Acute toxicity of Neonicotinoids and Carbamete on Honey Bee *Apis mellifera L*

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Abstract

Background and Objectives: The current study represents the first part of a Ph.D. research project conducted at The University of Guelph, Ontario, Canada in co-operation with the University of Babylon, Hilla – Iraq. The main objective of this part was to estimate the acute toxicity (LD 50) of clothianidin, imidacloprid (neonicotinoids), and carbaryl (Crabmeat) to honey bees. **Method:** The Three insecticides have been blamed for the colony collapse disorder (CCD) crisis, newly emerged adult bees were challenged orally and topically with a serial of doses of the three insecticides. Determination of the LD50 of each insecticide revealed that clothianidin was the most toxic insecticide to bees in oral and topical applications compared to the other two insecticides. **The findings:** The oral LD50 values were 0.004, 0.15, and 0.36 $\mu g/bee$ for clothianidin, imidacloprid, and carbaryl respectively. The topical LD50 values were 0.034, 0.085, and 0.24 $\mu g/bee$ for the three insecticides respectively Data were statistically analyzed by probit analysis using the US Environmental protection Agency statistical program (version 1-5).

***Keywords**: Honey bee, Neonicotinoids, Acute toxicity, LD50.*

Introduction

The massive loss of the honey bee colonies has been recorded worldwide and caused a decline in the abundance and diversity of the wide bees as well as honey bees [1]. Vanengelsdrop *et al.,*[2] called that phenomenon a colony collapse disorder (CCD). Many factors have been identified as potential causes or indicators of the colony collapse disorder, insecticides were the most to blame in crisis around the world [3]. The role of pesticides in the honey bee losses has recently regained consideration [4]. The neonicotinoids group of insecticides is relatively new and has increasingly being used worldwide – It is estimated that the current world annual sales of these compounds exceed one billion dollars and accounts for nearly 15% of the global insecticides market [5, 6]. More than (121) different pesticides were found in bee wax and pollen grains stored in combs [4]. Researchers considered neonicotinoids and carbamate the main factor for colony losses in many regions of the world [7, 8, 9]. Honey bees are regularly foraging 3-6 km from their colony [10], where they seek flowers, consequently, bees' exposure to insecticides is likely common Bees can be exposed to insecticides by two main exposure routes orally and contact. It was reported that the physiological fluids of the corn plants effectively transfer neonicotinoids such as (imidacloprid and clothianidin) from the coated seed to the guttation drops then to the foraging bees [11]. Contact exposure occurs when the flowers of treated plants are visited by bees [12]. Ninety percent of insecticides are neurotoxins, and neonicotinoids in particular, act as an agonist of acetylcholine occupying the binding site of nicotinic acetylcholine receptors (nAChRs) in the central nervous system, causing excitation and eventually paralysis leading to the death of affected insects [13, 14]. The present research represents part of a Ph.D. research project of Jarek (2013) the first author, conducted at the University of Guelph, Ontario, Canada in co-operation

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with the University of Babylon, Hilla, Iraq. The main objective of the present study is to determine the acute toxicity (LD50) of the three insecticides, imidacloprid, clothianidin, and carbaryl, that have been applied orally and topically on honey bees.

Material and Method

Honey bee colonies

Colonies of honey bees containing naturally mated queens of Buck fast strain were used as a source for bee workers. The colonies were free of brood diseases and were not treated with any chemicals before the experiments. Frames of late-stage capped brood were collected from the hives and placed individually in a wooden cage ($50 \times 7 \times 25\ cm$) with wire screen mesh on two sides. Cages were transferred to an incubator set at $(32 \pm 2°C \pm 10\% RH)$ .After 24 h, all newly emerged bees were brushed off the frames into a plastic container ($60 \times 38 \times 40\ cm$) and were kept in the container shortly to be used in experiment insecticides. Three insecticides (purity > 99%) were obtained from Sigma-Aldrich Company .These insecticides include imidacloprid (1-(6 chloro -3- pyridylmethgl) - N – nitroimda zolidin - Z – ylideneamine) , clothianidin (1- (2-chloro -1 , 3-thiazol -5-ylmethyl) -3- methyl - z – nitroguanidine) and carbaryl (1-napthyl - N – methyl carbamet . Insecticides were dissolved with sterile d H_2o to prepare a stock solutions (1000 μl, insecticide/ solvent) stocksolutions were diluted in serial dilutions to prepare 50% sucrose syrup for oral treatments and a preliminary test was conducted to choose a range of doses that were used later for estimating LD50 for the insecticides .For the doses of contact application the serial dilutions were made with d H_2o. For this test , low and high doses (oral 0.1 and 100 ppm , and contact 0.5 and 500 ppm) were applied on emerged bees for each insecticide.

