Biocatalysis and Agricultural Biotechnology 18 (2019) 101079

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

Agrochemical loaded biocompatible chitosan nanoparticles for insect pest management

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ARTICLE INFO

Keywords:
Chitosan nanoparticles
Drosophila melanogaster
Insect pest management
Permethrin
Spinosad
Toxicological evaluation

ABSTRACT

Pesticides are being used extensively all over the world to increase agricultural output. However, their use may have radical effects on humans and the environment. Thus, there is a dire need to develop new technologies so as to restrict the use of pesticides/agrochemicals to lower concentrations. In this regard, nanotechnology may provide promising solutions to the problems in the field of agriculture and food science. In the present study, we have synthesized chitosan and agrochemical loaded (spinosad and permethrin) chitosan nanoparticles and characterized them using XRD, FT-IR, UV–Vis, DLS/Zeta potential, FE-SEM, and HR-TEM. Chitosan is a skilled and resourceful polymer that has been used in the field of agriculture as nanoparticles. Chitosan nanoparticles are biodegradable in nature, non-toxic carriers for nucleotides and drugs, biocompatibility and have excellent adsorption abilities. After synthesis, the toxicity of chitosan and agrochemical loaded chitosan nanoparticles was checked on *Drosophila melanogaster* (model organism) at several concentrations (10, 50, 100 μg/mL) of nanoparticles. Assays such as survivability assay, climbing assay, and the larval crawling assay were performed to monitor and observe the toxicity of chitosan and agrochemical loaded chitosan nanoparticles and the results were compared with free agrochemicals. Results showed the agrochemical loaded chitosan nanoformulations to be more effective with a lasting residual effect as compared to the free agrochemicals. Thus, in conclusion, these nanoformulations may be used for insect pest management.

1. Introduction

Considering the current rise in global population, a large proportion of developing nations are facing food shortage on daily basis and thus it has become a big question on the food security of the nation. Agriculture is the backbone of many developing nations because it provides both food and gross domestic product. However, it is constrained by numerous biotic and abiotic factors (Mukhopadhyay, 2014; Scott and Chen, 2013). With overly increasing population, challenges like nutrient deficiencies, water scarcity, soil erosion, pests, and related diseases etc also tend to increase. So in order to overcome pest related challenges, there has been an inappropriate and excessive use of pesticides and fertilizers, which has resulted in creating the cataclysm for human and other life forms (eutrophication of aquatic habitats) (Chen and Yada, 2011; Rai and Ingle, 2012; Khot et al., 2012).

A pesticide is a broad name, comprising of compounds like insecticides, fungicides, herbicides, plant growth regulators etc. Precisely, a pesticide should be lethal to targeted pests and not to non-targeted pests but this proclamation has surfaced the controversy of use and abuse of the pesticides. A pesticide, which is a quick, easy and inexpensive solution towards pest control, have tremendous benefits in agriculture and also in other fields like forestry, public health etc. Apart from enormous benefits, pesticides also have hazardous nature and their use has posed risks to the environment and non-targeted species (Chhipa, 2017).

The need of the hour is to adopt smart ways to improve soil quality and pest control with the introduction of new technologies. With the recent advances in the field of nanotechnology, the domain has grabbed the attention and imagination of scientists and researchers in recent years. Nanotechnology, a fast, novel, and emerging field has fresh applications in the agricultural sector primarily in delivering the nutrients, pesticides, herbicides etc. as an active ingredient (Scott, 2007; Bharani et al., 2014). Previous studies demonstrated that PEG coated nanoparticles loaded with garlic essential oil have insecticidal activity against adult *Tribolium castaneum* (Herbst). Similarly, Badawy et al. reported that control release formulations of malathion and spinosad

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https://doi.org/10.1016/j.bcab.2019.101079
Received 16 January 2019; Received in revised form 25 February 2019; Accepted 4 March 2019
Available online 08 March 2019
1878-8181/

from chitosan/alginate/gelatin capsules to be more effective against *C. pipiens* larvae for a long period.

Polymeric nanoparticles have attracted a lot of attention among researchers and scientists as they are non-toxic, cost-effective, eco-friendly, effective at lower doses, and more importantly, can be used for controlled release formulations. Thus, protecting premature degradation of the active ingredients. Chitosan (from chitin: major constituent in the exoskeleton of arthropods and cell walls of fungi) is a partially deacetylated polymer of N-acetyl glucosamine that can be obtained through alkaline deacetylation of chitin. The amine and hydroxyl groups endow chitosan with many special properties, making it applicable in many areas and easily available for chemical reactions. This makes it safe, non-toxic and it can interact with polyanions to form complexes and gels. The positive amine group enhances the stability of nanoparticles with anionic material such as gene, drug, protein and small molecule via electrostatic interaction. Considering the above properties, chitosan has been used in the field of agriculture extensively (Bharani et al., 2014).

The spinosad and permethrin are active pest control agents against the Diptera family that includes flies, so the toxicological evaluations of these nanoformulations have been observed on the fruit fly (*Drosophila melanogaster*) (El-Say and El-Sawy, 2017). Spinosad (composed of two major active ingredients, namely, spinosyn A and spinosyn D) comes from a family called spinosyns, being produced by the actinomycetes bacterium *Saccharopolyspora spinosa* (Sparks et al, 1995, 2012). It has been used over a large scale for controlling pests against cotton and other pests effectively, but its extensive application have been shown to result in the selection of resistant strains of *Liriomyza trifolii* (Ferguson, 2004), *Plutella xylostella* (Zhao et al, 2002, 2006), *Musca domestica* (Shono and Scott, 2003; Khan et al., 2014), *Heliothis virescens* (Young et al., 2003), *Frankliniella occidentalis* (Loughner et al., 2005) and *Spodoptera litura* (Rehan and Freed, 2014). Permethrin is an insecticide of the pyrethroid chemical family, which is a type I pyrethroid and is an amalgam of two stereoisomers. Permethrin shows its effect on the nervous system of insects. It interrupts sodium channels and disrupts the function of neurons resulting in muscles spasm, paralysis and death (Arayne et al., 2011).

This study deals with the synthesis of agrochemical loaded chitosan nanoparticles, characterization of agrochemical loaded chitosan nanoparticles using FE-SEM, HR-TEM, DLS, Zeta potential, XRD, FT-IR, loading efficiency, release rate and checking their efficacy and biocompatibility against larva of fruit fly from an insect management point of view.

