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Lethal and Histological Effects of Neonicotinoids Insecticides on *Apis mellifera* (Hymenoptera, Apiadae)



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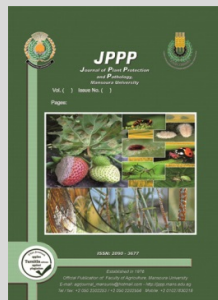
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ABSTRACT

Using of Neonicotinoids insecticides affect honeybees that visit field and horticultural crops for collecting nectar and pollen. Therefore, this investigation aimed to study the effect of three neonicotinoids insecticides (Imidacloprid, Acetamiprid and Dinotefuran,) with three (recommended, half, and quarter) doses on mortality rate of adult bees, also their effect on midgut tissues under laboratory conditions. The obtained results showed that a highly mortality% was recorded in Dinotefuran 100% after 48hr, while Acetamiprid revealed the lowest mortality% as 56, 51 and 33% with recommended, half and quarter dose, respectively. Imidacloprid recorded 81, 79 and 74% mortality for recommended, half and quarter dose, respectively. On the other hand, there was mild vacuolar degeneration with nucleus pyknosis in some cells in the lining mucosal epithelium of minimum concentration half recommended dose of Imidacloprid. Also, there was moderate proliferation of cells lining midgut with increasing number of goblet cells of the quarter recommended dose of Acetamiprid. It could be concluded that Dinotefuran is very dangerous and lethal on bees.

Keywords: *Apis mellifera*, Imidacloprid, Dinotefuran, Acetamiprid, midgut epithelial tissue, mortality%, histological effects.



INTRODUCTION

Honeybee has a great economic importance in agricultural productions because of its role in pollinating most of the flowering plants. (Potts *et al.*, 2006 and Tautz, 2008). The decrease in number of pollinators, especially honeybees, caused a big problem among beekeepers. In recent years, scientists have identified the phenomenon of the "colony collapse" as causing loss of numbers colony by 80 to 100%, the forager workers have no ability to fly, and they can't return to the hive in many cases. (Oldroyd, 2007).

There are many influential factors that work alone or together and cause weakness of colony. These factors included the excessive use of insecticides and intoxication of bee by this chemicals (El-Hefnawi, 2005; Ratnieks and Carreck, 2010; Tremolada *et al.*, 2010; El-Wakeil and Abdallah, 2012; Shebl *et al.*, 2013; Al Naggat *et al.*, 2015 and Codling *et al.*, 2018). Among the insecticides which used in agriculture, neurotoxins group that act as an agonist of the nicotinic acetylcholine receptors follows the neonicotinoids group. It includes Imidacloprid, Acetamiprid, Thiacloprid, Dinotefuran, and Thiamethoxam, (Matsuda *et al.*, 2001 and Elbert *et al.*, 2008).

Neonicotinoids Insecticides are widely used against sucking insects. Although, these compounds also affect untargeted insects such as bees. For this reason widespread of these compounds and have highly selective for the nicotinic receptors of insects preferably to use (El Hassani *et al.*, 2008). On the other hand, this insecticide is systemic, therefore, considered hazardous to pollinators whereas that nectar, pollen and exudates could become contaminated (Girolami *et al.*, 2009 and Stoner and Eitzer, 2012). Neonicotinoids Insecticides are accumulate in the colony and thus the young emerging honeybees can be chronically exposed to sub lethal

doses that effects of the short-term productivity on decreased colony performance and productivity (Pilling *et al.*, 2013 and Sandroek *et al.*, 2014).

Many workers studied the effect of neonicotinoids at different doses on longevity of bees and damage of the midgut cells when used orally. (Higes *et al.*, 2007; García-Palencia *et al.*, 2010 and Gregorc and Ellis, 2011). Midgut is the main site that secretes granules, digestion and absorption occur (Chapman, 1998 and Cruz-Landim, 1999). Kakmand *et al.*, 2008 reported that the acute exposure to Malathion, Thiamethoxam, and Deltamethrin caused several alterations in midgut cells and damage the midgut epithelium of honeybees

Some insecticides aimed specifically the midgut epithelium which is responsible for the detoxification of ingested xenobiotic (Mao *et al.*, 2011; Han *et al.*, 2012 and Vachon *et al.*, 2012). Aljedani, 2017 studied the toxicity of insecticide (Abamectin and Deltamethrin) on the lethal time and midgut of foragers honey bee *A. mellifera*. It was concluded that the Abamectin had high LT50 than Deltamethrin and reported a harmful effects on midgut cells.

