and the number of prey consumed by water bug was recorded. Predatory efficiency was calculated using the following formula:

$$\begin{aligned} \text{Predatory efficiency} \\ &= \left[\frac{\text{Number of consumed mosquitoes}}{\text{Number of predators}} \right] / \text{Total number of mosquitoes} \\ &\quad \times 100 \end{aligned}$$

Following the same method described above, the predation efficiency of *L. indicus* adults was assessed against *Cx. quinquefasciatus* larvae after a single mosquitocidal treatment with AgNP and CNP. In each treatment, 500 second- or third-instar larvae were introduced with two water bugs into a 5-l plastic filled with 5 l of dechlorinated water and 1 ml of the desired concentration of AgNP or CNP [i.e., one third of the LC_{50} (lethal concentration (ppm) that kills 50 % of the exposed organisms) calculated against first-instar larvae of *Cx. quinquefasciatus*]. Five replicates were conducted. Predation efficiency was calculated using the abovementioned formula.

Genotoxicity analysis

The goldfish, *C. auratus*, was chosen for this study due to its wide presence in common freshwater habitats and to its sensitivity to genotoxic chemicals (Deguchi et al. 2007). Juvenile individuals with average weight and length of 5 ± 1 g and 6 ± 1 cm, respectively, were purchased from a local market in Coimbatore and identified by the authors. Before the experiments, they were acclimated under laboratory conditions for 3 weeks at a population density of 15 specimens for 1 aquarium. Fishes were fed once a day with commercial fish food and exposed to 5, 10, 15, 20, and 25 ppm of CNP for 24, 48, 72, and 96 h.

Micronucleus assay

Experimental subjects were sacrificed in the ethical rules and regulations (see “Compliance with ethical standards” section). The frequency of micronuclei in the peripheral erythrocytes was evaluated according to the criteria described by Countryman and Heddle (1976) and Fenech (1993). Immediately after sampling, a drop of blood was smeared on clean slides (two per fish) and then dried at laboratory temperature. After 24 h, the samples were fixed using pure methanol for 10 min. Afterwards, they were stained with 4 % Giemsa solution for 10 min, air-dried, and prepared for permanent use. Cytological analysis was done under an optical microscope ($\times 1000$). A total of 2000 erythrocyte cells were examined per fish in coded slides. The presence of nuclear erythrocyte abnormalities was analyzed. The MN frequency was calculated as

$$\text{MN (\%)} = \frac{\text{Number of cells containing micronucleus}}{\text{Total number of cells counted}} \times 100$$

Single cell gel electrophoresis/comet assay

In fishes, about 98 % of total blood cells are erythrocytes. Therefore, no cell separation was performed and, hereafter, cells in the whole blood are referred to as erythrocytes (Theodorakis et al. 1994; De Miranda Cabral Gontijo et al. 2003). The alkaline comet assay was performed according to the method of Tice et al. (2000). The blood samples collected from gills of fish were diluted with 1 ml of PBS. Sixty microliters of the diluted sample was mixed with 200 μ l of 0.65 % low-melting-point (LMP) agarose. Seventy-five microliters of the mixture was then layered on the slides pre-coated with 0.5 % normal-melting-point (NMP) agarose and immediately covered with a cover slip and then kept for 10 min in a refrigerator to solidify. After gently

removing the cover slips, the slides were covered with a third layer of 90 μ l low-melting-point agarose and covered with cover slips again. After solidification of the gel, the cover slips were removed and the slides were immersed in cold lysing solution (i.e., 2.5 M NaCl, 100 mM Na₂-EDTA, 10 mM Tris, pH 10, with 10 % DMSO and 1 % Triton X-100 added fresh) and refrigerated at 4 °C for 2 h. After lysis, the slides were placed on a horizontal electrophoresis box side by side. The tank was filled with fresh electrophoresis solution (1 mM Na-EDTA, 300 mM NaOH, and pH 13.5) to a level approximately 0.25 cm above the slides. The slides were left in the solution for 20 min to allow the unwinding. Electrophoresis was performed using the same solution at 25 V and 300 mA for 25 min. The slides were then neutralized with 0.4 M Tris buffer at pH 7.5 and stained with 75 μ l ethidium bromide (20 μ g ml⁻¹). Then, the slide was photographed by fluorescent microscopy.