2. Materials and methods

2.1. Materials

All the chemicals, chitosan (degree of deacetylation ≥ 75%), sodium tripolyphosphate anhydrous (extra pure 94%), glacial acetic acid (99.5% pure), methanol (99.8% pure), propionic acid (99.0% pure), diethyl ether (> =99.0% pure), sucrose (99.5% pure) were purchased from Himedia, SRL, Fischer scientific, SDFCL, Qualikems, and EMPLVRA^{R}, respectively. The chemicals and reagents were of analytical grade and have been used without further purification.

2.2. Synthesis of chitosan nanoparticles

The chitosan nanoparticles were synthesized by ionic gelation method. The chitosan powder was dissolved in 2% (v/v) acetic acid solution followed by 15–20 min of constant stirring with the help of magnetic stirrer. After stirring, 0.8% (w/v) tripolyphosphate, the crosslinker was added in the chitosan solution (chitosan + acetic acid) and stirring for 5–10 min was done for homogenous mixing. The suspension was later centrifuged at 10,000 RPM (9520 g) for 30 min. The pellet was collected and later lyophilized to obtain chitosan nanoparticles (Vaezifar et al., 2013).

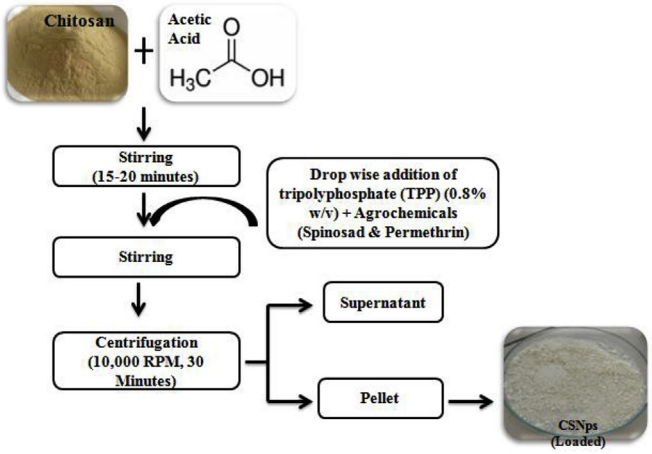

Fig. 1. Synthesis of agrochemical loaded chitosan nanoparticles via ionic gelation method.

2.3. Synthesis of agrochemical loaded (spinosad and permethrin) chitosan nanoparticles

The agrochemical loaded chitosan nanoparticles were synthesized by ionic gelation method shown in Fig. 1. The chitosan was taken and dissolved in the acetic acid (2% v/v) followed by continuous stirring with the help of magnetic stirrer for 15–20 min. The 0.8% (w/v) tripolyphosphate solution containing agrochemicals (spinosad and permethrin) was added to the chitosan solution (chitosan + acetic acid) followed by 5–10 min of stirring. The suspension was centrifuged at 10,000 RPM (9520 g) for 30 min. The pellet was collected and lyophilized to obtain agrochemical loaded (spinosad and permethrin) chitosan nanoparticles (Vaezifar et al., 2013).

2.4. Characterization of chitosan and agrochemical loaded chitosan nanoparticles

The chitosan and agrochemical loaded chitosan nanoparticles were characterized by using UV-VIS absorbance, XRD, FT-IR, FE-SEM, HR-TEM, DLS/Zeta potential. The characterization was done at Sophisticated Analytical Instrumentation Facility (SAIF), Panjab University, Chandigarh and Central Instrumentation Laboratories, NIPER Mohali.

2.4.1. UV–Vis absorbance

UV–Vis analysis was done using the UV–Vis–NIR Spectrophotometer Model Lambda 750 (Perkin Elmer). 5–10 ml of sample is required for sample analysis. The nanoparticles were analyzed by dispersing in water with uniform mixing before analysis (Vaezifar et al., 2013).

2.4.2. X-Ray diffraction

X-Ray Diffractometer (Powder Method) Panalytical's X'Pert Pro was used for XRD analysis of dried powdered samples carried out. 2 g fine powder sample on glass slide of size 3.5 cm × 2.5 cm and thickness 0.2 cm with uniform sample layer on one side was used for sample analysis (Qi et al., 2004).

2.4.3. Fourier transform infrared microscopy (FT-IR)

Fourier transform infrared spectroscopy was conducted using Perkin Elmer- Spectrum RX-IFTIR to obtain an infrared spectrum of transmission of the nanoparticles. The nanoparticles were analyzed in dried powdered form. The amount of sample required was 10 mg (Ray et al., 2010).

2.4.4. Field emission scanning electron microscope (FE-SEM)

The field emission scanning electron microscope images the sample surface by raster scanning over it with a high-energy beam of electrons. Field emission scanning transmission electron microscope with image analyzer was used for analyzing the structure of nanoparticles. The amount of sample required was 1 mg or less. The samples of nanoparticles were analyzed in dried form on copper (Cu) grid coated with carbon to increase the conductivity of the sample (Chodhury et al., 2012).

2.4.5. High resolution transmission electron microscope (HR-TEM)

It is an instrument for high-magnification studies of nanomaterials. High resolution makes it perfect for imaging materials on the atomic scale. The high-resolution transmission electron microscopy uses both the transmitted and the scattered beams to create an interference image. The lyophilized sample was dissolved in the water followed by sonication for 5 min and later placed onto a copper grid and were used for scanning (Chodhury et al., 2012).

2.4.6. DLS and zeta Sizer

Particle size and zeta potential of the powdered nanoparticles were measured by Malvern particle size analyzer (Model: Nano ZS), UK. All the nanoparticles were dispersed in a 1:1 ratio of ethanol: water and were sonicated for 5 min before analysis (Meng et al., 2011).

