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Original Article

Control of *Varroa destructor* in honey bees (*Apis mellifera*) with the aqueous extract of *Petiveria alliacea* and identification of its constituents by LC–MS

Controle de Varroa destructor em abelhas melíferas (*Apis mellifera*) com extrato aquoso de *Petiveria alliacea* e identificação de seus constituintes por LC-MS

J. C. Bracho-Pérez[a], I. Tacza-Valverde[a], D. Chávez-Rojas[a], C. L. Aquije-Dapozzo[a], J. L. Bringas-Salvador[a] and J. A. Vásquez-Castro[b*]

[a]Universidad Nacional Tecnológica de Lima Sur - UNTELS, SAICADES Research Group, Lima, Peru
[b]Universidad Nacional Agraria La Molina - UNALM, Department of Entomology, Lima, Peru

Abstract

The aqueous extract of *Petiveria alliacea* L. (Caryophyllales, Phytolaccaceae) was found to control varroosis caused by *Varroa destructor* in honey bees (*Apis mellifera*). Adult female *V. destructor* mites were immersed in solutions of the aqueous extract at different concentrations (24, 60, 100, 140, 180 and 200 mg L^{-1}). After 24h of exposure, mortality was assessed and the median lethal concentration (LC_{50}) was estimated to be 105.2418 mg L^{-1}. In the direct contact acute toxicity experiments, 0.5 µL of each of the above extract solutions was applied to the thoraces of *A. mellifera*, and the estimated LC_{50} was 165.4156 mg L^{-1}, demonstrating the minimal risk of the extract to this insect. Furthermore, the LC_{50} in *A. mellifera* after residual contact was 104.904 mg L^{-1}, indicating a greater risk of toxicity. Furthermore, oral toxicity, determined by incorporating various concentrations of the extract (12, 30, 60, 75 and 92 mg L^{-1}) into the diet for 24 h, 48 h, 72 h and 96 h of exposure yielded LC_{50} values of 153.1455, 12.0472, 50.5723 and 73.7513 mg L^{-1}, respectively, suggesting that ingestion poses the greatest risk of toxicity to bees. Finally, liquid chromatography–mass spectrometry (LC–MS) analysis revealed the presence of 16 compounds in the extract, of which 14 were identified, and the major compounds were two proline derivatives, trans-N-methyl-4-methoxyproline and N,N-dimethylproline, followed by N-α-tert-butyloxycarbonyl-valylproline-methyl ester. These results show the promising application potential of *P. alliacea* for the control of varroosis, but field bioassays are needed to establish the efficiency and possibility of using the acaricidal capacity of this plant to control *V. destructor*.

Keywords: bioassays, botanical biopesticide, aqueous extract, LC_{50}, chromatography.

Resumo

Foi demostrado que o extrato aquoso de *Petiveria alliacea* L. (Caryophyllales, Phytolaccaceae) controla a varroose causada por *Varroa destructor* em abelhas melíferas (*Apis mellifera*). Fêmeas adultas do ácaro *V. destructor* foram submersas em soluções do extrato aquoso em diferentes concentrações (24, 60, 100, 140, 180 e 200 mg L^{-1}). Após 24 h da exposição, a mortalidade foi avaliada e a concentração letal média (CL_{50}) foi estimada em 105,2418 mg L^{-1}. Nos experimentos de toxicidade aguda por contato direto, 0,5 µL de cada uma das soluções do extrato acima indicadas foram aplicadas no tórax de *A. mellifera*. Assim, a CL_{50} estimada foi de 165,4156 mg L^{-1}, demonstrando risco mínimo do extrato para esse inseto. Em adição, a CL_{50} em *A. mellifera* após contato residual foi de 104,904 mg L^{-1}, indicando maior risco de toxicidade. A toxicidade oral, determinada pela incorporação de várias concentrações do extrato (12, 30, 60, 75 e 92 mg L^{-1}) na dieta por 24, 48, 72 e 96 h de exposição, produziu valores de CL_{50} de 153,1455; 12,0472; 50,5723 e 73,7513 mg L^{-1}, respectivamente, sugerindo que a ingestão representa o maior risco de toxicidade para as abelhas. Finalmente, a análise por Cromatografia Líquida-Espectrometria de Massas (LC-MS) revelou a presença de 16 compostos no extrato, dos quais 14 foram identificados, e os principais compostos foram dois derivados da prolina – trans-N-metil-4-metoxiprolina e N,N-dimetilprolina –, seguidos por N-α-terc-butiloxicarbonil-valilprolina-metil éster. Estes resultados mostram o potencial promissor de aplicação de *P. alliacea* para o controle da varroose, mas bioensaios de campo são necessários para estabelecer a eficiência e a possibilidade de usar a capacidade acaricida desta planta para controlar *V. destructor*.

Palavras-chave: bioensaio, biopraguicida botânico, extrato aquoso, CL_{50}, cromatografia.

*e-mail: jaque@lamolina.edu.pe
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1. Introduction

Varroasis is a health problem caused by mites (*Varroa destructor*) that occurs bee colonies and constitutes the greatest threat to the development of beekeeping worldwide. The impact *V. destructor* has been of such great magnitude that no other parasite has generated so many studies, applied research and monetary investments than that of any other pest since the total collapse of bee colonies occurs withing two to three years after contracting varroosis (Anderson and Trueman, 2000; Rosenkranz et al., 2010).

V. destructor is globally recognized as the principal driver of honey bee colony losses. Once attached to the body of the bees, the mite extracts essential nutrients, suppresses immune function, and transmits viruses, particularly Deformed Wing Virus (DWV). Ultimately, its continuous parasitic activity leads to severe weakening and colony collapse, an epidemic phenomenon documented across multiple regions of the world (Jeyapriya et al., 2025). Molecular evidence clearly demonstrates that the mite *V. destructor* disrupts honey bee immunity, metabolism, and neuronal function, thereby promoting the proliferation of associated viruses—most notably Deformed Wing Virus (DWV). This interaction triggers epidemic-level outbreaks within colonies, leading to behavioral alterations, reduced resilience, and ultimately colony collapse (Morfin et al., 2023; Warner et al., 2024).

However, until now, varroosis has not been able to be eliminated, considering that *V. destructor*, the parasite that most impacts *Apis mellifera* colonies, is an obligatory parasite that can attack during different stages of its development, which has become a main reason for the drastic decreases in the numbers of beekeepers and honey bee colonies and cross pollination in Europe, Peru and other regions of the world (Dávila and Ortiz, 1987; De la Rua et al., 2009; Hristov et al., 2020; Morfin et al., 2023).

The search for a sustainable solution to control *V. destructor* has led to the consideration of plant resources. One such plant resource of interest is mucura (*P. alliacea*), a perennial and herbaceous plant that is native to the Amazon rainforest and is widespread in Peru. It has been proven that *P. alliacea* extracts can function as botanical biopesticides, exhibiting both insecticidal and acaricidal properties (Johnson et al., 1997; Rosado-Aguilar et al., 2010, 2017).

