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Botanical monoterpenes synergise the toxicity of azamethiphos in the vector of Chagas disease, *Triatoma infestans* (Hemiptera: Reduviidae)

Mercedes María Noel Reynoso[1], Emilia Ana Seccacini[1], Eduardo Nicolás Zerba[1,2] and Raúl Adolfo Alzogaray[1,2]

1 *UNIDEF-CITEDEF-CONICET-CIPEIN, Buenos Aires, Argentina*
2 *Instituto de Investigación e Ingeniería Ambiental, Universidad Nacional de San Martín, Buenos Aires, Argentina*

Abstract

OBJECTIVE To investigate what toxicological interactions occur when binary combinations of azamethiphos and botanical monoterpenes (eugenol, menthol or menthyl acetate) are applied to *Triatoma infestans*.

METHODS The toxicity of binary mixtures of azamethiphos and sublethal doses of a monoterpene (eugenol, menthol or menthyl acetate) was evaluated in nymphs of the first stage of *T. infestans*. Experiments using exposure to filter papers and topical application were carried out. Values of Lethal Concentration 50% (LC50) were calculated in the first case, and values of Lethal Dose 50% (LD50) in the second.

RESULTS The LC50 of azamethiphos applied on filter paper was 50.3 μg/cm^2. However, when it was simultaneously applied with a sublethal concentration of monoterpene, its toxicity increased (LC50 with eugenol = 11.20 μg/cm^2, LC50 with menthyl acetate = 5.30 μg/cm^2, LC50 with menthol = 7.26 μg/cm^2). When applied topically, the LD50 of azamethiphos was 7.85 μg/insect, but its toxicity drastically increased when it was applied together with sublethal doses of menthol (LD50 = 0.00016 μg/insect) or menthyl acetate (LD50 = 0.00051 μg/insect). The simultaneous application with eugenol did not significantly change azamethiphos toxicity (LD50 = 12.79 μg/insect).

CONCLUSIONS The toxicity of azamethiphos in *T. infestans* was synergised when it was applied together with eugenol, menthol or menthyl acetate on a filter paper. However, only menthol and menthyl acetate synergysed azamethiphos when mixtures were topically applied. The drastic effects of menthol and menthyl acetate in topical application experiments should be further studied as they could be the basis for developing more efficient triatomicidal products with a lower content of conventional insecticides than those currently used for controlling *T. infestans*.

keywords kissing bug, Chagas disease, synergism, monoterpene, insecticide

Introduction

Eugenol, menthol and menthyl acetate are botanical monoterpenes widely used for their medicinal, aromatic and pharmaceutical properties [1–4]. They also are insecticides and modify insect behaviour [2, 4]. In the blood-sucking bug *Triatoma infestans* (Klug), the main vector of Chagas disease in Argentina and limiting countries [5], some monoterpenes elicit repellency and hyperactivity [6, 7].

Toxicological interactions between conventional insecticides and botanical monoterpenes have been scarcely studied [8–10]. But they are worth considering as they may lead to the discovery of mixtures with a higher toxicity than that of their individual components (synergism). The addition of synergists to insecticides may allow reducing the use of conventional insecticides in pest control.

The objective of the present study was to investigate the toxicological interactions that occur when binary mixtures of a conventional insecticide (azamethiphos) and sublethal doses of botanical monoterpenes (eugenol, menthol or menthyl acetate) are applied to *T. infestans* nymphs. Azamethiphos is an organophosphorus insecticide (OP) commercialised for controlling flies, mosquitoes and cockroaches [11]. Although no previous studies mention

its use for controlling Chagas vectors, it could well be an answer to the increasing number of sources reporting pyrethroid resistance in *T. infestans* [12–16]. The OP fenitrothion and malathion have been successfully used for the control of pyrethroid-resistant populations of *T. infestans* in Argentina and Bolivia [17–19]. In this way, some OP appear to be the only viable alternative to pyrethroids to control resistant populations at present.

Material and methods

Biological material

The bioassays were performed with 5–7 day-old, fasted since eclosion, first instar nymphs of *T. infestans* from the CIPEIN colony. This colony is reared under controlled conditions of temperature (25 ± 2°C), RH (60-90%) and photoperiod (12:12 h L:D). Insect breeding follows a protocol approved by the CIPEIN Animal Care and Uses Committee (IACUC/CICUAL 1572/155).