2.4.7. Loading efficiency

The loading capacity of agrochemical (spinosad and permethrin) loaded chitosan nanoparticles were analyzed by first preparing a chitosan solution containing 250 mg chitosan mixed in 40 ml (2% v/v) glacial acetic acid followed by continuous stirring for 30 min at 600 RPM (34 g). Then, a tripolyphosphate solution of 20 ml (0.8% w/v) was prepared in which the agrochemical (spinosad and permethrin) was added at a concentration of 11 mg and 4 µl in amount. The tripolyphosphate solution was added in a dropwise manner into the solution. After addition of tripolyphosphate, the whole solution was continuously stirred for 1 h. The agrochemical loaded nanoparticles were later centrifuged at 4000 RPM (1523 g) for 30 min. The supernatant was taken out using a pipette and later optical density (OD) was measured from UV- Vis spectrophotometer at 540 nm. The pellet was discarded later (Bharani et al., 2014).

2.4.8. Release rate studies

The agrochemical (spinosad and permethrin) loaded chitosan nanoparticles were suspended in Tris-HCl buffer solution (5 ml, pH-7.4). The solution was stirred continuously for 5 h at 200 RPM (4 g) at 37 °C. At appropriate time intervals, the sample was centrifuged at 10,000 RPM (9520 g) for 20 min at 4 °C. The supernatant was decanted and was replaced with fresh buffer solution. Amount of released agrochemical in the supernatant was measured at 540 nm with the help of UV- Vis spectrophotometer (Dash et al., 2010).

2.5. Fly stock

Wild-type fruit fly was grown on food consisted of maize powder, sugar, yeast, agar, nipagin, and propionic acid. The flies were transferred to new bottles periodically and the stocks were maintained at 25 °C.

2.6. Survivability assay of Drosophila melanogaster

Chitosan and agrochemical loaded chitosan nanoparticles of different concentrations (10, 50, 100 µg/mL) along with the control were added onto food vials and left undisturbed for 24 h. Further, 15–20 flies were anesthetized in a bottle with diethyl ether and later transferred to food vials consisting of nanoparticles of each concentration. The observations were made on daily basis (till 14 days). The number of eggs was counted along with the number of hatched eggs and observations were made accordingly (Panacek et al., 2011).

2.7. Climbing assay of Drosophila melanogaster

Chitosan and agrochemical loaded chitosan nanoparticles of different concentrations (10, 50, 100 µg/mL) along with the control were added onto food vials and was left undisturbed for 24 h. Next day, twenty flies from these vials/bottles were anesthetized with diethyl ether and then transferred to a 100 ml measuring cylinder with a threshold mark of 10 cm. The mouth of the measuring cylinder was closed with the cotton plug along with tapping. The upward movement of flies was recorded for 1 min. The percentage of flies above the threshold line for 72 h was recorded and observations were made (Nichols et al., 2012).

2.8. Larva crawling assay of Drosophila melanogaster

The larval crawling ability has been widely used as a reliable parameter for analyzing any early stage changes in the crawling abilities of *Drosophila* larvae and also for determining the effect of drugs or particulate matter like nanoparticles on their locomotion. The larval crawling behavior was checked in triplicates by observing their locomotive movements on 2% agar plates featuring trail marks agar plates. Third instar larvae from each nanoparticles concentration were collected and later placed at the center of the plates. Graph papers as a representative scale were used to calculate distance and to analyze the speed in cm/min units. The pattern of crawling was observed following the marking of the trailing pattern (Sabat et al., 2016).

2.9. Statistical analysis

All the experiments were performed in triplicates and were repeated thrice. Mean values and standard deviations were calculated for all observations. Statistical analysis was performed in Microsoft Excel using one way ANOVA at significance value of 0.05 and 0.01 with the control values to determine the significant values. The values with $p < 0.05$ and $p < 0.01$ were considered to be statistically significant.

3. Results and discussion

3.1. Characterization of chitosan and loaded (spinosad and permethrin) chitosan nanoparticles

3.1.1. Fourier transform infrared spectroscopy (FT-IR) analysis

The FT-IR spectrum of chitosan nanoparticles and loaded chitosan nanoparticles (Table 1) is shown in Fig. 2(a and b, c). The broad band at around 3264.33 cm^{-1} is attributed to aliphatic primary amine (−NH) -

Table 1
Characteristic absorption and functional group of chitosan and agrochemical (spinosad and permethrin) loaded chitosan nanoparticles.

Characteristic Absorption (cm^{-1})	Functional Group
UC	
3264.33	Aliphatic primary amine (−NH) Alcohol (−OH) stretching vibration
2889.01	Alkyl C−H Stretch
1642.24	Alkenyl C=C
1535.27	Aromatic C=C bending
1320	S=O stretching
LCS	
1422.81	−OH carboxylic acid
1375.33	−OH phenol bending
LCP	
709.5	−CH aromatic bending

(UC-Chitosan nanoparticles; LCS-Spinosad loaded; LCP Permethrin loaded chitosan nanoparticles).

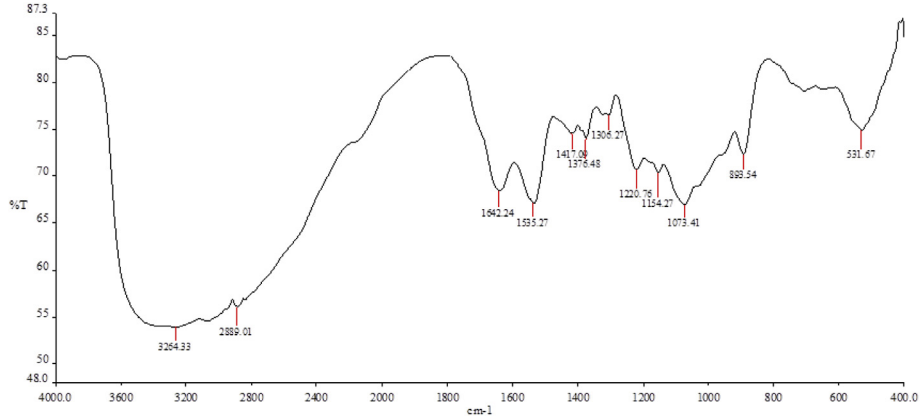

Fig. 2. a FT-IR Spectrum of chitosan nanoparticles.
b FT-IR Spectrum of spinosad loaded chitosan nanoparticles.
c FT-IR Spectrum of permethrin loaded chitosan nanoparticles.

