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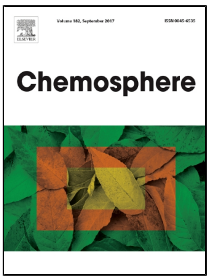

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PII:	S0045-6535(17)30947-5
DOI:	10.1016/j.chemosphere.2017.06.045
Reference:	CHEM 19439
To appear in:	*Chemosphere*
Received Date:	09 March 2017
Revised Date:	10 June 2017
Accepted Date:	12 June 2017

Please cite this article as: Yanmei Liu, Sihong Liu, Hui Zhang, Yanping Gu, Xuesheng Li, Mingyuan He, Huihua Tan, Application of the combination index (CI)-isobologram equation to research the toxicological interactions of clothianidin, thiamethoxam, and dinotefuran in honeybee, *Apismellifera* ., *Chemosphere* (2017), doi: 10.1016/j.chemosphere.2017.06.045

Application of the combination index (CI)-isobologram equation to research the toxicological interactions of clothianidin, thiamethoxam, and dinotefuran in honeybee, *Apismellifera*.

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Abstract: Due to complex pest control scenarios and the needs of agricultural production, different neonicotinoids may be used in certain agricultural applications. Consequently, honeybees may be exposed to these substances through distribution throughout plant tissues via the vascular system through several pathways, such as surface water, the exudates excreted from plants, and air pollution via drift of dust as well as contaminated pollen and nectar. In the current study, the single and combined toxicity of clothianidin, dinotefuran, and thiamethoxam to honeybees was examined after 48 h exposure by the acute oral method and combination index (CI)-isobologram equation. At the 48 h interval, our results showed that 1) the order of toxicities for the single insecticides was ranked as clothianidin > thiamethoxam > dinotefuran and that 2) all binary and ternary combinations showed synergism or additive effect at the effect (f_a) 0.5. Therefore, our results not only provided meaningful guidelines in evaluating the safety risk of the mixtures of the three neonicotinoids towards honeybees but also suggested that there is a significant interest in the study of mixture toxicities of neonicotinoids against honeybees because risk assessment of neonicotinoids against honeybees conducted only in individual insecticides may underestimate the realistic toxicity.

Key words: combined toxicity, acute oral toxicity, clothianidin, dinotefuran, thiamethoxam

1. Introduction

The honeybee, *Apismellifera,* plays a critical role in crop pollination. The pollination by honeybees commercially translates to billions of dollars yearly as pollination improves crop yield and maintains plant species in ecosystems (Klatt et al., 2014). The incidents of death of honeybees are of considerable concern since reductions in bee populations around the world may have adverse effects on agriculture and the environment. Therefore, numerous governments and research organizations have commenced programs and research to study the losses of bees to elucidate the underlying causes.

It is suspected that numerous stress ingredients are implicated in the decrease of honeybee populations. The use of agricultural chemicals has frequently been identified as a factor impacting on bee health (Goulson et al., 2015; Sanchez et al., 2016). In particular, neonicotinoids, which are systemic, have specifically been singled out for blame in several countries because they can have a wide-ranging adverse impact on pollinators (Blacquière et al., 2012; Sandrock et al., 2014). Importantly, neonicotinoids are used to control sucking insect pests as they can distribute throughout plant tissues via the vascular system (Elbert et al., 2008). Consequently, neonicotinoids can appear in pollen and nectar as well as in guttation fluids (Chen et al., 2014; Elbert et al., 2008; Goulson, 2013). Hence, honeybees may come in to contact with these substances transdermally, orally, and internally, following which these substances are transported to the hive through several pathways (Desneux et al., 2007): via water (Samson et al., 2014), via the exudate that is secreted from plants (Girolami et al., 2009), via atmospheric pollution via drift of dust which coats seeds during seeding (Alix et al., 2009), and via contaminated nectar and pollen (Botìas et al., 2015). To date, different neonicotinoids and metabolites have been identified in honeybees, honey, and bee bread, including clothianidin, thiamethoxam, dinotefuran, and imidacloprid (Chen et al., 2014; Kasiotis et al., 2014). Therefore, the possibility for synergistic interactions in the hive appears likely given the fact that adult bees are able to fly freely and to forage in the wild over a certain range within a