The purpose of this work is to study the effect of three neonicotinoids insecticides (Imidacloprid, Dinotefuran, and Acetamiprid) at three different doses (recommended, half and quarter doses) besides the control (without treated insecticides) on *A. mellifera* workers through mortality% and histological effects in midgut tissue.

MATERIALS AND METHODS

This investigation was carried out in the laboratory of the apiary yard, Agric. Exp. Sta., Fac. Agric., Cairo Univ. during the active period of 2019. In order to evaluate the adverse effects of three neonicotinoids insecticides (Imidacloprid, Acetamiprid and Dinotefuran) on workers of *A. mellifera* (older than 21 days).

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1. Experimental bioassay

Evaluation of the three doses of insecticides was used against older honeybee workers (≥ 21 days). The workers were collected under the normal conditions from hive entrance of strong healthy colony and tested for Nosema infection and Varroa mites according to Khoury *et al.*, 2011.

Each concentration for each insecticide had three replicates with 50 workers for each in addition to the control (sugar feeding without any treated insecticides) as shown in Table (1). Each replicate was put in a wooden cage (12×12×20cm). One face of this cage was covered with metal wire mesh according to Kakmand *et al.*, 2008. Honey bees of each cage were fed by 5ml sugar solution (1:1) according to Bortolotti *et al.* 2003. Each dose was diluted in sugar syrup. Each dose was allowed to feed on treating sugar syrup for 24 hr and then fresh untreated syrup was introduced to honey bees. Every day plastic petri dish, 3cm in diameter and 1cm in height was used for feeding each cage. Number of dead workers was recorded at 24, 48 and 72hr after treatments to

calculate the mortality percentages. For histological studies, samples (seven bees) of each treatment were collected after 48hr. The experimental cages were kept under temperature $27.9\pm 2^\circ\text{C}$ and humidity $58\%\pm 2$.

2. The histological studies

After 48hr, alive workers (seven bees) for all doses of Imidacloprid and Acetamiprid insecticides were collected as well as untreated workers honeybees. The samples were carried out in the laboratory of animal health research institute and fixed in 10% formulaic saline for 24 hr. Washing was done in tap water then serial dilutions of alcohol (methyl, ethyl, and absolute ethyl) were used for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56 degree in hot air oven for 24hr. Paraffin bees wax tissue blocks were prepared for sectioning at 4 microns thickness by slide microtome. The obtained tissue sections were collected on glass slides, Deparaffinized and stained by Hematoxylin and eosin stain for examination through the light microscope (Banchroft *et al.*, 1996).

Table1. The three neonicotinoid insecticides, its doses and control which were used in this study

Neonicotinoid insecticides	Active ingredient insecticides	Insecticides Formulations	Dose
1	Imidacloprid	(Imaxi 35%SC)	75cm ³ /100L (Recommended dose)
			37.5cm ³ /100 L
			18.75cm ³ /100L
2	Acetamiprid	(Mospilan 20%SP)	25gm/100L (Recommended dose)
			12.5gm/100 L
			6.25gm/100L
3	Dinotefuran	(Oshin 20%SG)	50gm/100L(Recommended dose)
			25gm/100 L
			12.5gm/100L
Sugar feeding without insecticide			Water tap(control)

4. Statistical analysis

Data were analyzed using SAS software program (SAS, 2001). Randomized complete design with three replications was used. Duncan's test was used to separate treatment means at $P \leq 0.05$.

RESULTS AND DISCUSSION

1. Toxic effects of Neonicotinoid insecticides on honey bee workers

Results presented in Table 2 show that the percentage of mortality after exposure to three doses of Neonicotinoid insecticides (Imidacloprid, Acetamiprid and Dinotefuran). Results referred to significant differences between all the

tested treatments in mortality. Dinotefuran treatments recorded the highest values of mortality (100%) after 48 hr. for recommended (50gm/100L), half (25gm/100L) and quarter (12.5gm/100L) doses.

