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Original Article

Toxicity of Mucura (*Petiveria alliacea*) extracts from the peruvian amazon against *Daphnia magna* for environmental protection and sustainable development

Toxicidade de extratos de Mucura (*Petiveria alliacea*) da Amazônia peruana frente a *Daphnia magna* para a proteção ambiental e o desenvolvimento sustentável

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Abstract

Natural products, specifically plant extracts with biological activity and the ability to act as botanical biopesticides are often mistakenly considered nontoxic. Scientific evidence indicates the contrary, and for this reason, the objective of this work was to evaluate the toxicity of extracts obtained from *Petiveria alliacea* L. (Caryophyllales, Phytolaccaceae) using *Daphnia magna* Straus (Cladocera, Daphniidae) as a bioindicator to identify the plant extracts and the respective concentrations that present the highest toxicity. Leaves of *P. alliacea* were collected in the Peruvian amazone. From this material, three types of extract (hexane, ethanolic and aqueous) were prepared, which were used in the bioassays with *D. magna* to find the least toxic extract. Acute toxicity bioassays with *D. magna* during 48 h of exposure to hexane, ethanolic, and aqueous extracts yielded median lethal concentration (LC_{50}) values of 26.9, 230.6, and 657.9 mg L^{-1}, respectively. The aqueous extract presented the lowest toxicity, causing minimal *D. magna* mortality in the range of 6.67 to 13.33% at concentrations of 10 and 100 mg L^{-1}. This result enables the efficient use of this plant species in a sustainable manner with a minimal environmental impact for the future development of natural products for pest control.

Keywords: bioassay, botanical pesticide, *Daphnia magna,* $LC_{50,}$ toxicity.

Resumo

A utilização de produtos naturais, especificamente extratos vegetais com atividade biológica e capacidade de atuar como biopraguicidas botânicos, é muitas vezes erroneamente considerada não tóxica. Evidências científicas demonstram o contrário e por isso o objetivo deste trabalho consistiu na avaliação da toxicidade de extratos obtidos de *Petiveria alliacea* L. (Caryophyllales, Phytolaccaceae) com a utilização de *Daphnia magna* Straus (Cladocera, Daphniidae) como bioindicador, que permitiu descartar os extratos vegetais e as respectivas concentrações que apresentaram maior toxicidade. As folhas de *P. alliacea* foram coletadas na Amazônia peruana. A partir deste material, foram preparados três tipos de extrato − hexânico, etanólico e aquoso −, que foram utilizados nos bioensaios com *D. magna* a fim de encontrar o extrato menos tóxico. Os bioensaios de toxicidade aguda com *D. magna* durante 48 h de exposição aos extratos hexânico, etanólico e aquoso permitiram estabelecer os valores da Concentração Letal Média (CL_{50}) de 26,9; 230,6 e 657,9 mg L^{-1}, respectivamente. O extrato aquoso apresentou a menor toxicidade, causando percentuais mínimos de mortalidade de *D. magna* na faixa de 6,7 a 13,3% nas concentrações de 10 e 100 mg L^{-1}. Estes resultados possibilitam o uso eficiente das espécies vegetais de forma sustentável e com mínimo impacto ambiental para o futuro desenvolvimento de produtos naturais para controle de pragas.

Palavras-chave: bioensaio, praguicida botânico, *Daphnia magna*, CL_{50}, toxicidade.

1. Introduction

The use of plants and plant extracts to reduce the levels of pest infestation constitutes a reality as a component of sustainable agroecological practices if one takes into account the experimental evidence confirming their effectiveness without affecting crop yields due to the presence of compounds or active ingredients (secondary metabolites) with the ability to control pests, i.e., botanical biopesticides or biochemical biopesticides, that could progressively replace synthetic pesticides (Deka et al., 2022; Fenibo et al., 2021; Glare et al., 2016; Kapila and Singh, 2021).

