



# Investigation of Pesticidal and Anti-biofilm Potential of *Calotropis gigantea* Latex Encapsulated Zeolitic Imidazole Nanoframeworks

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## Abstract

Zeolitic imidazole frameworks (ZIFs) are multifunctional and biocompatible material for biomedical applications. Herein we report for the first time *Calotropis gigantea* latex encapsulated ZIF-L nanoframeworks one-pot synthesis approach. The present study focuses on synthesis of latex encapsulated zeolitic imidazole frameworks (CG@ZIF-L) for elucidation of biofilm, mosquito and midge fly larvae. The physicochemical characteristic of as prepared CG@ZIF-L nanoframework was systematically investigated by microscopic and spectroscopic techniques. Encapsulation of *Calotropis gigantea* latex inside ZIF-L nanoframeworks shows notable toxicity against *E. coli*, *S. epidermis*. The larvicidal results revealed that CG@ZIF-L nanoframeworks beneficially kill the *Aedes aegypti* mosquito and midge fly (blood worm) larvae in a dose dependant manner. Over all the present study highlights the possibility of the multifunctional nature of CG@ZIF-L nanocomposite, which highly suitable material for biomedical applications.

**Keywords** Antibiofilm · Cytotoxicity · Encapsulation · Mosquitocidal · Zeolitic imidazole frameworks

## 1 Introduction

Plants are first resource for human utilized from the environment to cure ailments. Plants provide many leading compounds for the development of the new medicines [1–5]. *Calotropis gigantea* is a perennial herb which has long history in Indian Ayurvedic treatments. A wide range of phytochemicals from whole plant including flavonoids, tannins, cardiac glycosides, terpenoids has been used for various disease control and prevention techniques such as elephantiasis, cancer, ulcer and leprosy [6–9]. The latex is most prominent product and it has special chemical compounds like as calotropin, uscharidin, lupeol and calotoxin [10]. The emergence of life threatening diseases and drug resistant pathogens diverted the attention of researchers to plant compounds for drug development [11–14]. In the present scenario, pathogenic bacterial strains develop into multidrug resistance bacterial strain adapting to climatic changes [15]. Biofilm formation is one of the primary and notorious mechanisms of bacterial

adaptation in environmental condition. Formation of biofilm, provides more advantages to survival and adopt with environment [16, 17]. As bacterial infection and biofilm causing diseases are widely reported in animals and humans, it's a great challenge to maintain public health and hygiene [18, 19]. On a global scale, a mosquito has greatest concern about transmission of contagious diseases such as chickungunya, malaria, dengue and yellow fever [20–22]. In this context, *Bacillus thuringiensis* (Bti) is the most abundant pesticide used for mosquito and some pest control programs. The continuous use of Bti causes food-web related effects in wetlands and nature conservation areas [23, 24]. On the other hand, some mosquitos such as *Culex quinquefasciatus* and *Aedes aegypti* have developed resistance to Bti pesticide [25, 26]. These emergences of mosquito control have gained much attention on development of effective pesticides to control dengue virus transmitting vector *Aedes aegypti* mosquito [27, 28]. Furthermore, over population of midge fly larvae has developed into major problem for fresh water ponds and agriculture lands causing toxic shock syndrome (TSS) to humans. The control of mosquito vector and midge fly larvae is very difficult process for many countries [29, 30]. Biting midges are blood-sucking flies belonging to the family *Ceratopogonidae*, which includes

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more than 6,000 species available worldwide. The most important consideration of biting midges is medical and veterinary interest [31–33]. They transmit a number of protozoans, pathogens, viruses and filarial nematodes. In particular, veterinary research and wildlife conservation, they transmit many viral diseases to animals such as bluetongue disease, African horse sickness and epizootic hemorrhagic disease [34–36]. Because of the vast biodiversity of fauna and flora, plants are considered as an important source material for novel pesticides production. Furthermore, the natural components of plant extracts can provide an advantage to efficiently fight against pesticide resistance insects [37–39].

Recently, Plant derived compounds and nanomaterials play a major role in pest control with the help of nanotechnology. Plant compounds encapsulated nanomaterials are efficient in controlling the mosquito and some pest populations. Plant components together with nanomaterials exhibits, synergistic effect towards several molecular targets in pest control [39]. Zeolitic imidazole framework (ZIF-L) has emerged into promising class of nanocarrier in the field of biomedicine due to its unique physiochemical properties such as crystalline structure with exceptional thermal and chemical stability, high surface area with tunable pore size, good adsorption capacity and ease of functionalization. In addition, the biocompatibility, biogenic mode of synthesis and pH responsiveness has made it a suitable candidate for drug delivery specifically for cancer theranostics and as antimicrobial and pest control agents [18, 19, 39]. Hence, the present study focused on the fabrication of *Calotropis gigantea* latex encapsulated ZIF-L nanoframeworks and evaluate its mosquito, midge fly larvicidal activities and anti-biofilm potentials.

