Chemical and Biological Technologies in Agriculture

Assessment of the pesticidal behaviour of diacyl hydrazine-based ready-to-use nanoformulations

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Abstract

Background: Application of nanotechnology for crop protection in the form of nanopesticide has attracted significant interest in modern agriculture for the management of devastating polyphagous pests. In the present work, highly stable, ready-to-use water-based nanoformulations of hydrazine-based pesticides were evaluated for their Insect Growth Regulatory potential against the polyphagous insect pest—*Spodoptera litura*. Also, the nanoformulations were screened for their antifungal behaviour against plant pathogenic fungi; *Colletotrichum gloeosporioides, Rhizoctonia solani*, *Fusarium solani,* and *Alternaria solani*.

Results: Nanoformulation of sulfonyl acyl hydrazine derivative, NF7, emerged as the best insect growth regulator with GI_{90} value 0.010 mg L^{-1} followed by NF4 and NF6 with GI_{90} 0.012 and 0.013 mg L^{-1}, respectively. Results of diet incorporation method showed enhanced efficacy of nanoformulations when compared with topical application method. Antifungal screening showed that many nanoformulations displayed least 50% growth inhibition for treatment dosage 50–200 mg L^{-1} against the fungal pathogens tested (*C. gloeosporioides*, *R. solani*, *F. solani,* and *A. solani*). NF6, NF7, and NF8 were more potent antifungal agents at lower treatment dosages, while at high doses (400 and 800 mg L^{-1}), 100% growth inhibition at concentration was observed against *R. solani* and *F. solani*, except NF1 having 64% growth inhibition against *F. solani*.

Conclusion: Results presented here are very promising and deliver new nanoformulations of diacyl and sulfonyl acyl hydrazine-based derivatives to be employed as nanopesticide for sustainable crop protection.

Keywords: Diacyl hydrazine, Nanoformulation, Antifungal, Insect growth regulatory activity

Background

Over the few past decades, substantial active research has been reconnoitered on the possible application of nanotechnology for combating notorious pests in agriculture, and this included the development of novel and effective plant protection products [1, 2]. In recent years, a wide range of nanotechnological systems have been developed for agricultural applications, utilizing intensive research and development practices at both academic and industrial levels [3–6]. Nanotechnological innovations can help to mitigate the drawbacks associated with the classical pesticide formulation and application technologies, in particular to reduce the dose of pesticide, minimize nutrient loss, identify plant pathogens, detect pesticide residue, and increase crop yields without affecting non-target beneficial organisms and environment. Due to their small size and large surface area to-volume ratio, nanopesticides exhibit unique features compared to their bulk counterparts. Nanoformulation can upsurge solubility and dispersion of pesticide

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molecules in water. Nanodelivery systems can be engineered to overcome the various environmental and biological barriers helping pesticide formulations reaching their intended targets. Site-specific delivery of pesticides can greatly improve their efficiency with simultaneous reduction in effect on non-target organisms and ecosystem [7]. Such engineered nanopesticides have less environmental contamination due to reduced and targeted pesticide application, reduced losses, low susceptibility to photo degradation, and comparatively lower toxicity to their non-nano counterpart [7–9].

Diacyl hydrazine (DAH) class of insecticides (Fig. 1) has emerged as a promising group of chemically safe pest control agents that were discovered at Rohm and Haas Company (Presidential Green Chemistry Challenge 1998) [10]. These derivatives have been subjected to numerous chemical modifications due to their uniqueness and simplicity for optimizing new compounds with high pesticidal activity [11–13]. These active molecules can potentially mimic the action of two principle insect hormones, the steroidal moulting hormones, 20-hydroxyecdysone (20E) and the sesquiterpenoid juvenile hormone (JH), which regulate various stages of growth and development in insects [14]. In other words, they can mimic the action of moulting hormones and induce a precocious and incomplete moult in several insect orders via interaction with the ecdysteroid receptor proteins [15]. Diacyl hydrazine class of insecticide represents class of green compounds owing to their specificity to target pests and having almost no impact on most non-target and beneficial organisms. Also, their discerning insect toxicity makes these insecticides significant tools for insecticide resistance management (IRM) and integrated pest management (IPM) programs [16]. Presently, there are five registered diacyl hydrazine class of insecticides namely Tebufenozide (RH-5992), Methoxyfenozide (RH-2485), Halofenozide (RH-0345), Chromafenozide (ANS-118, CM-001), and Fufenozide (JS-118) (Fig. 1). Methoxyfenozide is most widely registered and used diacyl hydrazine class of insecticide with registrations in more than 50 countries for use on a several crops ranging from vegetables to forestry and tea plantation. It has been registered under the category of 'Reduced Risk Pesticide' by US EPA due to its novel mode of action, green chemistry, and reduced mammalian and ecological toxicity [15, 17].

Several nanopesticides developed from the existing commercial pesticides including Azadirachtin A, Carbofuran, Fenitrothion, Imidacloprid, Fipronil, α-Cypermethrin, Mancozeb, Thiram, Hexaconczole, and Carbendazim to name a few have been reported in the literature for their pesticidal behaviour against several insect pests and plant pathogenic fungus [18–27].

Recent work in the area highlighted the potential of nalidixic acid based diacyl and sulfonyl acyl derivatives as potential pesticide in combating insect pests and plant pathogenic fungus [28]. To the best of our knowledge, the Insect Growth Regulatory (IGR) behaviour of DAH class of nanoformulations has not been evaluated. With this background, we have developed environmentally safe, highly effective compared to their non-nano derivatives, stable, ready-to-use water-based nanoformulations of diacyl and sulphonyl acyl hydrazine derivatives of nalidixic acid [29]. In the present work, we have evaluated their IGR potential on the devastating polyphagous pest *Spodoptera litura* and antifungal efficacy on economically important plant pathogenic fungi *Colletotrichum gloeosporioides*, *Rhizoctonia solani*, *Fusarium solani,* and *Alternaria solani.*

Materials and methods

All the solvents and chemicals were purchased from Sigma-Aldrich, India, and were used without further purification. Methoxyfenozide, PESTANAL®, analytical standard and Hexaconazole, PESTANAL®, analytical standard were purchased from Sigma-Aldrich, New Delhi, India. Cypermethrin 25% EC was locally purchased from Silver Agro Chemicals, Rajkot Gujarat, India. Potato Dextrose Agar (PDA) medium used for antifungal assay was purchased from HiMedia Laboratories, New Delhi, India.

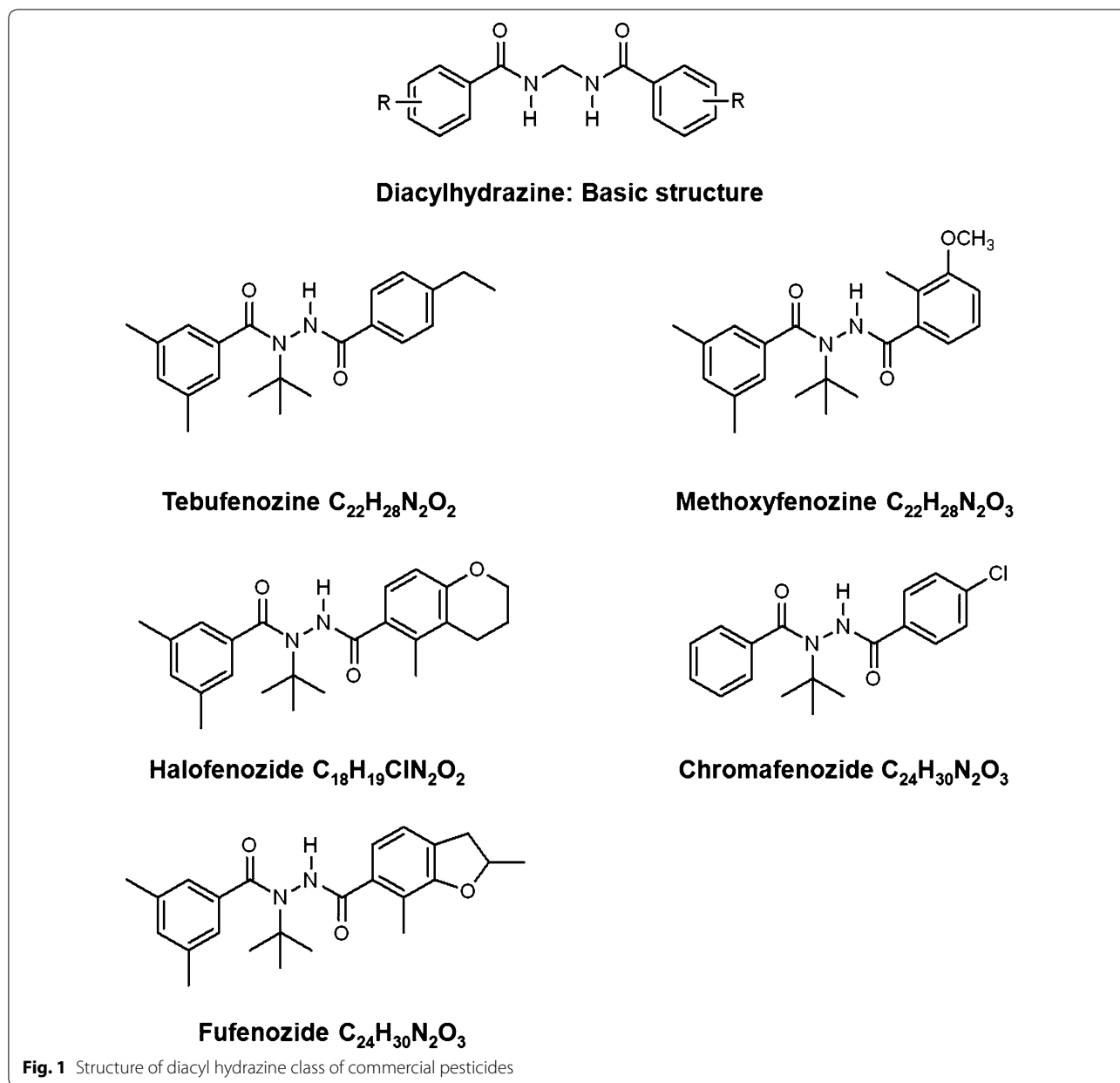### Synthesis of diacyl and sulphonyl acyl hydrazine derivatives

