Susceptibility of Apis mellifera (Hymenoptera: Apidae) to pellitorine, an amide isolated from Piper tuberculatum (Piperaceae)

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Original article

Susceptibility of *Apis mellifera* (Hymenoptera: Apidae) to pellitorine, an amide isolated from *Piper tuberculatum* (Piperaceae)

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Abstract – The acute toxicity of pellitorine, an amide isolated from *Piper tuberculatum* (Piperaceae) which is studied as a biopesticide in European corner borer, was evaluated on larvae and newly emerged adults of honeybee *Apis mellifera* by means of contact and ingestion bioassays. Workers in the larval and adult phase were separated in groups, which received pellitorine in different concentrations. The larvae were maintained in their own original cells, receiving feeding and normal care from the nurses. The adults were confined in wooden cages with screens, receiving artificial diet made up of sugar and water (1:1). The concentrations of 40, 200, 1 000, 5 000 and 25 000 ng a.i./individual were obtained diluting pellitorine in 98% ethanol. LD_{10} values of 39.14, 36.16 and 13.79 ng a.i./insect were determined for larvae, for adults by ingestion and adults by contact, respectively. The honeybee larvae were shown to be highly susceptible to the amide pellitorine.

***Apis mellifera* / toxicity / contact / ingestion / pellitorine / biopesticide**

1. INTRODUCTION

The utilization of insecticides in agricultural fields, particularly flowering fields visited by pollinating insects, has caused poisoning problems in apoid bees and in particular *Apis mellifera* L. Once contaminated by a certain insecticide, the bee may be killed by a lethal dose, or carry the insecticide to the beehive through contaminated nectar or pollen. In the former case, the sub-lethal dose of a given compound could be transferred to larvae via nurse bees. In addition, bee products obtained for human consumption can be contaminated with any insecticide compound. This situation could occur especially with formulations that adhere to the pollen collected by the bees (Johansen, 1977).

Before the systemic use of synthetic organic insecticides, the bioinsecticides were intensively

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used in agriculture, mainly for the control of leaf miner insects (Gallo et al., 1988). Currently, there is increasing concern to achieve highly selective products, demonstrating low toxicity to useful insects and humans. Bioinsecticides extracted from plants have shown potential insect control agents, such as azadiractin, extracted from *Azadirachta indica* (Butterworth and Morgan, 1971; Saxena and Khan, 1985; Schlüter et al., 1985; Schmutterer, 1990; Naumann et al., 1994), which presents only ecological selectivity to honeybees (Isman, 1993; Naumann and Isman, 1996).

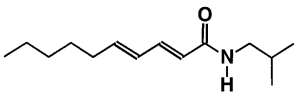
In this context, plants belonging to Piperaceae family present chemical compounds such as isobutyl amides, which have demonstrated potential insecticide activity toward sugar cane borer (*Diatraea saccharalis*) and velvetbean caterpillars (*Anticarsia gemmatalis*) (Navickiene, unpublished data), houseflies (*Musca domestica*) (Gbewonyo and Candy, 1992), European corn borer (O*strinia nubilalis*), as well as antifeedant properties (Matsui and Munskata, 1975; Miyakado et al., 1989; Bernard et al., 1995). In addition, the amides showed potent antifungal activity against phytopathogenic fungi *Cladosporium sphaerospermum* and *C. cladosporioides* (Alécio et al., 1998; Navickiene et al., 2000; Silva et al., 2002), and molluscicide activity against *Biomphalaria glabrata*, the vector responsible for schistosomose disease (Isao, 1984).

The honeybees *Apis mellifera* have an important economic role by producing honey, royal jelly, wax, propolis and by pollinating cultivated and wild plants. On account of these characteristics their susceptibility to agrochemicals has been studied for 50 years (Moraes et al., 2000). Considering the effectiveness of the amide pellitorine toward different insects and the possibility of its future commercial use in areas of economic interest, the purpose of this paper was to determine the toxicant effects of this amide isolated from *Piper tuberculatum* Jacq. on larvae and adults of honeybees *Apis mellifera*.

2. MATERIALS AND METHODS

2.1. Plant material

Piper tuberculatum Jacq. seeds, leaves and stems were collected in 1998 at Campus of INPA



(2*E*, 4*E*)-*N*-isobutyldecadienamide

Figure 1. Structural formula and chemical name (IUPAC) of the amide pellitorine isolated from *Piper tuberculatum.*

(Instituto Nacional de Pesquisa da Amazônia), Manaus, Brazil, and identified by Dr. Guillermo E.D. Paredes (Universidad Nacional Pedro Ruiz Gallo, Peru). The voucher specimen (Kato-163) is deposited at Herbarium of Instituto de Botânica, São Paulo – São Paulo State, Brazil.

