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Effect of Nanoemulsion of Hexanal on Honey bees (Hymenoptera; Apidae)

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Abstract
The effect of nanoemulsion of hexanal to honey bee species *viz*., *Apis cerana indica* (Fabricius), *Apis mellifera* (Linnaeus), *Apis florea* (Fabricius) was evaluated in the Department of Nano Science and Technology, Tamil Nadu Agricultural University (TNAU) Coimbatore during 2015-2016. Nano emulsion of hexanal at varying concentration was tested using dry film method against honey bees in a Completely Randomized Design (CRD) replicated three times. Nano emulsion of hexanal (0.06%) showed maximum mortality of worker bees up to 7.65 percent in *A. cerana indica*, 5.80 percent in *A. mellifera* and 5.20 percent in *A. florea* at 24 hours after treatment as against the recommended dose of 0.02 percent which effected on mortality of 4.33 percent in *A. cerana indica*, 4.75 percent in *A. mellifera* and 4.05 percent in *A. florea* at the same measure of exposure. The results clearly indicated that all the concentration of nano formulation of hexanal was harmless to worker honey bees which recorded a mortality of < 30 percent as per the threshold prescribed by IOBC.

Keywords: Safety, Nanoemulsion, hexanal, honey bees

1. Introduction

Mango ecosystem is known for the rich biodiversity of insects which includes pollinators *viz.*, *Apis cerana indica*, *A. florea*, *Syrphus*, *Chrysomya*, *Musca*, *Braunsapis hewitti* [19]. Among the various species of pollinators, *Apis* spp L. (Hymenoptera, Apidae) are common pollinator, with excellent adaptation to perennial fruit eco system responsible for fruit setting in many fruit plants [3, 9].

Hexanal is a plant derived biochemical molecule possessing properties to enhance shelf-life of fruits when applied externally as a pre-harvest spray or post-harvest dip or as a vapor treatment [16]. It is generally regarded as safe when used as a strong inhibitor of Phopholipase D and as technology to enhance shelf life and quality of fruits, vegetables and flowers [13-15] Nanoemulsion of hexanal would be more effective than the conventional macro emulsion form of treatment owing to extremely small droplet size less than 100 nm and uniform surface coverage[10]. In addition, high kinetic stability, low viscosity and optical transparency of nano emulsion make them very attractive system for product delivery. Biosafety is one of most important aspect of any nano product in agriculture which eliminates the undesirable effect to environment housed with beneficial and non-target organisms [10]. Hence, the present study was carried out to assess the toxicity of nano emulsion of hexanal formulation to common honey bee species visiting mango orchard.

2. Materials and Methods

The toxicity of nanoemulsion of hexanal to honey bee species *viz*., *Apis cerana indica Apis mellifera*, *Apis florea* was evaluated in the Department of Nano Science and Technology, Tamil Nadu Agricultural University (TNAU) Coimbatore during December-2016.Nano emulsion of hexanal at varying concentration was tested using dry film method against honey bees in a Completely Randomized Design (CRD) replicated three times.

2.1. Preparation of nanoemulsion of hexanal

Preparation of hexanal nanoemulsion involves mixing of hexanal: Tween 20 and ethanol in the ratio of 1:10:10 v/v basis and were sonicated using sonicator at 20 kHz for 15 min as per the standard method suggested by [8].

2.1.2. Preparation of varying concentration of hexanal nano emulsion

From the concentrated nano emulsion of hexanal 2ml, 4ml, 6ml was taken in separate standard volumetric flask of 100 ml capacity and the volume made up to 100 ml with double distilled water to prepare 0.02, 0.04, and 0.06 per cent concentration of nanoemulsion of hexanal respectively. Pure hexanal was used as standard check and its varying concentrations was prepared by taking 20 µl, 40 µl, 60 µl of pure hexanal in standard volumetric flask of 100 ml and the volume made up to 100 ml using ethanol (70 per cent) to prepare 0.02, 0.04, 0.06 per cent concentration of pure hexanal respectively. Absolute ethanol (99.9 %) 200 µl was taken and the volume made up to 100 ml in standard 100 ml volumetric flask using distilled water to prepare 0.2 per cent of ethanol. Similarly 200 µl of Tween 20 was taken and made up to 100 ml in a standard 100 ml volumetric flask using distilled water to prepare 0.2 per cent of Tween 20.

