Control of varroa jacobsoni in peruvian honeybees using a botanical Biopesticide

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Recibido: 27/11/2024 Revisado: 08/03/2025 Aceptado: 15/05/2025 Publicado: 30/06/2025

ABSTRACT

This study focused on the preparation of extracts from the leaves of *Petiveria alliacea* to find the most suitable extract with the least toxicity and the ability to control varroasis in honey bees. The hexanic, ethanolic, and aqueous extracts were subjected to acute toxicity bioassays with *Daphnia magna* for 24 h and 48 h of exposure. The aqueous extract proved to be the least toxic, causing minimal mortality and a median lethal concentration (LC_{50}) of 657.9601 mgL^{-1}. The acute toxicity bioassay with varroas showed that mortality is maximum at 200 mgL^{-1} (93.33%) during 24h of exposure and LC_{50} = 105.2418 mgL^{-1}. A chemical analysis using liquid chromatography coupled to mass spectrometry (LC/MS) allowed identifying that sulfur derivatives are the majority fraction in its composition (72.7%). The results suggest that the aqueous extract could have potential as a botanical biopesticide with acaricidal capacity, according to the fraction of quantified components and the background in the scientific literature.

Keywords: Bee, biopesticide, *Petiveria alliacea*, bioassays, *Daphnia magna,* Varroa

INTRODUCTION

Scientists from around the world, along with the United States' NASA Exoplanet Exploration Program, have discovered over 5000 planets beyond our solar system over the years. Despite these advancements, the search for a planet that rivals Earth in terms of complexity, biodiversity, and beauty remains unsuccessful. Our planet is a unique and perfectly balanced ecosystem where plant and animal species coexist in harmony (Budrikis, 2022; NASA, 2003).

Unfortunately, humans do not appreciate or understand the importance of the wealth that nature has provided them. We continue to make the same mistakes of the past and do not accept that if we could only understand a small part of the complex mechanisms and connections in ecosystems, we would have the tools and sustainable solutions for all the planet's problems.

The global concern about the coronavirus pandemic is only part of the problem. The connection between the pandemic and the protection of biodiversity, ecosystems, and their food chains is a sad reality that recalls Gabriel García Márquez's novel 'Chronicle of a Death Foretold'. In the novel, the foretold

death of Santiago Nasar, known to all, could not be avoided and finally materialized. If we look back in time, we find that many complaints have already been made about the need to take care of biodiversity. For example, in 1962, Rachel Carson published her work 'Silent Spring', in which she strongly denounced the profound impact of pesticides on nature and its endangered riches. Undoubtedly, a scientific chronicle of the current destruction of biodiversity foretold. (Carson, 1962; García Marquez, 1981).

Rachel Carson, in her work, convincingly presented scientific evidence of the damage that DDT and other synthetic pesticides cause to animal life and humans. Specifically, in Chapter 17, titled 'The Other Road', Carson (2020) clearly expresses:

> Chemical pest control in the forest is at best a stopgap measure bringing no real solution, at worst killing the fishes in the forest streams, bringing on plagues of insects, and destroying the natural controls... (p.276).

In that regard, the author continues with his terrible prediction and asserts that the phrase 'control of nature' is arrogant. This comes from a time when it was believed that nature existed for the benefit of man. The concepts and practices of applied entomology are mostly primitive, but they have been armed with modern and terrible weapons. By using them against insects, we have also directed them against the Earth (Carson, 2020, p. 277).

More than half a century later, in 2020, the World Wildlife Fund published a chilling report that solidly argues that the current crisis of the coronavirus pandemic is linked to a crisis on our planet, caused by the destruction of ecosystems and the loss of biodiversity. This has triggered worrying events, such as the transmission of more than 70% of human diseases that have occurred over the last forty years from wild animals, such as Ebola and AIDS. These diseases cause approximately one billion cases and millions of deaths worldwide every year. (Pratesi et al., 2020).

The truth is that the human species is directly responsible for this terrible situation. We continue to make the same mistakes and destroy our home, unlike the rest of the animal species. Therefore, it is our responsibility to correct the mistakes made. This research work, in the context of this current problem, tries to develop a botanical biopesticide from Peruvian biodiversity to sustainably protect one of the most important animal species for this planet: honey bees.