Data analysis

Mosquito mortality data were analyzed by probit analysis, calculating LC₅₀ and LC₉₀ (lethal concentration (ppm) that kills 90 % of the exposed organisms) following the method by Finney (1971). Water bug predation data were analyzed using a generalized linear model with two fixed factors: $y = X\beta + \varepsilon$ where y is the vector of the observations (i.e., the number of consumed preys), X is the incidence matrix, β is the vector of fixed effects (i.e., treatment and targeted instar), and ε is the vector of the random residual effect. A probability level of $P < 0.05$ was used for the significance of differences between values. Antioxidant data were transformed into arcsine proportion values and then analyzed by one-way ANOVA. The means were separated using Tukey’s honest significant difference (HSD) test. $P < 0.05$ was used for the significance of differences between means. JMP 7 was used for all analyses.

Results and discussion

Characterization of silver nanoparticles

The biosynthesis of AgNPs using the *M. oleifera* aqueous seed extract has been recently described in detail by Sujitha et al. (2015). In this paragraph, we confirmed the biosynthetic route by adding TEM analysis and briefly focusing on the biophysical characterization of *M. oleifera*-fabricated AgNPs. In our experiments, the biosynthesis of AgNPs was confirmed within 120 min and, after that the *M. oleifera* seed extract was added to the AgNO₃ solution. The color

changed from pale yellow to dark brown. The absorption spectra of AgNP at different time intervals showed highly symmetric single-band absorption peaks. A maximum absorption peak was observed at 450 nm; broadening of the peak indicated that the particles are polydispersed (Prasad and Elumalai 2011), and this may be due to excitation of surface plasmon vibrations in green-synthesized AgNPs (Shankar et al. 2004; Dinesh et al. 2015). SEM micrographs showed that the biosynthesized AgNPs were mostly spherical, highlighting a large distribution of sizes, mainly ranging from 35 to 55 nm. It is known that the shape of metal NP can considerably change their optical and electronic properties (Xu and Kall 2002; Chandran et al. 2006; Udayasoorian et al. 2011). TEM supported SEM results, highlighting that the green-synthesized AgNPs showed spherical and cubic shapes, with size ranging from 39 to 57 nm (Supplementary Material Fig. S1). Comparable TEM results were obtained for polydispersed AgNPs chemically reduced by Lee et al. (2004) or biosynthesized by Huang et al. (2007). Notably, biomolecules may act as reducing and capping agents, protecting AgNPs from aggregation (Shankar et al. 2004; Song and Kim 2009). EDX spectroscopy revealed a strong signal in the Ag region, confirming the presence of elemental silver. An XRD pattern of *M. oleifera*-synthesized AgNPs highlighted the presence of four high diffraction peaks indexing Bragg's reflection planes (111), (200), (220), and (311), respectively (Sudha Lakshmi 2011; Kumar et al. 2014; Phanjom and Ahmed 2015). These peaks may be due to the organic compounds which are present in the extract and responsible for the reduction of Ag ions and stabilization of AgNPs (Roopan et al. 2013). Thus, the XRD pattern showed that the AgNPs tested in this study are crystalline. An FTIR spectrum showed main peaks at 3336.758 cm^{-1} (–O–H stretch), 1637.519 cm^{-1} (aldehyde C–H stretch), and 597.916 cm^{-1} (C–Br bonds). The broad band appearing close to 3450 cm^{-1} can be assigned for O–H stretching vibration, indicating the presence of hydroxyl groups in the reducing agent (Das et al. 2013). Bands denote stretching vibration bands responsible for compounds like flavonoids and terpenoids (Huang et al. 2007).

Characterization of carbon nanoparticles

The UV–Vis spectrum of CNPs was reported in Fig. 1. The formation of CNPs was confirmed through the presence of an absorption peak at 235 and 335 nm, probably due to surface plasmon resonance (see also Murugan et al. 2015f) formed. In TEM assays, CNPs showed a mean size of about 3.3 nm and spherical morphology (Fig. 2). This indicates that low quantities of the extract can easily reduce carbon but do not

