LETHAL TOXICITY OF NEONICOTINOID INSECTICIDES TO INDIAN HONEYBEE, *APIS CERANA INDICA* F.

SHIRALLI, H.[1] – GURUPRASAD, G. S.[2*] – KANDKOOR, S.[3] – AKBAR, S. MD.[4] – NIRMALNATH, J.[5] – UDIKERI, S. S.[1] – RAYAR, S. G.[1]

[1]*Department of Entomology, College of Agriculture, Dharwad, Karnataka, India*

[2]*Krishi Vigyana Kendra, Hanumanmatti, Haveri, Karnataka, India*

[3]*Agriculture Research Station, Bailhongal, Karnataka, India*

[4]*Agriculture Research Station, Hebballi, Dharwad, Karnataka, India*

[5]*Department of Microbiology, College of Agriculture, Dharwad, Karnataka, India*

[*]*Corresponding author*
e-mail: guruento@gmail.com

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Abstract. Study assessed neonicotinoid toxicity to bees through oral, indirect contact, and topical application bioassays. All neonicotinoids demonstrated significant toxicity with LC_{50} values for imidacloprid, thiamethoxam, acetamiprid, dinotefuron, and clothianidin, respectively at 0.38, 1.11, 1.29, 0.26, 1.18 ppm (oral); 0.54, 1.33, 1.89, 1.22, 1.24 ppm (indirect contact); and 0.33, 0.40, 0.64, 0.47, 1.07 ppm (topical). Imidacloprid was found to be highly toxic with toxicities 3.39, 3.50, and 3.24 times higher and safety indices of 0.76, 1.08, and 0.66 for oral, indirect contact, and topical methods, respectively. Dinotefuron followed, with oral and indirect contact toxicities 4.96 and 1.54 times higher than that of imidacloprid and safety indices of 1.04 and 4.88, respectively. Thiamethoxam showed 2.67 times higher toxicity than that of dinotefuron with a safety index of 1.60 through topical application. Acetamiprid was the least toxic with safety indices of 6.45 and 9.45 for oral and indirect contact assays, while clothianidin had a safety index of 4.28 through topical application. These findings indicate that dinotefuron is the most toxic to bees in oral tests, while imidacloprid is highly toxic in both indirect contact and topical applications. Acetamiprid was found to be safer for bees in oral and indirect contact assays and clothianidin was safer in topical applications. The study reveals significant neonicotinoid toxicity to Indian bees with imidacloprid being particularly detrimental, emphasizing the need for mitigation strategies in Indian agriculture to protect bee populations.
Keywords: *oral ingestion, indirect contact, topical application, LC_{50}, safety index, relative toxicity*

Introduction

Pollination by insects is an important benefiting service, which account for 35 per cent of global crop-based food production benefitting through insect mediated pollination (Klein et al., 2007). Among insect pollinators bees occupy pivotal position as they are essential for qualitative and quantitative improvement of the production of various fruits, seeds, including oilseeds, nuts and fiber crops (Free, 1993; Pashte and Said, 2015; Pashte and Kulkarni, 2015). Further, bee pollination also enhances crop shelf life and commercial value (Klatt et al., 2014). Indian honeybee, *Apis cerana indica* Fabricius (Hymenoptera: Apidae) are the primary pollinators for many crops, with modified morphological, anatomical makeup and behavioral characters (Abrol, 2009; Pashte and Said, 2015).

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Unfortunately, honeybee populations are in decline since the 1990's, possibly due to a combination of pests, diseases, poor diet, colony collapse disorder and the increasing use of different pesticides (Potts et al., 2010; Goulson et al., 2015). However, colony collapse disorder is major concern. To date, there is no single factor that can explain colony loss in bees; however, one anticipated factor is the extensive application of chemicals for crop management (Naggar et al., 2015). Bee poisoning from pesticides is a serious problem worldwide (Barnet et al, 2007; Aktar et al., 2009; Potts et al., 2010; Goulson et al., 2015; Pashte and Patil, 2017).

Earlier assessments of insecticide toxicity for honeybee have mostly been undertaken with technical grade insecticides (Helson et al., 1994; Gradish et al., 2010). Such tests cannot always provide farmers with sufficient information about formulated insecticides against lethality or safety to bees. However, pest management decisions aimed at sustaining crop production by employing formulated pesticides, hence bee safety must be ensured for formulated pesticides. Previous studies have arrived toxicity of different formulated insecticides to Indian honeybees (Nadaf et al., 2013; Painkra et al., 2016; Vinothkumar et al., 2020; Gokulakrishna et al., 2021; Sowmiya et al., 2022).

Currently, an array of insecticides of different classes are available for pest management including pyrethroids, organophosphates, carbamates, neonicotinoids, botanicals and other novel insecticides of different origin acting on insect metabolism and regulation of growth and reproduction (Kodandaram et al., 2010).

Among different classes of insecticides, neonicotinoids are neurotoxic insecticides designed in the 80's, are systemic with long-term persistence. They permanently bind to nicotinic receptors of acetylcholine, blocking them and consequently the passage of nerve impulses (Tomizawa and Casida, 2005). Neonicotinoids have higher selectivity factors for insects versus mammals than most of insecticides apart from pyrethroids (Tomizawa and Casida, 2005). Several neonicotinoids indicated very strong toxicity to pollinating insects and in general Indian honeybee (*Apis cerana indica*), in particular causing other effects which are seldom easily identifiable, such as behavioural disturbances, orientation difficulties and impairment of social activities (Guez et al., 2001; Bortolotti et al., 2003; Medrzycki et al., 2003; Decourtye et al., 2004a,b; Desneux et al., 2007; El Hassani et al., 2008; Maini et al., 2010).

In Indian context, dependence on neonicotinoids is high for managing sucking pests in various field crops, vegetables and fruit orchards (Stanley et al., 2009, 2015; Gokulakrishna et al., 2021; Sowmiya et al., 2022). The proportion of neonicotinoids is more than 30% as compare to other group of insecticides among sucking pests protection strategies in India. At the same time there is paucity of scientific data on the harmful effects of neonicotinoids on honeybees with respect to Indian context. Hence, it is essential to know the toxicity levels of neonicotinoids to Indian bees, *A. c. indica*.