Honey bees, like many other species, are in danger of extinction due to the improper use of synthetic pesticides such as neonicotinoids and other pesticides that have caused the death of millions of bees and their colonies worldwide. These pesticides are used to control pests in general and to control *Varroa jacobsoni*, whose scientific name has been updated to *Varroa destructor* (Anderson and Trueman). This mite is the pest that most affects honey bees and can destroy their colonies. However, the consequences of losing the most important pollinator for the sustainable development of Earth's biodiversity could be disastrous and irreversible (Buszewski et al., 2019; Fikadu, 2020; Oruç et al., 2020).

The purpose of this research is to help protect honey bees (*Apis mellifera* L.) by studying the extracts obtained from the leaves of the botanical species known in Peru as mucura (*Petiveria alliacea* L.), identifying the extract that has the least environmental impact and is capable of providing the most effective

control against the pest *Varroa destructor* (Anderson and Trueman), a mite that destroys bee colonies. In addition, a quantitative determination of the family of secondary metabolites will be carried out using Liquid Chromatography-Mass Spectrometry (LC/MS).

METHODOLOGY

Processing of Plant Material. The collected plant material underwent a four-stage process: 1. Selection and Separation: The organ of interest (leaves) from the collected plant species was selected and separated. 2. Drying: The selected leaves were subjected to a drying process in an oven at a temperature of 40 ºC for a period of 48 h

Preparation of Extracts. The dry drug was crushed in a blade mill to obtain a fine powder, which was then sieved through a 100 µm sieve (Stage 3). Subsequently, the Organic Plant Extracts (Stage 4) were prepared. Two samples of 10 g each of the dry plant material were weighed. Each sample was placed in an amber glass jar and subjected to extraction with 100 mL of 96% ethyl alcohol and 100 mL of n-hexane in an ultrasonic bath at a temperature of 48 °C for 2 hours. The resulting extracts were filtered and concentrated under reduced pressure at a temperature of 38 °C using a rotary evaporator, thus obtaining the crude extracts. These were weighed and stored in amber glass jars at refrigeration temperature until their use in bioassays, at which time they were dissolved in propylene glycol.

Preparation of Aqueous Plant Extracts. 10 g of dry plant material were weighed and placed in an amber glass jar. Then, it was subjected to extraction in an ultrasonic bath at a temperature of 48°C for 2 hours with 100 mL of water. The resulting extract was filtered using Whatman No. 42 filter paper and stored in amber glass jars at refrigeration temperature. Subsequently, it was lyophilized and stored in a tightly closed amber jar at a temperature of -20°C until the development of the bioassays. 10 mg of the extract were used for its chemical composition study by Liquid Chromatography coupled to Mass Spectrometry (LC/MS).

Laboratory Bioassays. The methodology described by Bracho Pérez et al., (2019) was used to evaluate the sensitivity of D. magna (age<24h) using a reference test with sodium chloride (NaCl). The static toxicity test was carried out in plastic containers containing 4 mL of the extracts to be evaluated at five different concentrations. Five neonates were exposed to tests with five concentrations of the aqueous and organic extracts of mucura, ranging from 10 mgL^{-1} to 10000 mgL^{-1}, without feeding during the tests at 24 and 48 hours of exposure. Each concentration was evaluated in triplicate. The containers were incubated in the dark at a temperature of 20 ± 2 °C. Immobilization or absence of heart rhythm in the treated organisms, observed for 15 seconds under a stereomicroscope, was used as an indicator of mortality (Bracho Pérez et al., 2019).

Acute Toxicity Bioassay with *Varroa destructor* by Immersion. To evaluate the acute toxicity of the Mucura extract, adult females of Varroa from adult honey bees were collected using the roller method with powdered sugar, as described by Dietemann et al. (2015). The mites were collected before each bioassay. Contact toxicity bioassays were carried out by exposing the mites to six different concentrations of the *P. alliacea* extract, ranging from 2.4% (24 mg/L) to 20% (200 mg/L), over a period of 24 hours.