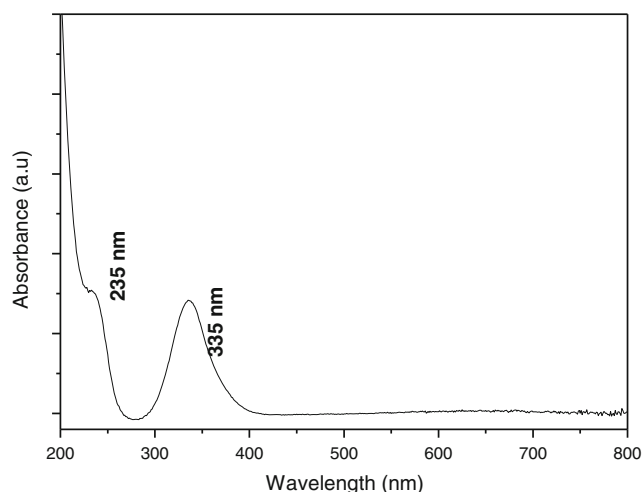


Fig. 1 UV–Vis spectrum of carbon nanoparticles

protect most of the quasi-spherical nanoparticles from aggregation, and this is probably due to the deficiency of biomolecules acting as capping agents (Kiruba Daniel et al. 2011). The XRD pattern of CNP is showed in Fig. 3; the peaks are indexed as (002) planes of FCC carbon by comparing with JCPDS data (Dubey et al. 2005). FTIR spectrum of CNP showed peaks at 3235 , 1688 , 1613 , 1200 – 1400 , and 850 cm^{-1} (Fig. 4); they denoted stretching in O–H, C=O, and C–O bonds of carboxylic acids and 850 -out-of-plane C–H vibration, respectively. Following Srinivas Naik et al. (2013), peaks at about 1019 cm^{-1} may be assigned to –C–O; peaks close to 1642 cm^{-1} may be assigned to chelated carbonyl group or –OH from carboxylic group in the sennosides. These absorbance bands are known to be associated with the stretching vibrations for –C–C–O, –C–C– [(in-ring) aromatic], –C–C– [(in-ring) aromatic], C–O (esters, ethers), and C–O (polyols),

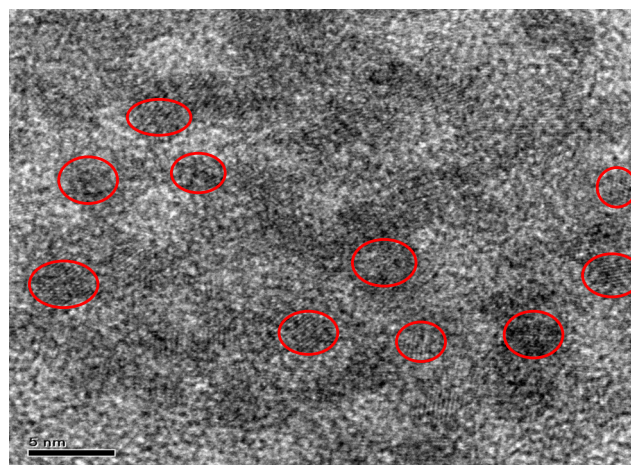


Fig. 2 TEM micrograph of carbon nanoparticles (highlighted by red circles)

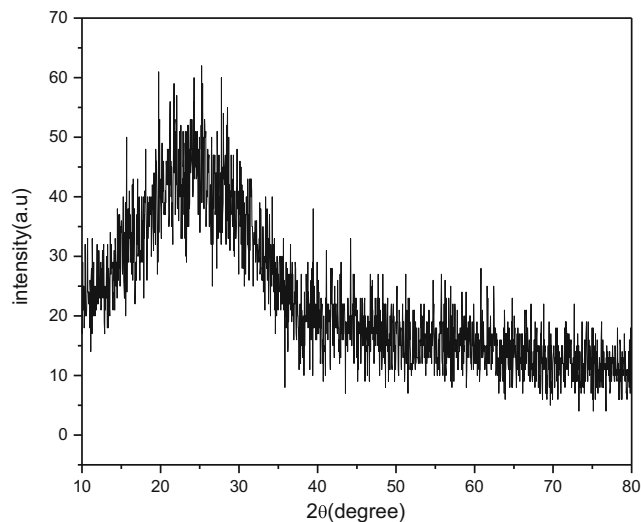


Fig. 3 XRD pattern of carbon nanoparticles

respectively (Das et al. 2013; see also Benelli 2016). The Raman spectrum of CNP showed two prominent peaks at 1340 and 1580 cm^{-1} which corresponded to the E_{2g} (G band) and disorder-induced (D band) modes of graphite, respectively (Fig. 5). Similarly, the Raman spectrum of soxhlet-purified mustard soot has two prominent peaks at 1590 and 1347 cm^{-1} which corresponds to the E_{2g} (G band) and disorder-induced (D band) modes of graphite (Dubey et al. 2005).