Materials and methods

In vitro toxicity assessment was made at Department of Entomology, College of Agriculture, University of Agricultural Sciences, Dharwad, Karnataka, India (Latitude: 15.4891º N, Longitude: 74.9813º E, with a subtropical climate and an average elevation of 695 m above MSL) where in Indian honeybee activity is common in fields and apiculture is a profession too.

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Test organism

Utilized foragers of Indian honeybee, *Apis cerana indica* Fabricius (Hymenoptera: Apidae), collected from five colonies each with a naturally mated queen. The hives were examined for the presence of diseases and pests during routine colony maintenance. Throughout the experiment, the colonies were free from diseases and pests. Therefore, no hive treatment of any chemicals was conducted prior to and during the studies.

Collection and inactivation of bees

Unspecified aged adult worker bees were collected from the frame that contained honey and pollen (with the exception of brood frames in order to avoid nurse bees) during morning hours (OECD, 1998) using glass test tubes (22.5 cm long and 6.25 cm diameter), then, mouth of test tube was closed with a cotton wad and same had been used for further in-vitro bioassay studies. These collected bees were preconditioned (starved) for 2 hr to enhance their appetite for food and anaesthetized by chilling in the refrigerator at 4ºC for 2 minutes to facilitate easy handling that makes them temporarily inactive (Thomas and Phadke, 1994; Human et al., 2013).

Laboratory bioassay with bees

Commercial formulations of neonicotinoid insecticides available in market were used (*Table 1*) for study. Three different bioassay methods *viz,* oral/ingestion test, indirect contact test and topical application have been deployed independently. Pilot toxicity tests were carried out for every insecticide to know the ranges of 20-80 per cent mortality through oral ingestion, indirect contact and topical application methods. Then six concentrations have been calculated for each insecticide limiting mortality > 20 to < 80 per cent. Concentration refers to the concentration of the formulated neonicotinoids in spraying water was used not with the active ingredient of the tested product. One treatment with water served as the untreated control and to correct the treatment mortalities with natural deaths. For each treatment, three replications were maintained with twenty bees each. The honeybees were considered “dead” when they remained absolutely still during a 10 seconds observation period (Iwasa et al., 2004).

Acute toxicity of insecticides to honeybees

Oral/ingestion tests

Each test group of bees were provided with 100-200 µl of 50 per cent sucrose solution in water, containing the test substance at the appropriate different concentrations of selected insecticides (*Table 1*). The amount of treated diet consumed per group was monitored. Once consumed (usually within 3-4 hours), the feeder was removed from the cage and replaced with one containing sucrose solution alone. The sucrose solutions are then provided *ad libitum*. For some compounds, at higher concentrations rejection of test dose may result in little or no food being consumed. After a maximum of 6 hours, unconsumed treated diet was replaced with the sucrose solution alone. Methodology followed according to OECD guideline 213 (Organization for Economic Co-operation and Development) of testing chemicals for bee safety

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Table 1. *Insecticides and their dosages used in the bioassay studies*

Sl. No	Insecticide	Oral/ingestion test	Indirect contact test	Topical application
1	Imidacloprid 17.8 SL (ppm = µl/ml)	0.50	1.00	0.15
		1.00	1.50	0.20
		2.00	2.00	0.25
		2.50	2.50	0.30
		4.00	3.00	0.35
		5.00	3.50	0.40
		Control		
2	Thaimethoxam 25 WG (µg)	1.00	2.50	0.50
		2.50	3.00	1.00
		3.00	3.50	1.50
		4.00	4.00	2.00
		5.00	4.50	2.50
		10.0	5.00	3.00
		Control		
3	Acetamiprid 20 SP (µg)	1.0	2.00	1.00
		2.0	4.00	1.50
		3.0	6.00	2.00
		4.0	8.00	2.50
		5.0	10.0	3.00
		10.0	12.0	3.50
		Control		
4	Dinotefuron 20 SG (µg)	10.0	10.0	5.00
		20.0	50.0	10.0
		30.0	75.0	15.0
		40.0	100.0	20.0
		50.0	150.0	25.0
		100.0	200.0	30.0
		Control		
5	Clothianidin 50 WDG (µg)	1.50	2.00	1.00
		2.00	2.50	1.25
		2.50	3.00	1.50
		3.00	3.50	2.00
		3.50	4.00	2.50
		4.00	4.50	3.00
		Control		

SL: Soluble liquid, WG: wettable granules, SP: soluble powder, SG: soluble granules, WDG: water dispersible granules, ppm: parts per million and mg: milli gram

Indirect contact test

Selected insecticides (*Table 1*) of different concentration based on pilot study were sprayed on buck wheat plant sowed in small pots (16 cm length and 20 cm diameter). Treatments were applied at bloom stage covering all over the surface of plant using high volume hand sprayer and left to dry in the shade for 2 hr. Pure water was treated as untreated control. The plants were then introduced into cages separately for selected insecticides of different concentration. Twenty bees were introduced separately for each cage to move freely on treated foliage for 3 hr. During the trail, the honeybees were fed with 50 per cent sucrose solution.

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Topical application

Anaesthetized bees were individually treated by topical application. The bees were randomly assigned to the different test doses and controls. A volume of 1 µl of solution containing the test substance at the suitable concentration was applied with a Hamilton micro applicator on to the dorsal side of the thorax of each bee. After application, the bees were allocated to test cages and supplied with sucrose solutions. Same procedure was followed for untreated control with pure water without insecticide (According to OECD guideline 214 of testing chemicals for bee safety).