This bioassay was performed by individually immersing each Varroa in different concentrations of the P. alliacea extract, using tweezers, and then placing them in glass vials. The vials were stored for an extended period to evaluate the acaricidal activity of the mucura extract over time. Five mites were exposed to each concentration and the bioassay was repeated three times. A control treatment and a solvent control used to prepare the extract were also included. The mites were kept in an incubator at 34 °C and without light. At the end of the exposure periods, the adult females of the mites were observed with a stereoscope. Three criteria were used to determine the final and effective lethality of the bioassays: 1. Mobile Varroas with coordinated or uncoordinated movement after stimulation, 2. Paralyzed Varroas with movement in one or more appendages, 3. Dead Varroas without need for stimulation. (Dietemann et al., 2015).

Chemical Study using LC/MS. The sample was dissolved in 100 µL of MeOH and analyzed using an Agilent 1200 Rapid Resolution HPLC connected to a Bruker maXis mass spectrometer. A sample volume of 2 µL was injected and a Waters Atlantis T3 column (4.6 x 100 mm, with a particle size of 5 µm) was used for separation. The mobile phase consisted of two solvents: solvent A, a mixture of H_2O:AcN (90:10), and solvent B, a mixture of H_2O:AcN (10:90). Both solvents contained 13 mM ammonium formate and 0.01% TFA. The mass spectrometer was set in positive ESI mode, with a capillary voltage of 4kV, a drying gas flow of 11 L/min at 200 ºC, and a pressure in the nebulizer of 2.8 bar. Chromatographic runs were processed using Bruker's internal algorithm for component extraction. The most intense peaks, both by TIC in positive and by absorbance at 210 nm, were considered for the interpretation of exact masses and molecular formula.

A combination of search criteria was used, including retention time and exact mass, in various specialized sources. These include the high-resolution mass spectrometry database, the Chapman & Hall natural products dictionary, the U.S. NIST Standard Reference Database Number 69, the NIST Chemistry WebBook, and the U.S. National Institutes of Health PubChem Chemicals Database. The official atomic weights of chemical elements were obtained from updates made by researchers from the IUPAC Inorganic Chemistry Division Committee (NIH) (Petersen & Amstutz, 2008; Buckingham et al., 2015; Meija et al., 2016; Kim et al., 2019; Linstrom & Mallard, 2020).

Statistical Analysis. The data obtained from the acute ecotoxicity bioassays on the effects of mucura (*P. alliacea*) extracts on *D. magna* were processed to calculate the Median Lethal Concentration (LC_{50}), which is expressed in mgL^{-1}, with a 95% confidence interval (α=0.05). Probit regression analysis was used to evaluate the significance and fit of the data. Statistical parameters such as the Slope Angular Coefficient, Chi Square, and Heterogeneity were determined using PoloSuite, an appropriate statistical program. From these results, the optimal concentration of mucura extract that would have the least environmental impact was determined. A similar analysis was performed to calculate the LC_{50} of the extract in acute toxicity bioassays on Varroa, using Probit analysis and following the same procedure as in the toxicity study on *D. magna*. (LeOra Software LLC, 1997; Robertson et al., 2017).

RESULTS

Acute Toxicity Bioassays

The results of the bioassays carried out with *D. magna* are shown in Table 1 and the statistical summary of the Probit analysis using the PoloSuite program is found in Table 2. These indicate a typical behavior of a toxicological study, where confidence intervals form hyperbolic curves that represent the dose-mortality relationship when exposing *P. alliacea* extracts to *D. magna* as a bioindicator. The trend towards the LC_{50} is observed in all cases, as the dose increases, the mortality rate increases logarithmically until it approaches the LC_{50} and causes the death of 50% of *D. magna* neonates (Figure 1), being the most notable and important aspect that the aqueous extract of *P. alliacea* has the highest value of LC_{50} = 657.9601 mgL^{-1}, that is, the least toxicological impact on *D. magna* and food chains (Robertson et al., 2017).

In that sense, organic extracts should be discarded for the control of varroasis in honey bees and only the aqueous extract should be applied. The development of bioassays with *V. destructor* recorded in Table 3 and its statistical summary in Table 4, show a more complex behavior where the dose-mortality relationship of biopesticide against Varroa denotes the mite's resistance to survive, but with a LC_{50} = 105.2418 mgL^{-1} (Figure 2), demonstrating the effectiveness of the biopesticide and harmlessness to control this pest with minimal impact on honey bees.