DPPH and ABTS radical scavenging and total antioxidant activity

We compared the radical scavenging activity of the *M. oleifera* seed extract (0.01 mg ml^{-1}) with BHT and ascorbic acid at the same concentration and expressed it as a percentage of inhibition against DPPH and ABTS,

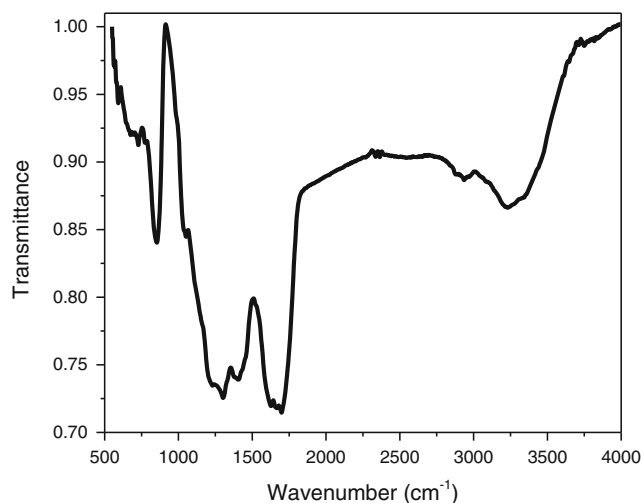


Fig. 4 FTIR spectrum of carbon nanoparticles

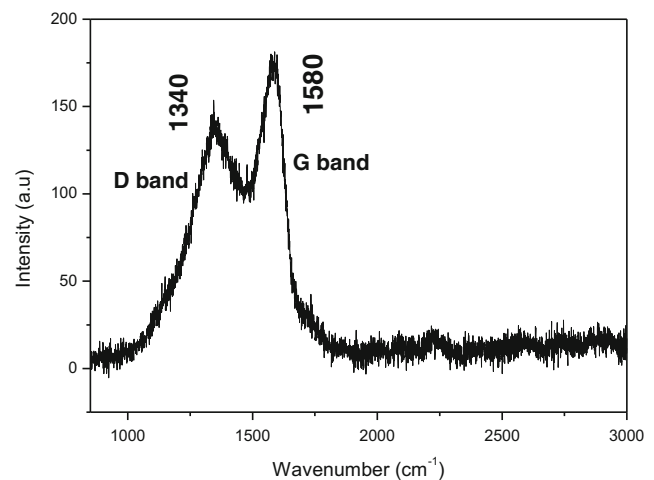


Fig. 5 Raman analysis of carbon nanoparticles

respectively (Table 1). Interestingly, the *M. oleifera* seed extract significantly reduced DPPH and ABTS (91.43 ± 0.68 and 94.15 ± 0.42 , respectively) if compared to ascorbic acid and BHT. Natural products are widely known for their antioxidant properties. For instance, Dimitrova et al. (2010) pointed out that *Hypericum* species also exhibited radical scavenging activity, with special reference to methanolic extracts of *Hypericum cerastoides*, *Hypericum perforatum*, and *Hypericum maculatum*. Furthermore, Liu et al. (2008) reported that the total phenolic content seems to be strongly related to the antioxidant potential of botanical preparations. Further research is urgently required to shed light on the compound(s) responsible of the high antioxidant activity of the *M. oleifera* seed extract, since this may have important connections with nutraceutical issues and human health.

Mosquitocidal activity

In laboratory conditions, the *M. oleifera* seed extract was toxic against the larval instars (I–IV) and pupae of *Cx. quinquefasciatus*. LC_{50} values were 112.151 ppm (I), 137.216 ppm (II), 146.540 ppm (III), 184.681 ppm (IV), and 240.111 ppm (pupae) (Table 2).

Table 1 DPPH and ABTS radical scavenging activity of the *Moringa oleifera* seed extract

Treatment	DPPH (%)	ABTS (%)
<i>Moringa oleifera</i>	91.43 ± 0.68^b	94.15 ± 0.42^b
BHT	36.22 ± 0.21^c	66.28 ± 0.28^c
Ascorbic acid	95.56 ± 0.18^a	98.63 ± 0.56^a

Within a column, means followed by the same letter(s) are not significantly different (Tukey's HSD, $P < 0.05$)

Table 2 Larval and pupal toxicity of the *Moringa oleifera* seed extract against the filariasis vector *Culex quinquefasciatus*