The preferred diacyl and sulfonyl acyl hydrazine derivatives were prepared by following simple synthetic route using Nalidixic acid, a well-known antibiotic, as starting material. The structures of all the synthesized compounds 1–8 were confirmed by their IR, ^{1}H, and ^{13}C NMR spectra [29].

Preparation of nanoformulations

Hydrazine class of pesticides inherently possesses low aqueous solubility. To enhance aqueous solubility and subsequent bioavailability of hydrazine derivatives, stable oil-in-water (O/W) nanoemulsions of diacyl and sulfonyl acyl hydrazine derivative were prepared by high-energy ultrasonic disintegration method [30] by suitably optimizing the ratios of various oils, surfactants, and cosurfactants. Water was used as continuous phase in all preparations as reported previously [29].

Characterization of nanoformulation

Droplet size and dispersity index (DI) of all eight prepared nanoformulations were measured in nanometer by Dynamic Light Scattering (DLS) Technique (Zeta

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Fig. 1 Structure of diacyl hydrazine class of commercial pesticides

Sizer Nano ZS90, Malvern, UK). The average diameter was determined by a program based on Stokes–Einstein equation. Each sample was diluted ten times before measurement. Hydrodynamic radii of lipid nanoemulsions were measured in triplicate as an average of 15–20 runs per measurement at 25 °C. Shape and morphology of all prepared nanoemulsions were envisioned by transmission electron microscopy (TEM) imaging (Tecni G² T20, TWIN, Electron source: Lab₆ filament, 60–200 kV). Also, the stability of nanoemulsions as a function of time was confirmed by measuring change in average droplet size, dispersity index, and size distribution of lipid droplets using DLS technique. Thermal stability of nanoformulations was monitored regularly for any change in their droplet size, dispersity index, average size distribution, pH, and conductivity as a function of time. The stability of active ingredient trapped in lipid droplets was determined by UV–Vis absorption spectrophotometer by following previously reported protocol [29].

Insect growth regulatory (IGR) activity
Rearing of test insects
The larvae of *S. litura* (Lepidoptera: Noctuidae) were reared for successive generations on the leaves of its

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natural host castor, *Ricinus communis*, at 26 ± 2 °C temperature and $70 \pm 5\%$ relative humidity with 18:6 h photophase:scotophase exposure. Utmost care was taken to avoid overcrowding and strict sanitation was maintained to prevent any infection. Larvae reared on the castor leaves were used for insect growth regulatory activities. All nanoformulations along with representative non-nanoderivatives were screened for their IGR activity by both topical application and diet incorporation method [31].

Topical application method

For testing acute toxicity of nanoformulations and representative non-nanoderivatives on *S. litura* larvae, various concentrations, viz. 0.1, 0.05, 0.01, 0.005, and 0.001%, of nanoformulations were prepared by serial dilution technique in water, respectively. Healthy, pre-starved newly moulted pre-weighed (30–40 mg) third instar larvae were treated topically with different concentrations of the nanoformulations. One microlitre of each solution was topically applied to the thoracic region of individual larva by using micropipette so as to receive the active ingredient at 1, 0.5, 0.1, 0.05, and 0.01 µg/larva. Ten replicates per concentration were used. Control larvae were treated with sterile RO water, while Methoxyfenozide was used as a standard. Treated larvae were released into clean and dried plastic containers individually containing fresh castor leaves for feeding. Every day, each container was cleaned to avoid any infection; the left-overleaf discs and excreta of the insects were cleaned and replaced with fresh leaves. After 24 h, larval mortality was observed. Observations of larval weight, every alternate day up to pupation; larval mortality, percentage pupation, pupal weight, deformed pupae, larval–pupal, and pupal–adult intermediates, per cent adult emergence and percent deformed adults were recorded. Percentage weight reduction in larva/pupa was calculated as [32]:

$$\text{Percentage weight reduction in larva/pupa}$$
$$= \frac{\text{Weight gained in Control} - \text{Weight gained in Treated}}{\text{Weight gained in Control}}$$
$$\times 100.$$

Diet incorporation method

The chronic exposure of nanoformulations and representative non-nano derivatives on *S. litura* larvae was determined by diet incorporation method. Test concentrations, viz, 0.1, 0.05, 0.01, 0.005, and 0.001%, were prepared by serial dilution in water. Leaf discs (90 mm) were punched from freshly washed and dried castor leaves dipped thoroughly in each concentration and air dried. The treated leaves were placed individually in petri plates (90 mm). Healthy, pre-starved, newly moulted pre-weighed third instar larvae (30–40 mg) were released into each petri plates. Fifteen replicates per concentration were used. Leaf discs treated with sterile RO water served as control and Methoxyfenozide was again used as a standard. After 24 h of feeding, the larvae were transferred to normal, untreated diet in a separate clean and dry container. Every day, the left-overleaf discs and excreta of the insects were removed, and fresh untreated clean leaves were provided for feeding. Larval mortality was observed after 24 h. Observations of larval weight, alternate days up to pupation, larval mortality, percentage pupation, pupal weight, deformed pupae, larval–pupal and pupal–adult intermediates, percentage adult emergence, and percentage deformed adults were recorded. Percentage weight reduction in larva/pupa was calculated as described previously in topical application method.

In vitro antifungal activity

The cultures of target phytopathogenic fungi, *Colletotrichum gloeosporioides* (MTCC9664), *Rhizoctonia solani* (ITCC 5563), *Fusarium solani* (ITCC6731), and *Alternaria solani* (ITCC 4632), were maintained on Potato Dextrose Agar (PDA) at 27 °C ± 2 °C, and were subcultured in Petri dishes prior to testing. The test fungi was routinely grown on fresh slant tubes of PDA and stored at 4 °C.

Antifungal behaviour of all nanoformulations along with corresponding non-nano derivatives was tested in vitro by poisoned food technique using readymade PDA medium [33]. Appropriate quantities of test formulations were added to the culture medium to get desired concentrations of 800, 400, 200, 100, and 50 mg L^{-1} by serial dilution method. Desired concentrations of non-nano derivatives was prepared in DMSO and then added into the molten medium for obtaining required concentrations as mentioned above for nanoformulations. RO water in case nanoformulations and DMSO in case of non-nano derivatives (1 ml) served as control. The medium poisoned with active ingredients was poured into a set of two petri dishes under aseptic conditions. When the culture medium in the plates solidified, a mycelial disc of 4 mm diameter from freshly grown fungal culture was inoculated at the centre of the each plate. The treated and control petri dishes were kept in BOD at 28 °C ± 2 °C until fungal growth in the control plates was almost complete (4–6 days). Hexaconazole served as standard. All the test formulations were tested in duplicates. The mycelia growth of fungi in both control (C) and treated (T) petri plates was measured diametrically in three different directions (mm) and percentage growth inhibition (*I*%) and corrected inhibition (IC) were calculated using the formula [32]:

$$I(\%) = (C - T)/C \times 100.$$

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The corrected percentage inhibition (IC) was calculated as follows:

$$IC = [(I(\%) - CF)/(100 - CF)] \times 100.$$

CF is the correction factor, derived from the equation;

$$CF = ((90 - Co)/Co) \times 100,$$

where 90 represents diameter (mm) of the Petri dish and Co is the growth of fungus in control plates (mm).