Chemicals

Eugenol (99%), menthol (99%) and menthyl acetate (98%) were purchased from Sigma-Aldrich (Buenos Aires, Argentina). Azamethiphos (99%) was a gift from Chemotecnica S.A. (Spegazzini, Argentina). Acetone (technical grade) and silicone oil (556 cosmetic fluid) were purchased from Merck (Darmstadt, Germany) and Daltosur S.R.L. (Buenos Aires, Argentina), respectively.

Bioassays

Exposure to impregnated papers. Nymphs were exposed to impregnated filter paper circles 11 cm in diameter (102 FAST, Hangzhou Xinxing Paper Industry & Co. Ltd., Funyang, China). Each circle was impregnated with 0.5 ml of a solution of azamethiphos alone or combined with a monoterpene (eugenol, menthol or menthyl acetate). An acetone:silicone (1:1) mixture was used as solvent. The solvent was left to evaporate for 24 h, and then, a glass ring was placed on the paper circle to prevent the nymphs from escaping. Ten first instar nymphs of *T. infestans* were then placed on the paper circle. Time of exposure was 2 h, following a protocol used at CIPEIN (unpublished). After exposure, the nymphs were transferred to a 250 ml plastic container with a folded piece of paper inside. The container was closed with a piece of gauze voile held in place with a rubber band and placed in a chamber under the same breeding conditions. Mortality was registered 24 h later. Nymphs that did not move after prodding them with a soft pair of tweezers were considered dead.

The following treatments were assessed: (a) solvent (acetone:silicone 1:1); (b) different concentrations of azamethiphos in acetone:silicone (1:1), ranging between 3.1 and 200 μg/cm^2; (c) a monoterpene solution in acetone: silicone (1:1) (390 μg/cm^2 of eugenol, menthol or menthyl acetate); and (d) the same concentrations of azamethiphos used in b), mixed with a constant concentration of monoterpene (390 μg/cm^2). The doses of monoterpenes used were the highest concentrations that did not produce intoxication symptoms in preliminary assays (incoordination, paralysis, lateral displacement, tremors, knock-down). Four independent replicates of each assay were carried out. Results were used for calculating Lethal Concentration 50% (LC50) values.

Topical application assays. A solution of azamethiphos in acetone, alone or combined with a monoterpene, was applied using a Hamilton microsyringe (Reno, NV). Each nymph received 0.2 μl on the dorsal region of the abdomen. The nymphs were immediately transferred to a chamber under the same breeding conditions as in the assays using impregnated papers. Twenty-four hours later, mortality was recorded. Nymphs that did not move after touching them with a soft pair of tweezers were considered dead.

The following treatments were assessed: (a) acetone alone; (b) different concentrations of azamethiphos in acetone, ranging from 0.04 to 4 μg/insect; (c) a monoterpene solution in acetone (eugenol: 2 μg/insect, menthol: 0.02 μg/insect, menthyl acetate: 20 μg/insect); and d) different concentrations of azamethiphos in acetone (ranging from 0.04 to 4 μg/insect) combined with a constant concentration of monoterpene in acetone (the same concentrations used in c). The doses of monoterpenes used were the highest that did not produce intoxication symptoms in preliminary assays. Four independent replicates of each assay were carried out, and the results were used to calculate Lethal Dose 50% (LD50) values.

Statistical analyses

Values of LC50 and LD50 were calculated with their respective 95% confidence intervals (CI 95%) using the software POLO PC [20]. This software does the computations described in Finney [21]. It tests (and corrects for) heterogeneity and analyses the chi-square goodness of fit. Differences between the values of LC50 or LD50 were considered significant when their CI 95% did not overlap ($P < 0.05$).

Results

In the first experimental series, first instar nymphs of *T. infestans* were exposed to filter paper circles impregnated with azamethiphos alone or in binary combinations with sublethal concentrations of eugenol, menthol and menthyl acetate (Table 1).

The LC50 of azamethiphos alone (50.30 μg/cm^2; 95% CI: 22.10–105.50) was significantly higher than the LC50 values of the combinations with eugenol (11.20 μg/cm^2; 95% CI: 1.80–21.60), menthol (5.30 μg/cm^2; 95% CI: 4.20–6.60) and menthyl acetate (7.26 μg/cm^2; 95% CI: 5.00–10.20) ($P < 0.05$). No significant differences were observed between the LC50 values of the three mixtures ($P > 0.05$).