Estudio Químico por LC/MS

The qualitative and quantitative analysis of the components of the aqueous extract of *P. alliacea* was carried out using the LC-UV-ESI-MS method (λ = 280 nm), resulting in a liquid chromatography profile with sixteen components at a wavelength of 210 nm. Sulfur and nitrogen organic compounds were the major metabolites, with an abundance of 5.3% and 72.7%, respectively (Figure 2).

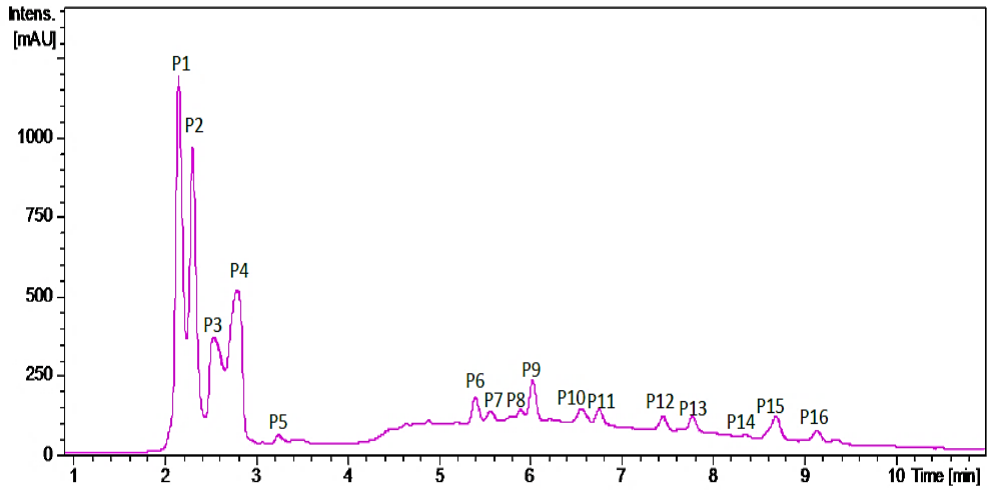

Figure 2. Liquid chromatography profile of the aqueous extract of mucura

DISCUSSION

The acute toxicity study of the leaf extracts of mucura with the bioindicator *D. magna* showed that the aqueous extract had the highest toxicity after 48 hours of exposure (see Table 1). In a concentration range between 10 and 100 mgL-1, the aqueous extract caused the least mortality in the bioindicator, with a mortality rate of 6.67% to 13.33%. Five concentrations (10.00, 100.00, 500.00, 1000.00 and 10000.00 mgL^{-1}) were used to establish the baseline susceptibility of *D. magna* to *P. alliacea* extracts. The resulting mortalities were recorded in Table 1.

El The study of lethal concentrations is an important parameter in the evaluation of the toxicity of a biopesticide. For this reason, a regression analysis was carried out using the Probit method with the PoloSuite program (LeOra Software LLC, 1997). The results are summarized in Table 2, where it can be observed that the calculated LC50 values confirm the greater toxicity of organic extracts. The order of toxicity is as follows:

$$\mathbf{CL_{50(hexanic\ ext.)} = 26.9103 > CL_{50(ethanolic\ ext.)} = 230.6366 > CL_{50(aqueous\ ext.)} = 657.9601}$$

The statistical results of the Probit analysis indicate that the aqueous extract has a range of LC50 capable of causing the lowest toxicity for the bioindicator between 305.2090 mgL^{-1} and 1562.8129 mgL^{-1}, with an average LC_{50} of 657.9601 mgL^{-1} unlike the organic extracts.

Figure 1 presents the dose/mortality regression curves corresponding to the three bioassays performed. These curves show a high sensitivity of D. magna neonates to P. alliacea extracts. According to the shape of the curves, it can be inferred that even minimal increases in the doses of the botanical biopesticide can trigger significant mortality responses in neonates. This characteristic satisfies one of the fundamental criteria of a bioindicator, which is its high sensitivity to the presence of chemical contaminants. This demonstrates the effectiveness of this toxicological bioassay in determining the susceptibility of *D. magna* neonates and the evaluation of xenobiotics capable of altering the balance of ecosystems and their trophic chains.