Oral application

Based on the results of the preliminary test , six doses were selected for each insecticides which are (0.001 , 0.01, 0.1 , 1.0 , 2.0 , 3.0 ppm) (1.0 , 5.0 , 15.0 , 20 , 0 , 25.0 , 50.0 ppm) and (1.0 , 5.0 , 15.0 , 25.0 , 30.0 , 50.0 ppm) .For clothianidin , imidacloprid , and carbaryl, respectively. The actual doses received by each bee in migrojams are (0.00001 , 0.0001 , 0.001 , 0.01 , 0.02 , 0.03 $\mu g/bee$) , (0.01 , 0.05 , 0.15 , 0.2 , 0.25, 0.5 $\mu g/bee$) and (0.01 , 0.05 , 0.15 , 0.25 , 0.3 , 0.5 $\mu g/bee$) for the three insecticides respectively .Bees of the control received only 10 Ml of 50% sucrose syrup. Three replications for each dose were used. Treated bees were maintained in cages provided with two gravity feeders containing 20 ml 50% sucrose syrup and the other contained d H_2O. Cages were incubated in $(32 \pm 2°C\ and\ 50 \pm 10\%\ RH)$ Bees were allowed to feed ad libitum. Mortality of bees was recorded 24 h after treatment [8].

Topical application

A direct topical application was utilized to measure the acute toxicity of the three insecticides in honey bees. Based on the results of the preliminary test, five doses of each insecticide were selected. The doses were (0.5 , 5.0 , 15.0 , 0.25 , 50.0 , ppm) , (5.0 , 25.0 , 50.0 . 125.0 , 250.0 ppm) and (5.0 , 50.0 , 125.0 , 150.0 , 250.0 ppm) for clothianidin , imidacloprid and carbaryl respectively .Insecticides solutions in d H_2o (vehicle) were administered individually as a single topical amount of 2μl (H_2o + insecticides) to the dorsal surface of the thorax of bees using micropipettes.The actual doses of each insecticides received by the bees corresponded to (0.001 , 0.01 , 0.03 , 0.05 , 0.1 $\mu g/bee$) ,(0.01 , 0.05 , 0.1, 0.25, 0.5 $\mu g/bee$) and (0.01 , 0.1 , 0.25, 0.3, 0.5 $\mu g/bee$) for clothianidin, imidacloprid and carbaryl respectively: Control bees only received the same amount of d H_2o . Treated bees were placed and maintained in a wooden cage and incubated as per oral applications. Bee mortality was recorded after 24 has above. Three replications per dose per insecticide were used.

Statistical Analysis

The estimation of LD50 and inverse 95% confidence limits (CL) for the three insecticides were determined by probit analysis, using the US Environmental Protection Agency statistical program (Version 1.5) (US EPA 1992). Bees mortality percentage was calculated as arcsine square root and subjected to analysis of variance using a completely randomized design.

Findings

Acute toxicity of insecticides to honey bees :

The LD50 for acute toxicity of clothianidin, imidacloprid, and carbaryl was investigated using two different applications, oral and topical. Bees exhibited neurotoxic symptoms, such as trembling, uncontrolled movements, and lack of coordination. The symptoms were more apparent in oral application trials. The highest doses of imidacloprid and clothianidin caused extensive vomiting in honey bees. All doses for both modes of applications of the insecticides caused a significantly higher mortality rate of bees compared to the control (Table 1). The LD50 of the three insecticides for the oral and topical applications are presented in (Table 2).

Table 1: Fad p values from ANOVA (analysis of variances) conducted on arcsine square-root transformed data for the average combined effect of 6 or 5 different doses of the three insecticides on the percent of honey bee mortality in 24 h post-treatment.