day or over a short period (Couvillon and Ratnieks, 2014), which results in honeybees coming into contract with toxicants. Numerous studies have suggested that neonicotinoids can seriously influence the health (Kasiotis et al., 2014) and performance (Sandrock et al., 2014) of bees, the population development of a colony (Rundlöf et al., 2015), and behaviors involved in foraging (Hladik et al., 2016), learning, and homing (Williamson and Wright, 2013). It is possible that those adverse influences on honeybees could result in damage of the colony structure as time passes. Few past pesticide toxicity studies have evaluated the risks of orally transmitted combined toxicity of neonicotinoids to adult bees (Christen et al., 2016; Zhu et al., 2014); however, most assessments of neonicotinoid toxicity to honeybees were based on exposing honeybees to single neonicotinoids, which might underestimate the real toxicity of neonicotinoids in agriculture. The results of surveys have shown that honeybees are unable to discriminate against neonicotinoids and have an appetite for foods containing neonicotinoids (Kessler et al., 2015). Consequently, co-occurrences of neonicotinoids might pose a higher risk to honeybees than single neonicotinoids or other chemicals. Therefore, the purpose of present study was to evaluate the mixture effect of ordinary neonicotinoids on adult bee survival. For the sake of simulating realistic exposure scenarios of honeybees to polluted food, we selected the three most continually detected neonicotinoids with which bees might come into contact: 1) clothianidin; 2) thiamethoxam, and; 3) dinotefuran (Chen et al., 2014). We tested these neonicotinoids solely and in total combinations through acute dietary exposure at concentrations ranging from those causing complete deaths of exposed honeybees to those concentrations that had little effect on survival.

Clothianidin with translaminar and root systemic activity can be used as soil, foliar, paddy, and seed treatment for control of sucking and chewing insects, such as planthoppers, stink bugs, aphids, and whiteflies in rice, maize, rape, fruit, vegetables, and citrus (ePM, 2011). Thiamethoxam with systemic activity is rapidly assimilated into the plant and transported acropetally in the xylem when it coming into contact with plant. This pesticide can be used as foliar and soil treatments for the control of flies, aphids, whiteflies, thrips, ricehoppers, and ricebugs, as well as some

lepidopterous species (ePM, 2011). Dinotefuran, which is a systemic pesticide with contact and oral action, can readily be assimilated into the plant and transported acropetally, and is used for controlling a series of sucking insects, including Coleopera, Diptera, and certain Lepidoptera in agriculture (ePM, 2011). In particular, the morality rate of honeybee was chosen as the toxicity endpoint. Moreover, for the purpose of identifying and quantifying the interactions between the three neonicotinoids, data were analyzed by the combination index (CI) equation (Chou, 2006). Finally, our results will provide valuable information for the conservation of honeybees and will contribute to the development of appropriate guidelines for testing the effect of these neonicotinoids in agriculture.

2. Materials and Methods

2.1 Test organisms

Young and healthy adult worker bees of the same colony, which were adequately fed, were gently captured from the same queen-right colony that had not been treated with chemical substances within 4 weeks. The collected bees were randomly assigned to wire mesh cages (dimensions: 12 cm × 8 cm × 8 cm) and randomly placed in an experimental room at 25 ± 2 °C and 60 ± 10% relative humidity in the dark. The bees were starved for 2 h prior to the beginning of the test.

2.2 Test pesticides

Dinotefuran (CAS-No. 165252-70-0, 95% TC), clothianidin (CAS-No. 210880-92-5, 96% TC), and thiamethoxam (CAS-No. 153719-23-4, 95% TC) were all supplied by the Hunan Research Institute of Chemical Industry (Changsha, China). Stock solutions of each of the aforementioned insecticides were dissolved in deionized water and diluted in 500 g/L (50% w/v) sucrose solution. Each stock solution was diluted to six test concentrations using calibrated micropipettes and volumetric flasks.

2.3 Toxicity test methods

The Organisation for Economic Co-operation and Development (OECD) guidelines for the testing of chemicals, honeybees, and acute oral toxicity tests were used (OECD, 1998). The procedure of the method is described below. First, six replicate test groups of fifteen bees were dosed with each concentration, including the control.

Each test group of bees was then administered a 300 µL test solution by a glass tube (dimensions: 40 mm long and 10 mm wide with the openendnarrowed to a diameter of approximately 2 mm). In addition, the weight of the treated diet per test group was recorded before providing the test solution to the groups. Once consumed, the feeder was withdrawn from the test cage and the feeder was replaced with that consisting of sufficient sucrose solution alone. For some groups, bees may have consumed little or no food at higher concentrations. After a maximum of 6 h, unconsumed treated diets were replaced with the sucrose solution alone. The weight of unconsumed treated diet was measured at the end of exposure. The intake of neonicotinoids by the bees was evaluated by measurement of the weight of treated food consumption, and finally converted to dose in µg active ingredient/ bee when conducted dose and mortality statistical analysis.