Low Environmental Risk Decision. Based on the findings, the aqueous extract of *P. alliacea* showed the lowest acute toxicity, indicating a possible weaker toxic effect on D. magna, recognized as a highly sensitive bioindicator. Organic extracts (ethanol, hexane) should be excluded to minimize future environmental toxicity risks and prevent potential damage to other food chains. Since no death of D. magna was observed at concentrations of 10 - 100 mgL^{-1}, the highest concentration of 100 mgL^{-1} could be used for the preparation of the aqueous extract.

The results obtained from the development of Acute Toxicity Bioassays with V. destructor are necessary to establish the concentration range capable of causing the best effectiveness on the parasite, where it can be verified that at doses above 140 $mg.L^{-1}$, mortality percentages capable of equaling or exceeding 80% are obtained. Mortality at a concentration of 100 mgL-1 of the aqueous extract of P. alliacea only reached 46.66%, making it vital to contrast with the results of field bioassays. The Probit regression analysis to establish the behavior and dose/mortality relationship resulted in LC50 = 105.2418 mgL^{-1} to reduce 50% of the infestation caused by Varroas, whose confidence interval at 95% probability was between 97.092-115.6 mgL-1, which includes the maximum acceptable concentration that guarantees the sustainable development of the

botanical biopesticide determined through the bioassay with D. magna, that is, 100 $mgL^{-1.}$

The quality of the fit to the Probit model can be evaluated through the parameters presented in Table 4. The slope's angular coefficient is 5.1872 ± 0.0057, which exceeds the value of 1.96 and demonstrates a good fit to the model. This indicates that the Probit analysis detected a significant response and suggests that the aqueous extract of P. alliacea may be effective in reducing infestation levels caused by V. destructor. In addition, the heterogeneity value is less than 1.0, which ensures a good fit to the Probit model and validates both the dose/mortality relationship and the veracity of the bioassay. (Robertson et al., 2017).

The dose/mortality regression curve of the bioassay is shown in Figure 2, illustrating one of the possible responses of a living system to a chemical controller. Despite the complexity of the mathematical behavior, the quadratic correlation coefficient shows that the best correlation is linear, with an R2 of 0.9597. However, when comparing the Probit analysis graph (left graph, Figure 2) with the graphs of the three possible mathematical trends (right graphs, Figure 2), it can be seen that at the beginning of the treatment, with the first two doses applied (24 and 60 mgL-1), the effect is minimal with a mortality of 6.63% to 20% and an exponential behavior (R2 = 0.882). This suggests that the mite is resistant to the applied dose.

Starting from the third dose of 100 mgL-1, the relationship between the dose and mortality becomes linear, with a high quadratic correlation value (R2 = 0.9597). Mortality increases rapidly, exceeding 46.66%, until reaching the final stage of treatments. At this point, the Probit analysis curve (right graph, Figure 2) fits a logarithmic behavior, specifically for the last three doses (140, 180, 200 mgL-1). The mortality values are the highest, but their differences are the smallest. This indicates that to significantly reduce Varroa infestation levels, adequate doses in the range of 100 mgL-1 to 200 mgL-1 of P. alliacea aqueous extract concentration are required. These doses are capable of causing the most intense responses in pest mortality and a prudent time of 24 hours is required to allow their proper interaction with the botanical biopesticide.

The choice of 75% formic acid as a positive control is not arbitrary. This organic acid has proven its effectiveness in controlling varroa at concentrations ranging between 60% and 85%, through vaporization, achieving a mite mortality rate of over 80%. In addition, it can also effectively reduce infestation in the cells of capped and sealed brood within the hive. (Calderone, 2000; Eguaras et al., 2003; Elzen et al., 2004; Mahmood et al., 2012; Satta et al., 2005; 2012; Ramírez & Calderón, 2017).

