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MEDICAL AND VETERINARY ENTOMOLOGY

# Biosynthesized Gold Nanoparticles Integrated Ointment Base for Repellent Activity Against *Aedes aegypti* L

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## Abstract

The present study focused on preparing a nano-ointment base integrated with biogenic gold nanoparticles from *Artemisia vulgaris* L. leaf extract. As prepared, nano-ointment was characterized by using Fourier-transform infrared spectroscopy, and the morphology of the nano-ointment was confirmed through a scanning electron microscope. Initially, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide results showed nano-ointment cytocompatibility at different concentrations (20–200 µg/mL) against L929 cells. The in vitro hemolysis assay also revealed that the nano-ointment is biocompatible. Further studies confirmed that nano-ointment has repellent activity with various concentrations (12.5, 25, 50, 75, and 100 ppm). At 100 ppm concentration, the highest repellent activity was observed at 60-min protection time against the *Aedes aegypti* L. female mosquitoes. The results indicated that the increasing concentration of nano-ointment prolongs the protection time. Moreover, the outcome of this study provides an alternative nano-ointment to synthetic repellent and insecticides after successful clinical trials. It could be an eco-friendly, safer nano-bio repellent, which can protect from dengue fever mosquitoes.

**Keywords** Gold nanoparticles · Scanning electron microscope · L929 cells · Repellent

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## Introduction

Nanoparticles were considered the fundamental building blocks of nanotechnology. In recent years, green-synthesized gold nanoparticles (AuNPs) have been proposed as a safer tool to combat various mosquito vectors. They are particularly important in medicine, parasitology, and pest management. Especially, nanobiotechnology is a branch of the physical and chemical approaches to produce nano-sized functional materials through biological principles (Ahmad et al. 2015; MubarakAli et al. 2011). The natural methods are preferred over chemical and physical approaches because of the elimination of toxic chemicals and nonpolar solvents in the synthesis procedure, limiting their applications in clinical fields (Narayanan and Sakthivel 2010).

Moreover, the biogenic synthesis route AuNPs are considered a compatible, nontoxic, and eco-friendly method. Nanoscale particles exhibit entirely new or improved properties based on specific characteristics such as size, distribution, and morphology (Khlebtsov and Dykman 2011; Kaushik et al. 2012). The NPs possibly were applied for vector control applications such as (a) the growth of new agents with higher significant activity, decreased toxicity, and sustained release and (b) to produce and control vectors by the use of NPs with repellent, insecticidal, or larvicidal activities (Magro et al. 2019). The NPs are found with potential importance in various pharmaceutical fields to manage various life-threatening diseases (Elechiguerra et al. 2005). The medical applications of AuNPs raised significantly due to their less toxicity throughout the whole body (Crooks et al. 2001). Khater et al. (2019) reported commercial mosquito repellent prevents mosquito-host interactions, including synthetic and natural repellents, which could potentially prevent mosquito-host interactions. In previous report, eco-friendly botanical extract synthesized with various nanoparticles was used for the different vector diseases and mosquito controls (Govindarajan et al. 2016a,b; Murugan et al. 2015; Roni et al. 2015).

*Artemisia vulgaris* L. essential oil was reported to exhibit mosquito repellent effect against yellow fever–transmitting *Aedes aegypti* L. mosquito vectors (Hwang et al. 1985; Ram and Mehrotra 1995; Nentwig 2003). It was also reported that *A. vulgaris* showed significant insecticidal action against the stored-product insect pest *Tribolium castaneum* (Herbst) (Wang et al. 2006). The leaves are used as cicatrizing for cuts and wounds (Singh and Chowdhery 2002), and paste or powder of the leaves is applied to cure various skin diseases (Kapoor 2000). However, in ancient times, Egyptians used various plant essential oils (EOs) for repelling insects, medicinal benefits, beauty care, and spiritual heightening, and in literally all features of their daily life (Khater 2017). Previous studies demonstrated that another species of *A. vulgaris* possess anthelmintic efficacy of crude aqueous leaf extracts of *A. herba-alba* (ACEA) compared to albendazole (ABZ) against the *Heterakis gallinarum* Schrank infected with turkey poults (Seddiek et al. 2011). Based on the reported studies, we carried out the experiments of nano-ointment base in repellent activity against the *Ae. aegypti* mosquitoes and performed toxicity studies by mouse dermal fibroblast (L929) cells (Divya et al. 2016). However, there is still a need for economic, commercially possible as well as environmentally clean synthesis route to synthesize AuNPs. Moreover, the integration of NPs with bioactive principles has to lead to the development of new vector control agents in biomedical applications.

