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ORIGINAL ARTICLE

Ecological risk assessment of *Piper aduncum* essential oil in non-target organisms

Patrícia Tidori MIURA[1], Claudio Martín JONSSON[2*], Sonia Claudia do Nascimento de QUEIROZ[2], Edsandra Campos CHAGAS[3], Francisco Célio Maia CHAVES[3], Felix Guillermo Reyes REYES[1]

1 Universidade Estadual de Campinas, Faculdade de Engenharia de Alimentos, Departamento de Ciência de Alimentos, Rua Monteiro Lobato, 80, CEP 13083-862 Campinas, SP, Brazil

2 Embrapa Meio Ambiente, Rodovia Governador Dr. Adhemar Pereira de Barros - km 127.5, Tanquinho Velho, CEP 13918-110 Jaguariúna, SP, Brazil

3 Embrapa Amazônia Ocidental, Rodovia AM-010, km 29, CEP 69010-970 Manaus, AM, Brazil

* Corresponding author: claudio.jonsson@embrapa.br; https://orcid.org/0000-0002-7630-2131

ABSTRACT

One possible alternative to chemotherapeutic agents in the treatment and prevention of diseases in fish farms is the use of *Piper aduncum* essential oil. However, ecotoxicological data are required to ensure its proper use and to prevent adverse effects on non-target organisms. These data are relevant since this essential oil is described as having insecticidal, molluscicidal and cytotoxic activitiy that may be associated with its chemical composition. Thus, the aim of this study was to evaluate the ecotoxicity of *P. aduncum* essential oil to five test organisms using the species sensitivity distribution (SSD) statistical approach. The chemical composition of the essential oil was characterized by means of gas chromatography coupled to mass spectrometry (GC-MS) and gas chromatography-flame ionization detection (GC-FID) for identification and quantitation purposes, respectively. The main component (75.5%) of the essential oil was dillapiole. The hazardous concentration for 5% of biological species (HC5) was calculated to determine the 95% protection level, resulting in a value of 0.47 mg L^{-1} (with a confidence interval of 0.028 - 1.19 mg L^{-1}.). A concentration range related to the level of protection for aquatic communities (the predicted no-effect concentration, PNEC) was determined through the application of safety factors to the HC5 value. The ecotoxicity parameters showed that *P. aduncum* essential oil can be used safely in water bodies at a concentration equal to or below 0.09 mg L^{-1}.

KEYWORDS: ecotoxicity, hazardous concentration (5%), predicted no-effect concentration, species sensitivity distribution, *spiked* pepper

Avaliação de risco ecológico do óleo essencial de *Piper aduncum* em organismos não alvo

RESUMO

Uma possível alternativa ao uso de fármacos veterinários no tratamento e prevenção de doenças na piscicultura é o uso do óleo essencial de *Piper aduncum*. No entanto, são necessários dados ecotoxicológicos para garantir seu uso apropriado sem causar efeitos adversos a organismos não alvo. Esta informação é relevante, pois esse óleo essencial é descrito como tendo atividades inseticidas, moluscicidas e citotóxicas, possivelmente associadas à sua composição química. Assim, o objetivo deste estudo foi avaliar a ecotoxicidade do óleo essencial de *P. aduncum* para cinco organismos-teste, usando o método estatístico da Distribuição da Sensibilidade das Espécies (SSD). A composição química do óleo essencial foi caracterizada por cromatografia gasosa acoplada a espectrometria de massa (GC-MS) e cromatografia gasosa com detector de ionização de chama (GC-FID), para fins de identificação e quantificação, respectivamente. O principal componente (75,5%) do óleo essencial foi o dilapiol. A concentração perigosa para 5% de espécies biológicas (HC5) foi calculada com um nível de proteção de 95%, resultando em um valor de 0,47 mg L^{-1} (com intervalo de confiança de 50% = 0,028 - 1,19 mg L^{-1}). A faixa de concentração relacionada aos níveis de proteção para comunidades aquáticas (concentração sem efeito previsto - PNEC) foi calculada através da aplicação de fatores de segurança ao valor de HC5. Os parâmetros de ecotoxicidade indicaram que o óleo essencial de *P. aduncum* pode ser usado com segurança em corpos d'água se a concentração for igual ou inferior a 0,09 mg L^{-1}.