A recent publication by Castillo-Pérez et al. (2022) showed the use of such products in Latin America motivated

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by the growing demand of consumers concerned about food, safety, and products used for pest control in the manufacture of food products (Khursheed et al., 2022; Souto et al., 2021).

It is very interesting that currently, botanical biopesticides are subject to and accepted for the management and control of pests in not only organic agriculture but also conventional agricultural production (Campos et al., 2019; Isman, 2015). The control of pests using plant secondary metabolites is part of the trend and need worldwide for new sustainable agroecological practices. In this sense, Pretty and Bharucha (2015) presented evidence and perspectives on the use of biopesticides (Neem) as a component of integrated pest management in Africa and Latin America. In addition, the trend toward the use of biopesticides plays an important role in the progressive reduction in synthetic chemical pesticide use, contributing to human health and care for the environment (Samada and Tambunan, 2020).

Botanical biopesticides are often considered nontoxic and safer than synthetic pesticides. However, the reality is far from the truth. Wiesbrook (2004) demonstrated that it is a very common misunderstanding to always consider them safer than synthetic insecticides, as some biopesticides have median lethal concentration (LD_{50}) values lower than those of synthetic pesticides and are therefore more toxic for fish, beneficial insects such as bees, and even mammals.

Rotenone is an example of a secondary plant metabolite that worries the scientific community, since despite being considered an efficient biopesticide, research has identified a possible contribution to Parkinson's disease. Various studies have demonstrated its ability to induce apoptotic cell death in experimental models with cells and animals and to cause cell necrosis through ATP depletion. This scientific evidence on the toxicity of a natural product brings attention to the false idea of their innocuousness (Betarbet et al., 2000; Li et al., 2003; Skulachev, 2006).

In this sense, a recent review by Ferraz et al. (2022) clearly demonstrated the urgent need to evaluate the toxicity of natural products, existing essential oils, and plant extracts with high toxicity levels using toxicity bioassays with *Daphnia magna* as a model to establish the median effective concentration (EC_{50}) or LC_{50} after 48 h of exposure to neonates. However, these plant products, used for their potential as botanical biopesticides, tend to be of low toxicity compared with synthetic pesticides, which, together with their rapid biodegradation, leads to minimal residuality, persistence, and environmental toxicity (Ferraz et al., 2022).

Invertebrates such as the microcrustacean *D. magna* are used not only because they meet the characteristics required for a sentinel organism but also because in terms of acute toxicity, they are considered highly sensitive analytical tools for determining the toxicity of environmental chemical pollutants and even neuroactive compounds, among others, which can disrupt the ecological pyramid and place human health at risk. Therefore, *D. magna* monitoring is important and contributes to protecting the environment and human health (Kikuchi et al., 2000; Olmstead and LeBlanc, 2000; Rawlings et al., 2019; Robertson et al., 2017; Teixidó et al., 2020; Zhou et al., 2008).

In an ecotoxicity test, the growth, reproduction, immobility, and mortality of *D. magna* are well studied and can be used as quantifiable health parameters. Dose–response data can help predict the consequences of exposure at other dose levels and life stages in other species or in susceptible individuals. Several studies have reported that *D. magna* has the ability to predictively establish levels of cytotoxicity comparable to those of mammals (Guilhermino et al., 2000; Kikuchi et al., 2000; Olmstead and LeBlanc, 2000).

Mucura (*P. alliacea*) is a perennial herbaceous plant native to the Amazon rainforest that is widely distributed in Peru. Preparations from this plant have been widely used in traditional medicine for the treatment of many ailments, taking advantage of its anti-inflammatory, antimicrobial, anticancer, and stimulating effects, among others. Mucura extracts have also been shown to have the ability to act as a botanical biopesticide, with insecticidal and acaricidal properties (Johnson et al., 1997; Rosado-Aguilar et al., 2010).