The detailed chemical characterization analysis of the secondary metabolites found and measured showed the existence of compounds containing nitrogen and sulfur. The latter are linked with the ability of P. alliacea to combat mites, according to several studies. In addition, the presence of aromatic components and phenolic derivatives, such as chalcones, may help to increase the acaricidal activity of the aqueous extract. Table 5 shows the percentage distribution of the secondary metabolites present according to the type of organic compound family. It is worth noting that a detailed chemical analysis will soon be published to determine the structure of the active principles and their biological implications. (Kim et al., 2006; Okada et al., 2008).

CONCLUSIONS

The development of acute toxicity bioassays with D. magna by exposure of neonates for 24h and 48h allowed to establish the aqueous extract of Mucura (Petiveria alliacea L.) with the minimum environmental impact at a concentration of 100 mgL-1. It was demonstrated that it can control at that concentration the infestation levels of V. destructor, the pest that most impacts the colonies of Apis mellifera L.

The study carried out using LC/ESI-MS allowed the quantification of secondary metabolites present in the aqueous extract of P. alliacea. The results showed that the fractions of sulfur and nitrogen organic compounds were the most abundant, with 5.3% and 72.7%, respectively. A detailed chemical analysis for the structural elucidation of the active principles mentioned in the aqueous extract will be published in the future.

The aqueous extract of P. alliacea meets the primary requirements to be considered as a botanical biopesticide. This extract has been subjected to acute toxicity studies with honey bees and has demonstrated its possible acaricidal activity to control the mite V. destructor, a pest that destroys the colonies of this hymenopteran, which is of vital importance throughout planet Earth. In addition, the results obtained indicate that the scaling and industrialization stages of the aqueous extracts of P. alliacea are viable, which could contribute to the development of Peruvian botanical biopesticides.

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TABLES

Table 1. Toxic effect of *Petiveria alliacea L.* (Mucura) extracts on the mortality of *Daphnia magna* during 48 hours of exposure.

Extract		Concentration (mg/L)	Number of *D. magna* neonates	48 h	
				Deaths	Mortality (%)
***Petiveria alliacea* L. (Mucura)**	**Aqueous**	10	15	1	6.67
		100	15	2	13.33
		500	15	5	33.33
		1000	15	10	66.67
		10000	15	14	93.33
	Ethanol	10	15	1	6.67
		100	15	4	26.67
		500	15	10	66.66
		1000	15	13	86.66
		10000	15	14	93.33
	Hexane	10	15	5	33.33
		100	15	11	73.33
		500	15	12	80.00
		1000	15	13	86.66
		10000	15	14	93.33

Table 2. Comparative statistical summary of the Probit Analysis for the Acute Toxicity Bioassay with *Daphnia magna.*

Extract *P. alliacea*	D. magna neonates	LC_{50} (mg/L) (CI 95%)	AC ± SE	χ^2	DF	Heterogeneity
Hexane	Susceptible	26.9103 (5.8320-65.2020)	3.5009 ± 0.3332	0.6244	3	0.2081
Ethanolic	Susceptible	230.6266 (90.4870-497.8240)	4.3539 ± 0.4746	1.3204	3	0.4401
Aqueous	Susceptible	657.9601 (305.2090-1562.8129)	4.1850 ± 0.4773	2.2375	3	0.7458

CI 95% - 95% confidence interval, *AC* - Angular coefficient, *SE* - Standard error of the mean, X^2 - Chi-square, *DF* - Degrees of freedom

Table 3. Acute toxicity bioassay with *Varroa destructor* by direct contact of the extracts of Mucura (*Petiveria alliacea* L.) during 24h of exposure.

Extract		Concentration (mgL^{-1})	Number of *V. destructor*	24 h	
				Deaths	Mortality (%)
Petiveria alliacea L. (Mucura)	*Aqueous*	24	15	1	6.66
		60	15	3	20.00
		100	15	7	46.66
		140	15	12	80.00
		180	15	13	86.66
		200	15	14	93.33
Control	Distilled Water	0.0	15	0	0.00
Control +	Formic Acid	75.0	15	15	100.00

Table 4. Statistical summary of the Probit Analysis for the Acute Toxicity Bioassay with *Varroa destructor*.