Insecticides	F value	P-value	Application
Clothianidin	166.8 39.12	0.0001 0.0001	Oral Topical
Imidacloprid	207.3 47.7	0.0001 0.0001	Oral Topical
Carbaryle	177.7 83.9	0.0001 0.0001	Oral Topical

Oral application

It was found that clothianidin was significantly more poisonous to honey bees than imidacloprid (>37 fold) and carbaryl (90 fold) and imidacloprid was significantly more toxic to bees than carbaryl (>2fold) at 24 h post-treatment (Table 2). The LD50 of clothianidin was 0.004 $\mu g/bee$while for imidacloprid and carbaryl these values were 0.15 and 0.36 $\mu g/bee$respectively .

Topical application

Similar to the oral application, clothianidin was significantly more toxic to honey bees compared to imidacloprid (>2 fold) and carbaryl (>7fold) at 24 h pt., but imidacloprid was significantly more than carbaryl (>2.5 fold). The LD50 of clothianidin was 0.034Mg/bee. The LD50 values for imidacloprid and carbaryl were 0.085 and 0.243 Mg/bee respectively (Table 2)

Table 2. LD50 of the three insecticides in two applications on bees after 24 h post-treatment. values were calculated by probit analysis 95% CL. in micrograms /bee

Insecticides	LD50 $\mu g/bee$	95% CL	Slope $\pm$SE	Intercept	Application
Clothianidin	0.004 0.034	$0.003 - 0.005$ $0.029 - 0.041$	0.002 ± 0.0001 0.005 ± 0.0006	0.003 -0.002	Oral Topical
Imidacloprid	0.15 0.085	$0.12 - 0.20$ $0.074 - 0.098$	0.005 ± 0.0007 0.003 ± 0.0003	0.007 -0.002	Oral Topical
Carbaryl	0.36 0.243	$0.032 - 0.038$ $0.222 - 0.262$	0.006 ± 0.0008 0.004 ± 0.0009	0.012 0.006	Oral Topical

The specific mortality rates for each dose of insecticide are presented in (Table 3-5). The current finding revealed that the toxicity of the three insecticides to bees varied according to the mode of application. Even though imidacloprid and clothianidin are both neonicotinoids, their toxicity to bees depended on the route of exposure. Imidacloprid exhibited higher toxicity in topical compared to oral application. In contrast, clothianidin showed more toxicity to bees in oral application than topical. Carbaryl was more toxic to bees in topical compared to oral application.

Table 3. mean percent mortality of honey bees in 24 h $(\pm SE)$ when treated orally or topically with six doses of clothianidin respectively.

Oral applications			Topical applications		
Doses $\mu g/bee$	N	% Bees mortality $\pm$ SE	Doses $\mu g/bee$	N	% Bees mortality $\pm$ SE
0.01	3	8.05 ± 4.2	1.0	3	19.01 ± 4.8
0.1	3	12.77 ± 1.4	10.0	3	25.45 ± 2.2
1.0	3	22.39 ± 6.3	30.0	3	41.52 ± 2.8
10.0	3	60.29 ± 1.9	50.0	3	63.71 ± 7.8
20.0	3	70.97 ± 3.77	100.0	3	83.44 ± 34
30.0	3	79.09 ± 2.01			
Control		000	Control	3	000

Table 4. Mean percent mortality of honey bees in 24 h $(\pm SE)$ when treated orally or topically with six and five doses of imidacloprid respectively

Oral applications			Topical applications		
Doses $\mu g/bee$	N	% Bees mortality $\pm$ SE	Doses $\mu g/bee$	N	% Bees mortality $\pm$ SE
10.0	3	3.32 ± 3.1	10.0	3	11.37 ± 1.4
50.0	3	20.44 ± 3.1	50.0	3	14.9 ± 2.5
150.0	3	42.3 ± 2.2	150.0	3	30.4 ± 0.2
200.0	3	44.79 ± 2.8	250.0	3	49.2 ± 2.5
250.0	3	58.95 ± 3.7	500.0	3	80.11 ± 0.9
500.0	3	73.2 ± 4.4			
Control	3	000	Control	3	000

Table 5. mean percent mortality of honey bees in 24 h $(\pm SE)$ when treated orally or topically with six and five doses of carbaryl respectively