Data analysis

Data on the mortality of test bees was converted into percentage of mortality and was calculated by adjusting for control mortality, and LC_{50} and LC_{90} for selected insecticides were determined with probit analysis by corrected mortality using below mentioned Abbott's formula (1925). PoloPlus software was used for probit analysis.

$$\text{Abbott's corrected mortality (\%)} = \frac{\text{\% treated mortality (T)} - \text{\% control mortality (C)}}{100 - \text{\% control mortality (C)}} \times 100 \quad \text{(Eq.1)}$$

where,
T: Per cent mortality in treatment,
C: Per cent mortality in controls.

The safety index of neonicotinoid insecticides was calculated by using the formula of Hameed et al. (1973):

$$S.\ I. = \frac{LC_{50}}{NRC} \quad \text{(Eq.2)}$$

where, S. I. is the safety index, LC_{50} is the median lethal concentration of insecticide (%) and NRC is the normal recommended insecticide concentration for crop pest control (%).

Relative toxicity of each tested neonicotinoids was calculated by following formula to know level of toxicity.

$$\text{Relative toxicity} = \frac{\text{Insecticide with high } LC_{50}}{LC_{50} \text{ of respective insecticide}} \quad \text{(Eq.3)}$$

Results

Acute lethal oral/ingestion toxicity

Toxicity assessment of selected neonicotinoids spray formulations trough oral/ingestion assess revealed that acetamiprid 20 SP and clothianidin 50 WDG had lower toxicity by indicating highest LC_{50} values of 1.29 ppm and 1.18 ppm, respectively. These two were followed by thiamethoxam 25 WG (1.11 ppm). Contrastingly, lowest LC_{50} values were noticed in imidacloprid 17.8 SL (0.38 ppm) and dinotefuron 20 SG (0.26 ppm) indicating their high toxicity to honeybees. Similar trend was noticed at LC_{90} also where imidacloprid 17.8 SL and dinotefuron 20 SG exhibited to highly toxic with lowest LC_{50} values (2.12 and 2.49 ppm, respectively) against

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highest LC_{90} values of 8.05 and 7.80 ppm in thiamethoxam 25 WG and acetamiprid 20 SP, respectively (*Table 2*). Probit mortality was regressed on the log values of insecticide concentration and calculated regression lines as given in the *Figure 1(A-E)* indicated a homogeneous response of honey bee population to insecticides through acute lethal oral assay after 48 hrs. Slope function is given in *Table 2*.

Table 2. *Oral/ingestion toxicity of neonicotinoid insecticides to foragers of A. cerana indica*

SL No.	Insecticides	LC_{50} (ppm)	95% Confidence limits (Fiducial limits)		LC_{90} (ppm)	95% Confidence limits (Fiducial limits)		Slope function (± SEm)	Chi-square	Heterogeneity
			Lower (%)	Upper (%)		Lower (%)	Upper (%)			
1.	Imidacloprid 17.8 SL	0.38	0.19	0.56	2.12	1.62	3.15	1.726 ± 0.263	4.30	1.07
2.	Thaimethoxam 25 WG	1.11	0.63	1.52	8.05	6.09	12.62	1.490 ± 0.253	3.61	0.90
3.	Acetamiprid 20 SP	1.29	0.86	1.67	7.80	5.77	12.96	1.644 ± 0.252	3.34	0.83
4.	Dinotefuron 20 SG	0.26	0.12	0.44	2.49	1.84	3.99	1.323 ± 0.246	2.45	0.61
5.	Clothianidin 50 WDG	1.18	0.75	1.56	7.00	5.29	11.16	1.663 ± 0.261	3.24	0.81

Acute lethal indirect contact toxicity

Impacts of neonicotinoids toxicity on honeybee was assessed through indirect contact test where acetamiprid 20 SP and thiamethoxam 25 WG had highest LC_{50} (1.89 and 1.33 ppm, respectively) and LC_{90} levels (15.42 and 11.46 ppm, respectively) and depicted to be least toxic. Clothianidin 50 WDG (LC_{50} 1.24 and LC_{90} 9.08 ppm) could stay next in the order of lower toxicity. Conversely, imidacloprid 17.8 SL and dinotefuron 20 SG exhibited least LC_{50} (0.54 and 1.22 ppm, respectively) and LC_{90} values (15.42 and 11.46 ppm, respectively) indicating quite higher toxicity to bees (*Table 3*). Probit mortality was regressed on the log values of insecticide concentration and calculated regression lines as given in the *Figure 2(A-E)* indicated a homogeneous response of honey bee population to insecticides through indirect contact toxicity test after 48 hrs. Slope function is given in *Table 3*.

Acute direct lethal toxicity (Topical application)

Toxicity response of bees was of similar kind when neonicotinoid formulations were administered topically. Highest LC_{50} and LC_{90} was registered with clothianidin 50 WDG and acetamiprid 20 SP indicating to be least toxic among the neonicotinoid as against lowest LC_{50} value of 0.33 ppm and LC_{90} value of 1.91 ppm in imidacloprid 17.8 SL and thiamethoxam 25 WG with lowest LC_{50} value of 0.40 ppm and LC_{90} value of 8.35 ppm (*Table 4*). Probit mortality was regressed on the log values of insecticide concentration and calculated regression lines as given in the *Figure 3(A-E)* indicated a homogeneous response of honey bee population to insecticides through acute direct lethal toxicity assay after 48 hrs. Slope function is given in *Table 4*.