2.2. Isolation of amide pellitorine

Analyses by High Performance Liquid Chromatography (HPLC) of the extracts of the whole plant (seeds, leaves and stems) showed that pellitorine was present in all tissues, but the highest concentration was observed in the seeds (15%).

Seeds of *P. tuberculatum,* immediately after plant collection, were dried at room temperature and powdered. The powder (24.33 g) was submitted to extraction using CH_2Cl_2:MeOH – 2:1 (2×600.0 mL) at room temperature. The resulting extract was concentrated in a vacuum to afford a green gummy resin (2.87 g). Part of the crude seed extract (2.00 g) was fractionated by column chromatography (silica gel, 40.00 g, C_6H_{14} – EtOAc in polarity gradient) yielding 36 fractions (15.0 mL). Fraction 13 (0.04 g) was washed with $CHCl_3$ to afford pellitorine (0.03 g), as described previously in the literature (Navickiene et al., 2000).

Structural identification of pellitorine

The amide had its molecular structure elucidated by ^1H and ^{13}C NMR, DEPT 135°, ^1H-^1H COSY, IR, mass spectral data analysis and by comparison with data described for pellitorine by Rosario et al. (1996: Fig. 1). In addition, the amide purity was verified by means of HPLC analysis, by detection of only one compound in the sample.

2.3. Materials and instrumentation

Silica gel (Merck 230-400 mesh) was used for all column chromatography unless otherwise stated and solvents were redistilled prior to use. The NMR spectra were measured on a Bruker spectrometer AC-200, using $CDCl_3$ as a solvent and TMS as

reference. The IR spectra were obtained on a Nicolet-730 FT-IR spectrometer using KBr disks. ES-MS was recorded on a VG Platform II spectrometer. HPLC analyses were performed on a Varian ProStar LC/PDA using a reverse phase C_{18} 5 µm column (Supelcosil C_{18}; 250 × 4.6 mm i.d.) with a precolumn (20 × 4.6 mm i.d.) purchased from Supelco INC (Supelco Park, Bellefonte, PA, USA). Chromatography was carried out under isocratic conditions with MeOH:H_2O (4:1, v/v) as a mobile phase at a flow rate of 1 mL/min. Total analytical run time for each sample (pellitorine and extracts from seeds, leaves and stems) was 30 min. The chromatograms were analyzed and plotted at 254 nm.

Solvents

All the solvents, including HPLC grade methanol, were purchased from Mallinckrodt (Mallinckrodt Baker, S. A. de C. V. 55320, Xalostoc, Edo. De Méx., México). Nanopure water (> 18 MOhm) was obtained using a Millipore (Millipore Corporation, 80 Ashby Road, Bedford, MA, USA) purifier. All solvents and samples were filtered through a 0.2 µm nylon membrane.

2.4. Site of the bioassays

The bioassays were carried out at the Apiculture Section of the Faculdade de Ciências Agrárias e Veterinárias of the Universidade Estadual Paulista, Jaboticabal, São Paulo State, Brazil, from March 25 to May 20, 2000.

2.4.1. Screening of doses

The amide pellitorine was diluted in solvent (98% ethanol) at concentrations of 80; 400; 2 000; 10 000 and 50 000 µL/L and the doses of 40, 200, 1 000, 5 000 and 25 000 ng a.i. were applied on larvae and adults of *Apis melliera*. This range of doses was chosen based on the reports of Navickiene et al. (unpublished data), where the potential insecticide action of this amide was evaluated against the sugar cane borer *Diatraea saccharalis*. In all experiments, the application of vehicle (98% ethanol) was used as control.

2.4.2. Acute toxicity in honeybee larvae

Very small larvae are frequently damaged when the treatment is applied. Also, previous investigation demonstrated that 98% ethanol at a dose of 0.5 µL was toxic to 1st instar larvae, causing high mortality (73%). For this reason, 3rd instar was chosen to assess the larvae acute toxicity. Each treatment was performed using thirty comb cells containing 3rd instar larvae (99.5 mg medium weight). The comb cells were labeled inside the colony with colored pins placed in the corners. Solutions of 0.5 µL ethanol containing different doses of pellitorine were manually applied into the larval food in each comb cell, using a micro-syringe. The treatments consisted of a combination of oral and topical administration; because the body of the larvae and the food were contacted with the solution. Larval mortality was evaluated 96 hours after exposure to the amide.