. The test duration of 48 h began with sucrose solution alone. If a rise in mortality exceeded 10% after the first 24 h, the duration of the test was prolonged to 96 h at most. Mortality was recorded at 24 h and 48 h after the administering of a given dose. For a valid experiment, the average mortality of the control was required to be within 10% at the end of the experiment.

In a pilot experiment, bees were exposed to a series of concentrations of individual insecticides to identify the range of concentration that produce 0–100% mortality at 48 h post exposure. Six desired concentrations and a control were then used for determining the medial lethal concentration (LD_{50}) value of each single insecticide at 48 h post exposure.

2.4 Mixture toxicity

The constant combination ratio LD_{50}:LD_{50} or LD_{50}:LD_{50}:LD_{50} was chosen to analyze the binary and ternary toxicity, respectively, so that the effects of individual insecticides within the combination would be approximately equal. In addition, insecticides were conducted at six concentrations with a dilution factor of 1.65. Tests with the same exposure duration were conducted simultaneously to avoid experimental variability.

2.5 Experimental design for insecticide combinations

Solutions of dinotefuran(D), clothianidin(C), and thiamethoxam(T), which were prepared as described above, were used in individual and binary combinations (C + D; D + T; C + T) and ternary combinations (C + D + T). Bees were exposed to a series of dilutions of individual insecticides and their binary and ternary combinations with a constant combination ratio LD_{50}:LD_{50} or LD_{50}:LD_{50}:LD_{50}, respectively.

2.6 Median-effect and combination index (CI)–isobologram equation for determining individual and combined toxicities

The individual and combined toxic effects of insecticides against honeybees were assessed using the median-effect equation given as follows (Chou and Talalay, 1984):

$$f_a / f_u = (D / D_m)^m, \quad 1$$

where D is the dose of an insecticide, f_a is the mortality influenced by D (percentage mortality / 100), f_u is the survival rate uninfluenced D($f_u = 1 - f_a$), D_m is the LD_{50}, m is the coefficient determining the shape of the dose-effect relationship, and $m < 1$, $m > 1$, and $m = 1$ signify flat sigmoidal, sigmoidal, and hyperbolic dose-effect curves, respectively. Rearranging Equation 1, we could obtain the following:

$$f_a = 1 /[\ 1 + (D_m / D)^m] \quad 2$$

$$D = D_m[f_a / (1 - f_a)]^{1/m} \quad 3$$

Therefore, if we know the values for m and D_m, we can easily determine the effect (f_a) for any given dose (D) in Equation 2, and the dose (D) for any given effect (f_a) in Equation 3.

In addition, by plotting $x = \log(D)$ versus $y = \log(f_a / f_u)$ according to the logarithm form of Equation 1, we obtain the median-effect plot as follows:

$$\log(f_a / f_u) = m \log(D) - m \log(D_m) \quad 4$$

In the median-effect plot (Equation 4), we can easily determine the (D_m) and m because the (D_m) is the antilog of the x-intercept and m is the slope. As described above, $m < 1$, $m > 1$, and $m = 1$ signify flat sigmoidal, sigmoidal, andhyperbolic dose-effect curves, respectively. In addition, the linear correlation coefficient (r) of the

median-effect plot can manifest the conformity of the data to the median-effect plot, and r = 1 shows excellent conformity.

Therefore, we can easily calculate the combination index (CI) values using the CI equation for a combination of n insecticides, which is given as

$$^{n}(\mathrm{CI})_x = \sum_{J=1}^{n} \frac{(D)_j}{(D_x)_j} = \sum_{J=1}^{n} \frac{(D_x)_{1-n}\ \{[\mathrm{D}]_j / \sum_{i}^{n}[\mathrm{D}]\}}{(D_m)_j\ \{(fax)\ j / [1-(fax)\ j]^{1/mj}\}}, \quad 5$$

where $^{n}(\mathrm{CI})_x$ is the combination index for n insecticides at x% effect (f_a), $(D_x)_{1-n}$ is the sum of the doses of n insecticides causing x% effect (f_a) in combination, $[D]_j / \sum_{1}^{n}[D]$ is the proportionality of the dose of individual n insecticides causing x% effect (f_a) in combination, $(Dm)j\{(fax)j / [1 - (fax)j]^{1/mj}\}$ is the dose of individual insecticides causing x% effect (f_a), f_{ax} is the fractional effect (f_a) at x% effect (f_a), D_m is the antilog of the x-intercept, and m is the slope of the median-effect plot mentioned above, where CI > 1, CI < 1, and CI = 1, indicating antagonism, synergism, and additive effect, respectively.