## 2 Materials and Methods

*Calotropis gigantea* latex, Nutrient agar, streptomycin, Zinc nitrate hexahydrate, 2-Methyl imidazole, Mueller Hinton agar, penicillin and Crystal violet were purchased in Himeidia, India. *Artemia salina* cysts were purchased in aquarium, Chennai, India. All the chemicals and reagents used are of analytical grade.

### 2.1 Collection of Latex from the Plant

The fresh latex was collected from exudation of aerial part of the plant in the natural population of *Calotropis gigantea* around Alagappa University, Science campus, Tamilnadu, India (10°05'32.6" N 78°47'17.2" E). The liquid latex was dried in hot air oven at 60 °C for 5 h. Dried latex was grinded

by mortar and the powdered sample were stored at refrigerator for further uses.

### 2.2 Synthesis of CG@ZIF-L Nanoframeworks

*Calotropis gigantea* latex encapsulated ZIF-L nanocomposite was prepared with slight modification based on the previous report of [18]. Briefly, 200 mg of zinc nitrate hexahydrate and 600 mg of 2-methyl imidazole dissolved in separate 45 ml of double distilled water. 5 mg of latex powder dissolved in 10 ml of methanol (0.5 mg/ml). Under magnetic agitation latex solution was slowly added into 2-methyl imidazole solution and the stirring was continued for 30 min. Zinc nitrate solution was slowly added into latex and 2-methyl imidazole mixture under magnetic agitation for another one hour until pure white colored colloid is formed which indicates the formation of CG@ ZIF-L framework. Finally, the excess amount of precursors was removed by washing with DD water by centrifugation at 8000 rpm speed. The hydrated sample was dried at 60 °C for 6 h.

### 2.3 Collection of Mosquito Larvae

The fourth instar stage of *Aedes aegypti* larvae were collected from the stagnant rain water drain in Peraiyur village, Ramanathapuram district, Tamilnadu, India (9°21'24.0" N 78°27'12.8" E). The collected larvae were maintained in plastic enamel trays containing dechlorinated tap water at room temperature for the further studies.

### 2.4 Collection of Midge Fly Larvae

The third instars stage of midge fly larvae were collected from field of sorghum cultivation ground in Peraiyur village, Ramanathapuram dist, Tamilnadu, India ((9°21'24.0" N 78°27'12.8" E). The collected midge fly larvae were maintained in plastic beaker containing dechlorinated tap water at room temperature for further experimental studies.

### 2.5 Mosquito and Midge Fly Larvicidal Bioassay

Mosquitocidal potential of CG@ZIF-L nanoframeworks against *Aedes aegypti* was evaluated based on OECD guideline standard method [40]. Five different groups of 20 mosquito larvae were transferred into glass beakers containing 200 ml of sterile double distilled water with various concentrations of CG@ZIF-L nanoframeworks (25–125 µg/ml). Double distilled water alone was used as negative control without addition of nanoframeworks. The 16:8 h light/dark cycle trials were used for assessment of larvicidal activity of CG@ZIF-L nanoframeworks. A Morphological and behavior change of treated groups was keenly watched at

distinct time intervals. Mortality rate was quantified after 24 h post exposure of treated larvae of *Aedes aegypti*. The biting midge fly larvicidal potential of CG@ZIF-L nanoframeworks was assessed by same above mentioned procedure and same concentrations. The lethality of larvae was quantified using the standard formula.

$$\text{Mortality (\%)} = \frac{X - Y}{Y} \times 100$$

X: Survival in the untreated control and Y: Survival in the treated sample.

## 2.6 Anti-biofilm Potential of CG@ZIF-L Nanoframeworks

Anti-biofilm efficacy of the CG@ZIF-L nanoframeworks were investigated against gram positive and negative bacterial strains (*E.coli*, *S.epidermis*) based on methodology of [19] with some modifications. Bacterial strains was allowed to grow over night and the O/N culture was subjected to dilution ( $10^8$  CFU/ml) in nutrient broth containing 0.5% glucose followed by treatment with different concentrations of nanoframeworks (25–100  $\mu\text{g/ml}$ ), for 24 h at 37 °C. The bacterial culture was coated on the surface of 1  $\times$  1 cm glass slides. A free floating liquid bacterial suspension was removed by phosphate buffer solution. The slides were stained with 0.5% crystal violet stain. Excess amount of stain was removed by washing thrice with sterile distilled water for 10 min; bacterial inhibition was measured as color intensity at 570 nm using multi plate reader.

Architecture and morphological changes of CG@ZIF-L nanoframework treated groups was observed and images were taken by using light microscope at  $\times 40$  magnification.

## 2.7 Statistical Analysis

The  $\text{IC}_{50}$ ,  $\text{LD}_{50}$  and  $\text{LD}_{90}$  values are calculated using Probit analysis software and comparison of variance in control and treated groups were carried out using one-way analysis of ANOVA software. Results were considered significant at the level of  $p < 0.05$ . A result was expressed as Mean  $\pm$  SD of triplicates.