On the other hand, individual bees which treated with Acetamiprid recorded lowest percentage of motility (0.56 ± 0.02 , 0.51 ± 0.01 and 0.33 ± 0.02 % for recommended (25gm/100L), half (12.5gm/100L) and quarter (6.25gm/100L) doses, respectively after 48hr. and increased to $88\% \pm 0.01$, $70\% \pm 0.01$ and $49\% \pm 0.02$ for recommended, half and quarter dose after 72hr, respectively. The rest bees were still alive until 14 days for quarter dose and 7 days for half dose.

Table 2. Mean of mortality % as resulted by three insecticides and its doses compared to control (sugar feeding only) after 24, 48 and 72 hr. under the laboratory conditions (temp. $27.9\pm 2^\circ\text{C}$ and humidity $58\%\pm 2$)

Treatments	Doses	Mortality % after 24hr.	Mortality % after 48hr.	Mortality % after 72hr.
Sugar feeding without insecticides	Control	3% \pm 0.00 ^f	6% \pm 0.01 ^g	8% \pm 0.00 ^g
	*RD, 75 cm ³ /100L	30% \pm 0.01 ^b	81% \pm 0.02 ^b	99% \pm 0.00 ^a
Imidacloprid (Imax35%SC)	HRD, 37.5 cm ³ /100L	28% \pm 0.01 ^{cb}	79% \pm 0.00 ^b	96% \pm 0.01 ^b
	QRD, 18.75cm ³ /100L	26% \pm 0.01 ^c	74% \pm 0.02 ^c	93% \pm 0.00 ^c
	RD, 25gm/100L	25% \pm 0.00 ^c	56% \pm 0.02 ^d	88% \pm 0.01 ^d
Acetamiprid (Mospilan 20 %SP)	HRD, 12.5gm/ 100L	17% \pm 0.02 ^d	51% \pm 0.01 ^e	70% \pm 0.01 ^e
	QRD, 6.25gm/100L	14% \pm 0.01 ^e	33% \pm 0.02 ^f	49% \pm 0.02 ^f
	RD, 50gm/100L	97% \pm 0.00 ^a	100% \pm 0.00 ^a	100% \pm 0.00 ^a
Dinotefuran (Oshin 20 %SG)	HRD, 25gm/100L	94% \pm 0.01 ^a	100% \pm 0.00 ^a	100% \pm 0.00 ^a
	QRD, 12.5gm/100L	94% \pm 0.01 ^a	100% \pm 0.00 ^a	100% \pm 0.00 ^a

Means followed by the same litter(s) in the same column are not significant differences ($P \leq 0.05$).

*RD: Recommended Dose, HRD: Half Recommended Dose and QRD: Quarter Recommended Dose.

While bees treated with Imidacloprid insecticide recorded 81% \pm 0.02% and 79% \pm 0.00 mortality % for recommended (75cm³/100L) and half (37.5 cm³/100L) doses, respectively compared with quartered dose (18.75cm³/100L) as 74% \pm 0.02. The mortality% increased to 99% \pm

0.00, 96% \pm 0.01 and 93% \pm 0.00 after 72hr for recommended, half and quarter dose, respectively.

Finally it could be concluded that Dinotefuran was the most dangerous insecticide on the honeybee and should not be used on flowering crops visited by bees. Imidacloprid

also showed high mortality rates as well as histological effects on the bee's midgut. Acetamiprid had the lowest effective pesticide in bee mortality, especially the quarter dose (6.25gm/100L).

These results are in agreement with those of Carvalho *et al.*, 2009 reported that regardless of the form of contamination found, Thiamethoxam (37.5 mg/ L) extremely poisonous to bees and causes more death More than 80% of samples after 3 days. Perry *et al.*, 2015 who found marked decrease in the survival of the workers, exposed to Thiametoxam and Picoxystrobin insecticides. They also reported that the very low doses of these insecticides had adversely effect on bees. Individual responses varied from stress to high mortality rates for workers, which affect functioning of the colony because of the breakdown in the division of labor of workers. Aljedani, 2017 in his study found that abamectin has a detrimental effect on forage workers and there is a significant effect on deadly time (LT50) and effects on midgut cells. This can cause indigestion in the midgut of the intestine and slow its effectiveness and thus affect the health and vitality of the bee colony. Domingues *et al.*, 2017 studied the survival rate and co-exposure to insecticides (Thiamethoxam, 1ppm and Picoxystrobin, 18 ppm) on *A. mellifera* workers. The results showed that low doses of these insecticides cause significant damage to bee workers and consequently affect their performance inter the colony.