PALAVRAS-CHAVE: ecotoxicidade, concentração perigosa (5%), concentração previsível sem efeito, distribuição da sensibilidade das espécies, pimenta-de-macaco

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INTRODUCTION

Essential oils have been considered as a possible method of disease prevention and treatment in aquaculture that causes less adverse environmental effects than veterinary drugs (Elumalai *et al.* 2020). Essential oils have shown interesting properties for aquaculture when administered correctly, such as sedative (Aydin and Barbas 2020), anesthetic (Hoseini *et al.* 2018), antimicrobial (Sutili *et al.* 2014), immunomodulation (Al-Sagheer *et al.* 2018), and stress reducing activity (Souza *et al.* 2017). However, it is important to consider that plant-derived active substances can be stressor factors or even toxic if the conditions (way of administration, concentration used, species-specific actions, chemotype and chemical composition) are not suitable (Souza *et al.* 2019).

Piper aduncum L. (Piperaceae) is a plant native to the Amazon region that stands out for its therapeutic action against fungi, stomach ache, rheumatism, fever and general infection (Mgbeahuruike *et al.* 2017), protozoan parasites (Dal Picolo *et al.* 2014), and for its repellent properties (Mamood *et al.* 2017). The chemical characterization of the metabolites present in *P. aduncum* reveals that the major compound is dillapiole (Gaínza *et al.* 2016).

The essential oil of *P. aduncum* has been used to treat fish diseases (Queiroz 2012; Corral *et al.* 2018), yet no information exists on its behavior against aquatic non-target organisms. Non-target organisms may eventually come into contact with the essential oil when it is used for therapeutic purposes in aquaculture production or when the aquaculture effluents reach the water bodies in the surrounding areas (Bártíková *et al.* 2016). Therefore, the ecotoxicological assessment is required to determine the parameters for the safe use of the essential oil in fish farming (Souza *et al.* 2019; Bashir *et al.* 2020).

Th ecotoxicological evaluation assesses the potential toxicity of the substance to aquatic life, how much of the substance is expected to get into the environment, and what are the potential effects of its use on the environment (FDA 2018). A useful parameter in this context is the hazardous concentration (HC5), which is the estimated concentration that protects 95% of the species in a community and can be predicted from a small number of toxicological data (OECD 1995). For this purpose, the species sensitivity distribution (SSD) is used, which can be estimated with data from at least five species belonging to at least four taxonomic groups (Batley *et al.* 2018), and considers the variation in the sensitivity to the tested substance among the species. SSD is a relevant tool in the definition of quality standards with respect to the maximum values of potentially toxic substances allowed in the environment in order to protect species diversity (Aldenberg and Slob 1993; OECD 1995). The determination of the "no observed effect concentration" (NOEC) is a necessary step to establish a tolerable concentration in the environment (OECD 1995).

In this study we aimed to assess the ecotoxicity of the essential oil of *P. aduncum*. The chemical composition of the essential oil sample was analyzed, and its HC5 was determined against five non-target organisms: a microalga (*Pseudokirchneriella subcapitata* Korshikov), lettuce (*Lactuca sativa* L.) seeds, a nematode (*Panagrolaimus sp.*) and two microcrustaceans (*Daphnia magna* Straus and *Artemia salina* L.). In addition, safety factors were applied to the HC5 value in order to establish levels of protection for aquatic communities.

MATERIAL AND METHODS

Origin of the material and essential oil extraction

Piper aduncum leaves, stems and inflorescences were collected from the medicinal plants and vegetables sector of Embrapa Amazônia Ocidental, in Manaus, Amazonas state, Brazil (03°06'23.04"S, 60°01'35.14"W). A voucher specimen was deposited on the herbarium of Instituto Nacional de Pesquisas da Amazônia (INPA 10480). Information on the voucher material is also contained in SISGEN access register # AA7B04F of the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (Brazilian Ministry of Environment).

The essential oil (EO) was extracted by hydrodistillation in the Laboratory of Medicinal Plants and Phytochemistry at Embrapa Amazônia Occidental, Manaus, Amazonas state, Brazil. The EO was stored in amber bottles at -18 °C.

Chemical EO composition

The ratio and type of substances present in EO of a species can vary as a function of several factors, such as growth conditions, altitude, soil type, agricultural methods and practices, developmental stage, part of the plant extracted, harvesting time, climate and fertilization (Moghaddam and Mehdizadeh 2017). Therefore, the chemical composition of the EO used in this study was determined, as a reference for the ecotoxicological analysis.