# 3 Results and Discussion

## 3.1 Powder XRD Analysis

The crystal structure of CG@ZIF-L nanoframeworks was evaluated by powder XRD spectroscopy technique. PXRD spectra (Fig. 1) shows sharp diffraction peaks at around 14.32°, 15.63°, 17.07°, 21.24°, 25.38°, 27.04°, 29.87°, 30.34°, 31.56°, 32.93°, 36.58° and 38.75° corresponding to

(100), (002), (101), (102), (110), (103), (200), (201), (004), (202), (104) and (203) Braggs reflection planes. Characteristic X-ray diffraction peaks at  $2\theta$  values and crystal planes confirmed that the hexagonal crystal structure according to JCPDS No. 01–1136. The crystalline size of CG@ZIF-L nanoframeworks was measured by Scherer's formula to be around 46.38 nm respectively.

## 3.2 UV-Vis Analysis

The characteristic UV- Vis absorption spectra of bare ZIF-L showed peak at 210 nm. Latex encapsulated CG@ZIF-L nanoframeworks (Fig. 2) showed absorption peak at 201 nm respectively. Shifting in the absorption spectra of

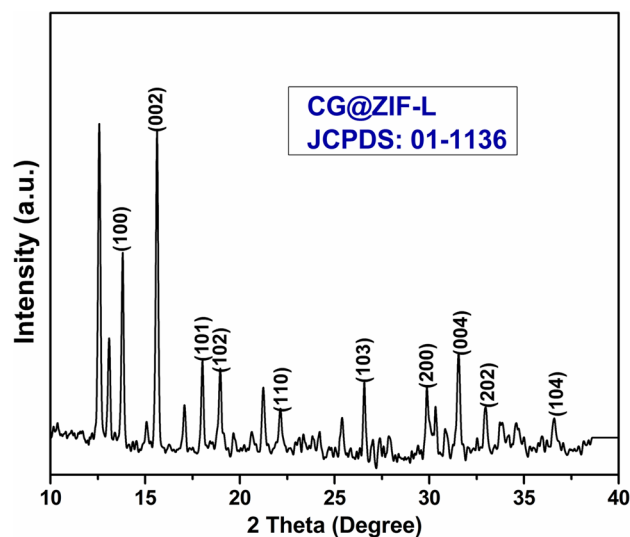


Fig. 1 Powder XRD analysis of CG@ZIF-L nanoframeworks

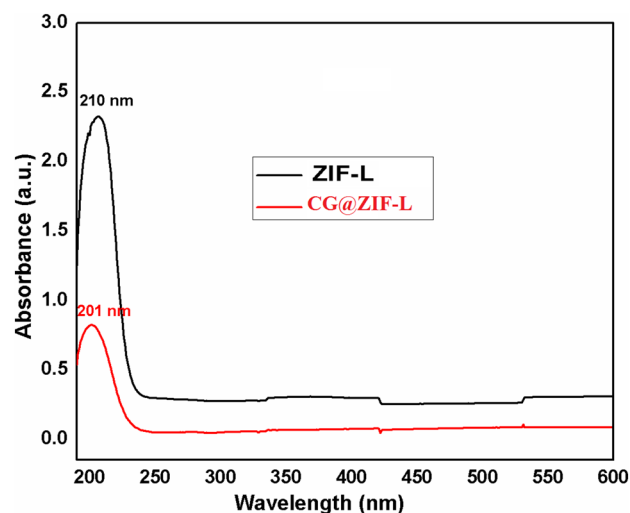


Fig. 2 UV-Vis spectra of CG@ZIF-L nanoframeworks

CG@ZIF-L nanoframeworks confirmed, that the encapsulation of latex within ZIF-L frameworks. Band gap energy of ZIF-L and CG@ZIF-L nanoframeworks was calculated to be 5.90 eV and 6.16 eV respectively. The band gap energy of synthesized nanoframeworks was increased by the encapsulation of latex into ZIF-L frameworks.

### 3.3 FTIR Analysis

The characteristic functional groups of synthesized CG@ZIF-L nanoframeworks were identified by FTIR spectroscopy. The spectra (Fig. 3) showed the intense peaks at around 683, 714, 949 and 1005  $\text{cm}^{-1}$  which attributes to C–H aliphatic stretching vibration of imidazole. Small peaks at around 1145, 1505  $\text{cm}^{-1}$  represented that the N–H stretching vibration of imidazole linker from zeolitic imidazole frameworks. The mild peak at around 1647 and 1756  $\text{cm}^{-1}$  corresponding to the axial deformation of C–O bending to C=O stretching vibrations of imidazole molecules.