has two stretching vibrations, that is, symmetrical and asymmetrical and alcohol (−OH) stretching vibration, relating to extra-molecular hydrogen bonding of molecules. It has been observed that in most of the spectra that the vibrations arose from −NH stretching vibrations and −OH stretching vibrations got merged and showed a single and broad curve in this region. The characteristic absorption bands emerged at 1642.24 cm^{-1} (alkenyl C=C), 1535.27 cm^{-1} (aromatic C=C bending) and 1320 cm^{-1} (S=O stretching) which is similar to other studies (Pawlak and Mucha, 2003; Agarwal et al., 2018). The loaded chitosan nanoparticles and the chitosan nanoparticles did not show any cardinal differences in FT-IR spectra. The only difference observed compared to chitosan and agrochemical loaded nanoparticles was the emergence of the peak in spinosad loaded chitosan nanoparticles at 1422.81 cm^{-1} (−OH carboxylic acid bending) and 1375.33 (−OH phenol bending-unbounded or free hydroxyl group of phenol. Intermolecular hydrogen bonding increases and additional bands start to appear at lower frequencies at the expense of free hydroxyl bond) along with the disappearance of the peak at 1154.27 cm^{-1} (C−O stretching). In the permethrin loaded chitosan loaded nanoparticles, there is an emergence of the peak at 709.5 cm^{-1} (−CH aromatic bending) due to sp^2 C−H bending and the disappearance of peaks at 1417.09 cm^{-1} (−OH alcohol bending) and 1514.27 cm^{-1} (−CO stretching). The C−O bonds depend on the primary, secondary and tertiary character of hydroxylated carbon (C) atom. C−O bonds are polar because of electronegativity difference between C and O and thus have a large dipole moment. Vibrations involve pulling and pushing of large dipole moment and hence large value and intense peaks which is the main distinguishing feature (Dounighi et al., 2012).

3.1.2. X-Ray diffraction (XRD) analysis

The X-Ray diffraction pattern of chitosan and loaded chitosan nanoparticles are shown in Fig. 3. The XRD pattern of chitosan nanoparticles exhibited a characteristic peak of about 20° indicating crystalline nature of chitosan of high degree, that is, very small amorphous polymer. The broad peaks of loaded chitosan nanoparticles suggest that these nanoparticles are of amorphous nature, which means incoherent scatter. The crystalline structure of chitosan nanoparticles has been fully destroyed after cross-linking with tripolyphosphate. So, the XRD patterns of loaded chitosan nanoparticles are of amorphous polymer (Lam et al., 2006).

3.1.3. Field emission scanning electron microscope (FE-SEM)

The morphology and size distribution of prepared nanoparticles were examined under field emission scanning electron microscopy (Figs. 4–6). The shape of the nanoparticles was found to be uniformly spherical. The dimension of the nanoparticles was 50–100 nm.

3.1.4. High resolution transmission electron microscope (HR-TEM)

The HR-TEM images (Figs. 7–9) of chitosan and agrochemical (spinosad and permethrin) loaded chitosan nanoparticles depicted the morphological and surface appearances. The nanoparticles were found to be spherical in shape of size 100 nm.

3.1.5. DLS/zeta potential

The average diameters of chitosan and agrochemical (spinosad and permethrin) loaded nanoparticles were 562.8 nm, 674.6 nm and 815.7 nm (Table 2) whereas the PdI value of chitosan nanoparticles was

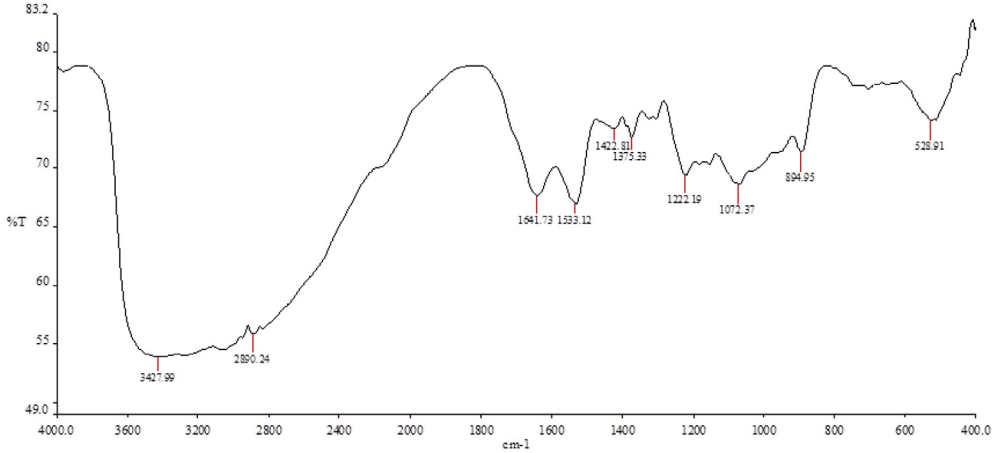

Fig. 2. (*continued*)

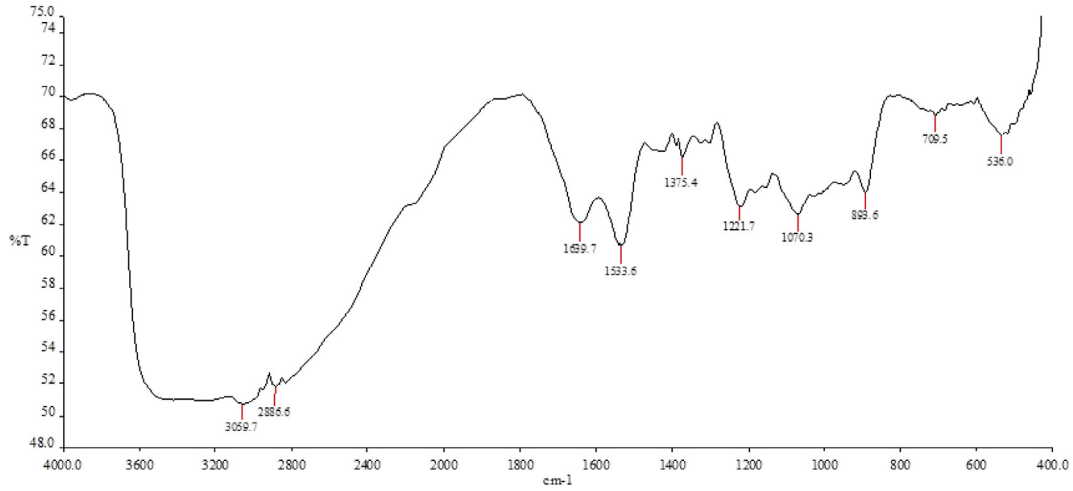

Fig. 2. (*continued*)