## Materials and methods

The AuNPs were synthesized by using leaf extract of *Artemisia vulgaris* L. and its larvicidal efficacy against the *Ae. aegypti* which was already reported elsewhere by Sundararajan and Ranjitha Kumari (2017) was evaluated. These prepared active NPs were used for the nano-ointment preparation. First, 3:2 ratio of polyethylene glycol (PEG) 3350 (3 g) and polyethylene glycol (PEG) 400 (2 g) was mixed with stearyl alcohol (5 g). Then, 1-mg biosynthesized nanoparticles were mixed with the above ointment base by using a mortar and pestle. Finally, the AuNP-bound ointment product was obtained. Nano-ointment (water-soluble bases) was prepared as per the earlier reported method (Allen and Ansel 2013). The as-prepared ointment was subjected to further characterization, toxicity evaluation, and repellent activity.

### Characterization of nano-based ointment

#### Fourier-transform infrared spectroscopy

Fourier-transform infrared spectroscopic (FTIR) study was carried out to identify the functional group stretching of biomolecules in the nano-ointment formulation. FTIR measurements were recorded in the wavenumber ranges from 4000 to 400 $cm^{-1}$ using a PerkinElmer spectrometer in diffuse reflectance mode at a resolution of 4 $cm^{-1}$ with KBr pellets.

#### Scanning electron microscope

Scanning electron microscope (SEM) analysis (JEOL 6360 TESCAN 10-kV machine) characterized morphological characteristics such as the shape and nature of nano-ointment. Five microliters of the sample was placed onto the carbon-coated copper grid, and a thin film of the sample was made on the grid. The extra sample was removed by keeping the sample under an incandescent light bulb.

## Cytocompatibility study

A murine fibroblast cell line (L929, NCCS Pune) was used to evaluate the cytocompatibility of formulated nano-ointment (Divya et al. 2016; Sathishkumar et al. 2016). Briefly, the cells were grown in DMEM medium appended with 10% fetal bovine serum (FBS), and antibiotic was grown in a culture flask and incubated in a $CO_2$ incubator (5% $CO_2$ and 37 °C) until attainment of 100% confluency. Furthermore, cell viability experiments were carried out on L929 cell lines 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as reported earlier. The L929 cells were seeded in a 96-well plate at the density of 10,000 cells/cm$^2$. After 24 h, different samples (20–200 µg/mL) were added and incubated in a $CO_2$ incubator for 24 h. Then, the medium was removed, and the cells were washed trice with PBS, and 20 µL MTT solutions (5 mg/mL in phosphate-buffered saline (PBS)) were added to each well. Finally, DMSO was added to dissolve the formazan crystals. The optical density was measured using a Beckmann Coulter Elisa plate reader (BioTek Power Wave XS) at a wavelength of 570 nm. On the other hand, the cytotoxicity experiments for rhodamine-1, -2, and -3 and Hoechst 33,258–tagged nano-ointment systems were conducted to confirm the treatment localization