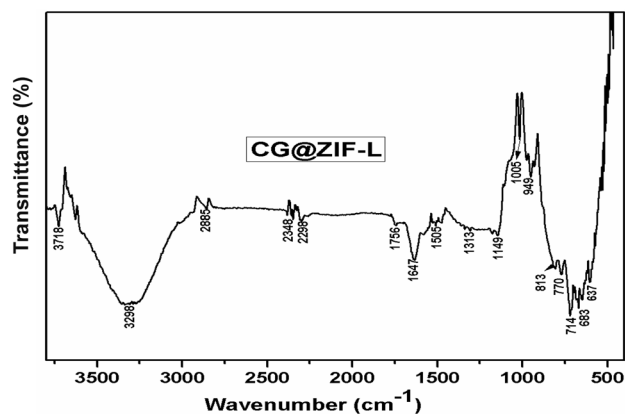


Fig. 3 FTIR spectra of CG@ZIF-L nanoframeworks

Large peaks at around 3298, 3718  $\text{cm}^{-1}$  confirmed that the bending vibration of hydroxyl group in the CG@ZIF-L nanoframeworks.

### 3.4 FE-SEM and EDX Spectroscopy Analysis

The crystal morphology and chemical composition of synthesized CG@ZIF-L nanoframeworks was studied by using FE-SEM and EDX spectroscopy analysis techniques. The results reveal that the encapsulation of *Calotropis gigantea* latex within the ZIF-L frameworks disturbed the crystal growth and leads lattice mismatch. The microscopic images concluded that the crystals are agglomerated (Fig. 4A). The results of EDX spectra of CG@ZIF-L nanoframeworks (Fig. 4B) shows higher amount of carbon and zinc ions which corresponds to the presence of zinc and organic components from 2-methyl imidazole and *Calotropis gigantea* latex. The notable amount of nitrogen represented that the formation of zinc and imidazole covalent bonding in ZIF-L frameworks.

### 3.5 TEM Analysis

The average particle size and crystal morphology was assessed by using HR-TEM imaging technique (Fig. 5A, B). Images of CG@ZIF-L nanoframeworks exhibited two dimensional spongy flakes and agglomerated spheres. The average particle size of CG@ZIF-L nanoframeworks was calculated to be 58.4 nm. The nano sized frameworks can be easily internalized to living cells either by endocytosis mechanism. As the prepared nanoframeworks are less than 100 nm sizes which gives more advantages to uses of biomedical applications.