Statistical analysis

All statistical tests, unless specified, were performed with SPSS Statistics V 22.0 (IBM, New York, USA). Prior to any statistical analyses, all data sets were tested for normality using Shapiro–Wilk's test and homogeneity of variance by Levene's test. Transformations were performed to meet the homogeneity of variance requirement (arcsine square root for percentage weight reduction of larvae, pupae, pupae–larval intermediate, and pupal–adult intermediate and abnormal adults). All recorded data after transformation were subjected to analysis of variance (ANOVA), wherever necessary in a complete randomized design (CRD). In the current study, we have presented data in the form of mean ± standard deviation (SD). Data obtained on the parameters examined that had homoscedastic variances were compared using one-way ANOVA with Tukey's HSD (Honestly Significant Difference), while data showing heteroscedasticity were analyzed by Games Howell's test at $p \leq 0.05$.

IGR activity was studied by performing one-way ANOVA to determine the effect of different formulations and their concentration on Percentage weight reduction of (i) larval and (ii) pupal for both topical application and diet incorporation methodologies. Further one-way ANOVA was also performed to study the impact of different formulations and their concentration on (i) percentage larval mortality, (ii) percentage pupal mortality, (iii) percentage larval–pupal intermediate formed, (iv) percentage pupal–adult intermediate formed, (v) percentage deformed adults formed, and (vi) percentage normal adults formed. To determine the inhibition of growth by 90%, GI_{90} was calculated using probit analysis [34].

Antifungal (percentage inhibition of fungal growth) activity of the prepared nanoformulations was also subjected to one-way ANOVA to study the impact of different formulations and their concentration.

Results and discussion

Synthesis of active ingredients

Six nalidixic acid diacyl hydrazine derivatives and two nalidixic acid sulfonyl acyl hydrazine derivatives were synthesised by method previously reported (Table 1). The

structure–activity relationships of substituted acylhydrazine molecules were well established [28]. Various factors including effect of nature of halogen atoms and its position on benzene ring, number of carbon atoms, and substitution of diacyl and sulphonyl acyl groups and their position significantly affect their bioefficacy potential which was discussed in the later sections.

Nanoformulation development and characterization

Six nanoformulations of substituted diacyl hydrazine derivatives, NF1, NF2, NF3, NF4, NF5, and NF6 and two nanoformulations of substituted sulfonyl acyl hydrazine derivatives, NF7 and NF8 of nalidixic acid were prepared by high-energy ultrasonic disintegration method. The prepared nanoformulations were observed to be stable at different storage conditions and were found stable for more than 12 months. The average droplet size of all 8 nanoformulations was found to be in the range of 42–138 nm with low dispersity index values indicating monodisperse behaviour of the droplets present in the system. TEM was used to confirm morphology of nanoemulsions and the droplets were found randomly dispersed thorough out the field. It has been noted that these nanoformulations also displayed enhanced stability when exposed to UV light (50 Hz) for 48 h as reported in the recently published work [29]. All prepared nanoformulations were kinetically stable systems with no creaming, phase separation, or amalgamation when kept at different storage temperatures of 4° and 25 °C for more than 12 months. Also, over a period of time, no significant change in pH and conductivity of nanoformulation was observed. The stability of active ingredient and entrapment efficiency of nanoemulsions was determined by UV–Vis absorption spectrophotometer. Absorption behaviour revealed that all eight entrapped active ingredients were stable for more than 12 months. All formulations possessed more than 80% entrapment efficiency, and nanoformulations NF2 and NF7 showed maximum entrapment efficiency of 91% and 88%, respectively [29]. The results further support higher stability of hydrazine class of pesticides. Moreover, these results confirms that the high energy generated by ultrasonicator probe do not have decorating effects on chemical entity of the molecules [35].

Insect growth regulatory (IGR) activity

The percentage growth inhibition by nanoformulation treatments observed in this study is greater than 50% inhibition, and hence, the inhibitory effect is expressed as 90% growth inhibition (GI_{90}). The results obtained by both topical application and diet incorporation method showed that all 8 nanoformulations displayed higher IGR activity compared to their representative

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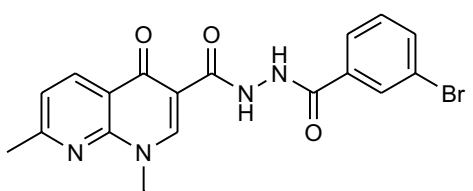**Table 1 Details of hydrazine derivatives used for preparing active nanoformulations**
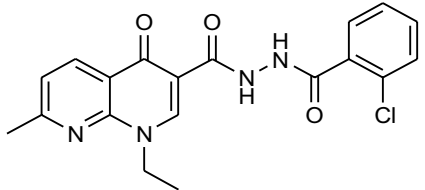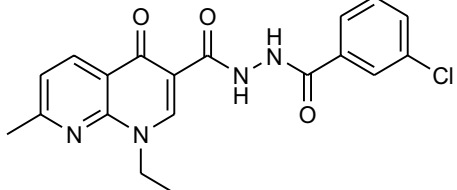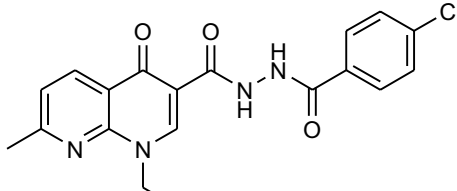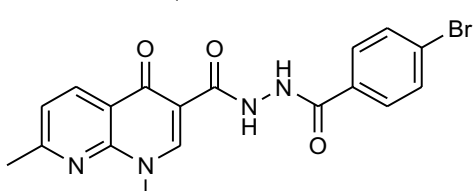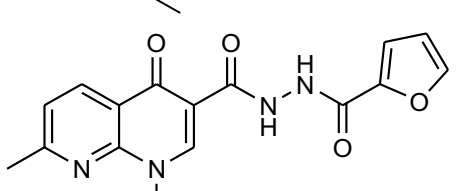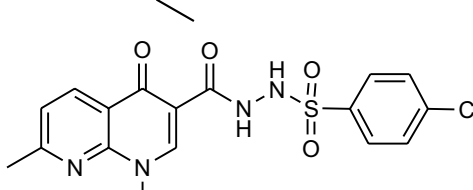
S. no.	IUPAC name	Structure
NF1	N′-(2-Chlorobenzoyl)-1-ethyl-7-methyl-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carbo hydrazide	
NF2	N′-(3-Chlorobenzoyl)-1-ethyl-7-methyl-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carbo hydrazide	
NF3	N′-(4-Chlorobenzoyl)-1-ethyl-7-methyl-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carbo hydrazide	
NF4	N′-(3-Bromobenzoyl)-1-ethyl-7-methyl-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carbo hydrazide	
NF5	N′-(4-Bromobenzoyl)-1-ethyl-7-methyl-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carbo hydrazide	
NF6	1-Ethyl-N′-(furan-2-carbonyl)-7-methyl-oxo-1,4-dihydro-1,8-naphthyridine-3-carbo hydrazide	
NF7	4-Chloro-N′-(1-ethyl-7-methyl-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carbonyl)-benzene sulphonohydrazide	

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Table 1 (continued)

S. no.	IUPAC name	Structure
NF8	*N'*-(1-Ethyl-7-methyl-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carbonyl)-4-nitrobenzene sulphonohydrazide	

NF nanoformulations

non-nanocounter parts. Different growth parameters in the life cycle of *S. litura* were used to assess the IGR activity of the nanoformulations including larval mortality, pupal mortality, larval–pupal intermediates formed, pupal–adult intermediates formed, abnormal pupae, deformed or abnormal adults and normal adults. Apart from these parameters, in both the methods percentage larval and pupal weight reduction in comparison with control were also determined. Furthermore, dead larvae, pupae, and other abnormal characters formed during the process were also recognized and compared with the normal ones. The treated larvae were considered dead when they were unable to respond to external stimulus of gentle pressure with a brush. Some of the larvae were unable to complete moulting resulting in the formation of larval-pupal intermediates, while others either died during the processor were unable to grow and remained smaller. In pupal stage, it was observed that at different test concentrations, pupae exhibit diverse degree of

chitinization, and most of the observed dead pupae were smaller in size and darker in color. Abnormal pupae were unable to complete moulting process resulting in the formation of pupal–adult intermediates or subsequent deformed adults. Abnormal adults formed had crippled wings and were unable to fly. In some of them, pupal exuviae was not removed completely; such deformed adults have short life span and die after some time.