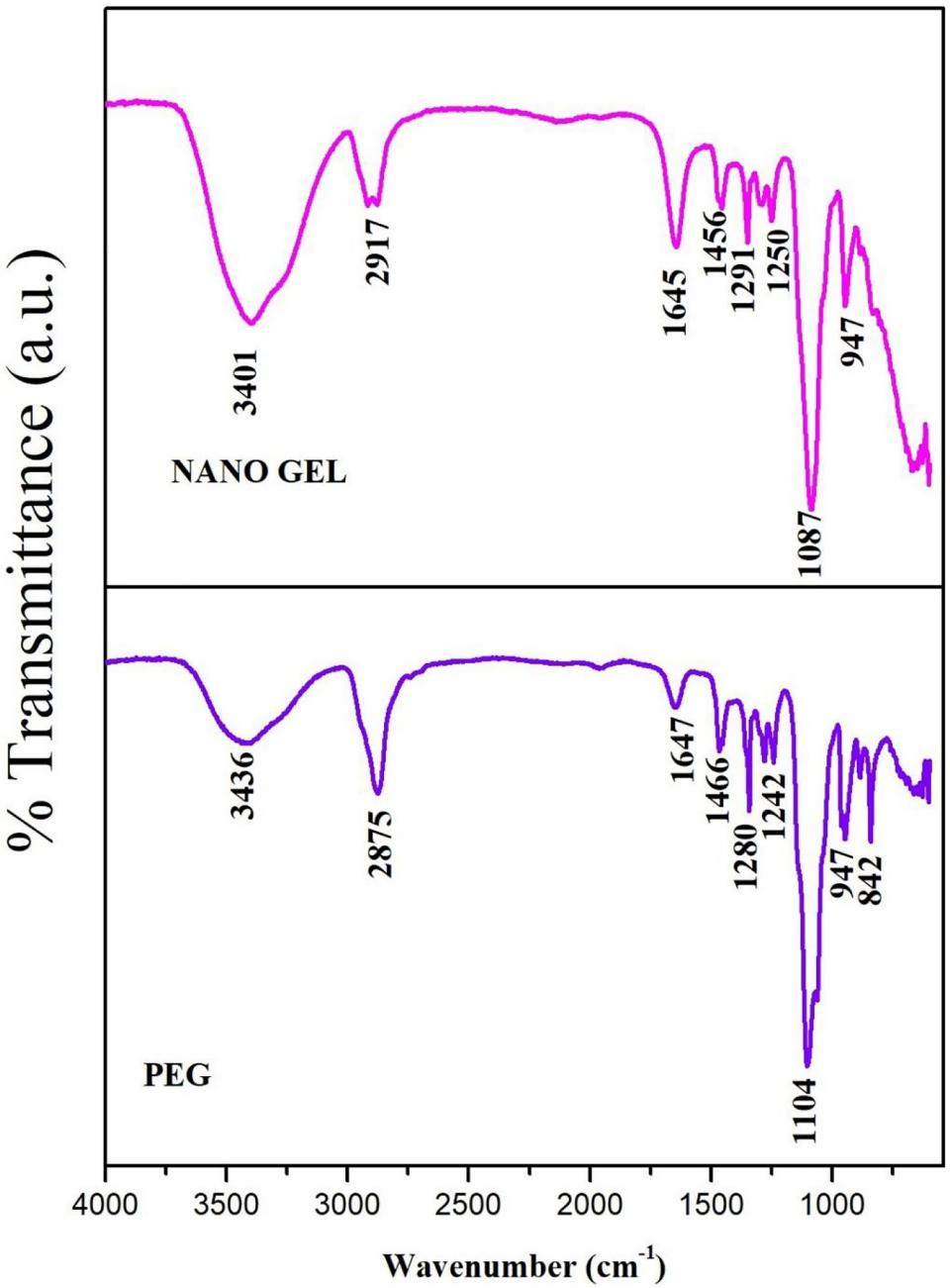


**Fig. 1** FTIR spectrum analysis of nano-ointment base with polyethylene glycol

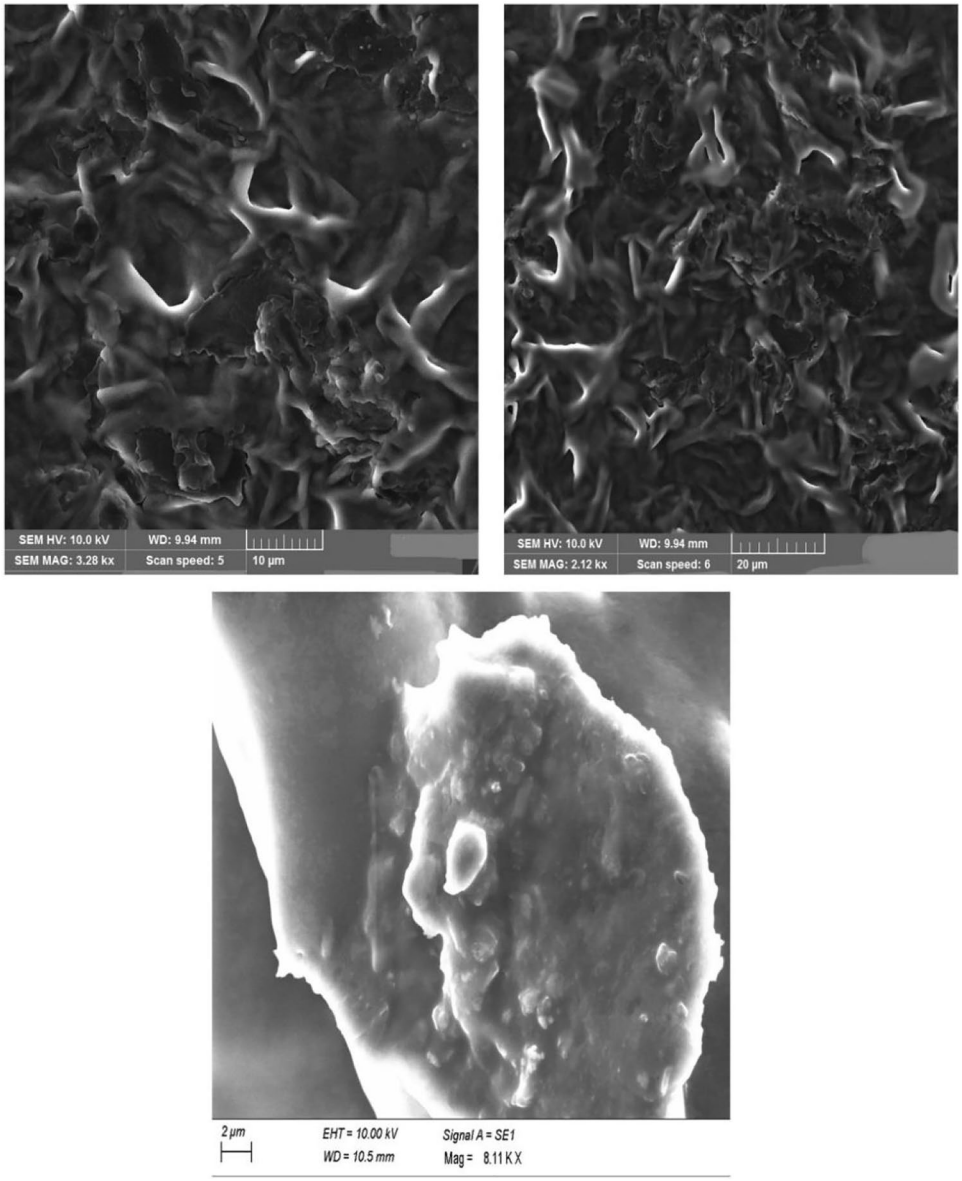


**Fig. 2** SEM image analysis of nano-ointment base with different magnifications

at different depths of skin layers. Briefly, nano-ointment (20 µL/mL)–treated cells were stained with rhodamine-1, -2, and -3 and Hoechst 33,258 (5 mg/mL) at 37 °C for 20 min. Then, it was mounted onto the glass slide using DPX for fluorescent imaging and viewed under the fluorescent microscope (Olympus-BX-51) (Divya et al. 2016).

## In vitro hemolysis assay

Hemolysis assay was performed with five replicates as per the earlier report with slight modifications (Sathishkumar et al. 2016). The nano-ointment was separately suspended in 10 mM phosphate-buffered saline. Fresh blood was collected from healthy volunteers in sterile lithium heparin vacutainers. Red blood cells (RBCs) were separated by centrifugation (1500 rpm for 10 min at 4 °C), and further diluted in 20 mM buffered saline (pH 7.4) to 5% v/v solution. A sample in the range of 5 to 50 µg/mL was added to the RBC suspension with 1% Triton X-100 added to phosphate-buffered saline and further incubated at 37 °C for 2 h and slightly vortexed. The suspension was incubated at static conditions for 4 h at 37 °C. After incubation, all the samples were centrifuged (Eppendorf) at 12,000 rpm at 4 °C and supernatants were transferred to a 96-well plate. The hemolytic activity was determined by measuring the absorbance at 570 nm (Biorad microplate reader model 550, Japan). Control samples of

0% lyse (in phosphate buffer) and 100% lysis (in 1% Triton X-100) were employed in the experiment. The per cent of hemolysis was calculated as follows:

$$\text{Hemolysis } \% = \frac{(\text{sample absorbance} - \text{Negative control})}{(\text{positive control} - \text{negative control})} \times 100\%$$