Target	LC ₅₀ (LC ₉₀) (ppm)	95 % confidence limit LC ₅₀ (LC ₉₀)		Regression equation	χ^2 (df=4)
		Lower	Upper		
Larva I	112.151 (311.503)	89.153 (273.433)	130.425 (373.920)	$y=0.721+0.006x$	0.480 n.s.
Larva II	137.216 (366.717)	114.668 (314.233)	157.636 (459.967)	$y=0.766+0.006x$	0.292 n.s.
Larva III	146.540 (399.252)	123.796 (335.296)	170.708 (517.063)	$y=0.743+0.005x$	3.707 n.s.
Larva IV	184.681 (435.508)	161.741 (364.581)	216.045 (566.167)	$y=0.944+0.005x$	2.714 n.s.
Pupa	240.111 (517.098)	207.644 (420.428)	297.098 (711.777)	$y=1.111+0.005x$	3.382 n.s.

No mortality was observed in the control. LC₅₀=lethal concentration (ppm) that kills 50 % of the exposed organisms LC₉₀=lethal concentration (ppm) that kills 90 % of the exposed organisms χ^2 =chi-square value df=degrees of freedom n.s.=not significant ($\alpha=0.05$)

A dose-dependent effect was found, as reported for a growing number of botanicals (Amer and Mehlhorn 2006c, d; Murugan et al. 2015a, b, c; Govindarajan and Benelli 2016; Govindarajan et al. 2016). Recently, Sujitha et al. (2015) reported that the *M. oleifera* seed extract is effective against young instars of the dengue vector *Aedes aegypti*.

AgNPs are highly toxic against *Cx. quinquefasciatus* larvae and pupae, with LC₅₀ of 8.752 ppm (I), 9.875 ppm (II), 11.719 ppm (III), 14.125 ppm (IV), and 18.676 ppm (pupae) (Table 3). Higher toxicity was obtained testing CNPs, since LC₅₀ values were 6.373 ppm (I), 7.710 ppm (II), 8.703 ppm (III), 11.482 ppm (IV), and 14.849 ppm (pupae) (Table 4). The mosquitocidal potential of CNPs has been scarcely investigated. To the best of our knowledge, only Saxena et al. (2013) highlighted that fluorescent water-soluble carbon nanoparticles (wsCNPs) formed from the carbonization of wood wool followed by oxidative treatment may impact on the life cycle of mosquitoes. In detail, they showed that at 3 mg l⁻¹ of wsCNPs, the growth of the *Aedes*, *Anopheles*, and *Culex* mosquitoes from the larval stage to adulthood is blocked. On the other hand, *M. oleifera*-synthesized AgNPs have been recently found toxic against *Ae. aegypti* larvae and pupae, with LC₅₀

ranging from 10.24 ppm (I) to 21.17 ppm (pupae) (Sujitha et al. 2015). Interestingly, these AgNPs also inhibited the growth of dengue virus, serotype DEN-2. More generally, in latest years, a growing number of green-fabricated AgNPs have been reported as effective pesticides against different mosquito vectors of medical and veterinary importance (e.g., Suganya et al. 2014; Veerakumar et al. 2014; Murugan et al. 2015a, b, c, d; see Benelli 2016 for a review).

Impact of nanoparticles on water bug predation

In standard laboratory conditions, the predation efficiency of *L. indicus* towards *Cx. quinquefasciatus* was 38.5 % (larva II) and 61.25 % (larva III). Post-treatment with AgNPs, *L. indicus* predation rates were boosted to 50.5 and 77.0 %, respectively. Post-treatment with CNPs, predation rates were boosted to 63.5 and 85.7 %, respectively (Table 5). Scarce information is available about how ultra-low dosages of nanoparticles impact behavioral traits of aquatic organisms sharing the same ecological niche as mosquitoes (reviewed by Benelli 2016; see also Murugan et al. 2015g). Pokhrel and Dubey (2012) studied the effect of AgNPs on the predator–prey relationship between *Daphnia magna* and dragonfly young instars,

Table 3 Larval and pupal toxicity of *Moringa oleifera*-synthesized silver nanoparticles against the filariasis vector *Culex quinquefasciatus*

Target	LC ₅₀ (LC ₉₀) (ppm)	95 % confidence limit LC ₅₀ (LC ₉₀)		Regression equation	χ^2 (df=4)
		Lower	Upper		
Larva I	8.752 (21.417)	1.227 (17.086)	12.342 (34.079)	$y=0.886+0.101x$	9.371 n.s.
Larva II	9.875 (24.394)	8.088 (22.139)	11.324 (27.640)	$y=0.872+0.088x$	4.440 n.s.
Larva III	11.719 (26.348)	10.146 (23.921)	13.090 (29.840)	$y=1.027+0.088x$	3.557 n.s.
Larva IV	14.125 (30.926)	12.528 (27.668)	15.655 (35.885)	$y=1.077+0.076x$	2.254 n.s.
Pupa	18.676 (38.077)	16.897 (33.244)	20.890 (46.032)	$y=1.234+0.066x$	0.384 n.s.