Extract *P. alliacea*	D. magna neonates	LC_{50} (mg/L) (CI 95%)	AC ± SE	χ^2	DF	Heterogeneity
Aqueous	Susceptible	105.2418 (97.092-115.6)	5.1872 ± 0.0057	0.6007	4	0.1502

LC_{50} – Median lethal concentration, *CI 95%* - 95% confidence interval, *AC* - Angular coefficient, *SE* - Standard error of the mean, X^2 - Chi-square, *DF* - Degrees of freedom

Table 5. Quantitative analysis of the components identified in the aqueous extract of mucura.

Comp.*	Area (%)	Type and Family of Organic Compounds
P1	72.7	Nitrogen derivative of the amino acid proline
P2		Nitrogen derivative of the amino acid proline
P4		Nitrogenated, dipeptide ester derived from the amino acid proline
P5		Nitrogenated, tripeptide derived from leucine
P6	2.8	Glucosylated diol
P13		Diterpenic glycoside
P7	3.0	Simple phenol (phenolic ester)
P15		Complex phenol (chalcone)
P8	5.3	Sulfurized, derived from thiophene
P9		Sulfurized, derived from the sulfonate
P10		Sulfurized, derived from thiophene
P11		Sulfurized, derived from thiophene
P14	0.2	Aromatic ester
P16	0.8	Cyclic polyol

*Comp. = Components of the aqueous extract separated and quantified in the liquid chromatography profile

FIGURES

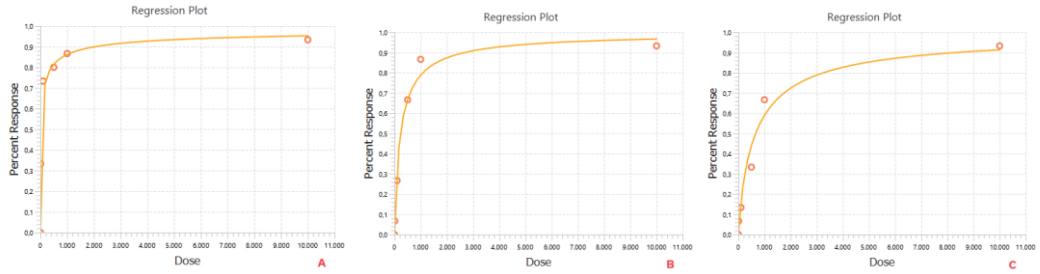

Figure 1. Graphical behavior of the dose/mortality relationship for the acute toxicity bioassay with *D. magna against hexane (A), ethanolic (B) and aqueous (C) extracts of P. alliaceae.*