### Repellent activity

The collected healthy *A. vulgaris* leaves were washed thoroughly under the running tap water and air-shade-dried for 1 or 2 weeks at room temperature. Then, the dried leaves were used to get oil from hydrodistillation using Clevenger type of apparatus after 4 h. The essential oil thus obtained was stored in an amber color glass vial (sealed with parafilm) held at 4 °C for further experiments, and AuNP nano-ointment was evaluated at various concentrations (12, 25, 50, 75, and 100 ppm) against the *Ae. aegypti* mosquito adults. Biting test time was observed for 15, 30, and 60 min through laboratory screen cages (diameter 40 cm × length 30 cm) containing 50 nulliparous, nonblood-fed, starved female mosquitoes (WHO 2009; Panneerselvam et al. 2012). Before starting the experiment, the volunteer's hands were cleanly washed with the distilled water, and both hands were covered with rubber gloves with a window (4 cm × 5 cm) on the forearm. After applying the sample on the skin of the gloved hand, the sample was allowed to dry for 2 min. The *Ae. aegypti* mosquito biting test was carried out in between 8:00 A.M. and 4:00 P.M., and a total number of mosquitoes biting were recorded on the forearm after 3 min of the study period. This study was carried out starting with the biting of at least two mosquitoes. The mosquito biting percentage was calculated for each test using the following formula:

$$\% \text{ of Biting} = \frac{(\text{No. of bites received by control arm} - \text{No. of bites received by the treated arm})}{\text{No. of bites received by the control arm}} \times 100$$

## Results

### Preparation of nano-based ointment

Combining PEG 3350 (solid) with PEG 400 (liquid) results in a very pliable semisolid ointment. The formula may be altered to contain up to equal parts of the two ingredients for the desired composition of firmer ointment. When aqueous solutions are to be combined with bending to the base, PEG 3350 with an equal amount of stearyl alcohol is advantageous in rendering the final product firmer.