No mortality was observed in the control. LC₅₀=lethal concentration (ppm) that kills 50 % of the exposed organisms LC₉₀=lethal concentration (ppm) that kills 90 % of the exposed organisms χ^2 =chi-square value; df=degrees of freedom n.s.=not significant ($\alpha=0.05$)

Table 4 Larval and pupal toxicity of carbon nanoparticles against the filariasis vector *Culex quinquefasciatus*

Target	LC ₅₀ (LC ₉₀) (ppm)	95 % confidence limit LC ₅₀ (LC ₉₀)		Regression equation	χ^2 (df=4)
		Lower	Upper		
Larva I	6.373 (18.108)	0.293 (14.917)	9.376 (25.025)	$y=0.696+0.109x$	5.797 n.s.
Larva II	7.710 (19.726)	0.600 (15.880)	11.066 (29.817)	$y=0.822+0.107x$	8.281 n.s.
Larva III	8.703 (23.262)	2.898 (19.106)	11.861 (33.243)	$y=0.766+0.088x$	5.587 n.s.
Larva IV	11.482 (28.332)	9.622 (25.370)	13.043 (32.831)	$y=0.873+0.076x$	1.987 n.s.
Pupa	14.849 (36.121)	12.880 (31.291)	16.796 (44.349)	$y=0.895+0.60x$	0.044 n.s.

No mortality was observed in the control. LC₅₀=lethal concentration (ppm) that kills 50 % of the exposed organisms LC₉₀=lethal concentration (ppm) that kills 90 % of the exposed organisms χ^2 =chi-square value df=degrees of freedom n.s.=not significant ($\alpha=0.05$)

showing that these AgNPs reduced the ability of *D. magna* to detect predators. Later on, some investigations revealed fascinating scenarios. In agreement with the present research, Murugan et al. (2015b) showed that very low doses (i.e., 1 ppm) of lemongrass-synthesized gold nanoparticles may control malaria and dengue mosquitoes by boosting copepod predation on early-instar mosquito larvae in a gold nanoparticle-contaminated environment. We hypothesize that low doses of green-synthesized AgNPs reduce the motility of mosquito larvae, thus enhancing the predation success of mosquito natural enemies (Murugan et al. 2015c, d).

Genotoxicity assay

C. auratus had normal erythrocytes, elliptical in shape with the centrally placed nucleus in the cytoplasm. These erythrocytes were observed in the blood taken from control individuals (Fig. 6a). After exposure to CNPs at doses lower than 25 ppm, no effects were found. After 24 and 48 h of exposure at 25 ppm, the cell membrane was bulged and some micronuclei were found without membrane (Fig. 6b, c). After 72 h, the cell membrane was damaged and numerous micronuclei were found without membrane (Fig. 6d). After 96 h, the cell membrane was fully damaged (Fig. 6e) and

micronuclei were observed in the erythrocytes of treated individuals. Concerning the comet assay, no impact on erythrocytes was observed at CNP doses lower than 25 ppm. After 24 h of exposure to 25 ppm, little erythrocyte damage was found (Fig. 7a, b). After 48 h of exposure, medium damage was found (Fig. 7c). After 72 and 96 h, high damage was reported (Fig. 7d, e). In agreement with our aim, Singh (2000) used the comet assay as a very sensitive tool for environmental monitoring which can detect ultra-low levels of DNA damage. Furthermore, Cavaş and Könen (2007) suggested that dose-dependent treatments with pollutants increase the frequencies of micronuclei, nuclear abnormalities, and DNA strand breaks.