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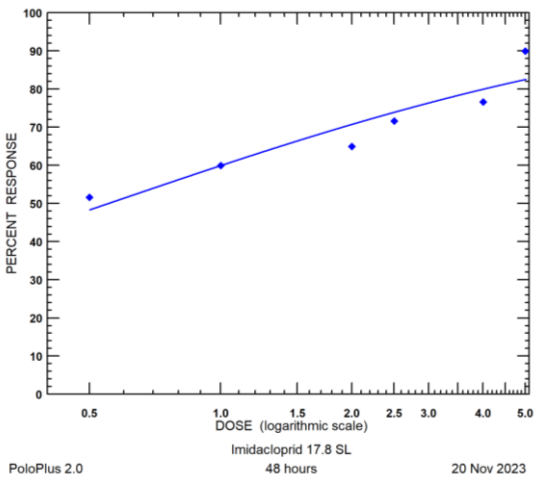
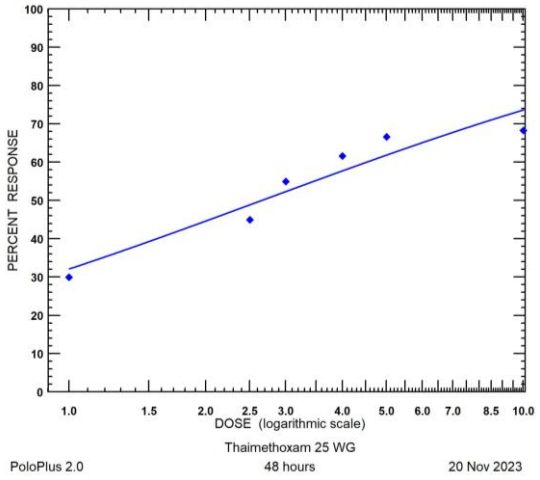
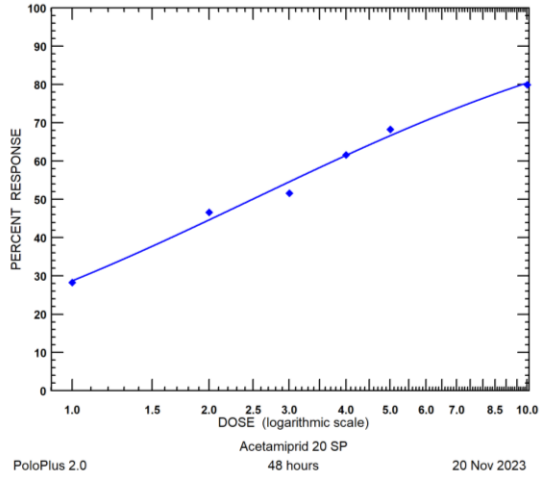


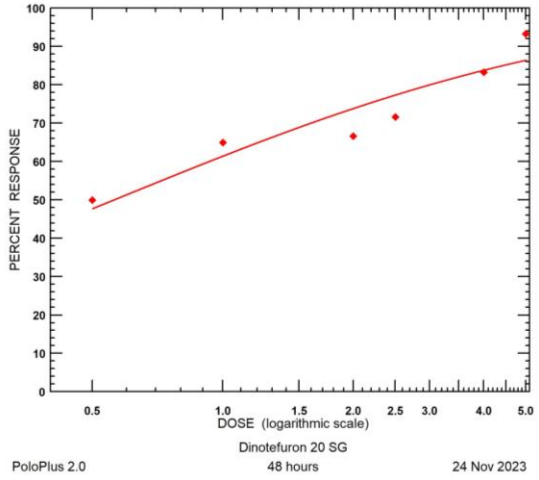
A) Imidacloprid 17.8 SL



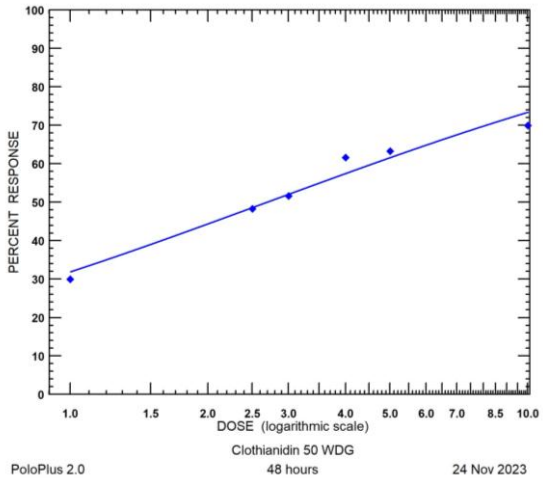
B) Thiamethoxam 25 WG



C) Acetamiprid 20 SP



D) Dinotefuron 20 SG



E) Clothianidin 50 WDG

Figure 1. *Concentration response mortality of honeybees to neonicotinoid insecticides (A-E) through oral or ingestion bioassay test (48 hr after treatment)*

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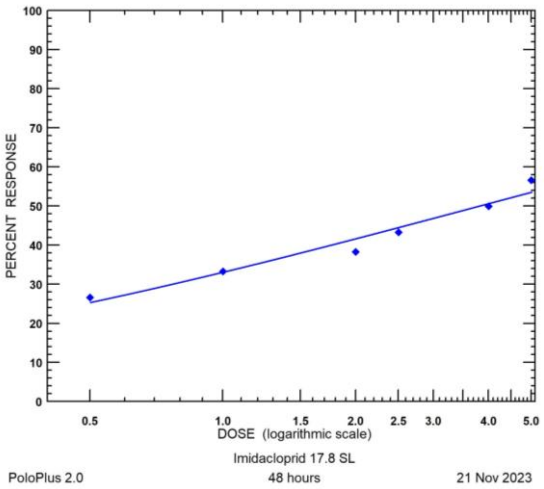
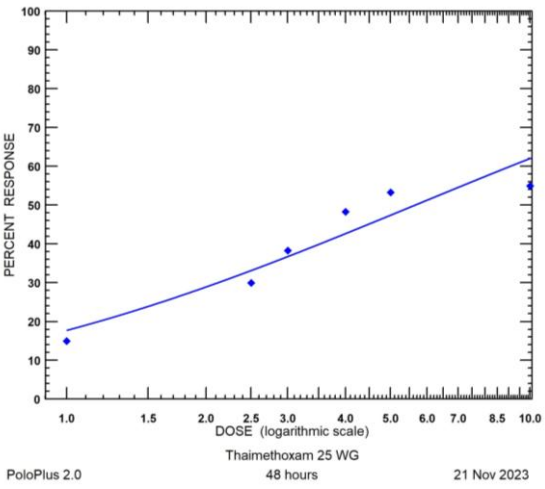
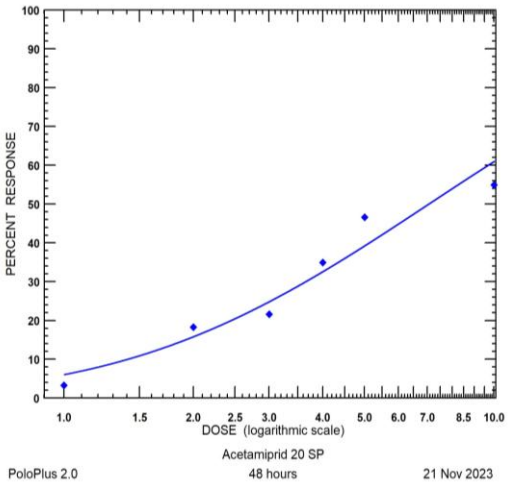