### Characterization of nano-based ointment

#### Fourier-transform infrared spectroscopy

The FTIR analysis revealed the chemical interaction between the AuNPs and the nano-ointment bases, which is shown in Fig. 1. FTIR spectra showed that AuNPs bind with nano-ointment bases with respective characteristic peaks at 3401 $cm^{-1}$ (N–H stretching primary amine), 2917 $cm^{-1}$ (N–H stretching amine salt), 1645 $cm^{-1}$ (C-H stretching aromatic compound), 1456 $cm^{-1}$ (O–H stretching carboxylic acid), 1291 $cm^{-1}$ (N = O bend nitro groups), 1250 $cm^{-1}$ (C-O stretching aromatic ester), 1087 (C-O stretching primary alcohol), and 947 $cm^{-1}$ (C = C bending alkene).

#### Scanning electron microscope

The surface morphology of nano-ointment was studied by SEM (Fig. 2). The SEM images revealed that spherical and hexagonal particles were homogeneously distributed without any aggregation.

**Cytocompatibility study** Cytocompatibility of prepared nano-ointment base formulations was evaluated using MTT assay on the L929 cell line. Figure 3 shows the percentage cell viability on L929 cells against different concentrations ranging from 20 to 200 µg/mL of the prepared systems. Our results confirmed that the nano-ointment showed no toxic effect on L929 cell lines. Even in the lowest concentrations, 20 and 40 µg/mL cell viability were recorded as 97% and 95% without affecting the healthy cells. The cell viability was investigated by using a fluorescent microscope in both controls as well as treated cell lines. Microscopic images of cultured L929 cells are shown in Fig. 4b; the rhodamine-123 dye was used to assess the membrane probable potential, which accumulates within the mitochondria in a potentially dependent manner. These results proved that the nano-ointment base, when treated with L929 cells, showed very low disturbance to mitochondrial membrane potential. While seen in Fig. 4c, the Hoechst staining showed that untreated control cells have normal morphology with an undamaged round nucleus which was emitted by a weak blue fluorescence. It is exciting to note that L929 cells were treated

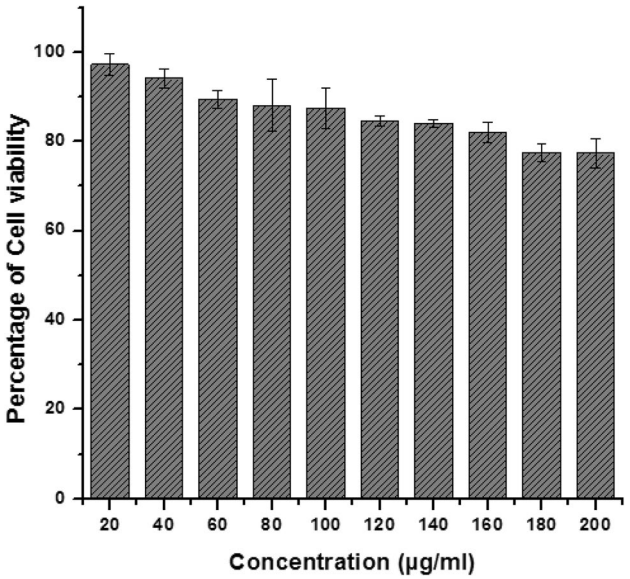


**Fig. 3** Cell viability studies of nanogel systems on L929 cell line by MTT assay. All of the obtained data were expressed as mean ± SD of the three experiments ($n = 3$)

with a nano-ointment base for 24 h, and exposed murine fibroblast cells showing bright blue color emission showed a significantly less disturbance in nuclear fragmentation condensation of chromatins after a 24-h treatment.

### In vitro hemolysis assay

Hemocompatibility of nano-ointment was studied to measure the damage to human RBCs. Our result revealed that nano-ointment at 5 µg/mL (0.1%) showed lower red hemoglobin compared with 50 µg/mL (0.5%). As shown in Fig. 5, in comparison with the positive control, the hemolysis activity of nano-ointment was less considerable; it involves its insecure nature in the application. The mechanism of direct hemolysis activity for similar toxic agents originated to be nonspecific.