As shown in Fig.1 a positive linear correlation ($r = 0.85\%$) was found between insecticide doses and mortality % after 48hr from exposure.

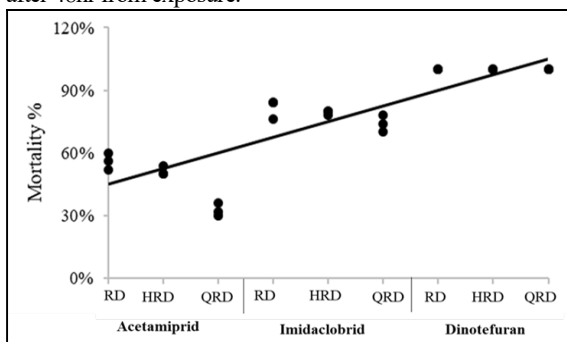


Fig. 1. Correlation between the mortality % and insecticides concentrations, $P < .0001$

2. Histological assessment in midgut tissues of honey bee workers using light microscope

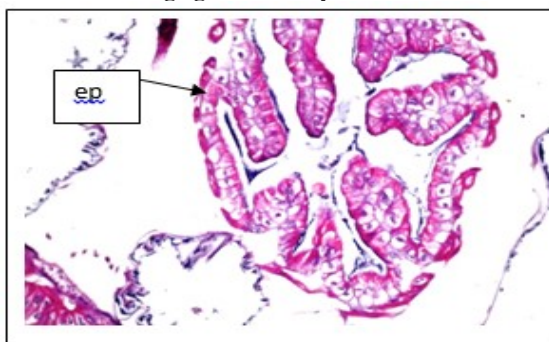


Plate A1. shows a normal histological structure of the control with high columnar epithelial cells with large round vesicular nucleus in the lining mucosa (H&E, X80)

The midgut epithelial cells of the bees fed by sugar solution (control) as shown in plates A₁ and A₂ possess large vesicular nuclei, homogenous cytoplasm with high columnar lining epithelial cells of mucosa; as recorded by (Cruz-Landim and Cavalcante, 2003; Dabour *et al.*, 2019). While midgut epithelial tissue of bees orally treated with the recommended dose of Imidacloprid appeared to be clear affected under the action of these insecticides (Plates B₁ and B₂). Their nuclei are abnormal, small size deep blue color (nucleus pyknosis), and most of lining mucosal epithelium showed vacuolar degeneration in the cytoplasm. This finding was in agreement with those of Kamand *et al.* 2008

There were mild vesicular degeneration with nucleus pyknosis in some cells in the lining mucosal epithelium of minimum concentration (half recommended dose of Imidacloprid) as shown in plates C₁ and C₂, in case of the recommended dose of Acetamiprid there was vacuolar degeneration detected in the cytoplasm of lining epithelium cells with intact nuclei (plates D₁ and D₂). While half recommended dose of Acetamiprid showed mild vesicular degeneration in some cells in the lining mucosal epithelium (plates E₁ and E₂). On other hand, the quarter recommended dose of Imidacloprid indicate sever proliferation of cells lining midgut with increase in number of goblet cells (mucinous cells) but the nuclei was a normal and also the were disorganization of cells and formation of irregular lumen. Where they were moderate proliferation of cells lining midgut with increase in number of goblet cells of the quarter recommended dose of Acetamiprid. Although the main target of the neonicotinoids pesticide group is acetylcholine receptors in the nervous system. However, secondary goals such as organs involved in the metabolism and more may also be affected. (Tomizawa and Casida, 2003). Furthermore, Cruz-Landim *et al.*, 1996 described the regenerative cells of the honey bee as a combination that consists of a group of nests located on the same level of the basement of the digestive cells. Such an observation is consistent with the findings of Serrão and Cruz-Landim, 1995; Cruz-Landim and Cavalcante, 2003. Africanized honeybees midgut treated with Thiamethoxam revealed that the number of regenerative cells in epithelium was reduction and inducing cytoplasmic vacuolization (Oliverira *et al.*, 2013). Forkpah *et al.* (2014) reported that some of the insecticides used accumulate in hives and have unknown physiological effects until now.