2.7 Analysis of results

The computer program CompuSyn (Chou and Martin, 2005) was used to calculate the parameters including the dose–response curve parameters, CI values, F_a-CI plot representing CI versus f_a, the fraction influenced by a special dose, and the polygonogram, which is a polygonal graphic representation describing antagonism, additive effect, or synergism for the insecticide combination.

3 Results

We studied the nature of interactions for a series of three neonicotinoids within the range of effect in total combinations in bees following the Honeybee Acute Oral toxicity test method and the CI-isobologram method (Chou and Talalay, 1984; Chou, 2006). The mortality rates of the controls were 6.67% in all acute toxicity tests, which demonstrated the reliability of the tests. The dose–response curve parameters (D_m, m,

and r) of the three neonicotinoids individually and their total combinations, and mean CI values of total combinations were concisely summarized (Table 1). All single neonicotinoids and their combinations could fit to the median-effect equation with sigmoidal shape dose-response curves (Fig. 2). The parameter D_m, which is analogously equal to the LD_{50} value, was the dose that produced a 50% mortality rate in honeybees. On the basis of the D_m values, the order of toxicities for the three single insecticides was ranked C > T > D at 48 h post exposure. The m was the Hill coefficient that is used for determining the shape of the dose-effect relationship: sigmoidal (m > 1), negative sigmoidal (m < 1), or hyperbolic (m = 1). In the present results, all single insecticides and their mixtures yielded sigmoidal shapes with analytical parameters m > 1. The r values, representing the linear regression correlation coefficient of the median-effect plots, exceeded 0.906, showing the good conformity of the data to the median-effect principle.

The D_m and m of all single insecticides and their binary and ternary combinations were used to calculate antagonism or synergism grounded on Equation (5) (Chou, 2006). CI values were recorded at LD_{10}, LD_{50}, and LD_{90}, indicating the doses required for producing 10%, 50%, and 90% mortality rates of honeybees, respectively (Table 1).

The F_a-CI plot, which depicts the CI values versus effect levels (f_a) affected by single insecticides or in combination with respect to controls for all binary and ternary combinations, also showed the interaction type of mixture insecticide(synergism, antagonism, and additive effect) against bees, revealed at 48 h post exposure, respectively (Fig. 2). The results of 48 h post exposure were described as follows:1) the ternary C + D + T combination exhibited antagonism at a effect (f_a) less than 0.25, approaching additive effect at effect (fa) between 0.25 and 0.55, synergism at higher effect (f_a); 2) the binary combination C + T showed a additive effect at effect (f_a) less than 0.35, which gradually became synergism at a higher effect (f_a), and; 3) the binary combination C + D exhibited a additive effect at effect (fa) less than 0.3, which showed synergism at a higher effect (fa); 4) the binary D + T combination showed an antagonism at effect (fa) less than 0.5, approaching additive effect beyond that effect

(fa), synergism at effect (fa) more than 0.8. The combined effect and selected average CI values includingLD_{10}, LD_{50}, and LD_{90} at three representative effects levels (f_a) were summarized at 48 h post exposure (Table 1).

The computer software CompuSyn is capable of simulating a graphic termed a polygonogram that can show combination results at any effect (f_a) by a semi-quantitative approach. In this way, we could intuitively inspect the effect of combinations of more insecticides before the experiments were conducted based on the combination results exhibited in the graphic. The trigonal polygonograms for the interactions of combinations were exhibited at 0.1, 0.5, and 0.9 representative effect levels (f_a), both at 48 h post exposure (Fig. 3). The polygonograms revealed interactions of all the binary and ternary combinations in the whole range of effect (f_a) at 48 h post exposure (Fig. 3).

4 Discussion

To date, few studies have investigated the joint toxicity of neonicotinoids on honeybees. The combination index (CI)–isobologram equation allows the prediction of the joint action at all effect levels for the combinations of several drugs without giving their chemical structure, mechanism of action, and any further information (Chou, 2006). Thus, this method has been widely used for studying pesticide interactions. For instance, the applications of the CI method in environmental risk assessment have been extended to different organisms, such as photosynthetic aquatic organisms, aquatic bioluminescent organisms, and earthworms (Chen et al., 2014; 2010; González-Pleiter et al., 2013; Rodea-Palomares et al., Wang et al., 2015). By using this method, we studied the nature of interactions for a series of effect levels of three neonicotinoids in total combinations in honeybees. The interaction of three neonicotinoids in bee workers has not previously been reported. The present results showed that the nature of all interactions indicated synergism or additive effect. This phenomenon was difficult to explain because the CI method only allows the quantitative determination of the interaction types, including synergism, additive effects, and antagonism; however, it does not elucidate how and why the interaction occurs and what the mechanism of the interaction is, which requires an alternative