Conclusions

This research focused on two eco-friendly nanosynthetic routes to fabricate mosquitocidal CNPs and AgNPs. UV–Vis spectrophotometry, FTIR spectroscopy, XRD analysis, SEM, TEM, EDX spectroscopy, and Raman spectroscopy confirmed the rapid and cheap synthesis of AgNPs and CNPs. The predation efficiency of the water bug *L. indicus* after a single treatment with

Table 5 Predation efficiency of *Lethocerus indicus* against *Culex quinquefasciatus* larvae in standard laboratory conditions and post-treatment with sublethal doses of silver and carbon nanoparticles (see text for dosages)

Treatment	Target	Exposure time				Total predation, n (%)
		0–6 h	6–12 h	12–18 h	18–24 h	
Control	Larva II	38±2.54	26±1.87	50±1.58	40±1.22	154 (38.5 ^c)
	Larva III	60±2.12	36±2.34	81±1.58	68±2.23	245 (61.25 ^c)
Silver nanoparticles	Larva II	54±1.87	32±2.00	70±1.22	46±1.41	202 (50.5 ^d)
	Larva III	69±2.34	52±2.12	98±1.00	89±1.87	308 (77.0 ^b)
Carbon nanoparticles	Larva II	68±1.44	44±2.78	82±1.55	60±1.37	254 (63.5 ^c)
	Larva III	85±2.22	67±1.34	97±1.55	94±2.21	343 (85.7 ^a)

Mortality is expressed as mean±SD of five replicates (two water bugs per replication). No mortality was observed in the control. Within a column, means followed by the same letter(s) are not significantly different (generalized linear model, $P<0.05$)

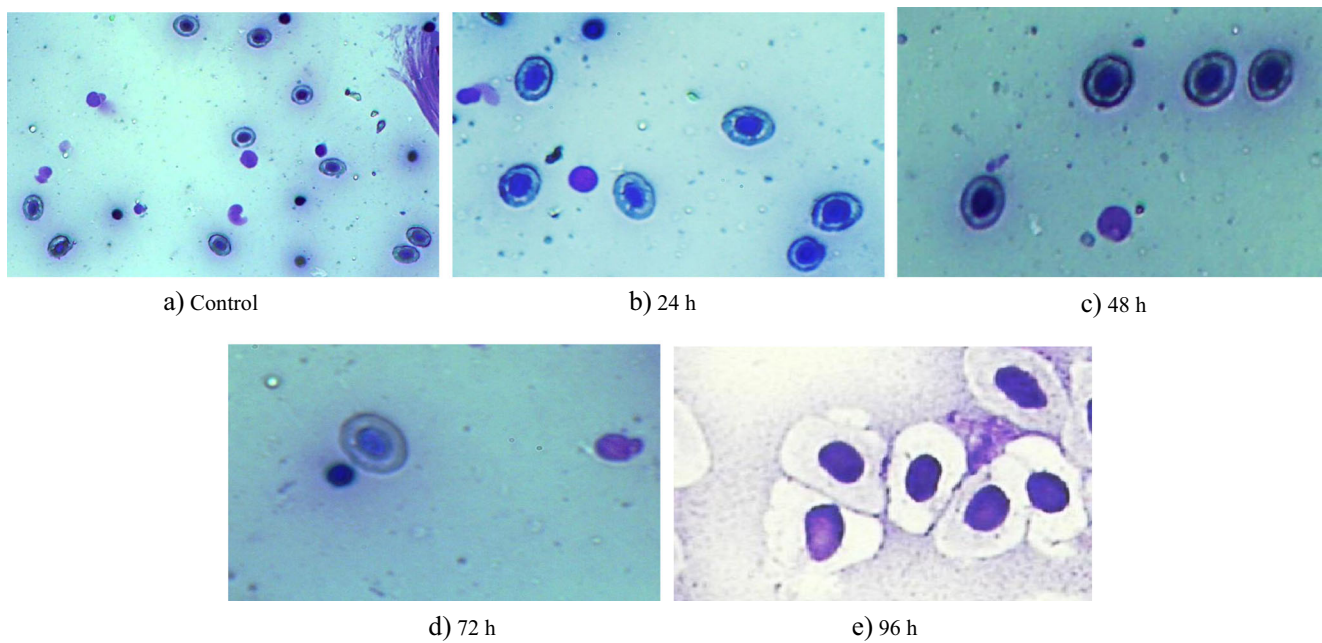


Fig. 6 a–e Photomicrographs of micronucleus analysis on erythrocytes of *Carassius auratus* exposed to carbon nanoparticles (25 ppm)

low doses of AgNP and CNP was boosted. Moderate evidence of genotoxic effects induced by exposure to high doses of CNPs was found on non-target goldfishes. The plant extract used for silver nanosynthesis showed excellent DPPH and ABTS radical scavenging activity. In summary, our results pointed

out that AgNPs and CNPs can be proposed as effective tools to reduce larval and pupal populations of filariasis vectors (see also Benelli 2016), with moderate genotoxicity and impact on behavioral traits of other aquatic organisms sharing the same ecological niche of *Cx. quinquefasciatus*. However, extensive field assays