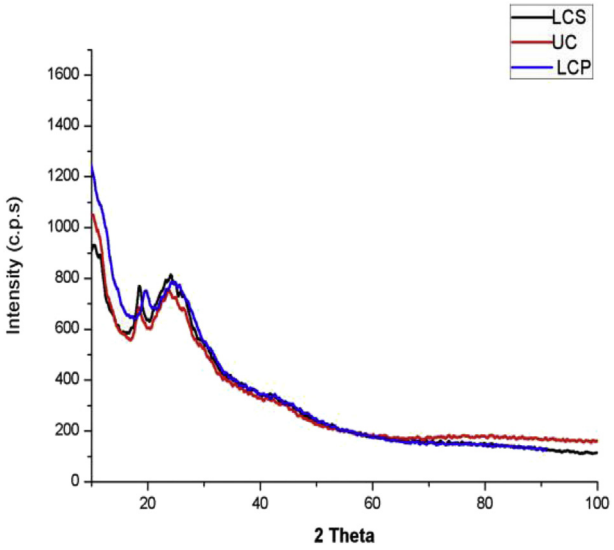

Fig. 3. XRD pattern of chitosan and agrochemical loaded nanoparticles (UC— Chitosan; LCS- Spinosad loaded; LCP- Permethrin loaded chitosan nanoparticles).

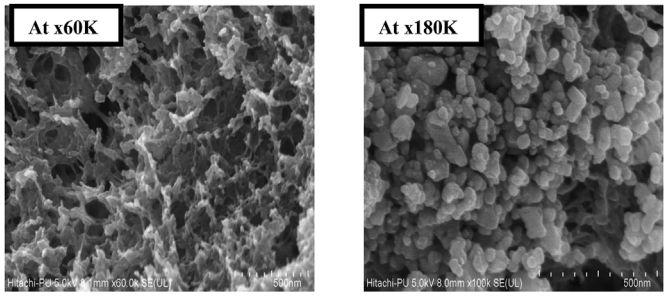

Fig. 4. FE-SEM image of chitosan nanoparticles.

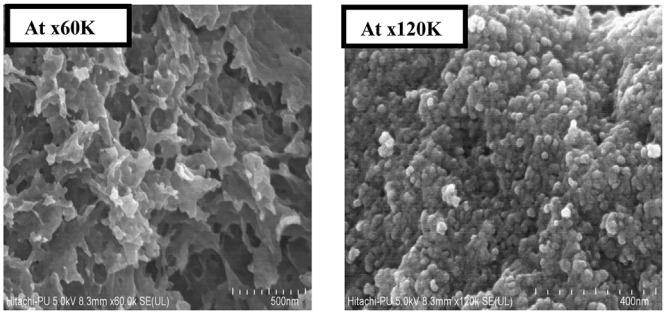

Fig. 5. FE-SEM image of spinosad loaded chitosan nanoparticles.

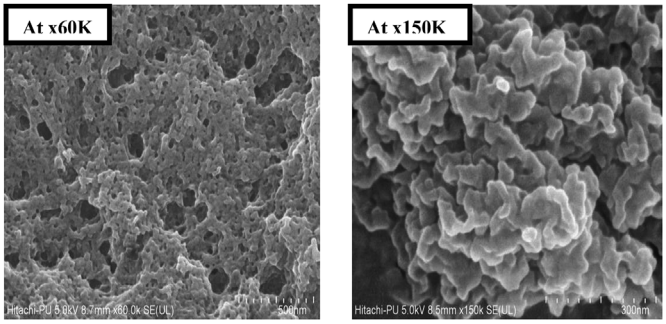

Fig. 6. FE-SEM image of permethrin loaded chitosan nanoparticles.

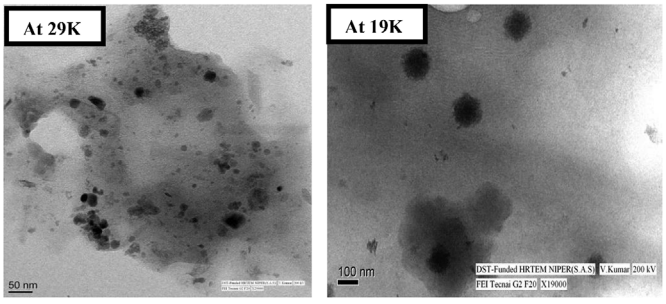

Fig. 7. HR-TEM image of chitosan nanoparticles.

0.276 while that of loaded chitosan nanoparticles were 0.336 and 0.451 (Table 2) depicting a narrow and favourable particle size distribution. The loaded nanoparticles were comparatively bigger than the chitosan nanoparticles due to the high molecular weight of agrochemicals. Similar results have been reported in previous studies (Campos et al., 2018).

The zeta potentials of chitosan and agrochemical (spinosad and permethrin) loaded nanoparticles were +14.4 mV, +13.5 mV and +11.9 mV (Table 2), suggesting that the agrochemical loading had led to minor reduction in particle's zeta potential. The positive zeta potential depicted that the nanoformulations were stable. The positive charge was because of the protonation of the amino groups of the

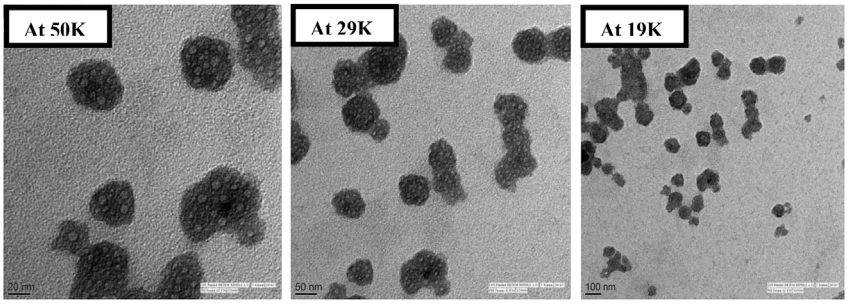

Fig. 8. HR-TEM image of spinosad loaded chitosan nanoparticles.

chitosan nanoparticles and such charge had made the electrostatic interactions with the cross linker (Tripolyphosphate) more favourable. During the synthesis of chitosan nanoparticles, there exist some residual ions from chitosan or tripolyphosphate, respectively. These residual ions, provides charge on the chitosan nanoparticles which is crucial for stability of the nanoparticles (Chuah et al., 2011). Similar studies have been reported using ganciclovir. The studies have suggested that isoelectric point and residual charges present on the surface of the chitosan nanoparticles are major contributors for zeta potential (Patel et al., 2016).