IGR activity by topical application method

The preliminary screening of all nanoformulations with representative non-nanoderivatives was carried out by topical application method. The percentage weight reduction in larvae and pupae increased along with the increasing treatment doses. The percentage weight reduction of larva after 24 h of treatment with nanoformulation and representative non-nanoderivatives is given in Table 2. A significant dose-dependent reduction in larval weight was seen after treatment with

Table 2 Percentage larval weight reduction by nanoformulation treatment against third instar larvae of *Spodoptera litura* by topical application method

Test formulations	Concentration (%)				
	0.1	0.05	0.01	0.005	0.001
Control	$0^{a,x}$	$0^{a,x}$	$0^{a,x}$	$0^{a,x}$	$0^{a,x}$
STD	$90.96 \pm 5.50^{a,z}$	$80.07 \pm 7.43^{b,xyz}$	$70.21 \pm 10.95^{ab,wxy}$	$65.91 \pm 6.77^{c,xy}$	$63.24 \pm 6.26^{c,xy}$
NF1	$87.51 \pm 3.50^{a,z}$	$85.66 \pm 2.45^{a,z}$	$84.14 \pm 6.38^{a,xyz}$	$72.68 \pm 9.72^{b,xy}$	$70.94 \pm 3.75^{b,xyz}$
1	$68.01 \pm 6.84^{a,x}$	$65.16 \pm 15.81^{a,w}$	$59.81 \pm 8.56^{a,w}$	$57.75 \pm 10.27^{a,x}$	$56.51 \pm 9.46^{a,x}$
NF2	$87.49 \pm 3.19^{a,z}$	$85.40 \pm 2.83^{ab,z}$	$81.69 \pm 7.42^{ab,xyz}$	$77.28 \pm 7.93^{ab,xy}$	$75.43 \pm 11.22^{b,yz}$
NF3	$88.0 \pm 2.91^{a,z}$	$86.67 \pm 5.32^{a,z}$	$81.14 \pm 6.27^{a,xyz}$	$79.23 \pm 9.51^{a,y}$	$77.49 \pm 8.07^{a,yz}$
NF4	$75.76 \pm 5.64^{a,xy}$	$68.51 \pm 4.29^{ab,wx}$	$67.68 \pm 8.55^{ab,wx}$	$65.27 \pm 8.15^{ab,xy}$	$63.25 \pm 5.95^{b,xy}$
NF5	$85.30 \pm 1.87^{a,yz}$	$82.05 \pm 7.77^{a,yz}$	$77.25 \pm 10.45^{a,wxy}$	$74.98 \pm 7.70^{a,xy}$	$72.98 \pm 12.7^{a,xyz}$
NF6	$74.91 \pm 5.04^{a,xy}$	$70.93 \pm 6.43^{a,wxy}$	$68.75 \pm 10.03^{a,wx}$	$65.59 \pm 14.81^{a,xy}$	$64.06 \pm 7.39^{a,xy}$
NF7	*$92.06 \pm 2.70^{a,z}$*	*$88.39 \pm 5.72^{a,z}$*	*$86.21 \pm 6.75^{a,yz}$*	*$82.53 \pm 10.26^{a,y}$*	*$81.10 \pm 9.88^{a,z}$*
7	$63.62 \pm 12.04^{a,x}$	$61.22 \pm 5.23^{a,w}$	$60.60 \pm 5.26^{a,w}$	$58.53 \pm 11.87^{a,x}$	$56.86 \pm 12.47^{a,x}$
NF8	$90.28 \pm 4.77^{a,z}$	$88.77 \pm 2.43^{a,z}$	$85.46 \pm 9.36^{a,z}$	$81.92 \pm 8.55^{a,y}$	$79.56 \pm 9.04^{a,yz}$

Data are represented as a mean $\pm$ SD of each of ten replicates ($n = 10$) in percentage. Same letters indicate no significant differences ($p \leq 0.05$) between different test formulations (a, b, c) and concentrations (x, y, z) used in our study according to Tukey's HSD and Games Howell's test. Italics show formulation and concentrations which can be selected for further investigation studies. *NF* nanoformulations; 1: non-nanoparent compound 1; 7: non-nanoparent compound 7. Methoxyfenozide was used as the standard (STD) in this study

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nanoformulations. At highest concentration of 0.1%, NF7 showed maximum percentage larval weight reduction of 92.06% followed by NF8, NF3, NF1, and NF2 having 90.28, 88.0, 87.51, and 87.49% weight reduction, respectively, in comparison with control. The standard methoxyfenozide displayed slightly lower larval weight reduction of 90.96%. Both non-nanorepresentatives 1 and 7 presented lower larval weight reduction in comparison to their nanoformulations. Similar trend was observed at lowest concentration of 0.001%. Also, it has been noted that even at lowest concentration more than 50% larval growth inhibition was witnessed. Similar weight reduction behaviour was found in case of pupa as depicted in Table 3. NF4 and NF6 showed 100% weight reduction at highest concentration on 0.1% in comparison to control. NF7 and NF8 showed 90.46 and 81.79% pupal weight reduction, respectively. Almost similar behaviour was observed at lowest concentration of 0.001% with highest weight reduction in NF7 followed by NF6, NF8, NF3, and NF2 showing 67.52, 65.75, 60.46, and 60.06% pupal weight reduction, respectively.

The larval mortality, pupal mortality, and abnormalities including larval–pupal and pupal–adult intermediates, and percentage abnormal and normal adults formed by topical application method are shown in Additional file 1: Figure S1 and Fig. 2 Concentration-dependent uniformity in percentage mortality and deformities was not seen in both topical application and diet incorporation method; this may be attributed to various factors like food, rearing temperature, and humidity. At higher concentration of 0.1% NF6 showed maximum percentage larval mortality

of 80% followed by NF4 and NF7 having 60% and 50% larval mortality individually, in comparison to 30% mortality with standard methoxyfenozide. The larval mortality decreased with decreasing nanoformulation concentration. Chemically, NF1, NF2, and NF3 possessing chloro group attached at *ortho*, *meta,* and *para* positions of the phenyl ring, respectively, displayed relatively similar mortality behaviour. The presence of heterocyclic ring in the molecule in NF6 is probably responsible for improved mortality in comparison with halogen groups. However, NF4 and NF5 containing bromo group attached at *meta* and *para* positions displayed better larval mortality.
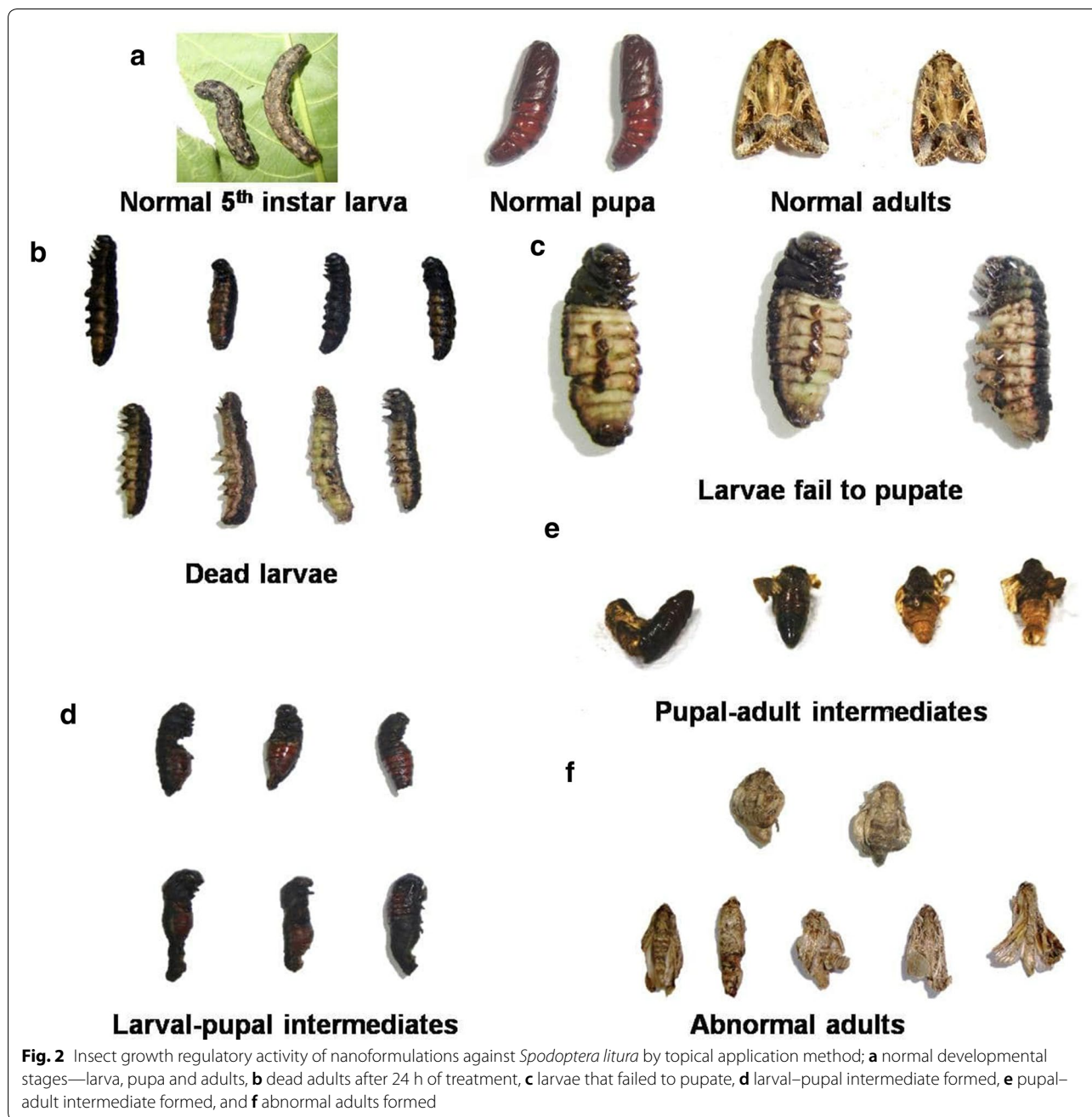
Pupal mortality results showed that NF8, a sulfonyl hydrazine derivative displayed maximum pupal mortality at 0.1% concentration followed by NF1, NF2, and NF3 with chloro group attached to the molecule. These results were in agreement with the reported literature for non-nanoderivatives [27] and further confirmed that the structure and type of functional group attached to the parent nucleus contributes to the IGR behaviour of the active ingredient. However, similar toxicity behaviour was observed for nanoformulations, but the results were more pronounced in comparison to non-nanoderivatives, suggesting that nanotization results in better penetration of active molecules inside insect body owing to their small size in comparison to their corresponding non-nano derivatives.