In the second experimental series, first instar nymphs of *T. infestans* were topically treated with solutions of azamethiphos in acetone and binary combinations of azamethiphos with a monoterpene (Table 2). The LD50 of azamethiphos alone was 7.85 μg/insect (95% CI: 3.92–16.15). The LD50 of the binary combinations were 0.00016 μg/insect (95% CI: 0.00014–0.0012) for menthol and 0.00051 μg/insect (95% CI: 0.00014–0.0016) for menthyl acetate. Both were significantly lower than the LD50 for azamethiphos alone ($P < 0.05$). The LD50 of the mixture containing azamethiphos and eugenol (12.79 μg/insect; 95% CI: 7.87–25.03) was not significantly different from that of azamethiphos alone ($P > 0.05$).

In both experimental series, no mortality was observed when the insects were treated with neither solvent alone nor solution of monoterpenes without azamethiphos.

Discussion

The object of this study was to evaluate the toxic effect of binary mixtures combining the insecticide azamethiphos and sublethal concentrations of one botanical monoterpene (eugenol, menthol or menthyl acetate) *in T. infestans*. When nymphs were exposed to impregnated filter papers, the three monoterpenes synergised the toxic effect of azamethiphos. However, when the mixtures were topically applied, only menthol and menthyl acetate produced a synergic effect.

There are very few studies on binary, ternary and quaternary mixtures of monoterpenes [22–26]. There are also very few references on mixtures of monoterpenes and conventional insecticides. Among those available, there are reports on the synergic effects of terpinen-4-ol and γ-terpinene on the toxicity of the organophosphorus profenofos and the carbamate methomyl in larvae of the Egyptian cotton leafworm, *Spodoptera littoralis* (Boisduval) [9]. Linalool and thymol also showed synergic effects on the neonicotinoid imidacloprid in the green peach aphid, *Myzus persicae* (Sulzer) [10].

The synergic effects of eugenol, menthol and menthyl acetate on azamethiphos reported in this study could be the result of interactions that modify some toxicokinetic or toxicodynamic process of this insecticide. A synergic effect would be expected, for example, if the monoterpenes increase the rate of absorption of azamethiphos, decrease its rate of detoxification or increase its interaction with its primary site of action.

The three monoterpenes are lipophilic [27], a property which should allow for a high rate of cuticular absorption. Hence, their presence in binary mixtures may facilitate the rate of cuticular absorption [28].

From a metabolic point of view, monoterpenes might synergise azamethiphos by inhibiting enzymes that detoxify it or compete with it over the same enzymatic activity. In insects, azamethiphos is a target for hydrolysis, oxidations and conjugations [29–31], but there is no literature connecting these monoterpenes to the inhibition of insect detoxifying enzymes. There are no references on their metabolism in insects; however, the presence of metabolisable groups in their chemical structures (such as methyl, methoxyl and ester bonds) suggests that eugenol, menthol and menthyl acetate could be the substrates of different insects detoxifying activities [32].

Table 1 LC50 for azamethiphos alone or in binary combinations with monoterpenes in first instar nymphs of *Triatoma infestans* exposed to impregnated filter papers

Treatment	*n*	LC50 (CI 95%) (μg/cm^2)	Slope ± SE	X^2
Azamethiphos	120	50.30a (22.1–105.5)	2.05 ± 0.41	18.81
Azamethiphos + eugenol	200	11.20b (1.8–21.6)	1.52 ± 0.47	7.93
Azamethiphos + menthol	200	5.30b (4.2–6.6)	4.00 ± 0.67	7.74
Azamethiphos + menthyl acetate	200	7.26b (5.0–10.2)	3.12 ± 0.48	19.37

CI 95%, Confidence interval 95%; LC50, Lethal concentration 50%; SE, Standard error.
To assess the toxicity of the mixtures, different concentrations of azamethiphos were used combined with a constant concentration of monoterpene (390 μg/cm^2). LC50 followed by different letters are significantly different ($P < 0.05$).

Table 2 LD50 for azamethiphos alone or in binary combinations with monoterpenes in first instar nymphs of *T. infestans* topically treated

Treatment	*n*	LD50 (CI 95%) (μg/insect)	Slope ± SE	X^2
Azamethiphos	200	7.85a (3.92–16.15)	1.20 ± 0.12	48.47
Azamethiphos + eugenol	200	12.79a (7.87–25.03)	1.26 ± 0.18	26.17
Azamethiphos + menthol	200	0.00016b (0.00014–0.0012)	0.24 ± 0.06	9.18
Azamethiphos + menthyl acetate	200	0.00051b (0.00014–0.0016)	0.49 ± 0.07	24.89

CI 95%, Confidence interval 95%; LD50, Lethal dose 50%; SE, Standard error.
Solutions were applied topically (1 μl/insect in the dorsal region of the abdomen). To assess the toxicity of the mixtures, different doses of azamethiphos were used combined with a constant dose of monoterpene (eugenol: 2 μg/insect, menthol: 0.2 μg/insect, menthyl acetate: 20 μg/insect). LD50 followed by different letters are significantly different ($P < 0.05$).