The acaricidal activities of the crude extracts, fractions, essential oils and their components of *P. alliacea* have been demonstrated against the larvae and adults of the cattle tick *Rhipicephalus (Boophilus) microplus* and the two-spotted spider mite (*Tetranychus urticae*) (Rosado-Aguilar et al., 2010; Neves et al., 2011; Arceo-Medina et al., 2016; Flota-Burgos et al., 2021).

A recent publication that evaluated the toxicity of extracts obtained from *P. alliacea* against neonates of *Daphnia magna* used as a bioindicator revealed that the aqueous extract was the least toxic. Thus, given the importance of protecting the environment, the use of this aqueous extract as an acaricidal agent represents a sustainable alternative (Bracho-Pérez et al., 2024).

The objective of this work was to evaluate the use of the aqueous extract of *P. alliacea* to control the *V. destructor* mite, which damages honey bee (*A. mellifera*) colonies, as well as to study its chemical constituents via liquid chromatography–mass spectrometry (LC–MS).

2. Materials and methods

2.1. Plant material

P. alliacea leaves were collected in the district of Castillo, province of Leoncio Prado, in the Department of Huánuco (09° 16 'S, 76° 00' W), in August 2016. A sample was placed in the Herbarium of the National Agrarian University La Molina with voucher number 012-2019-HM-UNALM.

2.2. Biological material

2.2.1. V. destructor (Anderson and Trueman)

Adult female varroas were collected from hives with varroosis in an apiary containing 20 colonies via the roller method with the use of powdered sugar according to Dietemann et al. (2013).

2.2.2. A. mellifera Linnaeus

The bees that were used in this experiment were from 10 European honey bees (*A. mellifera*) colonies that are part of a healthy apiary and were free of varroosis and had not been subjected to any disease treatments. In addition, the apiary is located in a healthy ecosystem away from industrial and environmental pollution. The hives that are in this apiary are of the American standard type with a single body.

2.3. Methodology

2.3.1. Acute toxicity to V. destructor determined by immersion

The acute toxicity of the *P. alliacea* extract was determined via the use of adult female *V. destructor* mites collected from adult honey bees using the roller method with powdered sugar. Mites were collected before each toxicity bioassay. Contact toxicity bioassays were performed by exposing the mites to six different concentrations, ranging from 2.4% (24 mg L^{-1}) to 20% (200 mg L^{-1}), of the *P. alliacea* extract selected for 24 h (Dietemann et al., 2013).

In this bioassay, groups of five mites were immersed in the *P. alliacea* extract at various concentrations in glass vials followed by storage for a long duration to evaluate possible time-dependent acaricidal activity. Each group consisted of triplicate samples, including the control treatment and solvent control groups. Adult female mites were kept in a dark incubator at 34 °C and were observed with a stereoscope at the end of the exposure periods. Three types of mites were considered to evaluate final and effective lethality: 1. mobile varroas with coordinated or uncoordinated movement after stimulation; 2. paralyzed varroas with movement of one or more appendages; and 3. dead varroas without stimulation requirements (Dietemann et al., 2013).

2.3.2. Acute toxicity to A. mellifera

To estimate the acute toxicity of the *P. alliacea* extract to honey bees, individual bees were collected from the brood frames of a bee hive and kept in the dark overnight in a 50% sucrose solution at 34 °C. After harvesting, the bees were exposed to the *P. alliacea* extract by three methods: i. direct contact using a microapplicator; ii. residual contact; and iii. oral exposure by incorporation into the diet.

The bioassays were carried out by exposing the bees to six different concentrations of the *P. alliacea* extract for the established durations.

i. Direct contact bioassay using a microapplicator

The direct contact bioassays were performed using serial dilutions (2.6–20% for a total of six concentrations) of the extract in the chosen solvent. The solutions (0.5 µL each) were topically applied to the dorsal thorax of the insects using a Burkard Arnold microapplicator. Control treatment consisted of applying 0.5 µL of solvent. Ten insects were treated with each concentration of extract or the solvent control, and the experiment was repeated six times. The treated and control insects were transferred to glass vials, which were incubated at 29-30 °C with 70–80% relative humidity, and mortality was evaluated after 24 h. The data were subjected to a probit analysis to determine the LD_{50} values (Sakuma, 1998; Vu, 2016).

ii. Residual contact bioassay

The methodology proposed by the United States Environment Agency (USEPA, 2012) was applied, with modifications. Bees were anesthetized with carbonic anhydride for 10 s, after which 10 individuals were placed in glass Petri dishes lined internally with filter paper that had been pretreated with the *P. alliacea* extract at concentrations ranging from 2.4% (24 mg L^{-1}) to 20% (200 mg L^{-1}) and dried by evaporation for 1 h. The bees were kept in environmental chambers at 34 °C and provided 50% sucrose syrup. Lethality was considered the endpoint for each bioassay, considering individuals that were unable to stand on their own, after 24 h of exposure.

iii. Oral toxicity bioassay by incorporation into the diet

OECD test no. 213 was applied to determine the acute oral toxicity of the extract to both young and healthy adult *A. mellifera* collected in the morning. The bees were placed in a stainless steel mesh cage (40 cm × 20 cm × 20 cm), provided ad libitum access to an aqueous solution of sucrose (500 g L^{-1}, 50% w/v) in glass capillary tubes (50 mm long × 10 mm wide). The bees were deprived of food 2 h before the toxicity tests, and those that died were replaced before the experiment. The experiment was conducted in the dark at 25 ± 2 °C with a relative humidity of approximately 50–70% (OECD, 1998).

The aqueous extract was lyophilized until a dry powder, which was considered the ai, was obtained. Different amounts of the powder were mixed with a sucrose solution to obtain five ai solutions with concentrations of 12 mg L^{-1}, 30 mg L^{-1}, 60 mg L^{-1}, 75 mg L^{-1} and 92 mg L^{-1} supplied to the bees at a dose of 120 µL until they were entirely consumed, after which the sucrose solution was supplied alone ad libitum. Each bioassay was performed in triplicate. Mortality was recorded at 24 h, 48 h, 72 h, and 96 h, in which endpoint was recorded for those individuals who were completely immobile (OECD, 1998).

2.3.3. LC–electrospray ionization (ESI)–MS analysis

2.3.3.1. LC system conditions

Each sample was dissolved in 100 µL of methanol (MeOH) and analyzed on an Agilent 1200 Rapid Resolution high-performance liquid chromatography (HPLC) system connected to a Bruker maXis mass spectrometer. A Waters Atlantis T3 column (4.6 × 100 mm, 5 µm particle size) was used for component separation. The mobile phase was composed of two solvents, solvent A (H_2O:ACN (90:10)) and solvent B (H_2O:ACN (10:90)), both of which contained 13 mM ammonium formate and 0.01% trifluoroacetic acid (TFA). Gradient elution was performed as shown in Table 1. The injection volume was 2 µL.