296 method(Chou, 2006). To begin with, the phenomenon that antagonism and synergism
297 showed at effect (fa) 0.1 and effect (fa) 0.9 respectively is difficult to explain due to
298 the fact that it is difficult to observe an antagonism and a synergism when the
299 mortality is close to 0% and 100%, respectively. In addition, the additive effect
300 related to the binary combination D+T that exhibited nearly additive effect and
301 ternary combination C+D+T that showed nearly slight synergism, might be explained
302 by the competitive interaction between dinotefuran and thiamethoxam, suggesting
303 dinotefuran and thiamethoxam binding to the same site, but clotuianidin binding in a
304 way, or to a site different from that of dinotefuran and thiamethoxam (Kayser et al.,
305 2004). Moreover, the synergistic effect of a toxicant within honeybees may be
306 associated with the following described below.

307 First, it is plausible that a mixture of neonicotinoids in sugar might be more
308 attractive and consumed by honeybees than single neonicotinoids in sugar when
309 honeybees are first exposed to the toxicant. This hypothesis is supported by the study
310 of Kessler et al.(2015), who showed that honeybees preferred to consume sucrose
311 solutions including thiamethoxam and imidacloprid across a broad range of
312 concentrations over sucrose alone, in spite of the high concentrations of
313 thiamethoxam and imidacloprid that increase their morality. Consequently, honeybees
314 experienced more rapid poisoning with the accumulation of toxicants and experienced
315 greater mortality than by exposure to the single toxicants under the same conditions.
316 This does not reflect an enhancement of the consumption of the drug at the end of the
317 test because the consumption of these mixture compounds by honeybees accelerated
318 the process of poisoning, leading to a decrease in the food consumed. The preference
319 of the honeybees for solutions containing a mixture of neonicotinoids is perhaps
320 related to the pharmacological action of their compounds on nicotinic acetylcholine
321 receptors (nAChRs) in the brains of the honeybees.

322 Second, one rationale behind these synergistic interactions is that the parent of the
323 drugs might be quickly metabolized into other chemicals, which perhaps shows high
324 affinity to nAChRs in the brains of bees. For example, thiamethoxam was rapidly
325 metabolized to clothianidin, the predominant neonicotinoid, which showed a high

affinity to nAChRs when orally administered to 5th instar *Spodopterafrugiperda* larvae (Nauen et al., 2003). Additionally, it is reported that thiamethoxam, at concentration of 0.3 mM, exhibited no response to neurons isolated from the *Heliothis virescens* nerve cord; whereas clothianidin exhibited high activity at concentration as low as 30 nM (Nauen et al., 2003). This result indicated that clothianidin had a higher agonist efficacy than thiamethoxam in *H. virescens* nAChRs. In addition, Brown et al.(2006) found that clothianidin is a "super" agonist of *Drosophila* nAChR. Therefore, when the thiamethoxam and clothianidin appeared concurrently in combination in our present test, it was possible that thiamethoxam was gradually metabolized into clothianidin. Consequently, with the concentrations of clothianidin accumulated and improved in combination with thiamethoxam and clothianidin, it could elicit its toxic response more quickly on honeybees within the same time as the sum of thiamethoxam and clothianidin, and consequently expressed a synergistic effect. The analogy to binary combinations C+D is that synergism can possibly exist with similar mechanism. In future work, further relative studies are necessary to comprehend the mechanism responsible for this phenomenon.

Third, synergism may also be explained by "facilitating actions," meaning that the secondary action of one insecticide can enhance the activity or level of the other insecticide in combination, or alternatively, "complementary actions," which refers to when insecticides influence the same target at different sites, at overlapping sites, or at different targets of the same pathway (Jia et al., 2009). In previous studies on the aphids *Myzuspersicae* and *Aphiscraccivora,* and the locust *Locustamigratoria* L, results suggested that thiamethoxam and imidacloprid may combine with different sites at the nicotinic receptor complex or in different modes (Wiesner and Kayser, 2000; Wellmann et al., 2004). Subsequently, when research about analyzing the mode of inhibition by neonicotinoids was conducted on the aphids *M. persicae* and *A. craccivora* Koch, it was discovered that the examined neonicotinoids of [^{3}H]imidacloprid binding can be split to two classes. One kind of compounds called direct competitors of [^{3}H]imidacloprid binding, which show competitive interaction with imidacloprid, implying binding to the same site, ie to the site of imidacloprid,