Therefore, evaluating the toxicity of *P. alliacea* plant extracts through bioassays using *D. magna* constitutes a guarantee of environmental protection, care for the trophic chains, and the elimination of possible environmental impacts in general as a preventive starting point for possible future product development for both human health and pest control applications. The objective of this work was to identify the mucura (*P. alliacea*) extract with minimal environmental impact through acute toxicity bioassays with *D. magna* by exposure to neonates for 48 h.

2. Materials and Methods

2.1. Plant material

Leaves of the species *P. alliacea* were collected in the district of Castillo, province of Leoncio Prado, located in the department of Huánuco (09° 16' S, 76° 00' W), in August 2016. A specimen was placed in the Herbarium of the National Agrarian University La Molina. The voucher number is 012-2019-HM-UNALM.

2.2. Biological material

The bioassays were carried out with *D. magna* neonates from the Institute of the Sea of Peru (Instituto del Mar del Perú - IMARPE). The specimens were kept at a temperature of 20 ± 2 °C in water with a pH of 7.14, an electrical conductivity of 10 µS cm^{-1}, an alkalinity of 93.5 mg CaCO3 L^{-1}, and a total hardness of 45.6 mg $CaCO_3$ L^{-1}. *D. magna* was fed with the algal species *Pseudokirchneriella subcapitata* (Korshikov) (Sphaeropleales, Selenastraceae) according to the international standards ISO 6341:2012 (ISO, 2012), OECD (2004), and USEPA (2002).

2.3. Methodology

2.3.1. Acute toxicity bioassay with Daphnia magna

The methodology published by Bracho-Pérez et al. (2019) consisted of performing a reference test with sodium chloride (NaCl) to evaluate the sensitivity of *D. magna* neonates (< 24 h). The static toxicity test was carried out in plastic containers by adding 4 mL of the extracts to be evaluated at five concentrations. Five neonates were exposed to five concentrations of the aqueous and organic extracts of *P. alliacea* ranging from 10 to 10,000 mg L^{-1} without feeding during the test at 24 and 48 h of exposure in darkness at 20 ± 2 °C. For each concentration, the bioassays were performed in triplicate. Immobilization of the treated organisms or the absence of a heart rate for 15 s under a stereomicroscope was used as an indicator of mortality (Bracho-Pérez et al., 2019).

2.3.2. Low environmental risk decision

Considering the results obtained from the acute ecotoxicity test, the same criteria of Bracho-Pérez et al. (2019) were applied, which consisted of excluding the plant extracts and the concentrations that presented the highest toxicity against *D. magna*, allowing the adequate determination of a possible botanical biopesticide for eco-friendly and sustainable pest control.

2.3.3. Processing of plant material

The *P. alliacea* leaves were dried in an oven at a temperature of 40 °C for 48 h. Subsequently, they were pulverized using a blade mill until they were reduced to a fine particulate material that was sieved using a 100 µm sieve.

2.3.4. Preparation of plant extracts

Ten grams of the plant material was weighed in triplicate. They were placed separately in amber glass flasks and subjected to extraction with 100 mL of n-hexane, 100 mL of 96% ethyl alcohol, or 100 mL of distilled water in an ultrasonic bath at a temperature of 48 °C for 2 h. Subsequently, the extracts were filtered with Whatman No. 42 filter paper. The organic extracts were concentrated under reduced pressure at a temperature of 38 °C with the use of a rotary evaporator until crude extracts were obtained, and the aqueous extract was lyophilized with the use of a Telstar Model Lyo Quest-55 lyophilizer at a freezing temperature of -20 °C and vacuum lyophilization at -55 °C for a period of 48 h. Finally, the concentrates obtained were redissolved in propylene glycol and distilled water for bioassay development.

2.4. Statistical analysis

The results from the acute ecotoxicity bioassays of *P. alliacea* extracts against *D. magna* were processed to determine the LC_{50}, expressed in mg L^{-1}, for a confidence interval of 95% ($\alpha = 0.05$). The significance and adjustment to the probit regression analysis was carried out through determination of the angular coefficients of the slopes, chi-square (X^2), and heterogeneity using the statistical program PoloSuite. This result allowed the establishment of the mucura extract type and the appropriate concentration capable of causing the least environmental impact (LeOra Software LLC, 2016; Robertson et al., 2017).