Another possible explanation of the synergism could be related to the site of action. Some monoterpenes, among them eugenol and menthol, inhibit the activity of acetylcholinesterase, which is the site of action of azamethiphos. But inhibition only occur when very high concentrations of the monoterpenes are applied; thus, it cannot be considered their primary site of action [33–36]. Nevertheless, an interaction involving this enzyme, which subsequently modifies its interaction with azamethiphos, cannot be overruled.

Finally, another type of synergism recently reported for eugenol arises by the way this monoterpene modifies the behaviour of third instar nymphs of *T. infestans* [37, 38]. When exposed to a surface treated with the pyrethroid permethrin, nymphs hyperactivated by eugenol pick up more permethrin and consequently become intoxicated faster than nymphs that are not hyperactivated. Therefore, hyperactivity could be responsible for the increase in toxicity of azamethiphos, at least in the case in which it was applied together with eugenol by exposure to impregnated papers. The absence of synergism when the mixture was topically applied supports this explanation, as in this case hyperactivity would have no effect on the dose of azamethiphos received. To test this hypothesis, the toxicological interaction between eugenol and azamethiphos on third instars of *T. infestans* should be evaluated.

Menthol also hyperactivates *T. infestans* [6], but in this case synergised azamethiphos both by exposure to impregnated papers and topical application. This suggests that even though hyperactivity was related to the synergism, it would not be its sole cause.

What mechanism is responsible for the fact that menthol and menthyl acetate synergise azamethiphos under conditions where eugenol does not synergise it? The answer could be in the nicotinic acetylcholinesterase receptors (nAChR), which are inhibited by both menthol and OP [39, 40]. Then, it is possible that if menthol and azamethiphos bind in a non-competitive way on these receptors, their joint application would cause synergism.

The simultaneous application of an OP and menthyl acetate could give a similar result, because it is probable that in *T. infestans* this monoterpene acts *in vivo* as a 'pro-menthol', because carboxyesterases could hydrolyse it to menthol when entering the insect. This speculation is based on the fact that *T. infestans* shows significant esterase activity [41, 42]. The strategy of esterifying a molecule to improve its absorption through the insect cuticle, turning it into a 'pro-insecticide' that by hydrolysis is easily regenerated within the body, is a practice whose effectiveness has already been demonstrated in *T. infestans* [43].

This hypothesis about a toxicological interaction between menthol and azamethiphos in *T. infestans* is highly speculative, because the above-mentioned research was performed using vertebrates nAChER. When more information on the toxicokinetics and toxicodynamics of monoterpenes is available, the mechanisms that cause the toxicological interactions in which they participate will be better understood.

The discovery of the synergic effects of monoterpenes on the toxicity of conventional insecticides could be the first step towards a highly desirable reduction in the use of conventional insecticides. Given the novelty of these results, and their potential applicability, it is important to further study about the mechanism producing the exceptionally high synergism of mentol and menthyl acetate on azametiphos toxicity in *T. infestans*. These monoterpenes increased the toxicity of azamethiphos by more than four orders of magnitude. An unusual result, considering that other synergic effects reported on binary mixtures containing a monoterpene and a conventional insecticides, is generally much lower. For example, the joint toxicity of binary mixtures of imidacloprid, profenofos and methomyl with different monoterpenes was between 1.8 and

4.8 times higher than the individual toxicity of each insecticide [9, 10].

It should also be investigated whether menthol synergises the OP fenitrothion and malathion, since both of them have been used successfully to control pyrethroid-resistant bugs [17–19]. Finally, it would also be interesting to investigate whether these synergic interactions also occur in other insect pests, especially in those already resistant to other insecticides.

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Corresponding Author Mercedes María Noel Reynoso, UNIDEF-CITEDEF-CONICET-CIPEIN, Juan B. de La Salle 4397, Villa Martelli, Buenos Aires, Argentina. Email: get_juy87@hotmail.com