3. Bioassay

Bioassay to assess the toxicity of nanoemulsion of hexanal on honey bees *viz., Apis cerana indica, Apis mellifera, Apis florea* was carried out using dry film toxicity method established by the "Organisation for Economic Cooperation and Development [12 & 17].

The actively foraging worker bees (~ 21 days old) of the three different test species were obtained from insectary, Department of Agricultural entomology, Tamil Nadu Agricultural University, Coimbatore. Filter paper discs treated with different concentrations of nano emulsion of hexanal (as detailed in Table 1.) were allowed to dry in shade. The shade dried filter paper discs was placed in circular plastic container (500 ml) with perforations in the lid and the honey bees *viz*., *A. cerana indica, A. mellifera* and *A. florea* were released at the rate of 10 per container after calming them by keeping in refrigerator for 4 to 6 minutes. After 1 h of exposure with treated filter paper disc in plastic container, the honey bees were transferred to a perforated nylon net bag (20 X 30 cm) provided with 40 per cent sucrose solution as adult diet. The mortality of bees was observed at 6, 12 and 24 hours after treatment and per cent mortality worked out.

$$\text{Per cent mortality} = \frac{P_0 - P_c}{100 - P_c} \times 100$$

P_0 - Observed mortality in treatment

P_c - Observed mortality in untreated check

Table 1: Treatments tested for biosafety

T_1-Nanoemulsion of hexanal @ 0.02%	T_6- Pure hexanal @ 0.06%
T_2-Nanoemulsion of hexanal @ 0.04%	T_7- Ethanol @ 0.2%
T_3- Nanoemulsion of hexanal @ 0.06%	T_8- Tween 20 @ 0.2%
T_4- Pure hexanal @ 0.02%	T_9- Control
T_5- Pure hexanal @ 0.04%	

4. Statistical analysis

The data were analyzed using SPSS® (Statistical Package for Social Science release 15.0 – SPSS Inc., Chicago, IL). The means were separated using LSD test at 5% level.

5. Results and Discussion

5.1. Effect of nano emulsion of hexanal to honey bees

5.1.1. Indian bee–*Apis cerana indica* F.

The effect of nano emulsion of hexanal to *A. cerana indica* was assessed in the laboratory and the results are presented in Table 2. Nano emulsion of hexanal was found to cause mortality of worker bees up to 7.65 percent in higher concentration of 0.06 percent at 24 HAT. Mortality at field recommended concentration of 0.02% was found less toxic to *A. cerana indica*, with minimum mortality of 1.10, 4.25 and 4.33 per cent respectively in 6, 12 and 24 HAT. The standard check, pure hexanal @ 0.02, 0.04 and 0.06 percent had higher mortality range of 4.55 to 11.10 percent, up to 24 HAT (Table 2). The surfactant Tween 20 @ 0.2 percent and solvent absolute ethanol 0.2 percent showed lesser mortality of 0.00, 3.33, 3.33 and 0.00, 1.10 and 2.20 percent respectively at 6, 12 and 24 hours after exposure. The untreated check showed no mortality of bees during the observation period up to 24 hours after treatment.

5.1.1.2. Italian bee *Apis mellifera* L.

The effect of nano emulsion of hexanal to *A. mellifera* was assessed in the laboratory and the results are presented in Table 3. Nano emulsion of hexanal was found to cause mortality of worker bees up to 5.80 percent in higher concentration of 0.06 percent. Mortality at field recommended concentration of 0.02% was found less toxic to the *A. mellifera* with minimum mortality of 2.05, 3.30 and 4.75 per cent respectively at 6, 12 and 24 HAT. The standard check, pure hexanal showed maximum mortality of 6.67, 8.90 and 9.10 per cent, respectively in 0.02, 0.04 and 0.06 percent concentration at 24 HAT. (Table 3). The surfactant Tween 20 @ 0.2 percent and solvent absolute ethanol @ 0.2% recorded mortality 1.10, 3.30, 2,58 and 1.10, 2.20, 2.58 percent respectively at 6,12 and 24 hours after treatment. The untreated check showed no mortality of bees during observation period up to 24 hours after exposure.