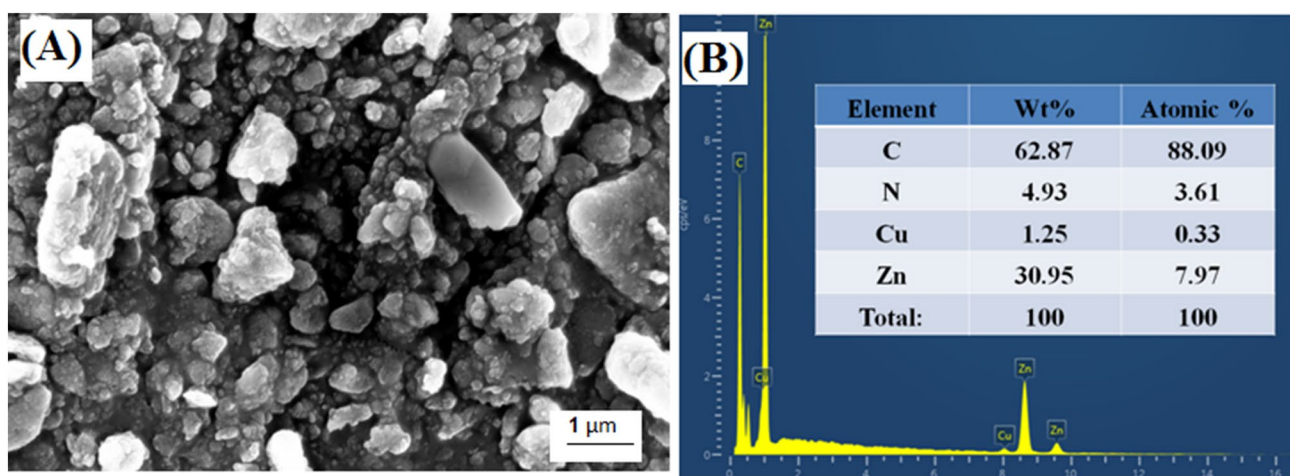


Fig. 4 SEM image and EDX spectra of CG@ZIF-L nanoframeworks

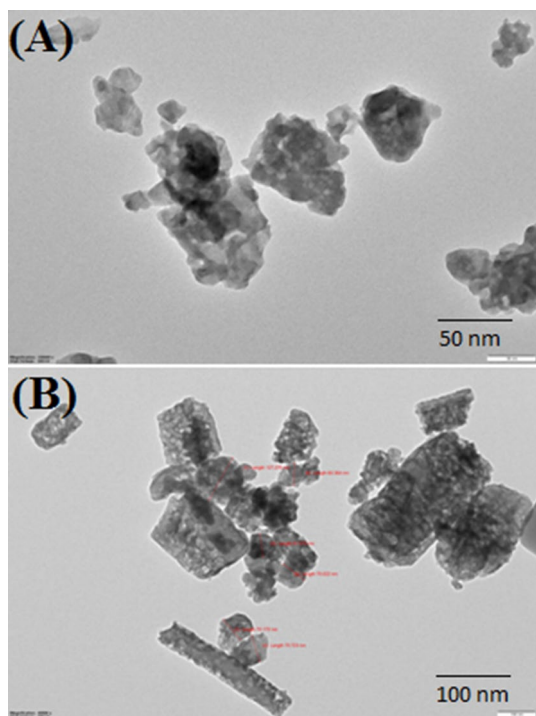


Fig. 5 TEM image of CG@ZIF-L nanoframeworks

### 3.6 Assessment of Particle Size and Thermal Stability Analysis of CG@ZIF-L

Size of the CG@ZIF-L was evaluated using DLS analysis and the results were shown in Fig. 6A. Results revealed the average particle size of CG@ZIF-L was observed to be 163 nm, which in accordance to the previous reports [41, 42] is suitable for cellular uptake and internalization of nanocomposite either by endocytosis or adsorption mechanism.

Thermal stability of ZIF-L and CG@ZIF-L was evaluated by thermo gravimetric analysis and the results are shown in Fig. 6B. Results revealed that ZIF-L is thermally stable up to 500 °C with no significant weight loss. ZIF-L alone showed mild weight loss of 4.04% at 100–500 °C due to loss of water and second plateau with 25.94% weight loss between 500–700 °C indicating the degradation of ZIF-L frameworks as reported by Prabhu et al., [18]. TGA of CG@ZIF-L showed weight loss of 19.5% in the region 250–500 °C exemplifying the decomposition of *Calotropis gigantea latex* from CG@ZIF-L nanocomposite. Further reduction in total mass by 49.9% was observed at temperature range 500–800 °C indicating complete degradation of ZIF-L frameworks.

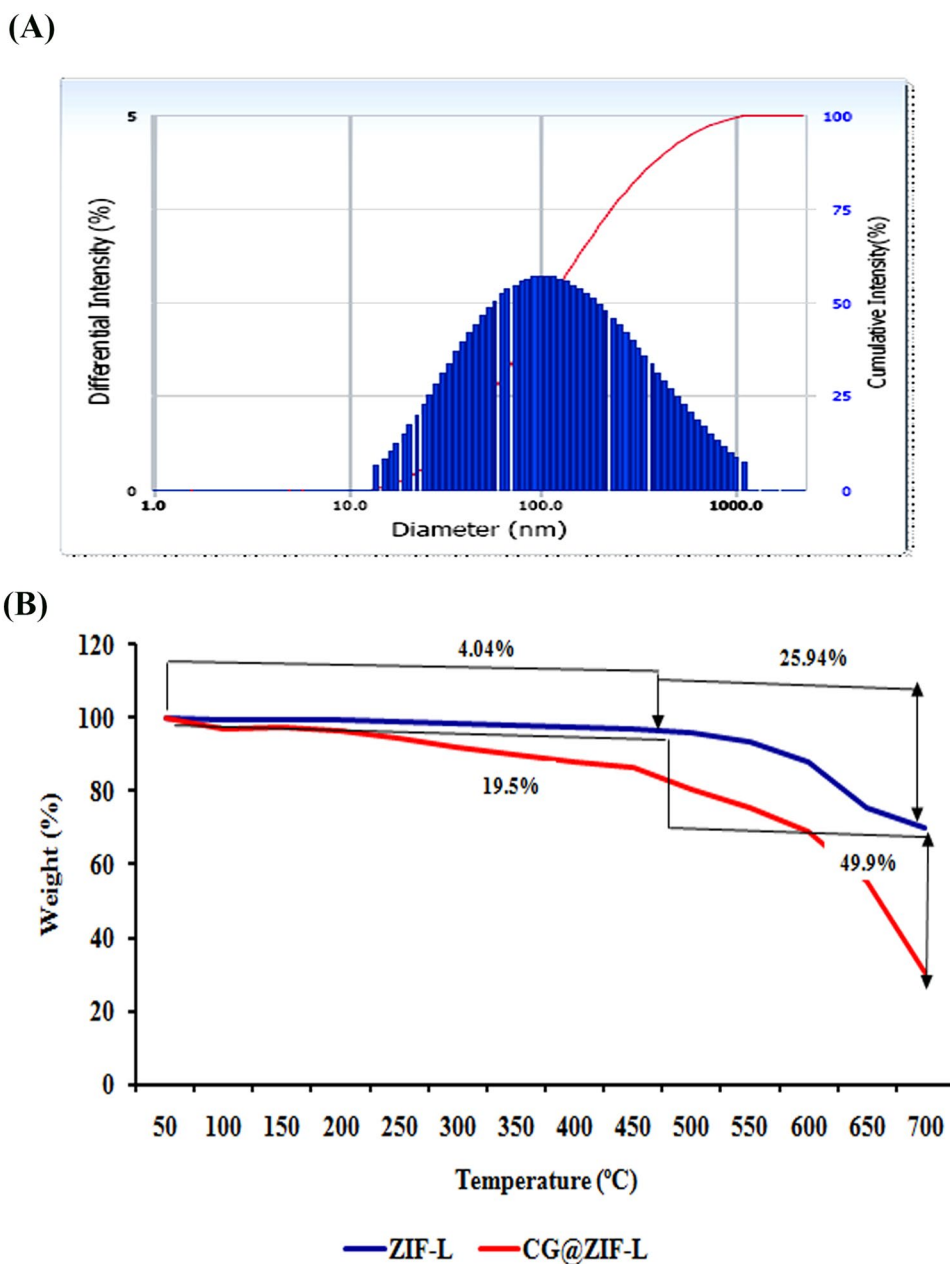
### 3.7 Anti-biofilm Efficacy of CG@ZIF-L Nanoframeworks