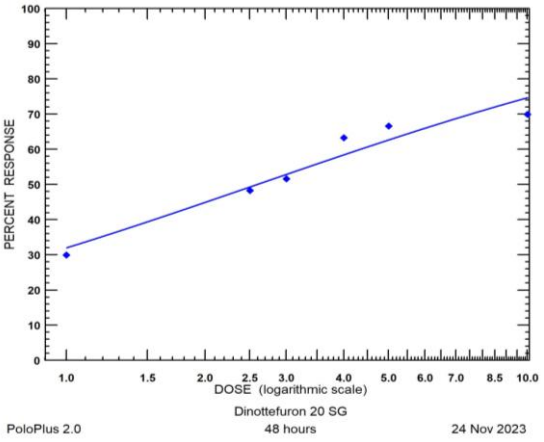
A) Imidacloprid 17.8 SL



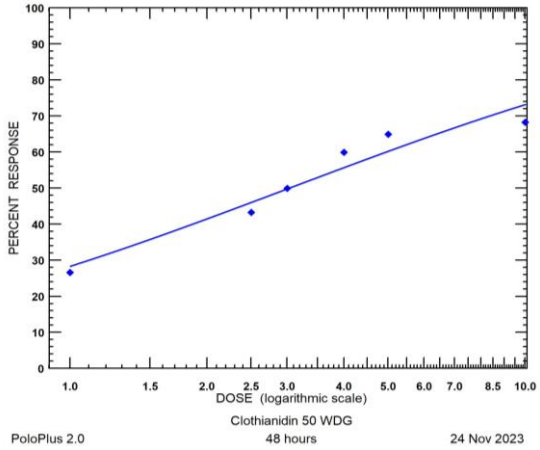
B) Thiamethoxam 25 WG



C) Acetamiprid 20 SP



D) Dinotefuron 20 SG



E) Clothianidin 50 WDG

Figure 2. *Concentration response mortality of honeybees to neonicotinoid insecticides (A-E) through indirect contact bioassay test (48 hr after treatment)*

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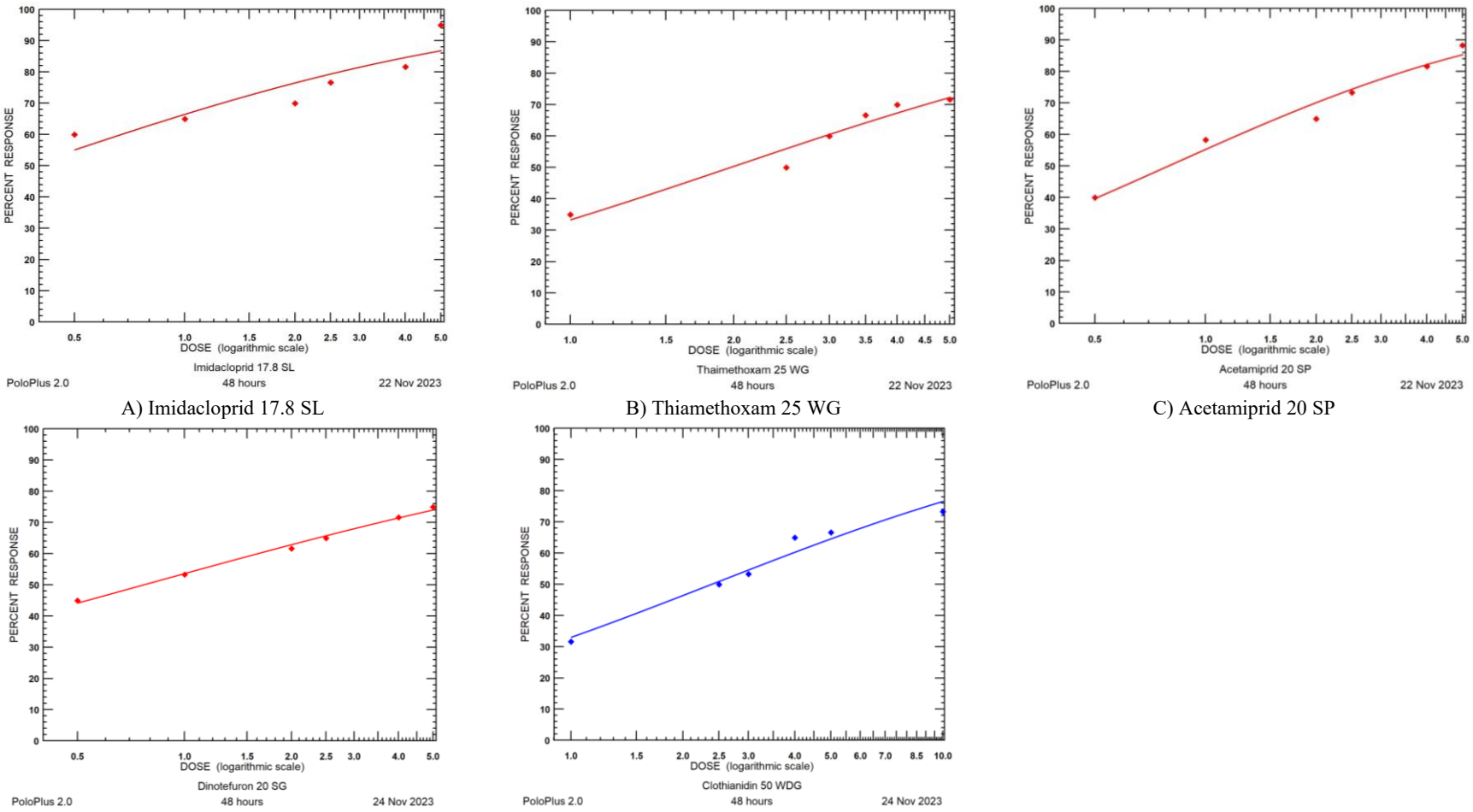