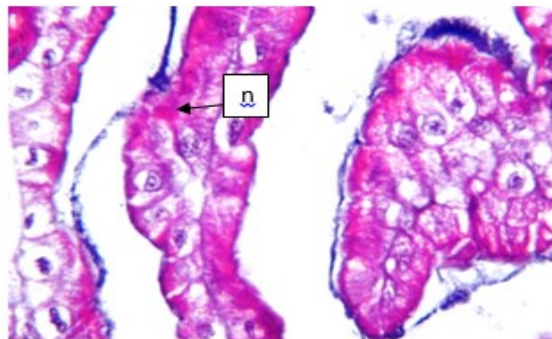


Plate A2. shows wing the magnification of Plate A1 to identify the normal histological structure of lining columnar epithelial cells with large round vesicular nucleus (H&E, X160).



Plate B1. shows vacuolar degeneration in cytoplasm of lining epithelium with nucleus pyknosis of the recommended dose of Imidacloprid (H&E, X80)



Plate B2. shows the magnification of Plate B1to identify deeblyish small size nucleus pyknosis with vacuolar cytoplasm cell over the cells of the recommended dose of Imidacloprid (H&E, X160)

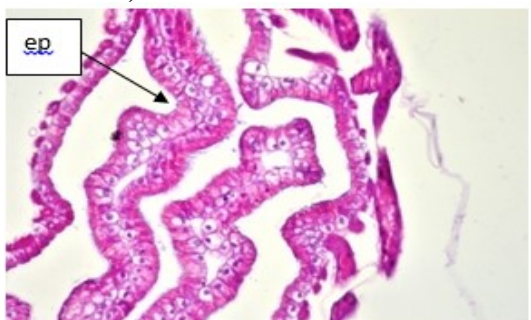


Plate C1. shows pyknosis of nuclei all over the lining mucosal epithelium of the half recommended dose of Imidacloprid (H&E, X80)



Plate C2. shows the magnification of plate C1to identify pyknosis of nuclei all over the lining mucosal epithelium of the half recommended dose of Imidacloprid (H&E, X160)



Plate D1. shows vacuolar degeneration in the lining mucosal epithelium with intact nuclei of the recommended dose of Acetamiprid (H&E, X80)



Plate D2. shows the recommended dose of Acetamiprid with magnification of Plate D1



Plate E1. shows mild vacuolar degeneration with intact nuclei with the lining epithelium cells of the half recommended dose of Acetamiprid ((H&E, X80)

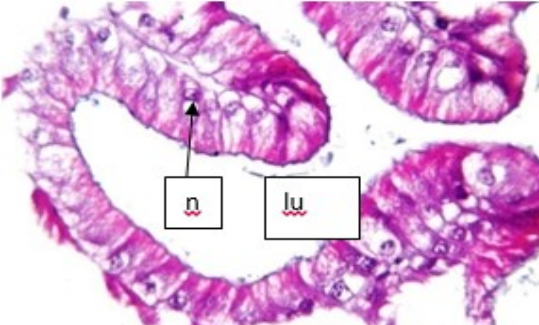


Plate E2. shows the magnification of Plate E1: to identify the mild vacuolar degeneration with intact nuclei with the lining epithelium cells of the half recommended dose of Acetamiprid (H&E, X160)

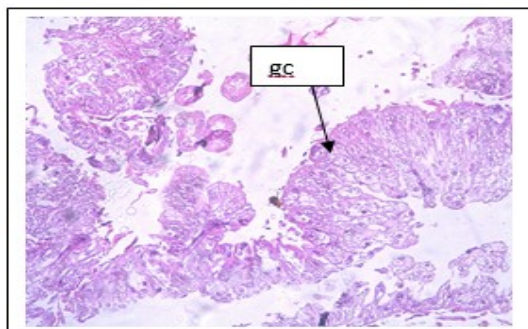


Plate F. shows sever proliferation of cells lining midgut with increase in number of goblet cells (mucinaus cells) of the quarter recommended dose of Imidacloprid (H&E, X200)