5.1.1.3. Little bees – *Apis florea* F.

The effect of nano emulsion of hexanal to *A. florea* was assessed in the laboratory and the results are presented in Table 4. The mortality of bees recorded at 6 HAT was low as compared to 12 and 24 HAT in all the treatments tested. The treatment of nano emulsion of hexanal caused at 0.06% a maximum bee mortality of 5.2 percent at 24 HAT, whereas the lower concentration of nano emulsion 0.02% and 0.04% recorded lesser mortality range of 2.58 to 4.20 percent in all the period of observation up to 24 HAT. The standard check, pure hexanal showed maximum mortality of 6.67, 9.60 and 12.80 per cent, respectively in 0.02, 0.04 and 0.06 percent concentration at 24 HAT. (Table 4). The surfactant Tween 20 @ 0.2 percent and solvent absolute ethanol 0.2 percent showed mortality of 1.10, 3.30, 2.58 and 1.10, 2.2, 2.65 percent at 6, 12 and 24 hours after treatment. The untreated check showed no mortality of bees during the period of observation up to 24 hours after treatment.

Based on the toxicity levels of the newer molecules tested against beneficial organisms, they are classified as harmless (mortality <30%), slightly harmful (> 30% and <79%), moderately harmful (> 80% and <99%), and harmful (>99%) [5, 6]. The mortality of honey bees recorded in nano emulsion of hexanal 0.02% at field recommended dose being significantly lesser than the standard check and control with mortality < 30 percent very close to the threshold prescribed by IOBC for the test product shall be claimed as harmless [1, 5, 18].

All the tested nanoemulsion of hexanal formulation and standard check pure hexanal were less toxic to honey bees. The results obtained in this study also agree with those of Karthika *et al*., (2015) [10] who also found that exposing of nano emulsion of hexanal formulation to honey bees *Apis* *cerana indica* were safe with no mortality.

The plant origin products like rotenone and azadirachtin were relatively non-toxic to *A. mellifera* [7]. Neem oil formulation at different concentration tested against honey bees *A. mellifera* was safe [2]. He safety of six different insecticides on *A. mellifera* under laboratory condition with no adverse effect when tested at field recommended dose [4]. These findings indicated the safety of plant product to honey bees which endorse the present findings on the harmless nature of nano emulsion of hexanal to honey bees being plant derivatives.

6. Conclusion

All the concentration of nano formulation of hexanal (0.02-0.06%) tested against worker honey bees of *A. cerana indica*, *A. mellifera* and *A. florea* were found harmless with the minimum mortality range of 4.05- 7.65 percent which is far below the threshold value of < 30 percent measured by IOBC.

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Table 2: Toxicity of nanoemulsion of hexanal to Indian bees – *Apis cerana indica* F.

Treatments	Mortality of worker bees (%)		
	6 HAT	**12 HAT**	**24 HAT**
T_{1}- Nano emulsion of hexanal @ 0.02%	1.10 (6.08)^{a}	4.25 (11.90)^{b}	4.33 (12.01)^{ab}
T_{2}- Nano emulsion of hexanal @ 0.04%	2.29 (9.65)^{ab}	4.55 (12.32)^{b}	4.75 (12.59)^{b}
T_{3}- Nano emulsion of hexanal @ 0.06%	3.60 (10.94)^{bc}	5.0 (12.92)^{bc}	7.65 (16.06)^{c}
T_{4}- Pure hexanal @ 0.02%	4.55 (12.32)^{c}	6.40 (14.65)^{bc}	8.20 (16.64)^{c}
T_{5}- Pure hexanal @ 0.04%	5.89 (14.05)^{cd}	7.65 (16.6)^{e}	10.00 (18.39)^{cd}
T_{6}- Pure hexanal @ 0.06%	6.20 (14.42)^{d}	7.90 (16.32)^{d}	11.10 (19.47)^{d}
T_{7}- Tween 20 @ 0.2%	0.00 (0.19)^{a}	3.33 (10.25)^{ab}	3.33 (10.25)^{ab}
T_{8}- Ethanol @ 0.2%	0.00 (0.19)^{a}	1.10 (6.02)^{a}	2.20 (8.53)^{a}
T_{9}- Control	0.00 (0.19)^{a}	0.00 (0.19)^{a}	0.00 (0.19)^{a}
Mean	2.63	4.46	5.73
S. Ed	0.29	0.43	0.56
CD (p= 0.05%)	0.62	0.90	1.18

HAT- Hours after treatment

Mean of three observations. In a column means followed by a common letter are not significantly different at p = 0.05 by DMRT

Figures in parentheses are arcsine √ P transformed values

Table 3: Toxicity of nano emulsion of hexanal to Italain bees – *Apis mellifera* L.