3.1.6. Loading efficiency (%LE)

The loading efficiency of chitosan nanoparticles was determined by the formula:

$$\%\,\text{LE} = \frac{[\text{Total pesticide used to prepare NPs} - \text{Free pesticide in the supernatant}]}{\text{Weight of the nanoparticles}} \times 100$$

The loading efficiency (Table 3) of spinosad and permethrin loaded chitosan nanoparticles are 95.4% and 99.6%, respectively. Some similar studies have been done with chlorogenic acid (CGA) loaded on the chitosan nanoparticles. It was investigated that the molecular weight of the chitosan nanoparticles have regulated the loading efficiency with the involvement of mechanisms like electrostatic interactions during loading (Nallamuthu et al., 2015).

3.1.7. Release rates studies

The release rates studies suggested that within 5 h, 30% (spinosad) and 75% (permethrin) of agrochemical was released from the loaded nanoparticles following the slow release (Fig. 13). The swift burst was observed because of the breaking of molecules which were bound to the surface of chitosan nanoparticles. Similar release rate kinetics has been reported for malathion and spinosad from chitosan/gelatin/alginate capsules in previous studies (Badawy et al., 2016). It has been proposed in the earlier studies that charge attraction between drug and chitosan plays a significant role in sustained release of drug and the slow release contributes to the slow degradation of molecules (Almoustafa et al., 2017).

Table 2
DLS/Zeta Potential of chitosan and agrochemical loaded chitosan nanoparticles.

Nanoparticles	DLS (nm)	Zeta Potential (mV)	PdI
UC	562.8	14.4	0.276
LCS	674.6	13.5	0.336
LCP	815.7	11.9	0.451

(UC-Chitosan nanoparticles; LCS-Spinosad loaded; LCP Permethrin loaded chitosan nanoparticles).

Table 3
Loading efficiency of agrochemical loaded chitosan nanoparticles.

Nanoparticles	Loading Efficiency (%LE)
LCS	95.4%
LCP	99.6%

(LCS-Spinosad loaded; LCP Permethrin loaded chitosan nanoparticles).

3.2. Toxicological evaluation of nanoparticles on Drosophila melanogaster

3.2.1. Survivability assay of Drosophila melanogaster

The effect of chitosan and agrochemical loaded chitosan nanoparticles on the egg laying capability of adult flies was monitored by counting their eggs after feeding different doses of chitosan nanoparticles supplemented with food for 14 days (Table 4). With the increasing number of days as well as an increase in the concentration of the nanoparticles, there was a decrease in the number of eggs hatching. With respect to untreated flies and control, chitosan and agrochemical loaded chitosan nanoparticles showed statistically significant less number of eggs hatching ($p < 0.05$).

Survivability assay is one such test to investigate toxicity studies of chemicals and in this study; it has been used to screen the toxicity of chitosan nanoparticles (Sabat et al., 2016; Jovanović et al., 2016; Mihajilov et al., 2014). Despite being biodegradable in nature, chitosan nanoparticles showed toxicity towards fruit fly as compared to untreated flies. The toxicity of chitosan was dependent on the applied

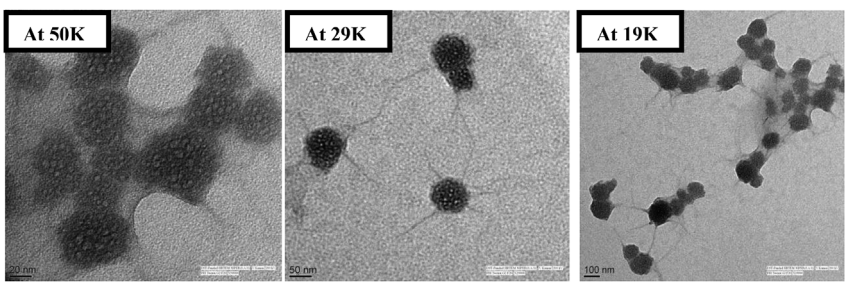

Fig. 9. HR-TEM image of permethrin loaded chitosan nanoparticles.

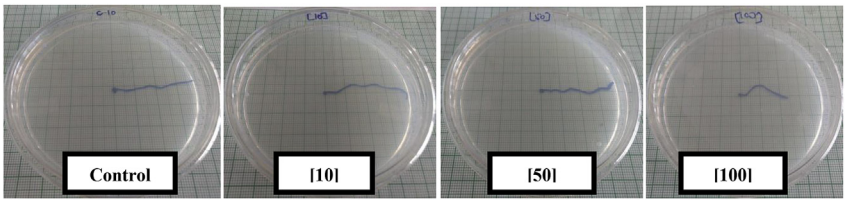

Fig. 10. Larval crawling assay of chitosan nanoparticles.

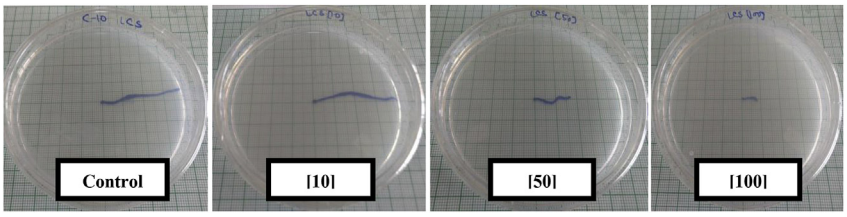

Fig. 11. Larval crawling assay of spinosad loaded chitosan nanoparticles.