3. Results and Discussion

The results from the acute toxicity study of *P. alliacea* leaf extracts against the bioindicator *D. magna* showed the organic extracts to have the highest toxicity. The hexane extract presented the highest toxicity, causing 33.33% neonate mortality at the lowest concentration of 10 mg L^{-1}; in contrast, the ethanolic and aqueous extracts at that same concentration only caused 6.67% mortality (Table 1). The ethanolic extract maintained a similar trend up to 100 mg L^{-1}, reaching 26.67% mortality. However, the aqueous extract at 500 mg L^{-1} caused less than 50% neonate mortality and generally caused the lowest mortality, 6.67 to 13.33% in a concentration range between 10 and 100 mg L^{-1} (Table 1).

Table 1. Toxic effect of *P. alliacea* extracts on the mortality of *D. magna* after 48 h of exposure.

	Type of extract	Concentration (mg L^{-1})	Number of *D. magna* neonates	48 h	
				Deaths	Mortality (%)
P. alliacea (Mucura)	Aqueous	10	15	1	6.67
		100	15	2	13.33
		500	15	5	33.33
		1000	15	10	66.67
		10 000	15	14	93.33
	Ethanolic	10	15	1	6.67
		100	15	4	26.67
		500	15	10	66.66
		1000	15	13	86.66
		10 000	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
		10 000	15	14	93.33

Decreasing toxicity was recorded in terms of the mean lethal concentration at 48 h of exposure: hexane extract (LC_{50} = 26.91 mg L^{-1}) > ethanolic extract (LC_{50} = 230.64 mg L^{-1}) > aqueous extract (LC_{50} = 657.96 mg L^{-1}) (Table 2). The mortality percentages for the three botanical extracts are indicated in Table 1, corroborating that the aqueous extract was approximately 3–25 times less toxic than the organic extracts based on the LC_{50} values obtained. The safest concentration range was between 10 and 100 mg L^{-1} with a minimum mortality percentage, a condition to be taken into account in the development of pest control products with minimal environmental impact.

Robertson et al. (2017) presented the characteristic behavior of a toxicological study with confidence intervals that yield hyperbolic curves and represent the dose vs. mortality when exposing *D. magna* to *P. alliacea* extracts (Figure 1). The angular coefficients of the slopes in Table 2 presented values greater than 1.96, ensuring the quality of the study carried out. In addition, heterogeneity below 1.0 was also adequate, indicating that the dose-mortality curve conformed to the probit analysis model and that the toxicity values obtained were highly significant.

The dose–response regression curves in Figure 1 indicate a high sensitivity of *D. magna* neonates to *P. alliacea* extracts, reflecting the fact that small increases in the botanical biopesticide dose yield intense mortality responses.

According to the Globally Harmonized System of Classification and Labelling of Chemicals proposed by the United Nations (UN, 2019) and the processing carried out by Ferraz et al. (2022), the toxicity of the hexane extract of *P. alliacea* (LC_{50} = 26.91 mg L^{-1}) was categorized as a category 3 acute short-term hazard (LC_{50} > 10 ≤ 100 mg L^{-1}), which means that its discharge or interaction with the environment is dangerous. The ethanolic (LC_{50} = 230.64 mg L^{-1}) and aqueous (LC_{50} = 657.96 mg L^{-1}) extracts were categorized as LC_{50} > 100 mg L^{-1} according to this harmonized system and should be considered practically nontoxic chemicals.

These results coincide with the evidence presented by Hernandez and Vendramim (2008) as part of solvent management for botanical biopesticide production, which established that the use of low-polarity organic solvents such as hexane and even medium-polarity solvents such as ethanol had a greater capacity to extract secondary metabolites capable of causing sudden death by acting as insecticides, while highly polar solvents such as distilled water were related to the ability to extract secondary metabolites capable of affecting biology, physiology, and growth, among other processes, denoting the ability to act as an insectistatic.