Oral applications			Topical applications		
Doses $\mu g/bee$	N	% Bees mortality $\pm$ SE	Doses $\mu g/bee$	N	% Bees mortality $\pm$ SE
10.0	3	11.7 ± 6.2	10.0	3	11.3 ± 1.4
50.0	3	25.9 ± 1.8	100.0	3	24.6 ± 5.5
150.0	3	49.2 ± 0.4	250.0	3	38.1 ± 3.7
200.0	3	75.9 ± 1.2	300.0	3	48.4 ± 1.2
300.0	3	77.5 ± 1.3	500.0	3	82.9 ± 3.7
500.0	3	79.6 ± 1.3			
Control	3	000	Control	3	000

Discussion

Acute toxicity of insecticides to bees:

Oral or contact intoxication by three insecticides induces rapidly neurotoxic symptoms such a movement co-ordination problems, trembling, and tumbling. The same symptoms had already been reported for various neonicotinoids[13, 15]. The highlighted disabling behavior could irreversibly affect honey bee survival in the field due to external dangers such a cold, prediction, and diseases. Bee memory and communication abilities might be impaired [13, 16]. Clothianidin was highly toxic to honey bees after 24 h of exposure (oral LD 50=0.004 $\mu g/bee$; topical LD 50=0.034 $\mu g/bee$) .Colthianidin exhibited higher mortality rates of bees compared to imidacloprid and carbaryl , regardless of the method of application . This finding is similar to that of Bailey *et al*.,[17]. The calculated LD50 for the acute oral toxicity of clothianidin of the present work is with in ranges reported in literatures (0.002-0.004 $\mu g/bee$) at 24 h and 0.003 $\mu g/bee$ [7]. In topical application clothianidin was less toxic to bees compared to that reported by Iwasa *et al*., [8], their LD50 was 0.022 μg/bee at 24 h pt. The higher level of toxicity of clothianidin to the bees compared with imidacloprid and carbaryl 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 transmitter (Ach), forming a complex with one or both sites [18]. Clothianidin has a great ability to join nACHR sites, while imidacloprid has a partial agonist action on nicotinic acetylcholine receptors [19]. Brown *et al*., [18] compared the agonist action of imidacloprid, clothianidin, and acetylcholine on nACHR, they found that clothianidin had a super-efficiency action on insects neurons with maximal currents evoked of 56% larger than those evoked by acetylcholine and imidacloprid. Therefore, clothianidin act antagonist to acetylcholine blocking its action on nACHRs which caused toxic symptoms and death As neonicotinoids, compound imidacloprid has a similar mode of action that of clothianidin, however, the present study showed that imidacloprid was relatively less toxic to bees compared with clothianidin in oral and topical applications due to the partial agonist and the chemical structure of the insecticide. Imidacloprid caused higher toxicity to bees by topical exposure than by oral exposure.

The present finding coincides with Suchail *et al*.,[9] but is not in agreement with Decourtye *et al*.,[20] who had recorded higher toxicity of imidacloprid via ingestion route than by contact route. The differences in LD50 values recorded for oral or topical application with those reported by other researchers could be attributed to the age of the treated bees which was older than that used in the present study.Older bees have a rigid cuticle that exhibits resistance to the penetration of insecticides. The relatively less toxic mechanism of carbaryl to bees compared to that of the other two Insecticides in both applications could be attributed to the chemical

component, action groups, and metabolism by insects. In general, the difference in oral and contact toxicity values exhibited in the present study and those reported by other studies could be associated with the variation in detoxification capacity of the honey bee colonies. Thus intracolony and intercolony variations for insecticides can result in differences in oxidative metabolism between honey bee .

The calculated LD50 values of the three tested insecticides were similar or lower than the residual amounts of these pesticides that have been detected on bees collected in field poisoning cases in crops treated with the same products [21] . Reported 0.674 and 3.66 $\mu g/bee$ as residues of clothianidin and imidacloprid in dead bees respectively. The residue of carbonyl was 21.4 $\mu g/bee$. This indicates that the results of the present study represent realistic expectations of the effects of these insecticides on honey bee health. The use of neonicotinoid insecticides should be banned due to their high toxicity and long residual effects on the bee's diversity.

Conflict of Interest: None declared.

Source of Funding: Nil.

Ethical Clearance: University of Guelph, Ontario, Canada, and University of Babylon, Hilla – Iraq

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