such as imidacloprid, N-desmethyl thiamethoxam, nitenpyram, acetamiprid. The other kind of compounds defined as non-competitive compounds of [^{3}H]imidacloprid binding, which do not directly compete with [^{3}H]imidacloprid binding and bind to in a way or a site, different from that of imidacloprid, such as thiamethoxam and the chloropyridyl analogue of thiamethoxam. Moreover, two sub-classes of non-competitive compounds of [^{3}H]imidacloprid binding may be defined in accordance with Hill coefficients of a drug. In addition, low Hill coefficients indicate different features of neonicotinoid binding (Kayser et al., 2004). The pharmacophore may react with the structural information for the mode of inhibition of [^{3}H]imidacloprid binding. For instance, the clothianidin with a-NH-methyl group and an acyclic pharmacophore displayed as a competitor of [^{3}H]imidacloprid binding. However, two close analogues thereof exhibited differences. The *N*-methyl clothianidin with a -N-dimethyl group showed as a pure non-competitive compound of [^{3}H]imidacloprid binding, whereas *N*-desmethyl clothianidin with a $-NH_2$ behaved as a mixed form of non-competitive compound of [^{3}H]imidacloprid binding (Kayser et al., 2004). Furthermore, although dinotefuran had some differences in structure of other neonicotinoids, no relative reports currently exist that show whether it acts at the same mode of binding as some already-known neonicotinoids to the target receptor. As we know, a structural modification of the pharmacophore can change the affinity of a drug and thus, can alter its mode of binding. Therefore, it is possible that dinotefuran might act at the different mode of binding and ultimately lead to the combined toxicity increased.

Therefore, the synergistic interaction may be due to specific pharmacokinetic behavior, a different binding site on the nicotinic receptor, or receptor isoforms.

5 Conclusions

We studied the nature of interactions for a series of three neonicotinoids within the range of effect in total combinations in bees following the honeybee acute oral toxicity test method and the CI-isobologram method. At 48 h post exposure, our results showed that: 1) the order of toxicities for the single insecticides was ranked as clothianidin > thiamethoxam > dinotefuran; 2) all the binary and ternary combinations showed synergism or additive effect at the effect (fa) 0..5. Our present results

386 indicated that the synergistic combinations may pose a potential risk to honeybees
387 whenever the neonicotinoids appeared concurrently in agriculture, and also indicated
388 that there is an urgent need to study the mixture toxicity of neonicotinoids in
389 honeybees because the assessment of the risk of insecticides towards honeybees,
390 conducted only in individual neonicotinoids, may underestimated the toxicity in
391 realistic conditions.
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395 **Acknowledgments**

396 This work was supported by National Natural Science Foundation of China
397 (31301689).

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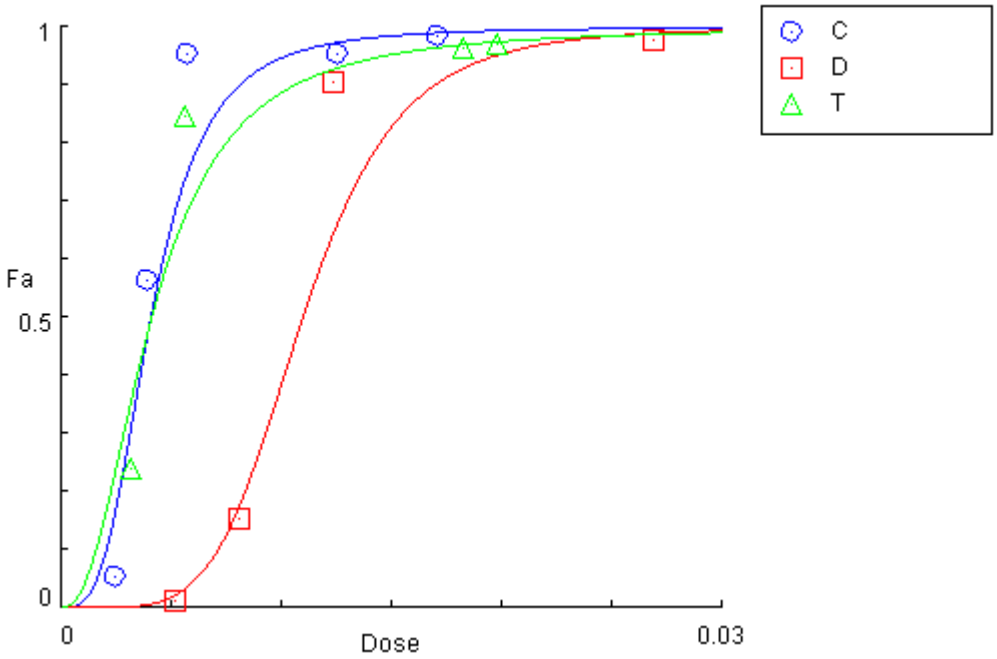
Table 1

Dose-effect relationship parameters and mean combination index (CI) values of dinotefuran (D), clothianidin(C), and thiamethoxam (T) singly and their binary and ternary combinations within honeybee tests after 48 h exposure.