Similar to mortality data, dose-dependent pattern was not observed in the formation of intermediates and abnormalities. Very few intermediates were formed in NF4, NF5, NF6, and NF7 (Additional file 1: Figure S1). It has been observed that no intermediates were formed in

Table 3 Percentage pupal weight reduction by nanoformulation treatment against third instar larvae of *Spodoptera litura* by topical application method

Test formulations	Concentration (%)				
	0.1	0.05	0.01	0.005	0.001
Control	$0^{a,z}$	$0^{a,z}$	$0^{a,z}$	$0^{a,z}$	$0^{a,z}$
STD	$88.26 \pm 1.36^{a,xy}$	$80.71 \pm 2.75^{b,x}$	$70.30 \pm 7.22^{c,wx}$	$67.69 \pm 2.88^{cd,wxy}$	$62.04 \pm 2.53^{d,vwx}$
NF1	$79.88 \pm 5.85^{a,w}$	$77.83 \pm 4.12^{a,x}$	$73.32 \pm 7.84^{ab,xy}$	$65.51 \pm 1.89^{bc,vwx}$	$59.25 \pm 2.39^{c,uv}$
1	$67.51 \pm 5.97^{a,v}$	$62.44 \pm 1.91^{ab,w}$	$60.05 \pm 5.96^{abc,w}$	$56.52 \pm 2.9^{bc,u}$	$53.62 \pm 4.71^{c,u}$
NF2	$86.79 \pm 3.28^{a,wxy}$	$80.49 \pm 3.38^{ab,x}$	$73.04 \pm 7.42^{bc,xy}$	$65.28 \pm 5.43^{cd,uvw}$	$60.06 \pm 3.07^{d,uvw}$
NF3	$82.14 \pm 4.90^{a,wx}$	$80.52 \pm 3.88^{a,x}$	$76.81 \pm 5.32^{a,xy}$	$65.14 \pm 3.09^{b,uvwx}$	$60.46 \pm 2.88^{b,vw}$
NF4	$100^{c,u}$	$76.36 \pm 5.13^{a,x}$	$72.23 \pm 3.11^{a,xy}$	$61.91 \pm 6.42^{b,uvwx}$	$58.13 \pm 4.68^{b,uv}$
NF5	$79.21 \pm 3.72^{a,w}$	$77.04 \pm 4.90^{ab,x}$	$70.78 \pm 4.63^{b,wx}$	$59.69 \pm 1.56^{c,uvw}$	$56.48 \pm 2.51^{c,uv}$
NF6	$100^{c,u}$	$73.96 \pm 7.91^{a,x}$	$71.01 \pm 7.52^{ab,wx}$	$62.61 \pm 8.28^{b,uvwx}$	$67.52 \pm 3.29^{ab,xy}$
NF7	$90.46 \pm 5.17^{a,y}$	$89.17 \pm 2.10^{a,y}$	$82.33 \pm 4.87^{b,y}$	$75.40 \pm 2.97^{bc,y}$	$68.99 \pm 2.44^{c,y}$
7	$68.29 \pm 6.19^{a,v}$	$64.23 \pm 3.12^{ab,w}$	$60.28 \pm 5.20^{abc,w}$	$58.59 \pm 6.34^{bc,uv}$	$53.83 \pm 2.90^{c,u}$
NF8	$81.79 \pm 3.31^{a,wx}$	$79.38 \pm 2.42^{ab,x}$	$74.69 \pm 3.98^{bc,xy}$	$69.73 \pm 3.86^{cd,xy}$	$65.75 \pm 4.95^{d,wxy}$

Data are represented as a mean ± SD of each of ten replicates ($n = 10$) in percentage. Same letters indicate no significant differences ($p \leq 0.05$) between different test formulations (a, b, c) and concentrations (*u, v, wx, y, z*) used in our study according to Tukey's HSD and Games Howell's test. Italics show formulation and concentrations which can be selected for further investigation studies. *NF* Nanoformulations; 1: non-nanoparent compound 1; 7: non-nanoparent compound 7. Methoxyfenozide was used as the standard (STD) in this study

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Fig. 2 Insect growth regulatory activity of nanoformulations against *Spodoptera litura* by topical application method; **a** normal developmental stages—larva, pupa and adults, **b** dead adults after 24 h of treatment, **c** larvae that failed to pupate, **d** larval–pupal intermediate formed, **e** pupal–adult intermediate formed, and **f** abnormal adults formed

case of the standard methoxyfenozide. Percentage pupal–adult intermediate formation is significantly lower in almost all treatments. All formulations displayed formation of abnormal non-functional adults at higher concentrations; however, in case of the standard, the percentage abnormal adult formation was relatively lower. The overall growth inhibition was based on emergence of normal adults. It was observed that at higher doses, reduced number of normal adults was formed (as depicted in Additional file 1: Figure S1), while, with decreasing concentration, the percentage normal adult emergence increases. Although, in comparison with non-nano derivatives, 1 and 7 having 60% normal adult emergence at 0.001%, NF7, NF4, and NF5 displayed significantly lower 10% and 20% normal adult emergence, respectively. These results further emphasise the better penetration of nanopesticide inside the target insect.

IGR activity by diet incorporation method
In this method, different concentrations, viz., 0.1, 0.05, 0.01, 0.005, and 0.001%, of nanoformulations along with representative non-nano derivative and standard IGR

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methoxyfenozide were applied on the 90 mm castor leaf surface. The larvae were then allowed to feed on treated dry leaves for 2 h. The chronic exposure of test formulations was studied till adult emergence based on the parameters previously described in the topical application method. Nanoformulations of each class, viz., derivatives having halogen group attached to the phenyl ring, heterocyclic ring, and sulfonyl hydrazine moiety showing

promising results in topical application method were further screened by diet incorporation method.