2.3.3.2. MS conditions

Mass spectra were acquired in positive ESI mode with the following parameters: capillary voltage, 4 kV; drying gas flow, 11 L min^{-1}; nebulizer temperature, 200 °C; and nebulizer pressure, 2.8 bar.

2.3.3.3. LC–MS data analysis and processing

The chromatographic data were processed using Bruker's internal algorithm to extract the components, and the most intense peaks, both in the positive mode total ion chromatogram (TIC) and by absorbance at 210 nm, were selected for exact mass and molecular formula determination. The retention time and exact mass combinations were used as the criteria when searching the high-resolution mass spectrometry database and the Chapman & Hall dictionary of natural products, as well as other specialized primary sources such as the Base US National Institute of Standards and Technology (NIST) Standard Reference Data Number 69, NIST Chemistry WebBook and the open access PubChem Chemical Database of the US National Institute of Health. The official atomic weights of the chemical elements were updated by the researchers of the Committee of the Inorganic Chemistry Division of the IUPAC (National Institutes of Health (NIH)) (Petersen and Amstutz, 2008; Buckingham et al., 2015; Wallace, 2023; Kim et al., 2020; Meija et al., 2016).

Table 1. Gradient elution profile of the *P. alliacea* aqueous extract.

Time (min)	Solvent (%)		Flow rate (mL min^{-1})
	A[1]	B[2]	
0.0	100	0	0.5
14.0	0	100	0.5
17.0	0	100	0.5
17.1	100	0	0.5
20.0	100	0	0.5

[1]H_2O:ACN (90:10); [2]H_2O:ACN (10:90).

2.3.4. Plant material processing and extract preparation

The sample was processed and the aqueous extract of *P. alliacea* leaves was obtained according to previously published experimental methods (Bracho-Pérez et al., 2024).

2.4. Statistical analysis

The acute toxicity bioassay results were processed to determine the median lethal concentration (LC_{50}), expressed in mg L^{-1}, with a confidence level of 95% ($\alpha = 0.05$). Statistical significance and probit regression analyses were carried out by determining the angular coefficients of the slopes, chi-square (X^2) values, and heterogeneity using the statistical program PoloSuite. These analyses determination of an appropriate effective concentration of the extract that would cause the least environmental impact (LeOra Software LLC, 2016; Robertson et al., 2017).

3. Results and discussion

3.1. Acute toxicity to V. destructor determined by immersion

Analyzing the plot of the bioassay results in Figure 1a, it can be observed that doses of *P. alliacea* extract above 140 mg L^{-1}, *V. destructor* mortality equaled or exceeded 80%, whereas at a dose of 100 mg L^{-1}, mortality reached values below 50% (46.66%). However, it is important to compare these data with those acquired via field assays.

Probit regression analysis revealed that the LC_{50} was 105.2418 mg L^{-1} with a 95% confidence interval of 97.092-115.6 mg L^{-1} (Table 2), and this range contains the maximum acceptable concentration that guarantees the sustainable development of biopesticide. A previous study with *D. magna* determined 100 mg L^{-1} *P. alliacea* aqueous extract as a suitable concentration (Bracho-Pérez et al., 2024).

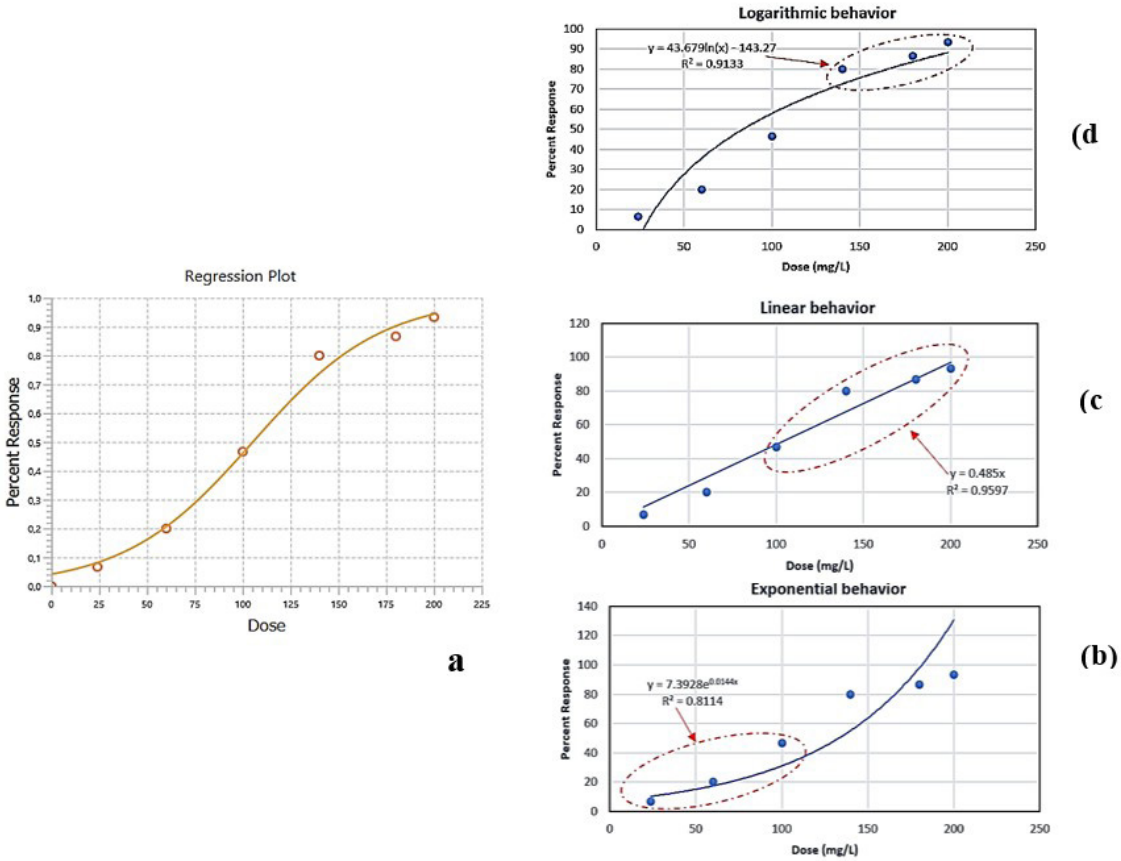

Figure 1. Dose–response curve of (a) *V. destructor* mortality caused by the *P. alliacea* aqueous extract and the exponential (b), linear (c) and logarithmic (d) segments.

Table 2. LC_{50} values and 95% confidence intervals (mg L^{-1}) obtained from the *Varroa destructor* acute toxicity bioassay.