2.4.3. Acute toxicity in adult honeybees

In order to verify dermal acute toxicity, 2 µL of pellitorine in different concentrations were deposited onto the thorax of newly emerged adult honeybees, with the aid of a micro-applicator Burkard 900-x (Burkard Manufacturing Co. Ltd.). We did not use CO_2 to anesthetize the workers, because they are very easily handled during the first day after emergence from the cell. The oral acute toxicity in adult workers was assessed using the same doses of pellitorine that were applied topically to adult honeybees. The doses of pellitorine were dissolved in 98% ethanol and sugar solution (50% granulated sugar and 50% water). In each treatment of both bioassays, 10 bees were confined in wood cages of 10 × 8 × 7 cm with a side cover nylon screen. The top face had two holes of 1.5 cm diameter where 10 cm^3 test tubes containing the sugar solution and water, respectively, were inserted. The cages were kept in the dark, in a Biological Oxygen Demand (B.O.D.) chamber at 30 ± 2 ºC, 70 ± 10% relative humidity. As the solutions were administered ad libitum to the group of bees in each cage, the amount of ingested volume was not measured. Dead honeybees were counted and removed every 24 hours and the experiment lasted 96 h.

2.5. Statistical analyses

In all the bioassays, a Randomized Design was performed with seven treatments (doses) and three replicates. The dose-response relationship was verified through the Probit analysis (Finney, 1971). The results were registered as mortality rates.

3. RESULTS

3.1. Acute toxicity in honeybee larvae

The honeybee larvae were shown to be highly susceptible to the amide pellitorine.

Table I. Mortality of *A. mellifera* workers at 96 hours after oral or topical application of different doses of pellitorine.

Dose	Larval mortality	Adult mortality (%)	
(ng a.i./insect)	(%)	Oral absorption	Dermal absorption
Control	3.3	4.2	3.8
40	17.9	18.8	28.9
200	22.8	27.6	39.1
1000	50.7	55.3	61.5
5000	70.8	74.5	83.2
25000	100.0	97.9	100.0

Table II. Lethal doses (LDs) at 96 hours obtained from Probit analysis for *A. mellifera* larvae and adults exposed to pellitorine.

	ng a.i./insect (sd)		
Lethal Dose	Larva	Adult, oral absorption	Adult, dermal absorption
LD_1	3.3 ± 0.6	3.0 ± 0.6	1.0 ± 0.2
LD_{10}	39.1 ± 13.5	36.2 ± 13.2	13.8 ± 4.8
LD_{20}	110.5 ± 49.7	102.8 ± 48.0	42.1 ± 18.9
LD_{50}	804.8 ± 501.4	759.0 ± 483.2	357.0 ± 229.5
LD_{90}	16550.0 ± 8178.6	15932.0 ± 8161.8	9241.0 ± 5068.2

Applications of doses from 40 ng a.i. pellitorine resulted in mortality rates higher ($P < 0.05$) than that observed for control (Tab. I). Linearly increasing mortality rates were observed with the increasing dosage. The LD_1, LD_{10}, LD_{20}, LD_{50} and LD_{90} values of pellitorine for *Apis mellifera* larvae were established by Probit analysis (Tab. II).

3.2. Acute toxicity in newly-emerged worker honeybees

Topical applications of pellitorine at doses ranging from 40 to 25 000 ng a.i. on newly-emerged worker honeybee promoted mortality rates higher ($P < 0.05$) than that obtained for the control (pure solvent) (Tab. I). The same trend was observed when the amide was diluted in the syrup supplied orally to the honeybees, causing a significant ($P < 0.05$) increase in the mortality when the smallest concentration was administrated. The high susceptibility of *Apis mellifera* workers to pellitorine was confirmed through Probit analysis, by means of oral and dermal LD_1, LD_{10}, LD_{20}, LD_{50} and LD_{90} values established for adult worker honeybees (Tab. II).