In addition, high-polarity water-soluble metabolites degrade rapidly, with very low persistence and residuality, causing minimal to no environmental contamination, unlike low-polarity organic extracts. This behavior coincides with the toxicity and chemical nature of the extracts evaluated with *D. magna* in this study (Hernandez and Vendramim, 2008).

In this sense, the ethanolic extract presented the lowest toxicity (LC_{50} = 230.64 mg L^{-1}), and according to Ferraz et al. (2022), it should be considered practically nontoxic. However, a study by Cal et al. (2022) demonstrated its high

Table 2. LC_{50} and 95% confidence interval (mg L^{-1}) obtained in the bioassays.

Extract *P. alliacea*	***D. magna* neonates**	**LC_{50} (mg/L)**	**AC ± SE**	**X^2**	**DF**	**Heterogeneity**
Hexane	Susceptible	26.9103	3.5009 ± 0.3332	0.6244	3	0.2081
		(5.8320-65.2020)				
Ethanolic	Susceptible	230.6266	4.3539 ± 0.4746	1.3204	3	0.4401
		(90.4870-497.8240)				
Aqueous	Susceptible	657.9601	4.1850 ± 0.4773	2.2375	3	0.7458
		(305.2090-1 562.8129)				

AC - Angular coefficient; *SE* - Standard error of the mean; X^2 - Chi-square, *DF* - Degrees of freedom.

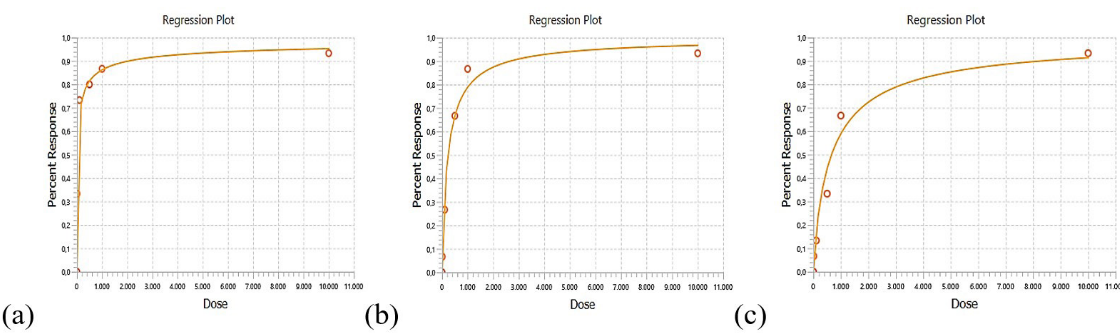

Figure 1. Dose-percent response curves for the mortality of *D. magna* against hexane (a), ethanolic (b), and aqueous (c) *P. alliacea* extracts.

toxicity and mutagenicity as well as its ability to induce oxidative lesions in the mitochondrial and genomic DNA of S. cerevisiae strains. This scientific evidence clearly demonstrates the high toxicity of this species and its ethanolic extracts, constituting essential evidence that they should be used with great prudence in pest control, and our opinion is that its use should also be avoided until more advanced toxicity studies with different nontarget species demonstrate the possibility of safe use.

The use of *D. magna* for the development of ecotoxicological studies with plant extracts and essential oils and research and development on botanical biopesticides for eco-friendly and environmentally safe pest control are being carried out widely throughout the world to prevent environmental impacts and damage to biodiversity (Ferraz et al., 2022; Griffiths et al., 2021; Li et al., 2015). Since 2002, the development of ecotoxicological bioassays using *D. magna*, specifically acute and chronic toxicity bioassays, has been officially established in Peru and other countries belonging to the Andean Community of Nations (Peru, 2002).