Extract *P. alliacea*	*Varroa destructor*	LC_{50} (mg L^{-1})	AC ± SE	X^2	DF	Heterogeneity
Aqueous	Susceptible	105.2418 (97.092-115.6)	5.1872 ± 0.0057	0.6007	4	0.1502

AC, angular coefficient; SE, standard error of the mean; X^2, chi-square, DF, degrees of freedom.

The fit to the probit model was optimal, as indicated by the angular coefficient of the slope being greater than 1.96, showing that the response detected by probit analysis was significant, as was the reduction in *V. destructor* infestation upon application of the *P. alliacea* aqueous extract. In addition, the heterogeneity value was less than 1.0, showing the validity of the dose–mortality relationship and therefore the validity of the bioassay results (Robertson et al., 2017).

The dose–mortality regression curve constructed from the data from this bioassay (Figure 1) constitutes one of the possible responses of an organism upon the application of a chemical control agent. The probit analysis (Figure 1a) suggested three possible mathematical trends (Figure 1b, 1c, 1d), wherein at the beginning of treatment with the lowest two doses (24 and 60 mg L^{-1}), the effect was small, with mortality rates of 6.63% to 20%, followed by an exponential trend, indicating that the mite was resistant to the dose used.

At the third lowest dose (100 mg L^{-1}), the curve was linear with the highest quadratic correlation coefficient value (R^2 = 0.9597), and mortality increased rapidly to greater than 46.66% until the final stage of treatment, that is, the final portion of the curve (Figure 1a) exhibits logarithmic behavior. At the three highest doses (140, 180 and 200 mg L^{-1}), the mortality rates were the highest but the differences among them were the smallest. However, it is evident that, on the basis of the *V. destructor* mortality data, dosages of the *P. alliacea* aqueous extract in the range of 100 mg L^{-1} to 200 mg L^{-1} are needed to significantly reduce varroa infestation, adequate concentration of the aqueous extract of *P. alliacea* capable of causing the most intense mortality responses of *V. destructor*, requiring a reasonable time of 24 h, which allows adequate interaction of the mite with the botanical biopesticide.

3.2. Acute toxicity to A. mellifera

3.2.1. Direct contact bioassay using a microapplicator

In this bioassay, 0.5 µL of each of the six concentrations, ranging from 24 mg L^{-1} (2.4%) to 200 mg L^{-1} (20%), of the *P. alliacea* extract was applied to the thoraces of the bees with a Burkard Arnold microapplicator. The assay was performed as described below.

1. The *P. alliacea* aqueous extract was serially diluted with distilled water to obtain solutions with the following concentrations (doses): 24 mg L^{-1} (C1), 60 mg L^{-1} (C2), 100 mg L^{-1} (C3), 140 mg L^{-1} (C4), 180 mg L^{-1} (C5), and 200 mg L^{-1} (C6);
2. Each solution (0.5 µL) was applied to the thoraces of the bees with an Arnold hand-held microapplicator (Burkard, England);
3. Ten bees were placed in each semi-open test tube, with 6 replicate tubes at each concentration. The tubes were placed in an incubator at 29–30 °C with 70–80% relative humidity for 24 h;
4. After 24 h, the tubes were removed from the incubator and placed in different test tubes on the basis of the aqueous extract concentration (C1-C6) (Figure 2);
5. The LC_{50} values were determined via probit analysis.

Qualitative analysis of the representative data in Figure 2 indicated the low susceptibility of the bees to C1, C2 and C3 on the basis of the low mortality rates, as most the bees climbed through the inner walls of the test tube. However, the bees treated with C4, C5 and C6 lost vitality and did not climb the inner wall of the test tube. Additionally, the highest mortality rates (30 - 70%) were observed at concentrations ranging from 140 – 200 mg L^{-1} (Table 3 and 4).

Figure 2. Bees were removed from the incubator for evaluation.

Table 3. Results from the direct and residual contact toxicity bioassay with the aqueous extract of *P. alliacea* and *A. mellifera* after 24 h of exposure.

Treatment		Concentration (mg L^{-1})	Number of *A. mellifera* individuals	Direct contact (24 h)		Residual contact (24 h)	
				Deaths	Mortality (%)	Deaths	Mortality (%)
Petiveria alliacea L. (mucura)	*Aqueous extract*	24	10	0	0.00	1	10.00
		60	10	1	10.00	2	20.00
		100	10	2	20.00	5	50.00
		140	10	3	30.00	7	70.00
		180	10	5	50.00	9	90.00
		200	10	7	70.00	10	100.00
Control	Water ed water	0.0	10	0	0.00	0	0.00

Table 4. LC_{50} values and 95% confidence intervals (mg L^{-1}) obtained from direct contact, residual contact and oral acute toxicity bioassay after incorporation into the diet with the aqueous extract of *P. alliacea*.

Acute toxicity bioassay	*Apis Mellifera*	LC_{50} (mg L^{-1})	AC ± SE	X^2	DF	Heterogeneity
Direct contact (24 h)	Susceptible	165.4156 (152.651-180.765)	3.0087 ± 0.0075	0.2237	3	0.0746
Residual contact (24 h)	Susceptible	104.904 (96.647-113.293)	3.6499 ± 0.0077	0.1246	3	0.0415
Oral (48 h)	Susceptible	112.0472 (85.511-193.363)	3.2802 ± 0.0042	1.1477	3	0.3826
Oral (96 h)	Susceptible	73.7513 (60.429-96.462)	3.361 ± 0.0062	0.7483	3	0.2494

AC, angular coefficient; SE, standard error of the mean; X^2, chi-square, DF, degrees of freedom.

Probit analysis of these data gave an LC_{50} value of 165.4156 mg L^{-1}. The angular coefficient of the slope was acceptable (3.0087 ± 0.0075) and higher than 1.96, meeting the probit model conditions for a significant toxicological response upon application of the *P. alliacea* aqueous extract. The heterogeneity result was also acceptable and less than 1.0 (0.0746). These results demonstrate the good fit to the probit model and confirms the dose–mortality relationship and validity of the bioassay results, as shown in Table 4 (Robertson et al., 2017).

The plot of the bioassay results indicting the dose–mortality relationship shows that appreciable mortality occurred only at does much higher than 140 mg L^{-1} after 24 h of exposure (Table 4 and Figure 3a).

An analysis of the results of this bioassay revealed that the risk for the species *A. mellifera* is minimal and that very high doses are required to exceed the LC_{50}. Additionally, 100 mg L^{-1} was considered the most appropriate dose on the basis of the previous bioassay data, which would lead to no more than 20% mortality in bees (Bracho-Pérez et al., 2024).

3.2.2. Residual contact bioassay

This bioassay consisted of placing filter papers that had been saturated with 6 concentrations of the *P. alliacea* extract and then dried in Petri dishes; the control treatment was distilled water. Bees (10 individuals) sedated with carbon dioxide before being placed in the Petri dishes. The bees were then maintained at 34 °C and assessed for mortality after 24 h of exposure. Individuals that were unable to stand on their own after treatment were also considered dead. High toxicity was observed at a dose of 140 mg L^{-1}, similar to those obtained from the direct contact bioassay, as shown in Table 4 and Figure 3b.