The effect of nanoformulation treatment on larval growth is given in Table 4. All nanoformulations showed dose-dependent percentage weight reduction; NF4, NF6, and NF7 showed 100% larval weight reduction at 0.1% concentration. These formulations act as very strong anti-feedant and deter larvae from feeding

Table 4 Percentage larval weight reduction by nanoformulation treatment against third instar larvae of *Spodoptera litura* by diet incorporation method

Test formulations	Concentration (%)				
	0.1	0.05	0.01	0.005	0.001
Control	$0^{a,z}$	$0^{a,z}$	$0^{a,z}$	$0^{a,z}$	$0^{a,z}$
STD	$88.94 \pm 8.41^{a,y}$	$68.24 \pm 8.64^{b,y}$	$63.66 \pm 18.66^{b,y}$	$58.94 \pm 9.79^{bc,wx}$	$38.97 \pm 12.99^{c,v}$
NF4	$100^{b,w}$	$74.02 \pm 7.72^{a,y}$	$70.35 \pm 6.02^{a,y}$	$69.47 \pm 5.07^{a,xy}$	$65.88 \pm 8.03^{a,xy}$
NF6	$100^{b,w}$	$100^{b,x}$	$65.65 \pm 10.05^{a,y}$	$61.70 \pm 8.99^{a,wx}$	$58.33 \pm 12.22^{a,wx}$
NF7	$100^{b,w}$	$100^{b,x}$	$100^{b,x}$	$80.33 \pm 6.90^{a,y}$	$77.12 \pm 7.81^{a,y}$
7	$77.84 \pm 3.63^{a,x}$	$76.45 \pm 12.48^{a,y}$	$56.55 \pm 15.61^{b,y}$	$48.81 \pm 14.35^{b,w}$	$45.99 \pm 8.90^{b,vw}$

Data are represented as a mean ± SD of each of fifteen replicates (*n* = 15) in percentage. Same letters indicate no significant differences (*p* ≤ 0.05) between different test formulations (a, b, c) and concentrations (*w, x, y, z*) used in our study according to Tukey's HSD and Games Howell's test. Italics show formulation and concentrations having highest potential as a nanopesticide. *NF* nanoformulations; 7: non-nanoparent compound 7. Methoxyfenozide was used as the standard (STD) in this study
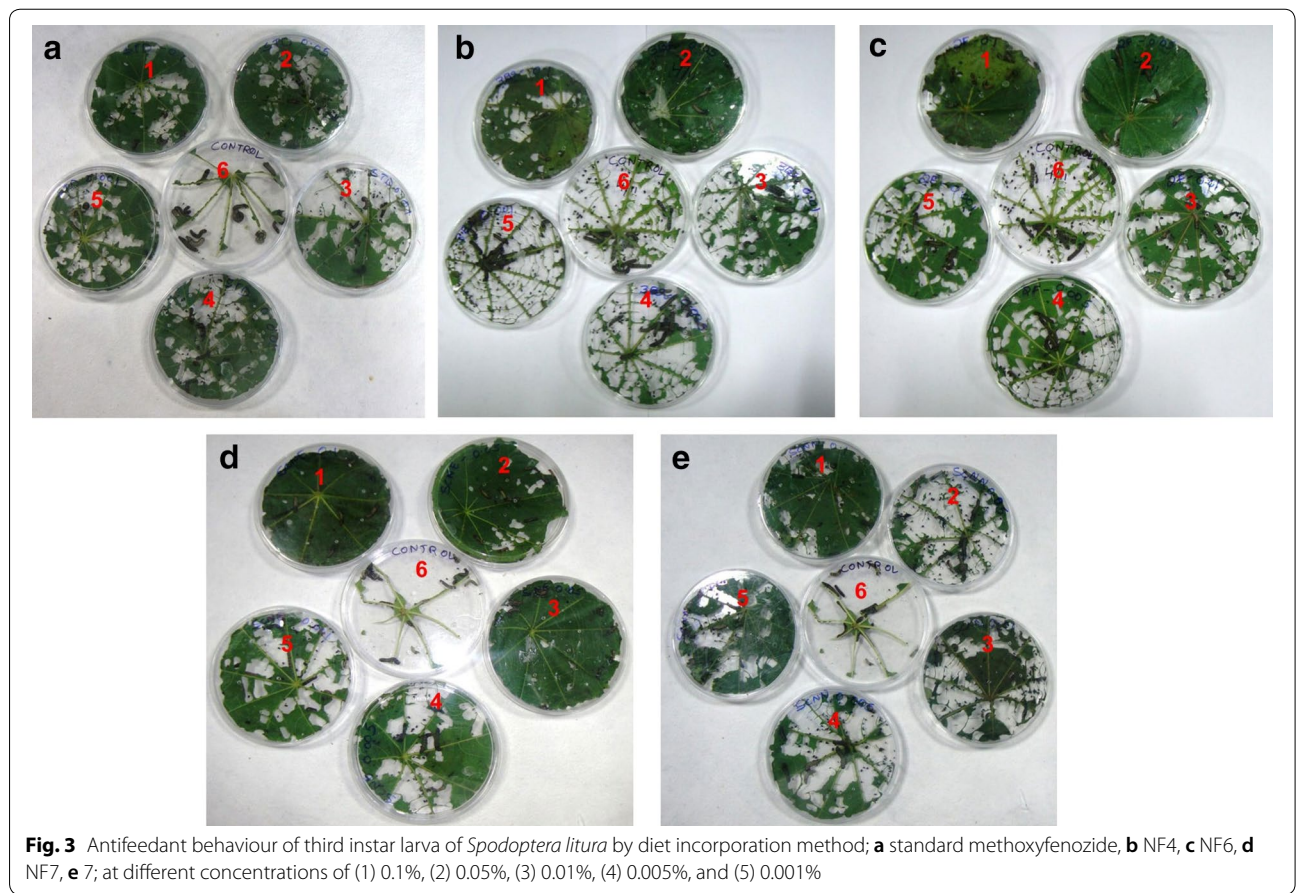

Fig. 3 Antifeedant behaviour of third instar larva of *Spodoptera litura* by diet incorporation method; **a** standard methoxyfenozide, **b** NF4, **c** NF6, **d** NF7, **e** 7; at different concentrations of (1) 0.1%, (2) 0.05%, (3) 0.01%, (4) 0.005%, and (5) 0.001%

treated leaves as evident from Fig. 3. Consequently, due to reduced or no feeding, NF7 displayed 100% larval growth inhibition up to lower concentration of 0.01%. Also, in case of all nanoformulations, more than 50% weight reduction was observed at lowest concentration. Surprisingly, standard methoxyfenozide showed lower larval weight reduction of 38.97%, in comparison with non-nanoderivative 7, having 45.99% weight reduction at 0.001% concentration. Relatively similar behaviour was observed in percentage pupal weight reduction pattern. Both NF7 and standard showed 100% pupal weight reduction at 0.005% concentration followed by NF4 and NF6 having 68.84 and 66.98% weight reduction, respectively, as depicted in Table 5. These results were in agreement with topical application method and clearly indicated that sulfonyl hydrazine derivative NF7 was most active compound.

Mortality data showed that nanoformulations prepared in our study impart maximum effect on larvae, as 100% larval mortality was recorded for all treatments at 0.1% concentration except for non-nanoderivative 7 having 46.7% larval mortality. However, with descending concentration, decrease in larval mortality was seen. NF7 showed maximum larval mortality of 73.5% at lowest concentration of 0.001% as shown in Additional file 1: Figure S2. Consequently, pupal mortality was significantly lower in all most all test formulations and ranged from 6.7 to 40%. Results by diet incorporation method showed lower number of intermediates formed. Also, the percentage abnormal adults formed were lower in number and ranged between 6.7 and 40%. A uniform dose-dependent behaviour was not observed. Only 6.7% normal adults was formed at lowest concentration of 0.001% in comparison to 40% against non-nanoderivative followed by NF6, NF4, and standard methoxyfenozide which gave 20% normal adult emergence in each case. Figure 4 shows IGR activity by diet incorporation