A) Imidacloprid 17.8 SL B) Thiamethoxam 25 WG C) Acetamiprid 20 SP

D) Dinotefuron 20 SG E) Clothianidin 50 WDG

Figure 3. *Concentration response mortality of honeybees to neonicotinoid insecticides (A-E) through topical bioassay test (48 hr after treatment). Neonicotinoid insecticides were tested at different concentrations and probit mortality was regressed on the log values of insecticide concentration. Calculated regression lines as given in the figures 1-3 indicate a homogeneous response of honeybee population to insecticides. Slope function is given in Table 2-4*

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Table 3. *Indirect contact toxicity of neonicotinoid insecticides to foragers of A. cerana indica*

SL No.	Insecticides	LC_{50} (ppm)	95% Confidence limits (Fiducial limits)		LC_{90} (ppm)	95% Confidence limits (Fiducial limits)		Slope function (± SEm)	Chi-square	Heterogeneity
			Lower (%)	Upper (%)		Lower (%)	Upper (%)			
1.	Imidacloprid 17.8 SL	0.54	0.27	0.79	6.35	4.18	13.83	1.205 ± 0.212	0.42	0.10
2.	Thaimethoxam 25 WG	1.33	0.78	1.80	11.76	7.84	25.59	1.355 ± 0.241	3.47	0.86
3.	Acetamiprid 20 SP	1.89	1.33	2.39	15.42	9.91	34.95	1.406 ± 0.231	1.62	0.40
4.	Dinotefuron 20 SG	1.22	0.56	1.75	6.40	4.47	13.76	1.781 ± 0.265	4.60	1.15
5.	Clothianidin 50 WDG	1.24	0.75	1.66	9.08	6.47	16.69	1.483 ± 0.248	3.23	0.80

Table 4. *Topical application toxicity of neonicotinoid insecticides to foragers of A. cerana indica*

SL No.	Insecticides	LC_{50} (ppm)	95% Confidence limits (Fiducial limits)		LC_{90} (ppm)	95% Confidence limits (Fiducial limits)		Slope function (± SEm)	Chi-square	Heterogeneity
			Lower (%)	Upper (%)		Lower (%)	Upper (%)			
1.	Imidacloprid 17.8 SL	0.33	0.16	0.49	1.91	1.49	2.67	1.688 ± 0.269	1.68	0.42
2.	Thaimethoxam 25 WG	0.40	0.13	0.68	8.35	4.82	28.50	0.978 ± 0.209	0.71	0.17
3.	Acetamiprid 20 SP	0.64	0.40	0.86	4.79	3.47	8.09	1.469 ± 0.219	2.21	0.55
4.	Dinotefuron 20 SG	0.47	0.21	0.72	6.20	4.01	14.41	1.147 ± 0.214	3.55	0.88
5.	Clothianidin 50 WDG	1.07	0.32	1.66	5.25	3.56	13.72	1.858 ± 0.279	6.80	1.70

Poisoning symptoms of selected formulated insecticides on Indian honeybees

Insecticide poisoning symptoms for selected test insecticide to Indian honeybee (*A. c. indica*) were documented during oral ingestion bioassay methods. Initially, bees were normal with uncoordinated movement after exposed to selected test insecticides but exhibited different kind of symptoms at 12 hr of post exposure including extended proboscis, full wing expansion, unhooked wings, extended legs, and rapid death (*Fig. 4(A, B, C)*).

Safety indices and relative toxicity of neonicotinoids to bees

Honeybees are essential pollinators for many cultivated crops and understanding the safety of insecticides during flowering is crucial to maximize their service. The safety index of neonicotinoid insecticides was assessed based on recommended spray

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concentrations provided by Central Insecticide Board and Registration Committee (CIB & RC) of government of India and LC_{50} values assessed in the present study. For oral ingestion, the safety indices of imidacloprid (0.76), dinotefuron (1.04), thiamethoxam (4.44), clothianidin (4.72) and acetamiprid (6.45) were assessed; imidacloprid and dinotefuron insecticides were demonstrated to be most toxic (*Table 5*). In indirect contact tests, imidacloprid(1.08) exhibited to be most toxic as compared to dinotefuron (4.88), clothianidin (4.96), thiamethoxam (5.32) and acetamiprid (9.45). For topical application, the indices of imidacloprid (0.66), thiamethoxam (1.60), dinotefuron (1.88), acetamiprid (3.20) and clothianidin (4.28); imidacloprid remained as most toxic. Acetamiprid was generally safer in oral and indirect contact tests, while clothianidin was safer in topical application.