The dose–mortality relationship was much more intense in the residual bioassay than in the direct application bioassay, which may be related to the chemical composition of the *P. alliacea* extract. The residual bioassay method involved placing 10 mL of the aqueous extract on filter paper, placing the filter papers in Petri dishes, and evaporating the solvent at room temperature (USEPA, 2012).

The flowers, leaves and roots of *P. alliacea* contain sulfur-containing volatile organic compounds, such as dibenzyl trisulfide, allyl isobutyl sulfide, allyl propyl disulfide, diallyl disulfide, cis-dipropenyl disulfide, trans-dipropenyl disulfide, dibenzyl disulfide, allyl isobutyl disulfide, diisoamyl disulfide, allylmethyl trisulfide, propyl propenyl trisulfide, dibenzyl trisulfide, diallyl trisulfide, and allyl propyl tetrasulfide, as well as amino acid precursors and various benzyl and phenyl derivatives, such as S-benzyl-l-cysteine sulfoxide, S-benzylcysteine, S-methyl-cysteine, S-ethyl-cysteine, S-propyl-cysteine, S-(2-hydroxyethyl)-cysteine, S-(2-hydroxyethyl)-cysteine sulfoxide, and trans-N-methyl-methoxyproline, among others. These secondary metabolites are part of the volatile fraction *of P. alliacea* and are related to the insecticidal and acaricidal activities of this plant species (Sousa et al., 1990; Johnson et al., 1997; Kubec and Musah, 2005; Kubec et al., 2002; Das Gracas et al., 2002; Kim et al., 2006; USEPA, 2012).

The results of this bioassay (Table 4) were subjected to probit analysis, which yielded an LC_{50} value of 104.904 mg L^{-1}.

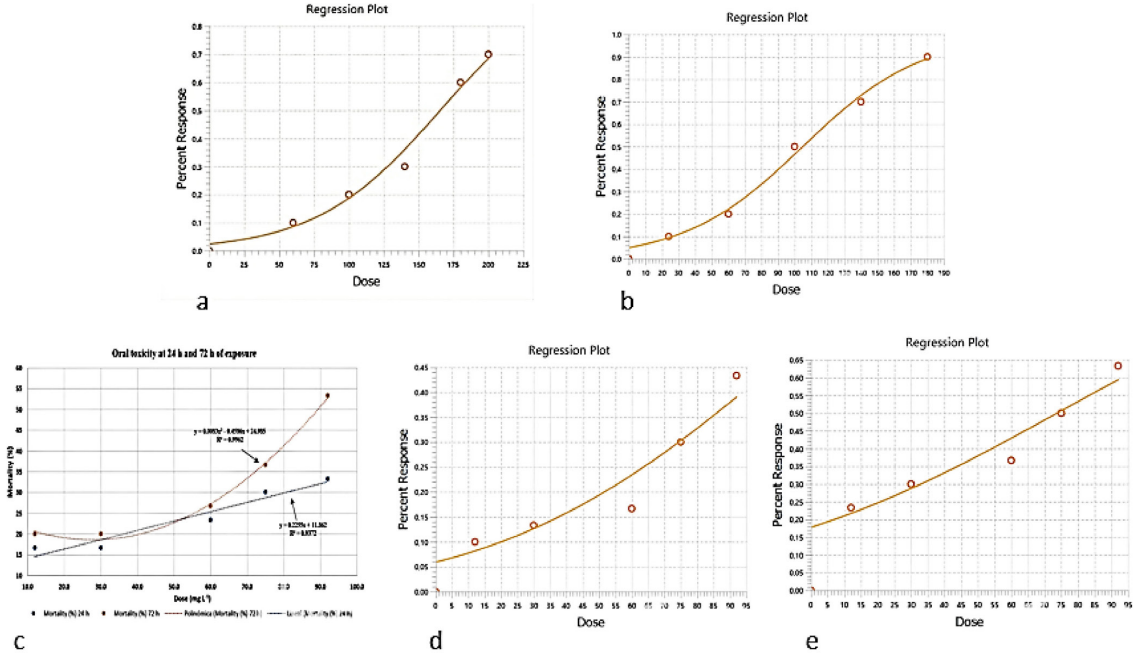

Figure 3. Dose–response curve of *A. mellifera* mortality in response to the *P. alliacea* aqueous extract in the direct contact (a), residual contact (b) and oral toxicity (c, d, e) bioassays after 24 h and 24 h, 48 h, 72 h, 96 h of exposure.

Compared with the results from the direct contact toxicity bioassay results, which gave an LC_{50} value of 165.4156 mg L^{-1}, it can be inferred that the aqueous extract of *P. alliacea* is more toxic after residual contact than direct contact. This difference in toxicity may be related to the biological activities of the components of the volatile fraction responsible for its insecticidal and acaricidal activity. Like the previous bioassays, the residual contact bioassay demonstrated acceptable fit to the probit model after statistical analysis (Sousa et al., 1990; Johnson et al., 1997; Kubec and Musah, 2001; Kubec et al., 2002; Das Gracas et al., 2002; Kim et al., 2006).

The residual contact toxicity bioassay results were considered valid on the basis of the angular coefficient of the slope (3.6499 ± 0.0077, which exceeded 1.96, a necessary condition to fit the probit model) and the significant toxic effect of the *P. alliacea* aqueous extract. In addition, the value obtained for heterogeneity was 0.0415 (less than 1.0), demonstrating the good fit to the probit model, as well as the general accuracy of the dose–mortality relationship determined from this bioassay (Robertson et al., 2017).

On the other hand, the plot shown in Figure 3b for probit analysis shows that the LC_{50} value of 104.904 mg L^{-1} is very close to 100 mg L^{-1}, which was considered the optimal dose according to previous studies with *D. magna*. These data suggest that residual contact poses a greater risk of toxicity to bees (*A. mellifera*) than direct contact does. Therefore, the dose should be less than 100 mg L^{-1}. However, field bioassays should be performed to establish the true impact of the aqueous extract on bees in their natural environment, as well as the appropriate doses and the most effective and safe application methods to minimize bee toxicity (Bracho-Pérez et al., 2019; Bracho-Pérez et al., 2024).

3.2.3. Oral toxicity bioassay by incorporation into the diet

The guidelines and methodology of OECD test no. 213 were used to determine the acute oral toxicity of the *P. alliacea* aqueous extract to honey bees. Young, healthy adult worker bees (*A. mellifera* L.) were collected in the morning and placed in a stainless steel mesh cage (40 cm × 20 cm × 20 cm) and provided ad libitum access to an aqueous solution of 500 g L^{-1} (50% w/v) sucrose, which was supplied in glass capillary tubes (50 mm long × 10 mm wide) with an open diameter of approximately 2 mm. The bees were deprived of food 2 h before the toxicity tests, and those that died were replaced before the test. The test was conducted in the dark at 25 ± 2 °C with a relative humidity of approximately 50–70% (OECD, 1998).