Fig. 4 Insect growth regulatory activity of nanoformulations against *Spodoptera litura* by diet incorporation method; **a** dead adults after 24 h of treatment, **b** larval–pupal intermediated formed, **c** pupal–adult intermediate formed, and **d** abnormal adults formed. For representative images of normal life cycle stages of *S. litura*, please refer to Fig. 3a
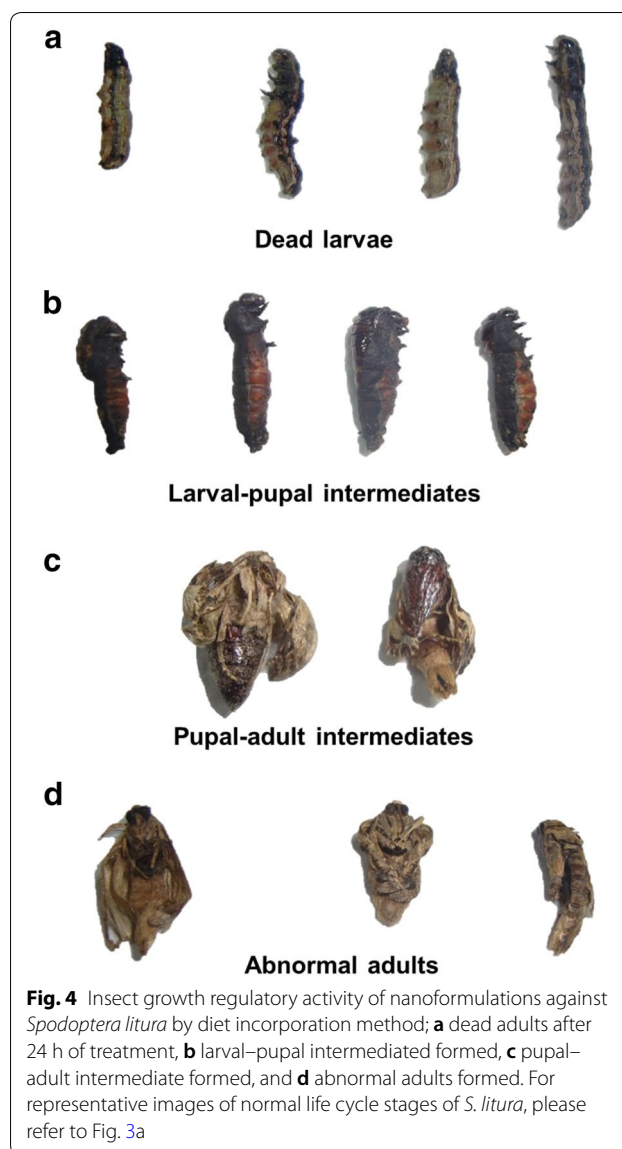
Table 5 Percentage pupal weight reduction by nanoformulation treatment against third instar larvae of *Spodoptera litura* by diet incorporation method

Test formulations	Concentration (%)				
	0.1	0.05	0.01	0.005	0.001
Control	$0^{a,z}$	$0^{a,z}$	$0^{a,z}$	$0^{a,z}$	$0^{a,z}$
STD	$100^{b,x}$	$100^{b,x}$	$100^{b,w}$	$100^{b,w}$	$61.46 \pm 6.34^{a,x}$
NF4	$100^{b,x}$	$100^{b,x}$	$70.50 \pm 5.16^{a,y}$	$68.84 \pm 4.66^{a,y}$	$65.09 \pm 3.35^{a,x}$
NF6	$100^{b,x}$	$100^{b,x}$	$69.02 \pm 2.94^{a,y}$	$66.98 \pm 4.09^{a,y}$	$64.94 \pm 3.09^{a,x}$
NF7	$100^{b,x}$	$100^{b,x}$	$100^{b,w}$	$100^{b,w}$	$74.45 \pm 4.94^{a,y}$
7	$50.46 \pm 3.81^{a,y}$	$47.62 \pm 5.88^{a,y}$	$44.83 \pm 8.02^{a,x}$	$43.85 \pm 4.19^{a,x}$	$42.26 \pm 6.98^{a,w}$

Data are represented as a mean $\pm$ SD of each of 15 replicates ($n = 15$) in percentage. Same letters indicate no significant differences ($p \leq 0.05$) between different test formulations (a, b, c) and concentrations (w, x, y, z) used in our study according to Tukey's HSD and Games Howell's test. Italics show formulation and concentrations having highest potential as a nanopesticide. *NF* nanoformulations; 7: non-nanoparent compound 7

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method having dead larvae, larval–pupal intermediates, pupal–adult intermediates, and abnormal adults formed.

To the best of our knowledge, effect of hydrazine-based nanopesticide on growth and subsequent development on *S. litura* for its effective control has never been explored. Careful analysis of results by both topical application and diet incorporation method showed that all prepared nanoformulations presented enhanced insect growth regulatory potential in comparison to non-nanocounterparts. Nanotization results in better incorporation and binding potential to the target site, thereby hampering normal growth and development process in insect. Out of all nanoformulations tested by topical application method, nanoformulation NF7, a sulfonyl acyl hydrazine derivative emerged as best IGR with GI_{90} value of 0.015 mg L^{-1}, in comparison the corresponding non-nano derivative 7 having GI_{90} value of 0.26 mg L^{-1} followed by NF6 with heterocyclic ring instead of phenyl

ring and NF4 and NF5 with bromo group attached at the *meta* and *para* positions of the phenyl ring having GI_{90} 0.024 mg L^{-1}, respectively (Table 6). The standard technical IGR methoxyfenozide showed lower GI_{90} value of 0.065 mg L^{-1} in comparison with nanoformulation NF7.

On the other hand, it has been found that the results of diet incorporation method depicted enhanced IGR efficacy of nanoformulations compared to topical application method, this result was in agreement with previously reported data for non-nano analogues showing higher efficacy by diet incorporation method in comparison to topical application method [27]. It has been observed that nanoformulation NF7 presented significantly higher GI_{90} of 0.010 mg L^{-1} compared to 0.122 mg L^{-1} for its corresponding non-nanoderivative 7, followed by NF4 and NF6 with GI_{90} 0.012 mg L^{-1} and GI_{90} 0.013 mg L^{-1}, respectively (Table 7). Overall results showed that all DAH-based nanoformulations displayed significantly higher GI_{90} values in

Table 6 Insect growth regulatory activity of nanoformulations against *Spodoptera litura* by topical application method

Test formulations	Heterogeneity		Regression equation	$b \pm SE$	R^2	GI_{90} (mg L^{-1})	Fiducial limit (95% confidence) (mg L^{-1})	
	df	χ^2					Min	Max
NF1	3	0.999	0.556x + 6.874	0.556 ± 0.366	0.962	0.080	0.017	0.451
1	3	0.997	0.652x + 6.664	0.652 ± 0.290	0.859	0.302	0.081	1.121
NF2	3	1	0.585x + 7.063	0.585 ± 0.362	0.976	0.046	0.009	0.238
NF3	3	1	0.629x + 7.308	0.629 ± 0.357	0.916	0.025	0.005	0.124
NF4	3	0.999	0.749x + 7.507	0.749 ± 0.297	0.962	*0.024*	0.006	0.090
NF5	3	0.999	0.646x + 7.314	0.647 ± 0.347	0.913	*0.024*	0.005	0.116
NF6	3	1	0.532x + 7.158	0.533 ± 0.426	0.935	*0.023*	0.003	0.158
NF7	3	1	0.691x + 7.543	0.691 ± 0.339	0.965	*0.015*	0.003	0.070
7	3	1	0.493x + 6.574	0.493 ± 0.393	0.969	0.262	0.044	1.545
NF8	3	1	0.528x + 7.179	0.529 ± 0.434	0.906	*0.021*	0.003	0.150
Methoxyfenozide	3	1	0.524x + 6.909	0.524 ± 0.399	0.956	0.065	0.011	0.393