In this sense, several bioassays have been carried out using *D. magna* to establish the toxicity of botanical species of Peruvian origin or introduced, but all have in common their insecticidal properties and traditional uses for pest control. The study of sachayoco (*Paullinia clavigera* Simpson, Sapindaceae) extracts showed the leaf chloroform extracts to be of the highest toxicity (LC_{50} = 237 mg mL^{-1}) and bark (LC_{50} = 398 mg mL^{-1}), allowing to establish hydroalcoholic extracts of less toxicity that are suitable for integrated pest management (Iannacone et al., 2007).

The evaluation of nine species, squash (*Cucurbita maxima* Duch. Ex Lam, Cucurbitaceae), eucalyptus (*Eucalyptus globulus* Labill, Myrtaceae), coca (*Erythroxylon coca* [Lamarck], Erythroxylaceae), Angel's trumpets (*Brugmansia candida* Pers., Solanaceae), rue (*Ruta graveolens* L., Rutaceae), castor bean (*Ricinus communis* L., Euphorbiaceae), garlic (*Allium sativum* L, Liliaceae), mint (*Minthostachys setosa* (Briquet) Epling, Lamiaceae), and pomegranate (*Punica granatum*, Punicaceae), demonstrated the usefulness of toxicity bioassays with *D. magna* (Iannacone et al., 2008). Five plants showed maximum acute toxicity with *D. magna* immobilization at 48 h of exposure: the leaves of *C. maxima*, *E. globulus* and *M. setosa*, the seeds of *C. maxima*, and the flowers of *P. granatum*.

Sandbox tree (*Hura crepitans* L., Euphorbiaceae) and elderberry (*Sambucus peruviana* HBK, Caprifoliaceae) are two Peruvian species that have been evaluated with *D. magna* due to their terrestrial and aquatic ecotoxicological risks. Both have powerful biopesticide activity, and their studies were very useful, since they allowed us to establish that their greatest toxicity was found in leaf and root extracts (Iannacone et al., 2014; Román-Farje et al. 2017).

However, very few acute toxicity studies of *P. alliacea* have been performed using *D. magna*. A study by Bracho-Pérez et al. (2019) allowed us to compare the toxicological effect of the hexane, ethanolic, and aqueous extracts of this species and *Clibadium peruvianum*; the organic extracts of both species had greater toxicity than the aqueous extracts. The aqueous extract of *P. alliacea* presented the lowest toxicity (LC_{50} = 711.18 mg L^{-1}, while the ethanolic (LC_{50} = 255.5 mg L^{-1}) and hexane (LC_{50} = 65.36 mg L^{-1}) organic extracts caused the highest mortality to *D. magna* neonates, corroborating the toxicological responses of *D. magna* to *P. alliacea* extracts from this study.

3.1. Low environmental risk decision

The hexane extract with high toxicity (LC_{50} = 26.91 mg L^{-1}) categorized as acute 3 toxicity was discarded to reduce future risks of environmental toxicity and avoid any possible damage to the trophic chains. The ethanolic extract was also discarded pending the development of other ecotoxicological studies that guarantee its safe use. Because the aqueous extract caused the lowest percentage of mortality to *D. magna* neonates (6.67-13.33%) in a concentration range of 10 to 100 mg L^{-1}, this extract was chosen as the safest for the preparation of botanical biopesticides from *P. alliacea* leaves.

4. Conclusions

Implementing acute toxicity methodologies with the use of a bioindicator such as *D. magna* made it possible to determine both the type of extract based on the physicochemical nature of the solvent used and the concentrations with minimal toxicity from *P. alliacea*, whose aqueous extract was the most appropriate because it caused the lowest mortality to *D. magna* neonates, with an LC_{50} = 657.96 mg L^{-1}.

It is interesting to note that the aqueous extract of *P. alliacea* opens up promising possibilities for the development of botanical biopesticides for pest control. Its evaluation for the control of the *Varroa destructor* mite, which damages bees, and an LC/MS chemical study will be the subjects of another study.

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