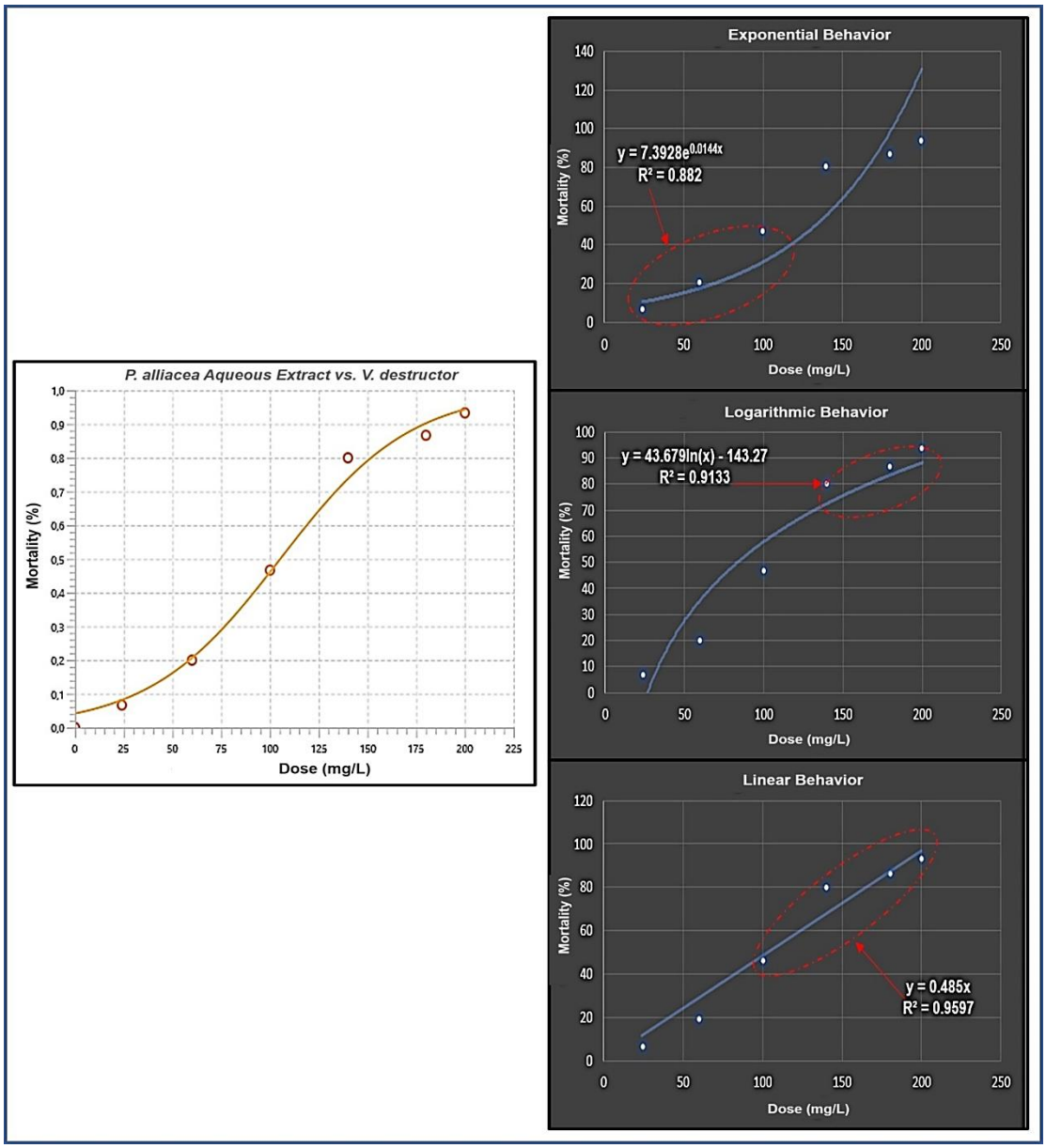

Figure 2. Graphical behavior of the dose/mortality relationship for the acute toxicity bioassay with *V. destructor* according to Probit analysis (left) and possible trend graphs (right).

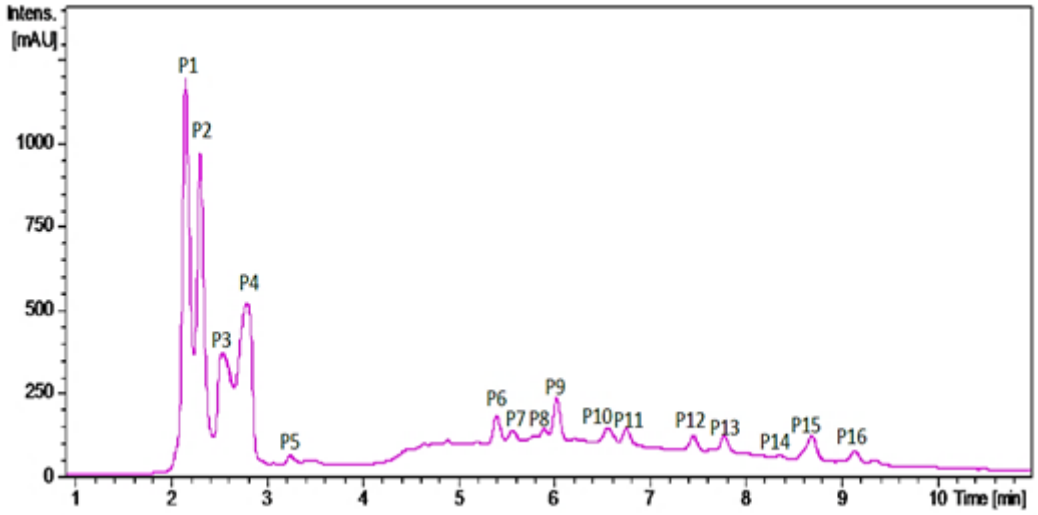

Figure 3. Liquid chromatography profile of the aqueous extract of mucura