### Repellent activity

In this study, nano-ointment base and essential oil repellent activity were carried out under the laboratory screen cage to avoid an allergic reaction to the tested person. There were differences in repellency among the essential oil and nano-ointment base repellents by *Ae. aegypti* mosquitoes. Present investigations revealed that the nano-ointment has significant repellent activity against *Ae. aegypti* mosquitoes. The highest (100 ppm) concentration of nano-ointment base and essential oil gave the highest repellent for the longest lasting period and protection against *Ae. aegypti* for 60 min (Table 1). The present study evaluates the increasing concentrations and protection time of nano-ointment base prolonged repellent against *Ae. aegypti* mosquitoes.

## Discussion

The results confirmed the conjugation of AuNPs with ointment by intermolecular hydrogen bonding and the formation of nanoparticle composites. Our products were in corroboration with the FTIR analysis of the nanoconjugates reported earlier (Tomuleasa et al. 2012). After preparation of nano-based ointment, the bond was observed in the range of 3200–3700 $cm^{-1}$, which was suitable to the presence of surface-bound –N and –OH groups. The peak shifted from 1647 to 1642 $cm^{-1}$ and represented C-H bonds' stretching vibrations from the rings. The obtained results were confirmed that the stretching vibrations of amine and the carboxylic acid–rich regions were involved in the formation of NPs and agglomeration of the composites in an ointment base. Therefore, the experimental results revealed that the flavonoid, phenolic, and terpenoid groups present in the plant extracts might be responsible for synthesis and an agglomeration of both AuNPs and ointment base. A very similar phenomenon was reported for cyclodextrin-modified nanogels, where the SEM micrographs showed that the increasing β-CD concentration decreases the size of nanogel (Khandelia et al. 2013). Nanogel particles are spherical and flat on the solid substrate after the elimination of water by evaporation. A similar study was reported by Jayakumar et al. (2012), where the cytotoxic activity of biosynthesized AuNPs from coconut water was quantified and written at a concentration of 100 µM; cell viability was observed 87% in HeLa and 85% in MCF-7 cancer cell lines. The cytotoxicity study of synthesized AuNPs from *Bacopa monnieri* L. leaf extract was also reported and checked the biocompatibility on HeLa and MCF-7 cell lines (Kettel 2011). Commercially available AuNPs were tested for MTT assay, and results showed that the highest concentrations range from 1 to 15 mg/mL more than 65% in Normal Human Dermal Fibroblasts (NHDF) cell lines (Babu et al. 2011). Curcumin-loaded chitin nanogels treated for skin cancer cell line showed cytotoxicity without affecting the healthy cells, which are a primary necessity for the cancer treatment modality (Mangalathillam et al., 2012). At the same time, cytotoxicity of gold nanoparticles was carried out by MTT assay and found that it reduced the viability of MDA-MB-231 cells in a dose-dependent manner at a concentration of 100 µg/mL (Babu et al. 2013). Synthesized gold nanoparticles using sodium citrate as a reducing agent and reduced viability of A549 and NCIH441 cell lines also affected the cellular proliferation where citrate acted as the capping agent (Mateo et al. 2015). Similarly, our data highly agree with a previously reported of synthesized AuNPs using by *Zingiber officinale* extract has been revealed the high-level