NF nanoformulations, 1: non-nanoparent compound 1, 7: non-nanoparent compound 7 (for further details, please see Pandey et al. [29])
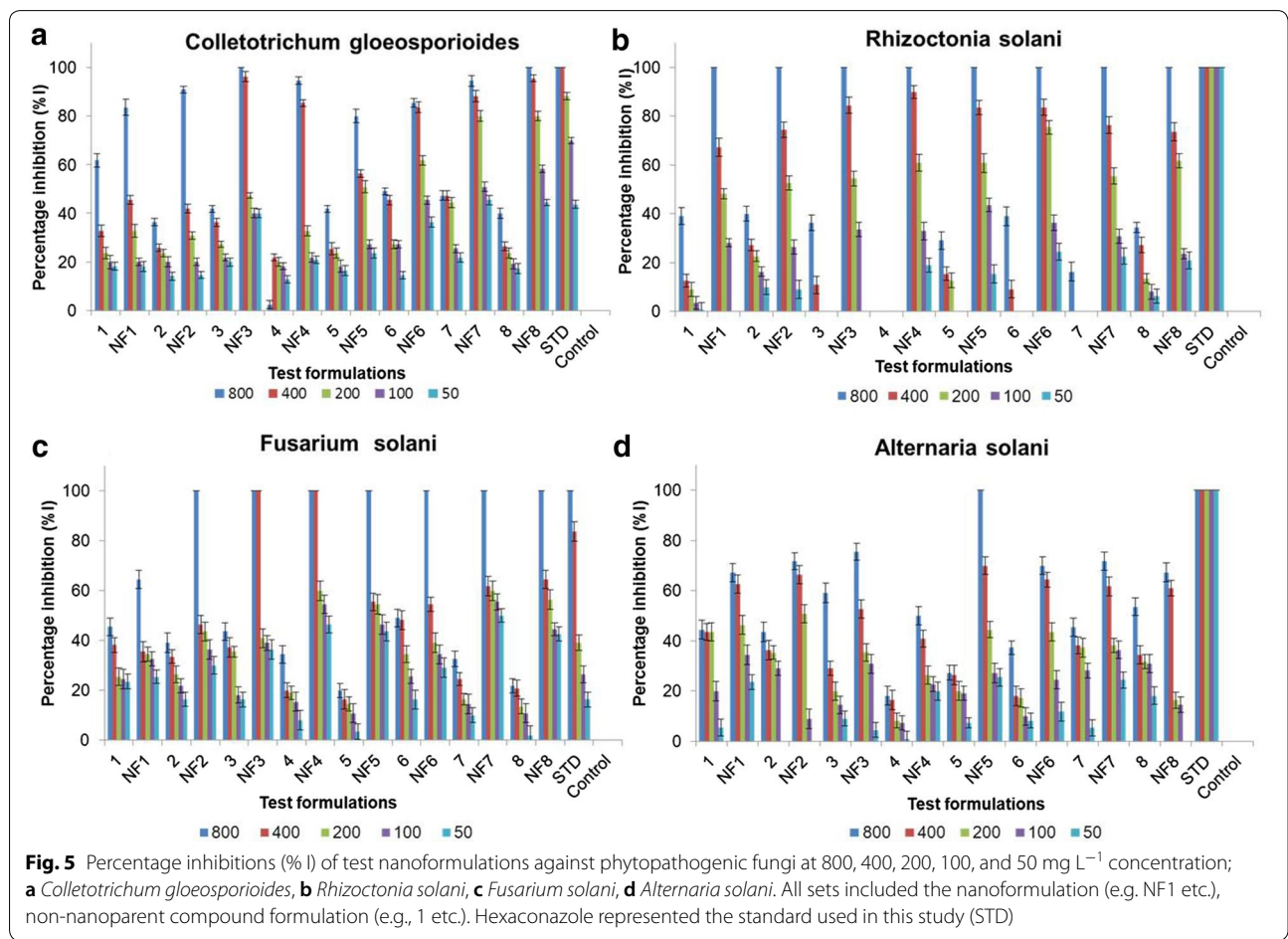
χ^2: goodness of fit, *b*: slope, SE: standard error, R^2: regression coefficient. Italics show nanoformulations having highest potential as a nanopesticide

Table 7 Insect growth regulatory activity of nanoformulations against *Spodoptera litura* by diet incorporation method

Test formulations	Heterogeneity		Regression equation	$b \pm SE$	R^2	GI_{90} (mg L^{-1})	Fiducial limit (95% confidence) (mg L^{-1})	
	df	χ^2					Min	Max
NF4	3	1	0.514x + 7.278	0.515 ± 0.470	0.977	*0.012*	0.001	0.098
NF6	3	1	0.699x + 7.621	0.699 ± 0.344	0.927	*0.013*	0.003	0.072
NF7	3	1	0.549x + 7.381	0.549 ± 0.448	0.917	*0.010*	0.001	0.078
7	3	1	0.500x + 6.741	0.501 ± 0.402	0.989	0.122	0.020	0.746
Methoxyfenozide	3	1	0.505x + 6.927	0.505 ± 0.420	0.912	0.055	0.008	0.366

NF nanoformulations, 7: non-nanoparent compound 7 (for further details, please see Pandey et al. [29])

χ^2: goodness of fit, *b*: slope, SE: standard error, and R^2: regression coefficient. Italics show nanoformulations having highest potential as a nanopesticide

comparison with their corresponding non-nanoderivatives and both methods of IGR evaluation showed that sulfonyl acyl hydrazine-based nanoformulation, NF7, appeared as the most promising IGR against *S litura*.

In vitro antifungal activity

The antifungal effect of the prepared nanoformulations and corresponding non-nanoderivatives were expressed in percentage inhibition (*I*%). All nanoformulations displayed moderate-to-good antifungal potential against the targeted plant pathogenic fungi. However, when compared with non-nano derivatives, a marked difference in efficacy was observed. The nanoforms showed enhanced efficacy at a given concentration and the effect of various treatments on fungal mycelium growth in comparison with non-nanocounterparts is shown in Fig. 5. Nanoformulation NF7 displayed 50% growth inhibition at lowest concentration of 50 mg L^{-1} followed by NF4 and NF8 having 46.4 and 42.7% inhibition when tested against *F. Solani*. Against *R. solani*, all formulations except NF1 displayed more than 50% fungal growth inhibition at 200 mg L^{-1} concentration. On the other hand,

nanoformulation NF4 presented 61% growth inhibition at 200 mg L^{-1}; however, its corresponding non-nanoderivative is absolutely non-toxic to *R. solani*. *C. gloeosporioides* also displayed enhanced sensitivity to all nanoformulations. NF3 and NF8 presented moderate toxicity of 40% and 44.5% inhibition at 50 mg L^{-1} concentration. Reasonable antifungal activity was observed against *A. solani* at all concentrations; however, with decreasing concentration, the efficacy weakened. The technical standard hexaconazole displayed 100% growth inhibition against all fungal stains at all concentrations. The nanoemulsion delivery system is likely to enhance the interaction of active ingredient with microbial cell membrane owing to its large surface area and passive cellular absorption mechanisms through the membrane [36], thereby decreasing mass transfer resistance and hence better antifungal profile [37], and the same mechanism may be responsible for antifungal behaviour of these nanoformulations. To best of our knowledge, antifungal behaviour of diacyl hydrazine and sulfonyl acyl hydrazine-based nanoformulations is reported for the first time in this paper. It has been observed that these nanoformulations displayed enhanced IGR potential in comparison to their antifungal

Fig. 5 Percentage inhibitions (% l) of test nanoformulations against phytopathogenic fungi at 800, 400, 200, 100, and 50 mg L^{-1} concentration; **a** *Colletotrichum gloeosporioides*, **b** *Rhizoctonia solani*, **c** *Fusarium solani*, **d** *Alternaria solani*. All sets included the nanoformulation (e.g. NF1 etc.), non-nanoparent compound formulation (e.g., 1 etc.). Hexaconazole represented the standard used in this study (STD)

behaviour. However, when applied, it may act as better crop protectant having dual properties.

Moreover, preliminary biosafety study of these nanoemulsions on Wax moth, *Galleria mellonella*, at 1000, 800, 400, and 200 µg mL^{-1} concentration showed no toxicity even after 72 h of treatment. Each treated larva displayed healthy growth and development for three subsequent generations at highest concentration of 1 mg mL^{-1} compared to 100% mortality within 1 min when treated with standard cypermethrin 25% EC [29].

Conclusions

The present study systematically demonstrates the remarkable pesticidal potential of eight stable water-based, ready-to-use, safe nanoformulations of a new DAH class of nanopesticide. All prepared nanoformulations, particularly sulfonyl acyl hydrazine-based, NF7 and NF8 displayed higher stability and efficacy over a period of time in comparison with their non-nano derivatives.

Nanoformulation NF7 displayed maximum potency with GI$_{90}$ values of 0.015 mg L^{-1} and 0.010 mg L^{-1} by topical application and diet incorporation method, respectively. Antifungal activity also highlighted good-to-moderate activity of nanoformulation NF7. The results showed that nano-based pesticide delivery could be an emerging technology with remarkable potential in enhancing agricultural productivity by overcoming crop losses by combating devastating and economically important pests with extremely reduced doses and targeted delivery of active molecules. Field trial and further dose optimization of these nanoformulations for subsequent product development are under progress. Preliminary toxicity studies on mammalian model *G. mellonella* displayed absolutely no toxicity of these nanoformulations. However, more in-depth understanding of toxicity at molecular level and evaluation of broad spectrum of efficacy against other notorious and economically relevant pests are emphasized. The overall results obtained by IGR activity analysis and antifungal behaviour indicated that such nanoformulations can be explored for better and safe crop protecting agents against devastating insect pests ensuring higher food security to growing world population owing to their relatively safer chemical profile against non-target organism and environment.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s40538-020-0177-9.

Additional file 1: Figure S1. Effect of nanoformulations on *Spodoptera litura* by topical application method. A) Percentage larval mortality, B) Percentage pupal mortality, C) Percentage larval-pupal intermediate formed, D) Percentage pupal-adult intermediate formed, E) Percentage deformed adults formed and F) Percentage normal adults formed. **Figure S2.** Effect of nanoformulations on *Spodoptera litura* by diet incorporation method. A) Percentage larval mortality, B) Percentage pupal mortality, C) Percentage larval-pupal intermediate formed, D) Percentage pupal-adult intermediate formed, E) Percentage deformed adults formed and F) Percentage normal adults formed.

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