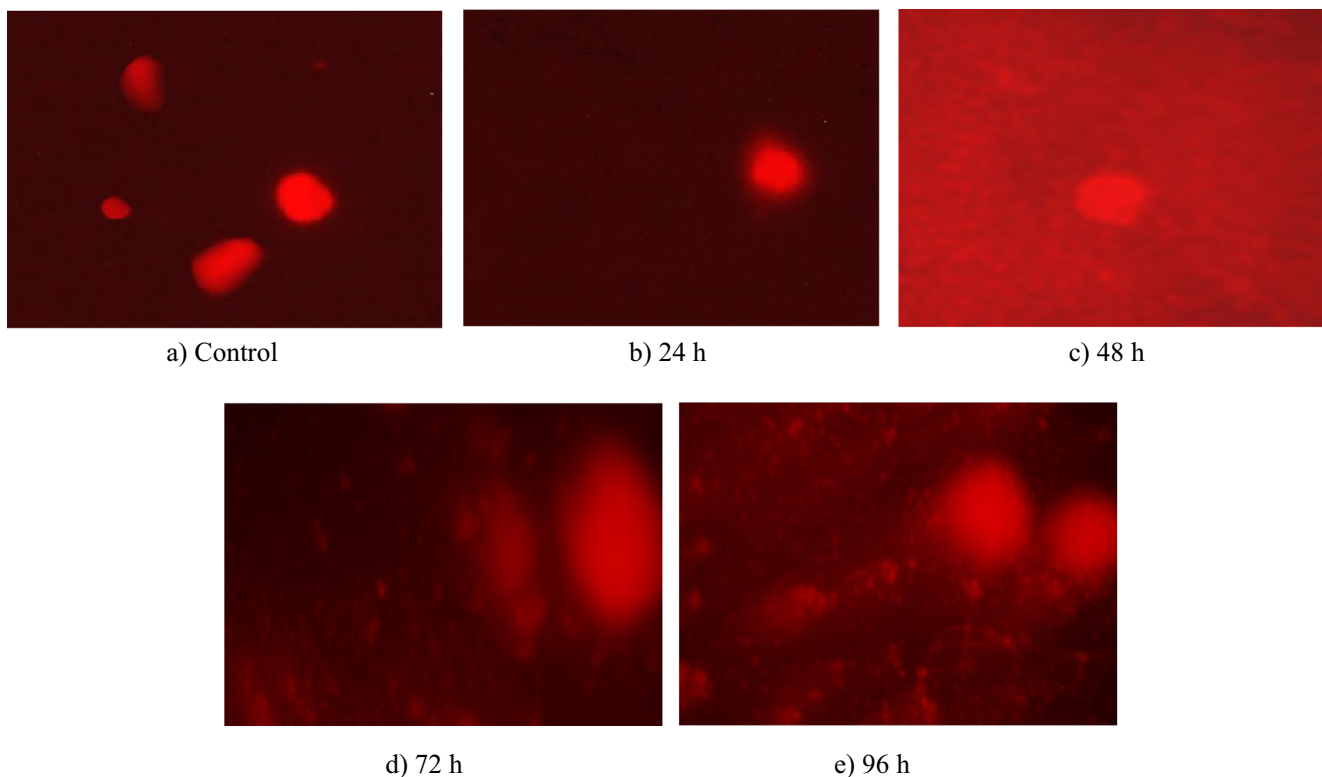


Fig. 7 a–e Comet assay photomicrographs of *Carassius auratus* erythrocytes post-exposure to carbon nanoparticles (25 ppm)

based on the employ of carbon and metal nanoparticles capped with plant-borne metabolites against mosquito vectors are urgently required.

Acknowledgments The authors would like to thank the financial support rendered by King Saud University, through the Vice Deanship of Research Chairs.

Compliance with ethical standards All applicable international and national guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

Conflict of interest The authors declare that they have no competing interests. G. Benelli is an Editorial Board Member of *Parasitology Research*. This does not alter the authors' adherence to all the *Parasitology Research* policies on sharing data and materials.

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