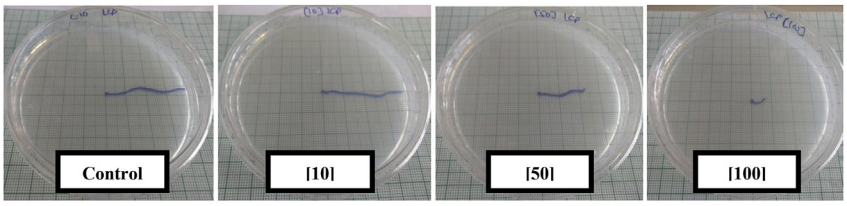

Fig. 12. Larval crawling assay of permethrin loaded chitosan nanoparticles.

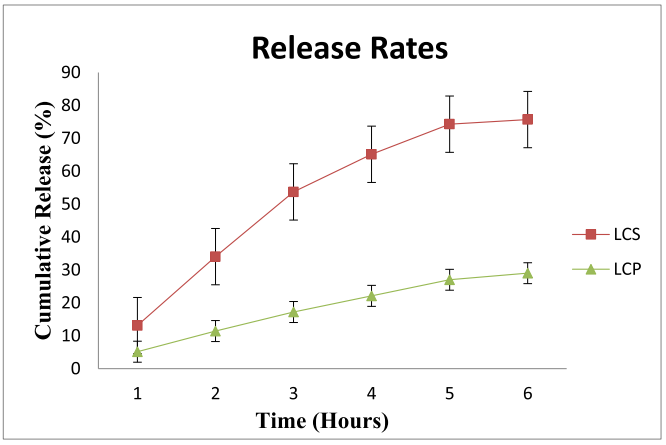

Fig. 13. Release rates of agrochemical (spinosad and permehrin) loaded chitosan nanoparticles (LCS- Spinosad loaded; LCP- Permethrin loaded chitosan nanoparticles).

concentration. So, with the increase in the concentration, there was an increase in toxicity and thus the reduction in the hatching of eggs at the end of day tenth. It has been previously reported that chitosan nanoparticles cause toxicity in a concentration dependent manner in insects of soyabean and animal models such as zebra fish model (Kitherian, 2017; Hu et al., 2011; Yang et al., 2016).

Considering the agrochemical loaded chitosan nanoparticles, the amount of agrochemical present in nanoformulations with respect to control are given in Table 7. From the table, it can be observed that 4 µg agrochemicals were used in a free form for positive control exposure. On the other hand very little amount of encapsulated agrochemicals are present (0.037 µg, 0.18 µg, 0.37 µg) in 10 µg/mL, 50 and 100 µg/mL of loaded chitosan nanoparticles, which thus depicts that with very less amount of agrochemicals; more effect on the egg laying capacity of the flies was observed and thus nanopesticide formulations were found to be more effective at lower doses with increased bioavailability. This is in accordance with previous studies, which states that these nanoencapsulated agrochemicals are able to reduce the dosage of these agrochemicals and even decrease the human exposure (Prasad et al., 2017). On the similar lines, Ziaee et al., reported that *Carum Copticum* oil loaded nanogels of chitosan/myristic acid were found to be many fold more toxic as compared to unencapsulated oil (Ziaee et al., 2014).

3.2.2. Climbing assay of Drosophila melanogaster

The only behavioral assay, which is performed for fruit fly, is the climbing assay. The climbing assay, depicts a strong and reproducible behavior, and is a useful in recognizing any defect in the locomotion of flies that can be represented by the height to which the flies are required to climb (Jovanović et al., 2016). Wild-type flies were used and were monitored for 72 h. While comparing with the untreated flies and control (Table 5), the chitosan nanoparticles and agrochemical (spinosad and permethrin) loaded chitosan nanoparticles had a significant effect on the climbing of flies ($p < 0.05$).

The toxicity of chitosan is dependent on the applied concentration, thus with the increase in the concentration; more toxicity and more decrease in the climbing of the flies was observed. At 10 µg/mL, the climbing of flies started to decrease slowly within 72 h whereas at 50 µg/mL, there had been a gradual decrease in climbing of flies and at 100 µg/mL, the climbing of flies decreased sharply. Even though, the agrochemicals were present in very less amount they significantly decreased the climbing ability of the flies (Table 5). Thereby, implying that the nanoformulations are effective at lower doses in a slow and targeted manner. In previous studies also, nanoparticles were found to interfere with the climbing assay as they caused a decrease in rate of climbing with increased concentrations of nanomaterials (Pappus and Mishra, 2018, Catherine et al., 2011).

3.2.3. Larval crawling assay of Drosophila melanogaster

A larval crawling assay is well-renowned assay to identify the neural damage at an early stage of development (Affleck et al., 2006). At different concentrations of chitosan and agrochemical loaded chitosan nanoparticles (10, 50, 100 µg/mL), numerous trailing patterns were observed (Figs. 10–12). It has been observed that with the increase in the concentration, the path covered by larvae fumbles and hence sluggish walk was observed (Table 6). The overall crawling rate decreased with increase in the concentration of the nanoparticles. The

Table 4
Survivability assay of chitosan and agrochemical loaded chitosan nanoparticles.

	No. of eggs hatched (Mean ± S.D) (* = $p < 0.05$ and ** = $p < 0.01$ compared to untreated control)		
Concentration (μg/mL))	**Day 3**	**Day 6**	**Day 10**
Untreated flies	775.2 ± 17.1	820.1 ± 16.9	890.4 ± 21.1
UC (Chitosan nanoparticles)			
Control- chitosan solution (10 μg/mL)	780.6 ± 16.3	730.3 ± 16.4	473.3 ± 20.5*
10	716.7 ± 24.9	656.7 ± 41.1*	400.5 ± 24.5*
50	580.4 ± 16.3	456.7 ± 65.9*	350.6 ± 29.5*
100	403.3 ± 36.8*	256.7 ± 36.8*	186.7 ± 20.8**
LCS (Spinosad loaded nanoparticles))	**Day 3**	**Day 6**	**Day 10**
Control- free spinosad (400 μg/mL)	730.2 ± 16.3	566.7 ± 28.7*	443.3 ± 33.9*
10	676.7 ± 20.5	520.3 ± 37.4*	330.5 ± 37.4*
50	550.5 ± 40.8	423.3 ± 41.9*	210.2 ± 32.6**
100	416.7 ± 28.7	250.4 ± 40.8*	121.7 ± 20.1**
LCP (Permethrin loaded nanoparticles))	**Day 3**	**Day 6**	**Day 10**
Control- free permethrin (400 μg/mL)	670.5 ± 28.2	606.7 ± 17.8	470.2 ± 25.5*
10	613.3 ± 24.8	500.4 ± 28.2*	376.7 ± 24.8*
50	550.6 ± 28.2	456.7 ± 28.6*	276.7 ± 21.6*
100	373.3 ± 35.6*	283.3 ± 38.9*	110.1 ± 7.1**

(UC-Chitosan nanoparticles; LCS-Spinosad loaded; LCP Permethrin loaded chitosan nanoparticles).
* Signifies significance level of 0.05.
** Signifies significance level of 0.01.