The previously prepared *P. alliacea* aqueous extract was lyophilized in the Environmental Chemistry Laboratory at the National Technological University of South Lima until a dry powder containing the ai was obtained. Different amounts of the powder were mixed with a sucrose solution to obtain five ai solutions with concentrations of 12 mg L^{-1}, 30 mg L^{-1}, 60 mg L^{-1}, 75 mg L^{-1} and 92 mg L^{-1}. These mixtures were supplied to the bees at a dose of 120 µL until they were entirely consumed, after which the sucrose solution was supplied alone ad libitum. Each bioassay was performed in triplicate. Mortality was recorded at 24 h, 48 h, 72 h, and 96 h, in which fatality was recorded for those individuals who were completely immobile (OECD, 1998).

The results presented in Table 5 from 48 h and 96 h of exposure were fit to the probit model, while the data from 24 h to 72 h of exposure were discarded.

i. Oral toxicity after exposure for 24 h and 72 h

The oral toxicity data acquired after 24 h and 72 h of exposure fit linear (R^2 = 0.8743) and polynomial (R^2 = 0.9962) functions, respectively, on the basis of their quadratic correlation coefficients. The oral exposure of bees to the *P. alliacea* extract in the diet did not fit the probit model (Figure 3c).

Using the respective equations for the 24 h and 72 h data, the LC_{50} values obtained did not comply with the probit model, and were as follows.

- Linear behavior (24 h): LC_{50} = 143.1455 mg L^{-1}
- Polynomial behavior (72 h): LC_{50} = 50.5723 mg L^{-1}

ii. Oral toxicity after exposure for 48 h and 96 h

The results after exposure for 48 h and 96 h (Table 5) were subjected to probit analysis, and the LC_{50} values were 112.0472 mg L^{-1} and 73.7513 mg L^{-1}, respectively. The LC_{50} determined from the 48 h data was lower than that obtained from the 24 h data (LC_{50} = 143.1455 mg L^{-1}) and much higher than that obtained from the 72 h data (LC_{50} = 50.5723 mg L^{-1}). These data suggest that as the exposure duration increases, the mortality caused by and the toxic effects of the aqueous extract included in the diet increase. However, after the longest exposure duration (96 h), the LC_{50} did not decrease. This is typical of a more severe toxic effect, which actually increased and then stabilized at a value slightly higher than that obtained at 72 h. However, in general, the highest mortality and toxicity were achieved after the longest exposure duration.

After 48 and 96 h of exposure, the behavior of the bioassays was optimal (Table 5), the angular coefficients of the slopes exceeded 1.96 (AC (48 h) = 3.2802 ± 0.0042; AC (96 h) = 3.361 ± 0.0062), which is necessary to fit to the probit model, and significant toxicity was observed. In addition, the heterogeneity (het.) values were also lower 1.0 after both exposure durations (het. (48 h) = 0.3826; het. (96 h) = 0.2494), demonstrating the good fit to the probit model and the general accuracy of the dose–mortality relationship in this bioassay (Robertson et al., 2017).

The plot constructed from the probit analysis after exposure for 48 and 96 h shows that the mortality increases with increasing applied dose, reaching 50% mortality at a dose of 112.0472 mg L^{-1} after 48 h and 73.3513 mg L^{-1} after 96 h. However, although these data are similar, at 96 h, there was a greater trend toward linearity, wherein the LC_{50} was much lower, indicating stronger toxicity (Figure 3d and 3e).

The LC_{50} after the first 24 h of exposure was the lowest (143.1455 mg L^{-1}); that is, the dose needed to induce death in 50% of the individuals was the highest. Subsequently, the LC_{50} values after 48 and 72 h of exposure reflect the toxicity of the extract to the internal organs of the bee after oral administration. After 72 h of exposure, the extract exhibited maximum toxicity (LC_{50} = 50.5723 mg L^{-1}) and consequently, the highest mortality rate. Finally, the decreasing trend in LC_{50} value stabilized at 96 h (73.7513 mg L^{-1}) while maintaining high toxicity.

These results demonstrate the importance of evaluating the effects of exposure every 24 h until 96 h because disturbances in living systems generally depend on the magnitude of the disturbance, which is related to the dose, time and duration of the disturbance and the susceptibility of the host to xenobiotics. Therefore, from these data, it can be inferred that supplying the aqueous extract to the colony feed is not a good option, as it may cause severe toxicity to this species.

Many biological systems can function normally within their homeostatic limits. When the dose of the aqueous extract is first low but then increases, clear biological responses, manifested as mortality, were produced. In this sense, the response obtained at 96 h (the longest exposure duration) is clearly the most important from the oral toxicity bioassay (Krewski et al., 2010).

3.2.4. Qualitative analysis of the components of the P. alliacea aqueous extract

The components of the *P. alliacea* aqueous extract were qualitatively examined via LC–UV–ESI–MS (λ = 280 nm). A representative HPLC chromatogram of the aqueous extract of *P. alliacea* is presented in Figure 4, where the identified peaks are numbered; their identities are presented in Table 6.

Qualitative analysis of the *P. alliacea* aqueous extract showed the presence of 16 components, of which 14 were identified, as shown in Table 6. The extract dominated by polar components capable of establishing hydrogen bonds,

Table 5. Results from the oral toxicity bioassay in which the *P. alliacea* aqueous extract was incorporated into the diet and evaluation every 24 h for a total of 96 h of exposure.

Treatment		Concentration (mg L^{-1})	Number of *A. mellifera* individuals	24 h		48 h		72 h		96 h	
				Deaths	Mortality (%)	Deaths	Mortality (%)	Deaths	Mortality (%)	Deaths	Mortality (%)
Petiveria alliacea L. (mucura)	Aqueous extract	12.0	30	5	16.66	3	10.00	6	20.00	7	23.33
		30.0	30	5	16.66	4	13.33	6	20.00	9	30.00
		60.0	30	7	23.33	5	16.66	8	26.66	11	36.66
		75.0	30	9	30.00	9	30.00	11	36.66	15	50.00
		92.0	30	10	33.33	13	43.33	16	53.33	19	63.33
Control	Distilled water water	0.00	30	0	0.00	0	0.00	0	0.00	0	0.00