Pesticides	Dose-effect parameters				CI values at		
	D_m	m	r		LD_{10}	LD_{50}	LD_{90}
D	0.0110	5.05	0.953		---		
C	0.00408	3.20	0.906		- --		
T	0.00411	2.31	0.952		- --		
D+T	0.00760	4.97	0.978		1.51	1.09	0.841
C+D	0.00581	5.55	0.999		1.01	0.833	0.698
C+T	0.00352	3.28	0.978		1.01	0.860	0.746
C+D+T	0.00548	5.83	0.987		1.33	0.928	0.673

The computer software Compusyn was used for calculating the parameters of D_m, m, r, and CI values. The parameters of D_m, m, and r are the antilog of the x-intercept, the slope, and the linear correlation coefficient of the median-effect plot, which indicates the potency (LD_{50}), the shape of the dose-effect curve, and the conformity of the data to the mass-action law, respectively (Chou and Talalay, 1984; Chou, 2006). D_m and m values are used to calculate the CI values (Equation 4), and $CI < 1$, $CI > 1$, and $CI = 1$represent synergism, antagonism and additive effect, respectively. LD_{10}, LD_{50}, and LD_{90} are the doses for producing 10%, 50%, and 90% mortality rate of honeybees, respectively.

Dose in µg active ingredient/ bee



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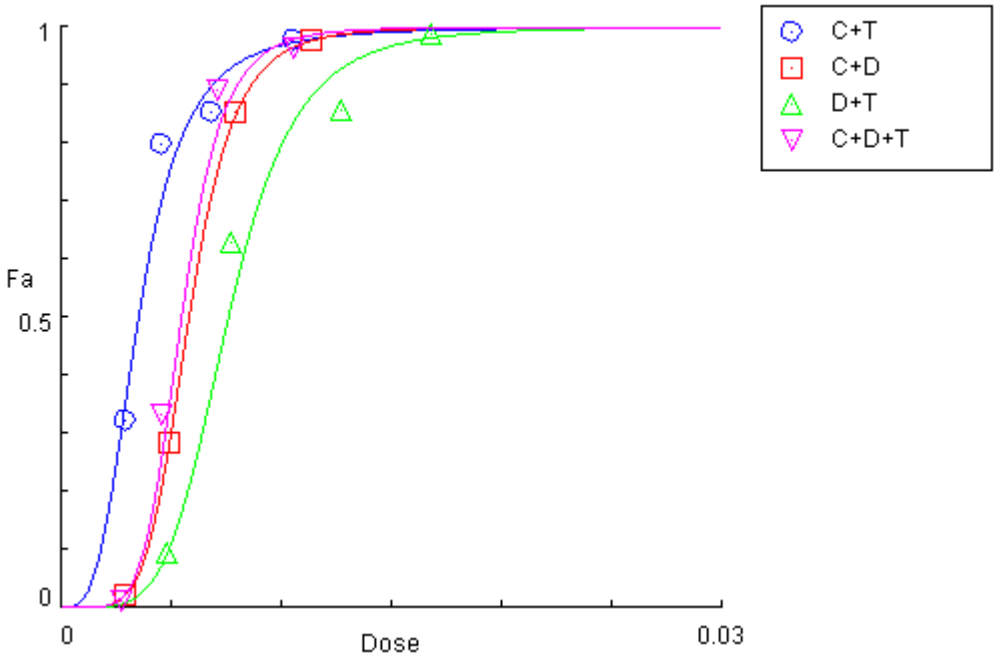

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527 Fig.1. Dose-effect curves of dinotefuran (D), clothianidin(C), and thiamethoxam
528 (T)and their total combinations for the mortality rate of honeybees after an exposure
529 of 48 h.