*Escherichia coli* and *Staphylococcus epidermis* are more dreadful nosocomial pathogen infecting humans which causing life threatening disorders. A bacterial bio-film formed by the aggregation of bacterial cells adjoining by self-production polymer matrixes adhered to surface of medical tools and tissues causing serious health problems to humans and animals [19, 43]. The biofilm inhibitory potential of synthesized CG@ZIF-L nanoframeworks was assessed by crystal violet staining assay. Results revealed that, the encapsulation of *Calotropis gigantea* latex efficiently control the bacterial growth compared to control sample and ZIF-L alone. Figure 7B illustrated that CG@ZIF-L nanoframeworks controlled the growth and eradicated the biofilm matrix in dose dependant manner, while negligible activity was observed in ZIF-L alone treated group. Furthermore quantitative study of Crystal violet binding assay results (Fig. 7A) revealed that CG@ZIF-L nanoframeworks inhibited biofilm forming bacteria *E.coli* and *S.epidermis* growth 92% at  $78 \pm 1.35$  µg/ml with IC<sub>50</sub> value of  $38.57 \pm 0.94$  µg/ml concentrations. ZIF-L alone exhibited 24% biofilm inhibition at maximum dose of 100 µg/ml. The presence of zinc and biochemical constituents of *Calotropis gigantea* latex is leading factors of enhanced anti-biofilm potential of CG@ZIF-L nanoframeworks.

### 3.8 Mosquito Larvicidal Efficacy of CG@ZIF-L Nanoframeworks

Results of mosquito larvicidal activity of CG@ZIF-L and ZIF-L alone showed maximum mortality in CG@ZIF-L at the dose of 60 µg/ml, while ZIF-L showed no mortality effect even at the highest dose of 125 µg/ml. The LC<sub>50</sub> and LC<sub>90</sub> values of CG@ZIF-L against *Aedes aegypti* larvae were observed to be  $39.43 \pm 1.16$  and  $72.53 \pm 1.84$  µg/ml respectively. Results reveal that CG@ZIF-L showed significantly effective larvicidal activity at low concentration when compared to CA@ZIF-L reported in our previous studies [18] The mosquitocidal potential of synthesized CG@ZIF-L nanoframeworks increased in higher concentration with reliable lethality and morphological changes in treated larvae groups. Most of the pesticides working mechanism are based on ROS mediated apoptosis leading to insects death [20, 24, 44]. Here, the presence of zinc ions in ZIF-L nanoframeworks, Calotropin and Calotoxin present in the CG latex might have induced ROS generation leading to apoptosis mediated cell death in mosquito larvae [45, 46]. The nanoscale CG@ZIF-L frameworks easily penetrate into the epithelial cells of treated larvae, which reduce the growth,