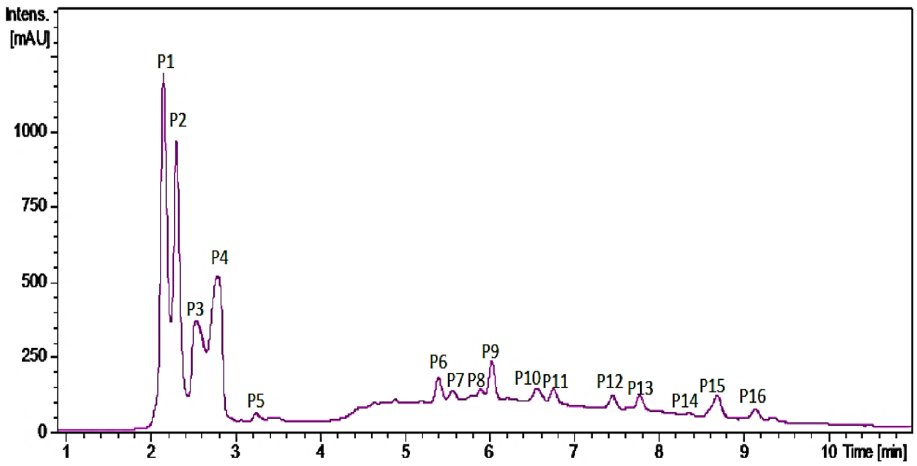

Figure 4. Representative LC–ESI–MS chromatogram of the aqueous extract of *P. alliacea* with detection at 280 nm. The identities of the peaks are given in Table 6.

which is typical after extraction into aqueous media and represents the physicochemical characteristics of these compounds that allows their entry into the aqueous fraction during extraction. The presence of four nitrogenous derivatives (P1, P2, P4, and P5), accounting for 25% of the total components of the aqueous extract, as well as four sulfur components equivalent to an additional 25% of extract components. These results are consistent with those of previous studies, such as that of Kim et al. (2006), which determined that sulfur derivatives, such as thiosulfonates, trisulfides and sulfinic acid derivatives, are responsible for the antibacterial and antifungal activity of the extract. Thus, it was established that thiosulfonates containing benzyl groups have the most potent biological activity. Other studies have also demonstrated the importance and presence of thiosulfonates in *P. alliacea* L. (Kim et al., 2006; Okada et al., 2008).

In addition to sulfur-containing amino acids, such as sulfur derivatives of cysteine and their sulfoxides, γglutamyl dipeptides were also identified, which have been isolated from the roots of *P. alliacea* and are present in the tissues of this plant species. The enzymatic breakdown of the cysteine derivatives in *P. alliacea* produces thiosulfonates in a similar way to that observed in Allium species. However, the biochemical role of dipeptides in plant tissue has not been fully established. These compounds are known only as intermediates in the formation of sulfoxides derived from cysteine present in *P. alliacea*, and various studies have associated them with nitrogen and sulfur storage in plants (Kubec and Musah, 2001, 2005; Kubec et al., 2002).

P8, P9, P10 and P11 in Table 6 were identified as secondary organosulfurized metabolites derived from thiophene, except for component P9, which is a sulfonate. Extracts of plants of other origins do not contain substituted sulfur derivatives of cysteine, which may be related to the direct dependence of the formation of these derivatives on various factors, such as the soil composition, climatic conditions and collection time. Thus, cysteine sulfoxides are not present because these compounds are precursors of the other organosulfides that are present in the leaves and are only found at very low concentrations, and the primary products of their decomposition, such as thiosulfonates, are formed by CS lyase (Fenwick et al., 1985; Block, 1992; Kubec and Musah, 2001; Kim et al., 2006).

On the other hand, Lyndon et al. (1997) demonstrated the powerful acaricidal activity of a fraction of the ethanolic extract of the roots of *P. alliacea* against ticks taken from infected cattle. Their bioassays revealed that the main component of this fraction was dibenzyl trisulfide, which has a high median lethal dose.

A subsequent study confirmed the acaricidal activities of extracts and fractions of the stems and leaves of *P. alliacea*, which led to the high mortality of cattle tick *R. microplus* larvae and adults after their immersion. The methanolic extracts of the stems and leaves of *P. alliacea* caused 100% mortality in these bioassays. Fractionation of the methanolic extract revealed that the best activity was achieved with the apolar n-hexane fraction, of which the main components were benzyldisulfide (BDS) and benzyltrisulfide (BTS), which may be responsible for the good acaricidal activity. These findings are consistent with the results obtained by Lyndon et al. with *P. alliacea* roots (Lyndon et al., 1997; Rosado-Aguilar et al., 2010).

Together, these studies demonstrate the powerful insecticidal and acaricidal activities of *P. alliacea*. However, the starting extracts in these studies were methanolic and ethanolic extracts, which were fractionated with low-polarity organic solvents to yield fractions enriched in DBD and DBT. However, this investigation with *D. magna* allowed the establishment of organic extracts as the most toxic extracts with the greatest effects on the environment; thus, these extracts have not been further applied, and the aqueous extracts have instead been used to control *V. destructor* due to their lower mortality and guarantee environmental safety. Furthermore, the most notable and novel finding of this study is that the aqueous extract has marked acaricidal activity, and LC–ESI–MS showed that the sulfurized components DBD and DBT are not in this extract (Table 6) (Lyndon et al., 1997; Rosado-Aguilar et al., 2010; Bracho-Pérez et al., 2019).

Table 6. Components identified in the mucura (*P. alliacea* L.) aqueous extract by LC–MS.

Peak	Compound	Concentration (mg kg^{-1})	t_R (min)	m/z
1	trans-N-methyl-4-methoxyproline	6455	2.17	158.9544
2	N,N-dimethylproline	5271	2.32	144.1010
3	not identified	3200	2.55	-
4	N-α-tert-butyloxycarbonyl-valylproline-methyl ester	4597	2.80	228.1465
5	aspartyl-leucyl-leucine	134	3.25	360.2205
6	(2,4)-2-O-β-D-glucopyranosyl-2,4-pentanediol	409	5.39	266.1433
7	N-propyl 3,4,5-trihydroxybenzoate	182	5.56	212.2081
8	Ethyl 2-thiophenecarboxylate	117	5.89	156.0313
9	allyl benzenesulfonate 2-propen-1-ol	592	6.02	198.0420
10	butyl 2-thiophenecarboxylate	275	6.55	184.0629
11	4-methyldibenzo-thiophene	235	6.74	198.0596
12	not identified	220	7.44	-
13	β-D-mannopyranoside-19-isopimarane-7,15-dien-3β-ol	235	7.76	466.3014
14	4-(4-hydroxy-3-methoxyphenyl)-2-butanone	40	8.34	194.1011
15	2'-hydroxy-2,3,4,4',5,5',6'-heptamethoxychalcone	500	8.67	434.1643
16	6-Methoxy-1,2,3,4,5-pentahydroxy-cyclohexane	0.8	9.12	195.0863

These results lead to two important findings. First, the aqueous extract of *P. alliacea* of Peruvian origin can act as a natural controller or acaricide of botanical origin of *V. destructor*, and second, the components of the aqueous extract of *P. alliacea* responsible for this biological activity are either new or related to the organic compounds (primary or secondary metabolites) previously identified in *P. alliacea*.