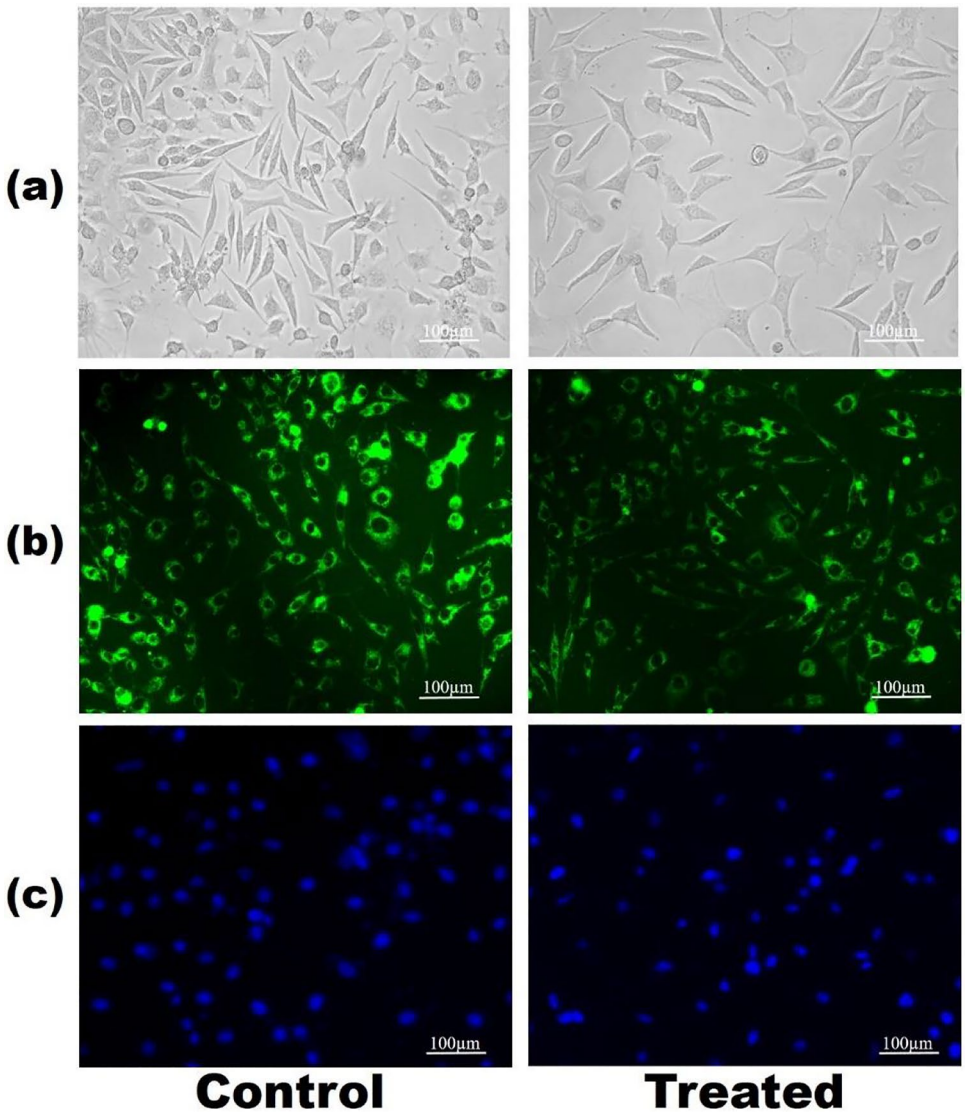


**Fig. 4** Fluorescent images of L929 cells treated with nano-ointment base in morphometric measurement of cell damage. **a** Normal view without adding dye. **b** Rhodamine-1, -2, -3 dye-treated. **c** Hoechst 33,258 dye-treated

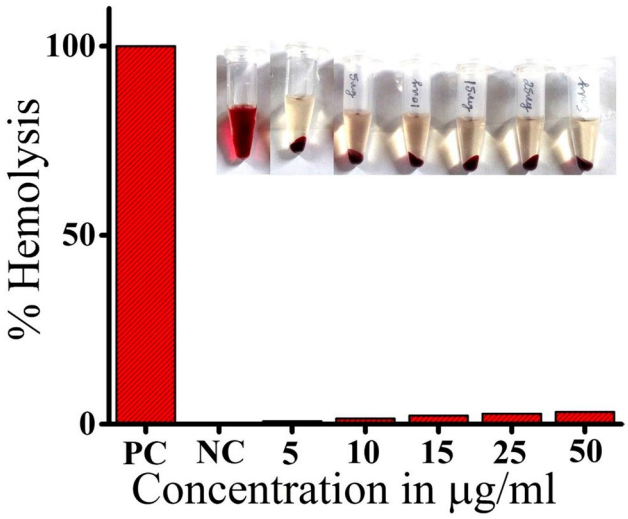


**Fig. 5** Percentage hemolysis of different concentrations of nano-ointment base (PC—positive control, NC—negative control)

compatibility with the blood cells that implement not initiate any aggregation of cells and the NPs do not appear to activate the platelets. It was conditional that the surface passivation of nanomaterials with different bioagents will develop their biocompatibility (Krishnaraj et al. 2014). In the present study, in comparison with earlier reports in *Citrus* plants, essential oil was reported against *Ae. aegypti* mosquitoes: protection time ranged from 10.0 ± 8.7 to 65.0 ± 22.9 min, biting rate ranged from 1.2 to 2.3%, and protection ranged from 98.3 to 98.8% (Kettel et al. 2011). The *A. vulgaris* benzene and methanol extracts were previously reported to have repellent activity against *Ae. aegypti* (Uboldi et al. 2009; Kumar 2011). Another study proved that the *Zingiber cassumunar* Roxb essential oil possess repellent activity against *Culex quinquefasciatus* Say (165-min protection time and 0.9% biting rate) and *Ae. aegypti* (90-min protection time and 0.8% biting rate) (Soonwera 2015). *Chrysanthemum cinerariaefolium* dry flower powdered has been used in ancient insecticide times