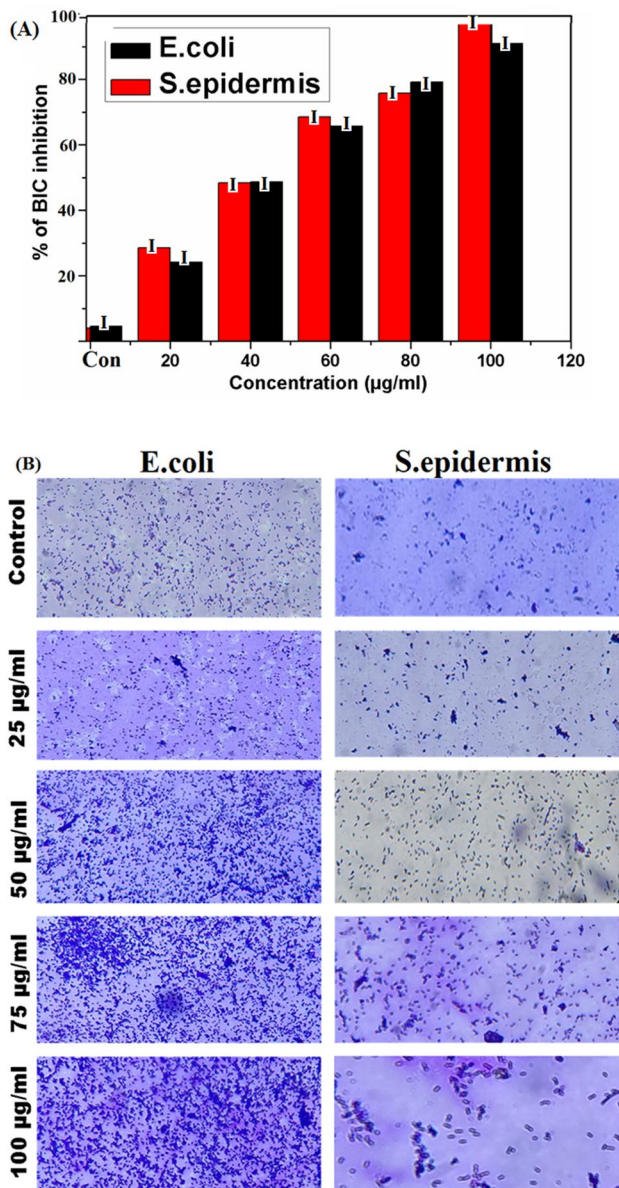
**Fig. 6** **A** Particle size analysis of CG@ZIF-L by DLS analysis; **B** Thermogravimetric analysis of ZIF-L and CG@ZIF-L



leading to malformation based death. Figure 8 exhibit significant morphological changes and growth control in treated larvae compared with control group. The organelle damage and growth retardation such as head, thorax was observed in treated larvae in accordance to the reports of Prabhu et al., [18]. Microscopic observation of treated larvae revealed loss of hair, epithelial cells and thorax damage.

### 3.9 Larvicidal Effects of CG@ZIF-L Nanoframeworks Against Midge Fly Larvae

Biting midge flies is a type of biting pests, their blood sucking habits raises great concern about transmission of disease agents in humans and animals [47, 48]. Biting midges are annoying species, but no report in transmission of human disease. They have a greater impact



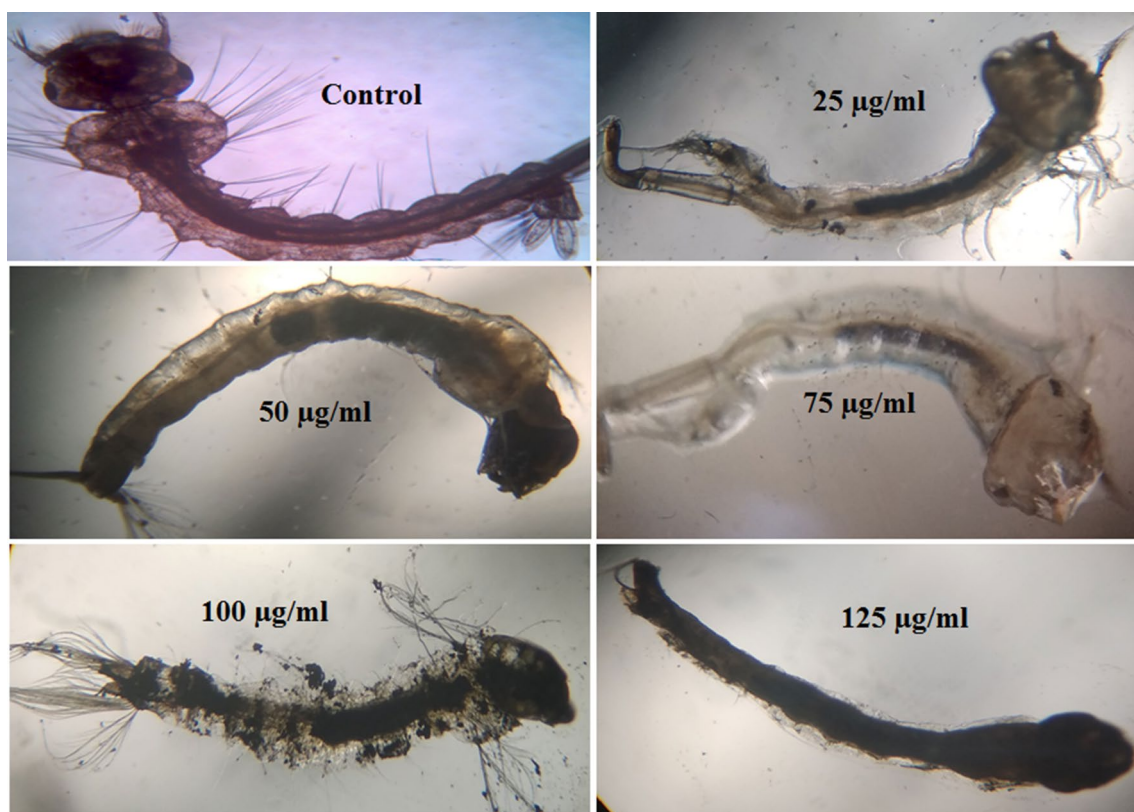
**Fig. 7** **A** Quantification of biofilm inhibition of CG@ZIF-L nanoframeworks by CV binding assay; **B** Light microscopic images of bacterial biofilms after treatment of CG@ZIF-L nanoframeworks