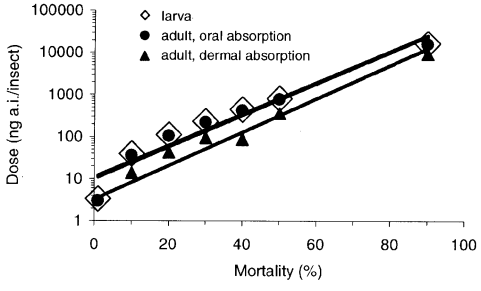


Figure 2. Linear regressions obtained from Probit analysis, showing the relationship between the dose of amide pellitorine and the mortality of *Apis mellifera.*

In all the bioassays, the linear regressions of the values show that the effects (mortality) were dose-dependent (Fig. 2).

The LD_{90} values established in the present work (Tab. II) for larvae (16.55 µg a.i./insect) and adult workers by oral absorption (15.93 µg a.i./insect) and dermal absorption (9.24 µg a.i./insect) were lower than the LD_{50} value (90.95 µg a.i./insect) obtained for 4th instar *Diatraea saccharalis* larvae by topical application

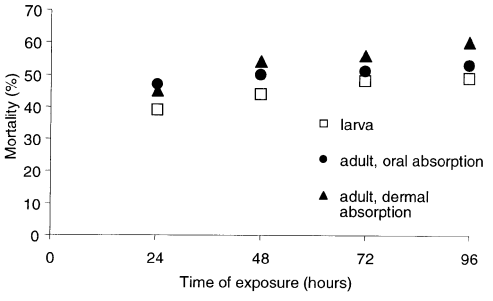


Figure 3. Time course of mortality of *Apis mellifera* exposed to amide pellitorine at dose of 1000 ng a.i./insect.

(Navickiene, unpublished data). Therefore, pellitorine at a dose considered as LD_{50} for *D. saccharalis* larvae could cause 100% mortality of honeybees by means of dermal administration (Fig. 2), demonstrating the high acute toxicity of pellitorine for honeybee larvae and workers. The comparison of data obtained in the present work and those obtained by Navickiene et al. (unpublished data) is valid as both used similar application techniques and measurements (as recommended by IRAC, 1990).

The action of pellitorine on the honeybees was observed over 24 h after the applications, independent of the method. At the intermediate dose of 1 000 ng a.i./insect, almost all the insects died after 24 hours of exposure. The mortality rate between 24 and 96 hours was less pronounced (Fig. 3). The lower mortality rate shown in the larvae might have happened due to different penetration speed through larvae and adults, a fact demonstrated in other cases of toxic effects of pesticide on bees. This phenomenon is not well understood. Certain morphological characteristics, such as differences in the insect cuticle, have been shown to be related to this type of insecticide resistance (Erickson and Erickson, 1986).

4. DISCUSSION

The amide pellitorine was shown to be more active when its absorption took place via dermal contact (topical application) in adult honeybee workers. The reason is a possible action of detoxification enzymes present in the digestive system, liver or Malpighi tubes of the insect. Guedes (1998) suggested that behavioral, physiological and biochemical mechanisms of the insect can influence the response to selective pressures exercised by an insecticide product, promoting an ability of the insects to avoid the lethal effects of the toxin. According to Dauterman (1994), by biochemical mechanism, a polar reagent group (e.g. -OH, -SH, $-NH_2$, -COOH) is introduced into the insecticide molecule, increasing the solubility in water and thus its susceptibility to the detoxification process. Such reactions have been demonstrated to several classes of insecticides (e.g. organophosphates, carbamates and pyrethroids) in which enzymatic biotransformation could be carried out.

Due to the peculiarity of the honeybee larval development in cells containing food supplied by the workers, it became difficult to separate the toxicity effects caused by ingestion or contact, since both process could occur simultaneously.

The susceptibility of *Apis mellifera* to chemical products is expected to be lower as individual weight increases (Ladas, 1972; Gerig, 1975), as has been observed for Africanized honeybees when exposed to the herbicide paraquat (Nogueira-Couto et al., 1996). However, in the present study, the noxious activity of pellitorine on the larvae was reduced when compared to that on the adults receiving topical application. A possible explanation for this result is related to the cleaning practice of larvae and cell, constantly done by 1-5 day old workers (Nogueira-Couto and Couto, 1996). The residues of pellitorine could be removed from the cell or the body of the larvae when workers clean them daily.

The acute toxicity of pellitorine to *A. mellifera* was evaluated under laboratory conditions. When exposed to the environment, the amide can presents different results, due to the influence of external factors, such as the temperature, ultraviolet light, pH of treated plant tissues, rain, etc. In a general way, bioinsecticides present limited persistence under field conditions (Schmutterer, 1990). The residual effect of the amide has not been determined yet; even so, it can be given a restricted estimative of a few days. Hence, a possible ecological selectivity of the product to the honeybees

cannot be discarded. The determination of half-life of pellitorine should be further performed in order to be used it as insecticide in the field conditions.