Previously published data have already shown the ability of the aqueous extract of this plant species of Peruvian origin to act as an insecticide, as it can cause appreciable mortality in third-stage larvae of *Musca domestica*; however, this is the first evidence of its ability to control *V. destructor* mites, which have caused the greatest infestation of honey bees worldwide. Unfortunately, there are very few molecules and products that are capable of at the very least reducing infestations in bee colonies with varroosis (Bracho-Pérez et al., 2019; Nöel et al., 2020).

The aqueous extract of *P. alliacea* contains nitrogen and sulfur compounds that constitute part of the nitrogen and sulfur reserves of the plant species. Among the four nitrogenous compounds present in the aqueous extract (P1, P2, P4, and P5), three are derivatives of proline (P1, P2, and P4). One of these identified derivatives, P1 (trans-N-methyl-4-methoxyproline), is a highly polar alkaloid previously isolated and identified by Sousa et al. (1990) from the inflorescences of *P. alliacea* collected in Minas Gerais, Brazil. The remaining two derivatives of proline are the previously unidentified N,N-dimethylproline (P2) and N-α-tert-butyloxycarbonyl-valylproline-methyl ester (P4), a novel dipeptide ester identified for the first time in *P. alliacea*.

The presence of γ-glutamyl dipeptides in the roots of *P. alliacea* shows that primary metabolites may exist in the tissue of this plant species. Thus, the identification of the dipeptide P2 and the tripeptide aspartyl-leucyl-leucine (P5) are novel constituents of this aqueous extract. The peptides in this plant species must have various biochemical roles, since peptides regulate a variety of processes related to plant development and defense. Specifically, peptides can act as molecular messengers during plant interactions with other organisms, alerting the plant to possible attacks and inducing defense mechanisms. Microbe-associated molecular patterns are molecular fragments recognized by plants that indicate a potential invasion, as peptides derived from microbial proteins are associated with plant-specific pattern recognition receptors, which elicit a cascade of defense responses (Zipfel et al., 2004, 2006; Matsubayashi and Sakagami, 2006).

In addition, peptides warn plants of attack by herbivorous insects; the inceptin peptide is one such molecular pattern that activates defenses in response to herbivore invasion. Thus, it is important to note the presence of endogenous plant peptides that regulate defenses and act as internal inducers (Schmelz et al., 2006; Ryan et al., 2007; Mithöfer and Boland, 2008).

The aqueous extract also contains aromatic compounds, such as n-propyl 3,4,5-trihydroxybenzoate (P7), 4-(4-hydroxy-3-methoxyphenyl)-2-butanone (P14) and 2'-hydroxy-2,3,4,4',5,5',6'-heptamethoxychalcone (P15), a simple phenolic ester, an aromatic ketone and a flavonoid precursor chalcone, respectively.

The constituents P7 and P14 are derivatives similar to phenylpropanoids that are found in the essential oils of the leaves and flowers of *P. alliacea*. Chalcone precursors of flavanones, such as leridal, have been widely found in the alcoholic extracts of *P. alliacea* aerial parts. However, the identified chalcone (P15) is often methoxylated and has not been previously identified in *P. alliacea* (Table 6) (Bastos Silva et al., 2018).

As part of their defense mechanisms, plants induce the biosynthesis of chalcones and flavonoids, which are generally found in the leaves of plant species, including *P. alliacea*, which specifically protect the plant from predatory insects. After a leaf has been damaged by an insect, a cascade of enzymatic reactions occur that alter and stimulate the biosynthesis of chalcones and flavonoids. The metabolism of these compounds by insects generates byproducts that alter the palatability and suitability of the plant for an insect, cause weight loss, interfere with oviposition, and modify larval food selection, as larvae have neuronal sensory receptors that respond to a range of phagostimulants and deterrents such as carbohydrates, amino acids, alkaloids, diterpenoids and phenols (Simmonds, 2001).

Thus, the presence of complex phenols such as chalcones (e.g., P15), simple phenols such as the phenolic derivative 3,4,5-trihydroxybenzoate of n-propyl (P7), other metabolites such as nitrogenous compounds derived from amino acids (P1, P2, P4, and P5), and the diterpene glycoside virescenoside Q (P13) demonstrate the ability of the plant to control pests as a general defense mechanism against insects that could be related to the acaricidal effects of *P. alliacea* against *V. destructor*.

Finally, (2,4)-2-O-β-D-glucopyranosyl-2,4-pentanediol) (P6) and β-D-mannopyranoside-19-isopimarane-7,15-dien-3β-ol (P13) are highly polar and high solubility in water due to the presence of OH groups that can form hydrogen bonds with water and indicates their propensity to be glycosylated. The component of the *P. alliacea* aqueous extract with the longest LC retention time (t_R = 9.12 min; Figure 4) was identified as 6-methoxy-1,2,3,4,5,6-pentahydroxy-cyclohexane (Table 6). This monomethylated cyclohexitol, also known as D-pinitol, exhibited the strongest affinity for the mobile phase because its five active hydroxyl groups (Sousa et al., 1990; Urueña et al., 2008).

4. Conclusions

The aqueous extract of *P. alliacea* exhibited an LC_{50} of 105.2418 mg L^{-1} against *V. destructor* and reduced *V. destructor* infestation by 46.66, 80.00, 86.66 and 93.33% concentrations of 100, 140, 180 and 200 mg L^{-1}, respectively, after 24 h of exposure.

The application of 100 mg L^{-1} aqueous extract, which has LC_{50} values after direct contact and residual contact of 165.4156 mg L^{-1} and 104.904 mg L^{-1}, respectively guarantees the minimum impact on honey bees (*A. mellifera* L.). The direct contact bioassays revealed minimal impact on bees, and when applied at a dose of 100 mg L^{-1}, a mortality rate of less than 20% is guaranteed. However, the results from the residual contact and oral toxicity by incorporation into the diet bioassays revealed a risk of toxicity to bees since the optimal dose (100 mg L^{-1}) caused 50% mortality in the residual contact bioassay and 63.33% mortality in the oral toxicity study after 96 h of exposure.

Therefore, we suggest that the aqueous extract of *P. alliacea* should not be supplied to bees in their food. However, it is vital to carry out field studies to establish how direct and residual contact with the extract components and application methodologies affect the colonies to reduce the impact on bees while adequately controlling *V. destructor*.

The chemical composition of the aqueous extract contains new components not previously identified in *P. alliacea* aqueous extracts, as well as other components that have been previously or are of a chemical nature similar to others previously identified, that demonstrate the acaricidal potential of the aqueous extracts of this plant species. However, it is critical to continue studies that establish a direct relationship between the chemical composition and the acaricidal activity to determine the biologically active components.

Therefore, field bioassays should be performed to determine the optimal dose of the extract and conditions to control *V. destructor* infestation with a minimal impact on *A. mellifera* colonies.

Data Availability Statement

All data analyzed in the research are available at Repositorio Institucional UNHEVAL and can be accessed via hyperlinks: https://hdl.handle.net/20.500.13080/7125

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