530 Dose in µg active ingredient/ bee

531

532

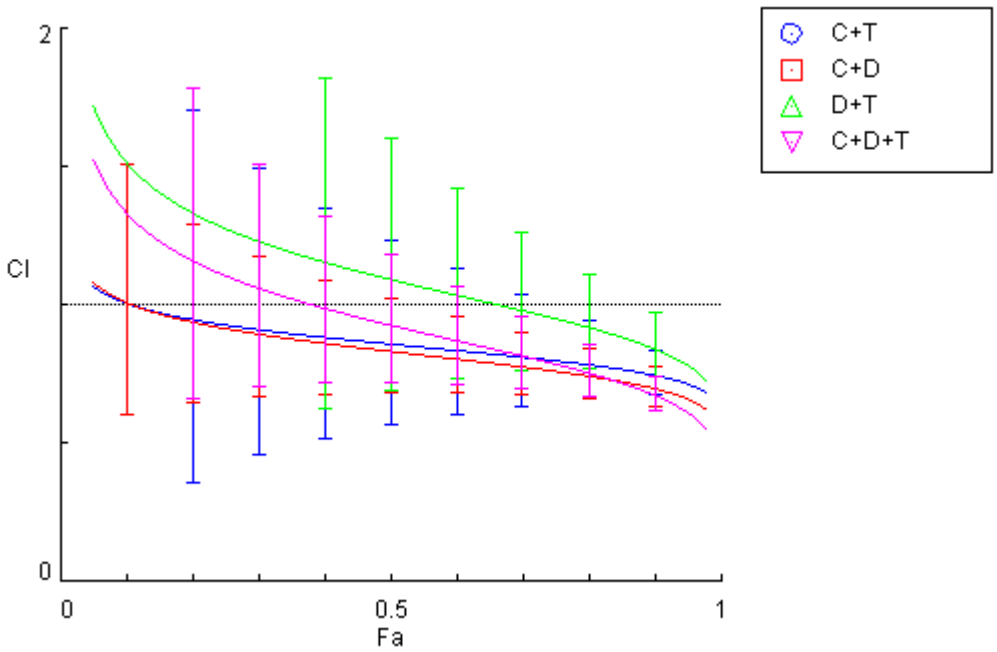

Fig.2. Combination index Plot (f_a − CI plot) for total combinations of dinotefuran (D), clothianidin(C), and thiamethoxam (T) for the mortality rate of honeybees after an exposure of 48 h. CI values are plotted as a function of the fraction affected (f_a) by the simulation of the computer software Compusyn. CI < 1, CI > 1, and CI = 1 represent synergism, antagonism, and additive effect, respectively. Three specialty experiments with two repetitions were used for the analysis. On the basis of SDA (Sequential Deletion Analysis), the vertical bars indicate 95% confidence intervals for CI values (Chou and Martin, 2005).

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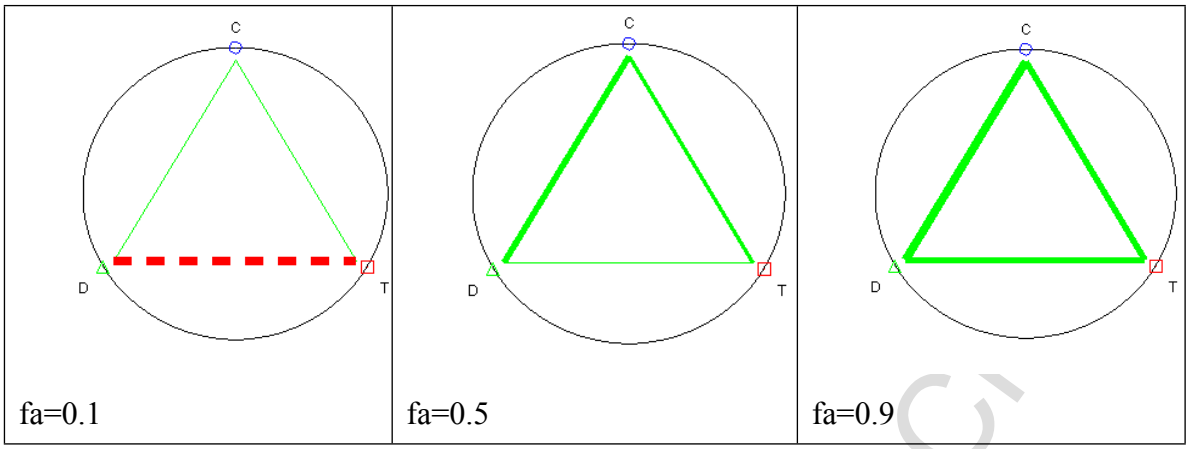

554 Fig.3. Polygonograms showing the toxicological interactions of dinotefuran (D),
555 clothianidin(C), and thiamethoxam (T) in total combinations when calculated by
556 Compusyn for the mortality rate of honeybees at three representative effect levels (f_a):
557 fa = 0.1, 0.5, and 0.9 after an exposure of 48 h. Solid line represents synergism, and
558 the strength of each synergism is indicated by the thickness of the line.

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Highlights

1. A handy method was used for studying the nature of interactions of three neonicotinoids.
2. Honeybee assay was conducted in acute oral toxicity test.
3. Synergisms and additive effect were observed for the combinations.