Treatments	Mortality of worker bees (%)		
	6 HAT	**12 HAT**	**24 HAT**
T_{1}- Nano emulsion of hexanal @ 0.02%	2.05 (4.83)^{a}	3.33 (10.51)^{ab}	4.75 (12.59)^{ab}
T_{2}- Nano emulsion of hexanal @ 0.04%	2.20 (8.53)^{ab}	4.05 (11.61)^{b}	5.20 (13.18)^{c}
T_{3}- Nano emulsion of hexanal @ 0.06%	3.25 (10.39)^{ab}	4.90 (12.79)^{b}	5.80 (13.18)^{c}
T_{4}- Pure hexanal @ 0.02%	4.20 (11.83)^{b}	5.89 (14.05)^{bc}	6.67 (14.96)^{cd}
T_{5}- Pure hexanal @ 0.04%	5.00 (12.92)^{b}	6.67 (14.96)^{bc}	8.90 (17.36)^{d}
T_{6}- Pure hexanal @ 0.06%	6.67 (14.96)^{c}	7.95 (16.38)^{c}	9.10 (17.56)^{d}
T_{7}- Tween 20 @ 0.2%	1.10 (6.08)^{a}	3.30 (10.51)^{ab}	2.58 (9.24)^{a}
T_{8}- Ethanol @ 0.2%	1.10 (6.08)^{a}	2.20 (8.59)^{a}	2.58(9.24)^{a}
T_{9}- Control	0.00 (0.19)^{a}	0.00 (0.19)^{a}	0.00 (0.19)^{a}
Mean	2.84	4.25	5.06
S.ED	0.29	0.40	0.48
CD (p= 0.05 %)	0.61	0.85	1.02

HAT- Hours after treatment

Mean of three observations. In a column means followed by a common letter are not significantly different at p = 0.05 by DMRT

Figures in parentheses are arcsine √ P transformed values

Table 4: Toxicity of nano emulsion of hexanal to little bees –*Apis florea* F**.**

Treatments	Mortality of worker bees (%)		
	6 HAT	**12 HAT**	**24 HAT**
T_{1}- Nano emulsion of hexanal @ 0.02%	2.58 (9.24)^{a}	3.33 (10.51)^{a}	4.05 (11.61)^{ab}
T_{2}- Nano emulsion of hexanal @ 0.04%	3.10 (10.14)^{ab}	3.45 (10.70)^{ab}	4.20 (11.83)^{ab}
T_{3}- Nano emulsion of hexanal @ 0.06%	3.25 (10.39)^{ab}	4.30 (11.97)^{ab}	5.20 (13.18)^{b}
T_{4}- Pure hexanal @ 0.02%	5.80 (13.94)^{b}	6.20 (14.42)^{b}	6.67 (14.96)^{b}
T_{5}- Pure hexanal @ 0.04%	6.20 (14.42)^{b}	6.67 (14.96)^{b}	9.60 (18.05)^{c}
T_{6}- Pure hexanal @ 0.06%	7.95 (16.38)^{c}	8.90 (17.36)^{c}	12.80 (20.96)^{d}
T_{7}- Tween 20 @ 0.2%	1.10 (6.08)^{a}	3.30 (10.51)^{a}	2.58 (9.24)^{a}
T_{8}- Ethanol @ 0.2%	1.10 (6.08)^{a}	2.20 (8.59)^{a}	2.65 (9.24)^{a}
T_{9}- Control	0.00 (0.19)^{a}	0.00 (0.19)^{a}	0.00 (0.19)^{a}
Mean	3.45	4.26	5.31
S.ED	0.36	0.41	0.54
CD (p= 0.05%)	0.75	0.87	1.14

HAT- Hours after treatment

Mean of three observations. In a column means followed by a common letter are not significantly different at p = 0.05 by DMRT

Figures in parentheses are arcsine √ P transformed values

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