ACKNOWLEDGEMENTS

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Résumé – Sensibilité d'*Apis mellifera* (Hymenoptera, Apidae) à la pellitorine, amide isolé de *Piper tuberculatum* (Piperaceae). Les abeilles domestiques (*Apis mellifera* L.) sont fréquemment exposées aux résidus d'insecticides dans les zones agricoles. Il est nécessaire d'étudier leur sensibilité à ces composés chimiques, sans parler de l'éventuelle contamination du miel. L'arrivée de nouveaux insecticides sur le marché pour lutter contre les ravageurs des cultures dépend de la connaissance de leur impact sur les population des insectes auxiliaires. Dans ce contexte nous avons évalué sur des larves et des abeilles naissantes la toxicité aiguë par contact et par ingestion de la pellitorine (Fig. 1), amide isolé de *Piper tuberculatum* Jacq. (Piperaceae) et utilisé comme biopesticide contre la pyrale du maïs (*Ostrinia nubilalis*). Les ouvrières, au stade larvaire et adulte, ont été réparties en groupes recevant chacun des doses différentes de pellitorine. Les larves ont été maintenues dans leur cellule d'origine où elles étaient nourries et soignées normalement par les nourrices. Les ouvrières adultes étaient confinées dans des cagettes en bois et grillagées et recevaient une nourriture artificielle composée de sirop de sucre (1:1). La pellitorine a été diluée dans de l'éthanol à 98 % aux doses de 40, 200, 1 000, 5 000 et 25 000 ng de matière active (m.a.) par individu. Nous avons trouvé une DL_{10} de 39,14 ng m.a./insecte pour les larves et de 36,16 et 13,79 pour les adultes, respectivement par ingestion et contact (Tab. II). La pellitorine est plus active lorsque son absorption se fait par voie cutanée que par ingestion. De par sa toxicité aiguë pour les larves comme pour les adultes, le produit est hautement dangereux pour les abeilles domestiques.

***Apis mellifera* / toxicité par contact / toxicité par ingestion / pellitorine / biopesticide**

Zusammenfassung – Schädigung von *Apis mellifera* (Hymenoptera: Apidae) durch Pellitorine, einem aus *Piper tuberculatum* (Piperaceae) isolierten Amid. Honigbienen sind häufig Insektizidrückständen in landwirtschaftlich bearbeiteten Flächen ausgesetzt. Eine Schädigung durch diese chemischen Verbindungen, zusätzlich zur Kontamination von Honig, muss überprüft werden. Die Einführung neuer Insektizide am Markt zur Bekämpfung von Schädlingen ist abhängig von Kenntnissen ihrer Wirkung auf die Population von Nutzinsekten. Die akute Toxizität von dem Amid Pellitorine, das aus der Pflanze *Piper tuberculatum* (Piperaceae) gewonnen und als Biopestizid in der Landwirtschaft gegen den Europäischen Maiszünsler (O*strinia nubilalis*) eingesetzt wird, wurde in Versuchen durch Kontakt und Fütterung von Larven und frisch geschlüpften Imagines von *Apis mellifera* bestimmt. Larven und adulte Arbeiterinnen wurden in Gruppen aufgeteilt, die verschiedene Dosen Pellitorine erhielten. Die Larven wurden in ihren ursprünglichen Zellen belassen und von Ammenbienen gefüttert und gepflegt. Adulte Bienen wurden in Holzkäfigen, versehen mit Gittern, gehalten und erhielten Zuckerwasser (1:1). Das Amid wurde in 98 % Ethanol für eine Dosierung von 40, 200, 1 000, 5 000 und 25 000 ng a.i./Individuum gelöst. Für Larven und für Adulte wurden LD_{10} Werte von 39,14, 36,16 und 13,79 ng a.i./Insekt gefunden (Aufnahme durch Nahrung und Kontakt). Es konnte gezeigt werden, dass Pellitorine aktiver ist, wenn die Aufnahme über die Haut als über die Nahrung erfolgte. Der Nachweis dieser hohen akuten Toxizität für Larven und adulte Bienen belegt die hohe Schädigung von Bienen durch Pellitorine.

***Apis melifera* / Kontakt / Nahrungsaufnahme / Pellitorine / Biopestizid**

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