**Table 1** Effect of nano-ointment base on repellent activity of *Aedes aegypti* L

| Various concentrations (ppm) | Protection % (min) | | |
|---|---|---|---|
| | **15** | **30** | **60** |
| Nano-ointment | | | |
| 12.5 | $55.57 \pm 2.15^{e}$ | $43.04 \pm 2.58^{d}$ | $29.05 \pm 1.16^{d}$ |
| 25 | $41.41 \pm 1.66^{d}$ | $32.38 \pm 2.52^{c}$ | $23.85 \pm 5.36^{c}$ |
| 50 | $34.91 \pm 1.71^{c}$ | $23.74 \pm 3.26^{b}$ | $12.58 \pm 2.30^{b}$ |
| 75 | $23.73 \pm 2.26^{b}$ | $14.85 \pm 1.71^{a}$ | $9.98 \pm 0.39^{b}$ |
| 100 | $15.97 \pm 1.58^{a}$ | $11.21 \pm 0.98^{a}$ | $1.16 \pm 1.04^{a}$ |
| *A. vulgaris* leaf essential oil | | | |
| 12.5 | $73.03 \pm 1.54^{ef}$ | $61.37 \pm 0.87^{f}$ | $50.48 \pm 0.42^{e}$ |
| 25 | $66.13 \pm 1.59^{d}$ | $52.51 \pm 1.85^{d}$ | $41.60 \pm 0.86^{d}$ |
| 50 | $60.11 \pm 0.64^{c}$ | $41.76 \pm 1.71^{bc}$ | $32.99 \pm 1.44^{cd}$ |
| 75 | $51.29 \pm 3.65^{b}$ | $36.86 \pm 1.35^{b}$ | $22.78 \pm 1.42^{b}$ |
| 100 | $38.12 \pm 3.05^{a}$ | $20.74 \pm 2.30^{a}$ | $16.17 \pm 1.57^{a}$ |

Means in each column followed by different letters are significantly different ($P < 0.05$, by one-way ANOVA and Duncan's Multiple Range test)

(Hwang et al. 1985). Some natural plant product plays a significant role in the development of commercial insecticide. Hence, one example shows that α-terthienyl has been used for its herbicidal activity (Amer and Mehlhorn 2006). The skin test for evaluating the repellency of catnip oil presented two primary active ingredients compounds of Z, E-nepetalactone, and E, Z nepetalactone which showed more repellent activity (Phasomkusolsil and Soonwera 2011). Earlier study information of *Camellia japonica* L. essential oils isolated from this compounds such as 3-career, alpha-terpinene, limonene, gamma-terpinene, terpinolene, and (-)-terpinen-4-ol against the *Ae. aegypti*, *Aedes albopictus* (Skuse) (Benner 1993) and confertifolin compound isolated from *Persicaria hydropiper* L. essential oil showed good repellent activity showed 100% adulticidal activity against *Ae. albopictus* (Lambert et al. 1991). The essential oil of *Chromolaena odorata* flowers has been reported to hold repellent activity against *Ae. aegypti* mosquitoe bites and two types of grain storage insects: *Sitophilus zeamias* Motschulsky and *Tribolium castaneum* (Herbst) (Zhu et al. 2006; Gu et al. 2009; Dua et al. 2008; Trongtokit, et al. 2005; Nerio et al. 2009; Caballero-Gallardo et al. 2012). In our repellent activity, the results are very promising to formulate a potent and affordable nano-ointment against the dengue fever disease–transmitting *Ae. aegypti* mosquito. In conclusion, We are reporting for the first time that nano-ointment base exhibits excellent repellent activity against *Ae. aegypti* dengue fever vector. MTT results confirmed that nano-ointment exhibits no toxicity on L929 cells, and in vitro hemolysis assay revealed that nano-ointment is biocompatible with the biologically synthesized active nanoparticles and may be used for dengue vector control after repeated clinical trials. Thus, this ointment is a promising candidate for better repellent activity against the dengue fever mosquito protection time more than 60 min and it can be used in the commercial and industrial production levels.

**Author Contribution** B.S. and B.D.R.K. conceived of the idea of the work. B.S. developed the study, performed the wrote the main manuscript. B.S., G.S., P.S., and S.S. carried out the experiments. A.K.M., S.M., A.T., and E.J. analyzed data interpretation. A.A.S.B.M., V.D.S., and Z.Z. revised the manuscript contents. All authors discussed the results and contributed to the final manuscript.

## Declarations

**Conflict of Interest** The authors declare no competing interests.

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