Table 5
Climbing assay of chitosan and agrochemical loaded chitosan nanoparticles.

	%ge of flies above 10 cm mark (Mean ± S.D) (* = $p < 0.05$ and ** = $p < 0.01$ compared to untreated control)		
Concentration (μg/mL))	24 h	48 h	72 h
Untreated flies	90.1 ± 3.7	93.1 ± 3.8	94.8 ± 4.5
UC			
Control- chitosan solution (10 μg/mL)	86.7 ± 3.5	80.7 ± 3.9	74.7 ± 4.9
10	76.7 ± 3.4	70.2 ± 4.1	64.3 ± 4.9
50	79.7 ± 4.5	64.7 ± 4.5	63.5 ± 5.7
100	68.3 ± 5.7	57.3 ± 6.1*	53.3 ± 6.2*
LCS			
Control- free spinosad (400 μg/mL)	85.7 ± 3.6	79.5 ± 1.4	60.7 ± 3.6*
10	81.3 ± 2.8	65.7 ± 3.6*	49.3 ± 4.9*
50	58.7 ± 5.3*	46.7 ± 6.9*	34.7 ± 4.7*
100	48.7 ± 5.3*	28.1 ± 3.7**	19.3 ± 4.5**
LCP			
Control- free permethrin (400 μg/mL)	89.7 ± 4.5	69.3 ± 4.4	44.2 ± 4.9*
10	80.2 ± 4.1	59.3 ± 5.4*	34.3 ± 4.9*
50	54.3 ± 4.8*	44.3 ± 5.4*	27.7 ± 6.2*
100	39.3 ± 4.9*	23.4 ± 6.2**	16.7 ± 6.3**

(UC-Chitosan nanoparticles; LCS-Spinosad loaded; LCP Permethrin loaded chitosan nanoparticles).
* Signifies significance level of 0.05.
** Signifies significance level of 0.01.

sluggish and zigzag walk of larvae indicates the toxic stress on the neural mechanism resulting in improper brain coordination, abnormal muscle contraction and crawling because of the defect in the cholinergic neurons (Roth and Lynch, 2009; Riedl and Louis, 2012). Larval crawling assay have also been used in earlier studies to evaluate the behavioral effect of various nanoparticles such as silver and titania and these nanoparticles have also been found to interfere with the larval crawling thereby proving their toxicities to *Drosophila* model (Debabrat et al., 2016;Raj et al., 2017a, b).

4. Concluding remarks

The chitosan and agrochemical (spinosad and permethrin) loaded chitosan nanoparticles were synthesized using ion gelation method and characterized using FT-IR, FE-SEM, HR-TEM, UV–Vis Spectroscopy, DLS/Zeta potential. The toxicity of nanoparticles was assessed using *Drosophila melanogaster* (fruit fly) from the insect pest management point of view. Assays like survivability assay, climbing assay, and the larval crawling assay were performed for the same at three concentrations, i.e., 10, 50 and 100 μg/mL. The results indicated that the chitosan nanoparticles despite being biodegradable have shown toxicity with respect to untreated flies and the toxicity was dependent on the concentration of the nanoparticles. From the survivability assay, it was countable that with increasing concentration of chitosan nanoparticles the toxicity on the egg-laying capacity of flies increased. Also, with the minute amount of agrochemical in the loaded suspension solutions, more effect were observed thus proving that the nanopesticide formulations were effective at lower doses as compared to free agrochemicals. The free pesticides had a residual effect that was not long lasting on the insect pests while on the contrary; they tend to create environmental havoc. On the other hand, the nanopesticide formulations were effective and had a lasting residual effect which was evident from the survivability assay and climbing assay. Thus, suggesting that the nanopesticide were released in a slow or targeted manner along with protection from premature degradation and were effective at much lower concentration as compared to free agrochemicals. Future studies should be done to check the efficacy of agrochemical loaded nanoformulations by spraying them on different parts of the plants and feeding the plant pest and checking their survival under laboratory conditions.

Table 6
Larval crawling assay of chitosan and agrochemical loaded chitosan nanoparticles.

Concentration (μg/mL)	Number of squares moved by larva (Mean ± S.D) (* = $p < 0.05$ and ** = $p < 0.01$ compared to untreated control)
Untreated flies	44.5 ± 1.8
UC	
Control- chitosan solution (10 μg/mL)	40.3 ± 1.2
10	37.7 ± 1.3
50	33.4 ± 2.5
100	19.7 ± 4.5*
LCS	
Control- free spinosad (400 μg/mL)	40.3 ± 1.4
10	38.7 ± 1.3
50	13.2 ± 2.2*
100	5.3 ± 2.1**
LCP	
Control- free permethrin (400 μg/mL)	38.2 ± 1.7
10	36.3 ± 1.9
50	21.2 ± 1.6*
100	5.7 ± 1.7**

(UC-Chitosan nanoparticles; LCS-Spinosad loaded; LCP Permethrin loaded chitosan nanoparticles).
* Signifies significance level of 0.05.
** Signifies significance level of 0.01.

Table 7
Amount of drug in loaded nanoformulations.

	Amount of agrochemical per treatment (μg)	
Nanoparticles concentration (μg/mL)	LCS	LCP
Control- free agrochemical (400 μg/mL)	4	4
10	0.037	0.037
50	0.18	0.18
100	0.37	0.37

(LCS-Spinosad loaded; LCP Permethrin loaded chitosan nanoparticles).

Data sharing

The nanoparticle characterization data used to support the findings of this study are included within the article. Other data used to support the findings of this study will be available from the corresponding author upon request.

Acknowledgement

This work was partially supported by the Wellcome Trust/DBT India Alliance Fellowship [IA/E/14/1/50177] awarded to Madhu Khatri.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101079.

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