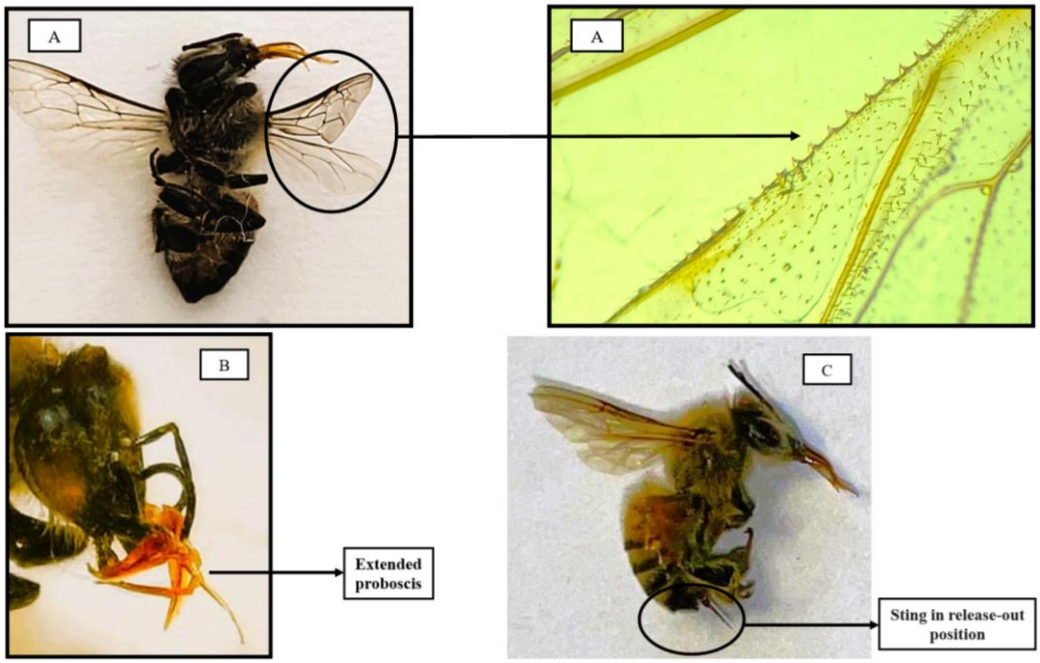

Figure 4. *(A) Full wing expansion (unhooked wings/disconnected hamuli), (B) Extended proboscis and (C) Sting in release-outposition in dead bees*

Table 5. *Safety indices of neonicotinoid insecticides against worker honeybees through topical application test*

SL No.	Insecticides	Recommended concentration*	Oral/ingestion tests		Indirect contact test		Topical application	
			LC_{50} (ppm)	Safety index	LC_{50} (ppm)	Safety index	LC_{50} (ppm)	Safety index
1.	Imidacloprid 17.8 SL	0.5 ml/l	0.38	0.76	0.54	1.08	0.33	0.66
2.	Thaimethoxam 25 WG	0.25 g/l	1.11	4.44	1.33	5.32	0.40	1.60
3.	Acetamiprid 20 SP	0.2 g/l	1.29	6.45	1.89	9.45	0.64	3.20
4.	Dinotefuron 20 SG	0.25 g/l	0.26	1.04	1.22	4.88	0.47	1.88
5.	Clothianidin 50 WDG	0.25 g/l	1.18	4.72	1.24	4.96	1.07	4.28

Note: * - recommended concentration as per Central Insecticide Board and Registration Committee (CIB&RC) of government of India.

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The relative toxicity of these neonicotinoids compared to the least toxic benchmarks showed that, for oral ingestion, clothianidin, thiamethoxam, imidacloprid, and dinotefuron were 1.09, 1.16, 3.39, and 4.96 times more toxic than acetamiprid (1.00), respectively (*Table 6*). In indirect contact tests, thiamethoxam, clothianidin, dinotefuron, and imidacloprid were 1.42, 1.52, 1.54, and 3.50 times more toxic than acetamiprid (1.00), respectively. For topical application, acetamiprid, dinotefuron, thiamethoxam, and imidacloprid were 1.67, 2.27, 2.67, and 3.24 times more toxic than clothianidin, respectively. These results highlight the varying degrees of toxicity among neonicotinoids with imidacloprid generally being the most toxic to bees across different exposure methods.

Table 6. *Relative toxicity of neonicotinoid insecticides against worker honeybees through topical application test*

SL No.	Insecticides	Oral/ingestion tests		Indirect contact test		Topical application	
		LC_{50} (ppm)	Relative toxicity	LC_{50} (ppm)	Relative toxicity	LC_{50} (ppm)	Relative toxicity
1.	Imidacloprid 17.8 SL	0.38	3.39	0.54	3.50	0.33	3.24
2.	Thaimethoxam 25 WG	1.11	1.16	1.33	1.42	0.40	2.67
3.	Acetamiprid 20 SP	1.29	1.00	1.89	1.00	0.64	1.67
4.	Dinotefuron 20 SG	0.26	4.96	1.22	1.54	0.47	2.27
5.	Clothianidin 50 WDG	1.18	1.09	1.24	1.52	1.07	1.00

Note: Relative toxicity = Insecticide with high LC_{50} /LC_{50} of respective insecticide

Discussion

Acute oral and contact toxicity of neonicotinoid spray formulations to honeybees

In the present investigation, the most common neonicotinoid spray formulations were evaluated *in vitro* for their toxicity to Indian bees through three different bioassay tests: oral, contact, and topical application. The study demonstrated a considerable effect of these methods on toxicity. Indian honeybees exposed to five selected neonicotinoids exhibited rapid movements during the initial hours after exposure followed by slow movements in later hours. Various poisoning symptoms were observed up to 12 hr after treatment in dead bees, including extended proboscis, full wing expansion, unhooked wings, extended legs, and rapid death. Similar symptoms have been documented for various neonicotinoids by earlier researchers (Bortolotti et al., 2003; Laurino et al., 2011; Pashte and Patil, 2017; Gonalons and Farina, 2018).

However, this is the first report on *Apis cerana indica*, which has significant applications in Indian subcontinental apiculture and pollination ecology. Variations in the toxicity within the group of neonicotinoids for different bioassay methods were also evident. In acute oral toxicity test, dinotefuron 20 SG and imidacloprid 17.8 SL was documented to be highly toxic to honeybees after 48 hr of exposure (LC_{50} value: 0.26 and 0.38 ppm, respectively) as against thiamethoxam and clothianidin which were exhibited least toxicity with higher LC_{50} value (LC_{50} value: 1.33 and 1.24 ppm, respectively). Present findings are conferred with earlier workers who registered less

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toxic of acetamiprid as it could belong to chloronicotinyls subclass contain N-cyano-amide radicle which gets metabolised in to less toxic metabolite for the bees and confers lower toxicity (Iwasa et al., 2004; Elbert et al., 2008; Tison et al., 2017).