on animal disease transmission through biting [49, 50]. In India, especially Tamil Nadu biting and non-biting midges multiply in the rainy season (July–December). They inflict a burning sensation in humans and small reddish welt at the bite site [51]. Furthermore the over population of non-biting midge larvae may cause water

contamination and air filtration blockage. The midge fly is one of the non-biting insect in genus Culicoides, larvae feed the active plankton in water, which leads to oxygen demand and provide opportunity for the growth of pathogenic bacteria [29, 52]. Some species of non-biting midges are highly vulnerable in the initial stages of crop growth [53–55]. Globally sorghum midges create major shoot damage affecting 85% of crop yield in *Sorghum bicolor* L. plants [56–59]. There is no more data and research available about biting midges in Tamil Nadu, India. This is first report regarding the control of non-biting midge flies (blood worm) larvae by using nanomaterial. The larvicidal bioassay results revealed that *Calotropis gigantea* latex encapsulated ZIF-L nanoframeworks effectively control the growth of midge fly larvae. The quantitative results concluded that the maximum inhibition appeared at  $80.25 \pm 1.05$  µg/ml concentrations and  $LC_{50}$  value against midge fly larvae was observed to be at  $39.68 \pm 1.23$  µg/ml. ZIF-L alone showed negligible activity against midge fly larvae at the maximum concentration of 125 µg/ml. Microscopic observation of treated larvae groups (Fig. 9) showed morphological changes and hemoglobin degradation increased with increased dose of CG@ZIF-L nanoframeworks.

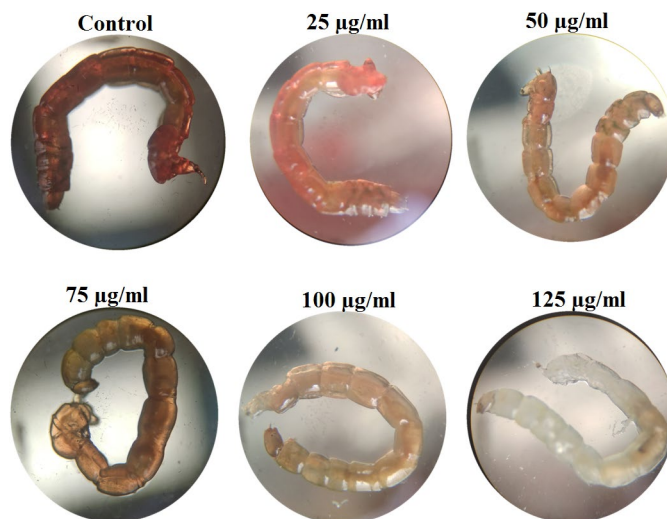
## 4 Conclusion

The present study successfully fabricated *Calotropis gigantea* latex encapsulated ZIF-L nanoframeworks by one-pot synthesis method. The synthesized CG@ZIF-L nanoframeworks showed excellent potent antibiofilm activity against *Escherichia coli* and *Staphylococcus epidermis* bacterial strains. Results indicates the growth inhibition of microcolonies and architectural deformation. Furthermore, CG@ZIF-L nanoframeworks exhibit reliable toxicity against *Aedes aegypti* and midge fly larvae in dose dependent manner. The larvicidal results concluded that the CG@ZIF-L nanoframeworks damaged the epithelial cells of mosquito and midge fly larvae. In midge fly larvicidal bioassay, CG@ZIF-L nanoframeworks induced the hemoglobin degradation and organelle malformation which leads to death of larvae. These findings are very new and provided initial evidence that CG@ZIF-L nanoframeworks might act as promising material for the designing of antibiotics and pesticides.



**Fig. 8** Light microscopic images of *Aedes aegypti* mosquito larvae after treatment of CG@ZIF-L at different doses (magnification  $\times 10$ );

**Fig. 9** Light microscopic images of midge fly larvae after treatment of CG@ZIF-L nanoframeworks at different concentrations (magnification  $\times 4$ )



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## Declarations

**Conflict of interest** On behalf of the authors declared that there is no conflict of interest.

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