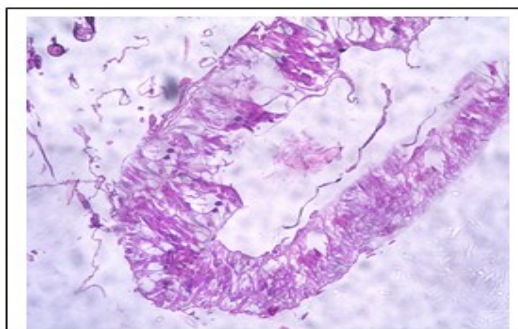


Plate G. shows moderate proliferation of cells lining midgut with increase in number of goblet cells of the quarter recommended dose of Acetamiprid (H&E, X200)

** Vacuolar (v) nuclei normal (n), lumen (lu); goblet cells (gc) Staining: Hematoxylin- Eosin (H&E): Bars: 50 mm.

Plate 1: Honeybees (*A. mellifera*) midgut after 48 hr. of exposure. (A₁ and A₂ control) ; (B₁ , B₂ exposed bees with the recommended dose of Imidacloprid) , (C₁ and C₂ exposed bees with the half recommended dose of Imidacloprid) , (D₁ and D₂ exposed bees with the half recommended dose of Acetamiprid) (E₁ and E₂ exposed bees with the half recommended dose of Acetamiprid);(F exposed bees with quarter recommended dose of Imidacloprid and G exposed bees with quarter recommended dose of Acetamiprid).

CONCLUSION

The results showed that, the neonicotinoids insecticides were lethal for workers of honeybee. The dinotefuran caused the higher in mortality than both Imidacloprid and Acetamiprid. The quarter recommend dose (6.25gm/100L) of Acetamiprid had little effect on midgut cells than recommended dose (25gm/100L. The pesticide Acetamiprid caused the lowest influential especially the concentration of a quarter. Finally, these results recommended that it must reduce the doses of Neonicotinoids insecticides especially in crop pollination and avoid spraying crops at the time of flowering, especially honey crops. Dinotefuran Very toxic for bees and kills all bees after 48hr.

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التأثيرات المميتة والهستولوجية للمبيدات الحشرية التابعة للنيونيكوتينويد على حشرة نحل العسل *Apis mellifera*

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تستخدم مبيدات النيونيكوتينويد بمدى واسع على المحاصيل الحقلية والبستانية التي قد تؤثر على حشرة نحل العسل أثناء زيارتها للمحاصيل سواء للتلقيح أو جمع الرحيق وحبوب اللقاح. ومن هنا جاء الهدف من إجراء البحث وهو دراسة تأثير ثلاثة أنواع من المبيدات الحشرية تابعة لمجموعة النيونيكوتينويد بثلاث جرعات مختلفة وهي الجرعة الموصى باستخدامها ونصف الجرعة وربع الجرعة وذلك تحت ظروف المعمل. تمت الدراسة في منحل كلية الزراعة جامعة القاهرة حيث تم استخدام ١٠ معاملات بـ ٣ تركيزات من ثلاث أنواع مبيدات بالإضافة إلى الكنترول الغير معامل بالمبيد. أوضحت النتائج المتحصل عليها أن مبيد الدينتوفيران شديد السمية بالنسبة لحشرة نحل العسل حيث سبب نسبة موت وصلت إلى ١٠٠% بعد ٤٨ ساعة من المعاملة به وذلك للثلاث تركيزات المستخدمة منه. تفاوتت نسب الموت بالنسبة لمبيد الأميذاكلوبريد حيث كانت نسب الموت بعد ٤٨ ساعة من التعرض للمبيد ٨١، ٧٤ و ٧٤ لكل من الجرعة الموصى بها والنصف جرعة والربع على التوالي. كما أوضح تأثيرات سلبية على أنسجة المعدة والخلايا الظلامية المبطن لها والسيترولازم والأنويه. من ناحية أخرى كان مبيد الأسيامبيريد الأقل تأثيراً على أنسجة معدة النحل وخاصة تركيز ربع الجرعة الموصى بها والذي سجل نسبة موت بعد ٤٨ ساعة من التعرض ٣٣% ووصلت إلى ٤٩% بعد ٧٢ ساعة .