In both indirect contact and topical application toxicity test, imidacloprid was found to be highly toxic to honeybees after 48 hr of exposure with LC_{50} value of 0.54 and 0.33 ppm and resulted higher mortality rates of bees compared to other neonicotinoids. Acetamiprid was found less toxic to bees when intoxicated through contact and it had exhibited highest LC_{50} value (0.64 ppm). Clothianidin was found less toxic to bees when they intoxicated through topical application due to partial agonist action on nicotinic acetylcholine receptors (Li et al., 2007).

Brown et al. (2006) compared the agonist actions of imidacloprid, clothianidin, and acetylcholine on nAChRs. As neonicotinoids, both imidacloprid and clothianidin have a similar mode of action. However, the present study showed that clothianidin was relatively less toxic to bees compared to imidacloprid in topical application, due to its partial agonist nature and chemical structure. Imidacloprid caused higher toxicity to bees through oral, indirect contact and topical application.

However, neonicotinoids with radicle N-nitro guanidine (dinoteuron, imidacloprid, thiamethoxam and clothianidin) are reported to be highly toxic to non-target insects (Elbert et al., 2008; Decourtye and Devillers, 2010). The highest LC_{50} value of acetamiprid for *A. c. indica*, indicated that acetamiprid could be the less toxic among neonicotinoids tested to bees. However, earlier worker called Suchail et al. (2000) who had documented oral LD_{50} value of imidacloprid at 48 hr were 4.80 ng/honeybee compared to other neonicotinoids.

The most toxic neonicotinoids contained a nitro substitution and within this group, imidacloprid was found more toxic to bees compare to other neonicotinoids. The higher level of toxicity of imidacloprid to the bees compared with other could likely be explained by the mode of action. The mode of action of neonicotinoids is by occupying the binding sites of nACHRs, blocking the neuro-transmitter acetyl choline (Ach), forming a complex with one or both sites (Brown et al., 2006) imidacloprid has a great ability to join nACHRs present in high density in insect nervous tissues with high agonist action.

A previous study on imidacloprid by Nauen et al. (2001) revealed an oral LD_{50} of 41 ng/honeybee at 72 hr of exposure for the highest dose resulting in 50 per cent mortality. Suchail et al. (2001) treated bees with imidacloprid at different doses ranging from 10 to 10,000 mg/kg resulting in LD_{50} values of 60 ng/honeybee at 72 hr and 40 ng/honeybee at 96 hr as the exposure period increased, the LD_{50} values decreased. Laurino et al. (2011) recorded oral LC_{50} and LD_{50} values of 0.07 ppm and 2.68 ng/honeybee for clothianidin and 0.12 ppm and 4.41 ng/honeybee for thiamethoxam, respectively at 48 hr of exposure.

Present findings are in line with the studies conducted by Bovi et al. (2018) where in contact lethal concentration of imidacloprid was 0.0308 ± 0.0218 µg/bee. Similarly, Laurino et al. (2011) recorded the indirect contact LC_{50} values at 48 hr of exposure as 2.96 ppm for clothianidin and 3.31 ppm for thiamethoxam. Khan (2005) obtained an LC_{50} value of 0.0035% for imidacloprid in *A. c. indica*. Costa et al. (2015) determined the topical LC_{50} value for *Melipona scutellaris* to be 2.01 ng a.i./µl for 24 hr and 0.81 ng a.i./µl for 48 hr. Tarek et al. (2021) reported a topical LD_{50} value of 0.034 µg/honeybee for clothianidin and 0.085 µg/honeybee for imidacloprid after 24 hr of treatment.

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It could be noted in present study that safety index values ranged from 0.76 (imidacloprid) to 6.45 (acetamiprid) in oral test, 1.08 (imidacloprid) to 9.45 (acetamiprid) in indirect contact test and 0.66 (imidacloprid) to 4.28 (clothianidin) in topical application. Thus, acetamiprid found to be the safer among the neonicotinoids in oral and contact exposure while clothianidin in direct contact (topical application) against foragers of honeybee. The safety index values for *A. cerana indica* observed here are comparable to *A. mellifera* (Pashte and Patil, 2017) safety indices for indoxacarb, dimethoate, cypermethrin, imidacloprid and fipronil with 0.443, 0.385, 0.370, 0.350 and 0.063, respectively. These results highlight the varying degrees of toxicity among neonicotinoids, with imidacloprid consistently being the most toxic to bees across different exposure methods. This underscores the need for more stringent regulations and careful application practices when using neonicotinoids, especially imidacloprid during the peak foraging activity of bees in general and Indian honeybee in particular. Moreover, the relative safety of acetamiprid in oral and indirect contact tests and clothianidin in topical applications, suggests their preference over neonicotinoids in field applications.

Conclusion

Study assessed the acute toxicity of various neonicotinoid insecticides to honeybees through oral ingestion, indirect contact and topical application revealing significant variations in toxicity levels among the tested neonicotinoids. Safety indices confirmed imidacloprid and dinotefuron as the most toxic across oral and contact exposure methods. Acetamiprid demonstrated higher safety through oral and indirect contact exposure and clothianidin in topical applications. Imidacloprid consistently emerged as the most toxic neonicotinoid. These findings underscore the need for careful selection and use of specific neonicotinoids, particularly during flowering periods when bees are most active to protect these essential pollinators. Although neonicotinoid spray formulations tested here are harmful to honeybees (*A. c. indica*), the wiseness lies in using comparatively safer ones. The data indicated that acetamiprid is relatively less toxic and imidacloprid is relatively more toxic to bees. Health risk assessment studies should be pursued to enhance understanding of the impact of neonicotinoids. Our data suggest that the chemical risk to bee pollinators can be minimized by choosing insecticides with lower toxicity for crop pest management.

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