Standards and solutions: To determine the chemical composition of the *P. aduncum* EO, analytical standards of n-alkane (C7-C40 49452-U SUPELCO, USA), octane ≥ 99% (74821-100 ML Sigma Aldrich) and eicosane 99% (219274-5G Sigma Aldrich) (Adams, 2007) were used. The EO was diluted with ethyl acetate (final concentration = 0.05 µL ml^{-1}).

Chromatographic method: The chromatographic conditions of the standard solutions and of the EO sample were the same. We used a Varian 3900 gas chromatograph coupled to a Varian Saturn 2100T mass spectrometer (GC-MS) and a Shimadzu 2010 (AOC 5000 injector) gas chromatograph with a flame ionization detector (GC-FID).

For chromatographic separation, we used an OV-5ms bonded column (30 m x 0.25 mm, 0.25 µm) (Ohio Valley Specialty Co., Marietta, OH, USA). In the GC-MS system, helium gas was used as the carrier gas, whereas in the GC-FID system, nitrogen was used, both with a flow rate of 1 ml min^{-1}. The optimized parameters were: injector temperature = 220 °C, initial temperature = 50 °C, temperature gradient = 3 °C min^{-1}, final temperature = 310 °C), range of mass/charge ratio analyzed = 41 - 450 *m/z*, and injected volume = 1 µl, splitless mode, electron impact at 70 eV.

Identification and relative area of EO compounds

The EO compounds were identified by GC-MS by comparison with the mass spectra of the NIST 2.0 library (NIST 2005), through retention index (RI) calculations according to Van den Dool and Kratz (1963) for chromatographic assays with linear temperature programming in relation to the hydrocarbon chain (C7-C40) (Equation 1) and by referring to data in the literature.

$$RI_x = 100n + 100(t_x - t_n)/(t_{n+1} - t_n) \quad \text{(Equation 1)}$$

where x = chemical compound; n = carbon number of the hydrocarbon eluting before compound x; t_x = retention time of compound x; t_n e t_{n+1} = retention times of the hydrocarbons eluting before and after compound x, respectively.

The identified compounds were quantified by GC-FID under the same analytical conditions used in the GC-MS by means of the percentage obtained from the area of each peak relative to the total area of the peaks of the compounds found.

Assay with *Pseudokirchneriella subcapitata*

In this assay, the effect of the EO on the growth of the microalga *Pseudokirchneriella subcapitata* was evaluated. The methodology followed Becaro *et al.* (2015). The algae were cultured in suspension in NPK medium under controlled light (1300 lux) and temperature (20 ± 2 °C) (Jonsson *et al.* 2015). The suspension was distributed in a 96-well polystyrene culture plate, with a final density in each well of 1.4 x 10^6 cells ml^{-1}. The algae suspensions were exposed to definitive EO test concentrations of 0.1, 1, 10 and 100 mg L^{-1}. Twelve replicates (wells) were used for each test concentration. Tween® 80 was used as a solubility adjuvant in the test solutions. Besides, in the same 96-well culture plate, six wells were filled with a control (250 µl of algae suspension and 50 µl of NPK medium + Tween® 80). Algae growth was estimated through cell suspension absorbance (750 nm) measured with a microplate reader (Tecan Sunrise) to calculate the growth rate. Readings were taken at 0, 24, 48, 72, 96, 120 and 168 h.

Assay with lettuce (*Lactuca sativa*)

This assay evaluated the effect of the EO on the growth of lettuce *(Lactuca sativa)* seedlings. The assay followed the methodology of Castro *et al.* (2018). Lettuce seeds were obtained commercially and the trials carried out in the dark for 168 h at (20 ± 1 °C) in 12-well polystyrene plates, according to Bautista et al. (2013). Seeds were exposed to four EO concentrations (0.1, 1, 10 and 100 mg L^{-1}) and two controls (distilled water and distilled water + DMSO). A total of 216 seeds were used. Seeds were placed individually in each well containing a Whatman no. 2 paper disk. Three plates were used per test condition, with a total of 18 plates. Dimethylsulfoxide (DMSO) was used as solubility adjuvant, and 0.4 mL test solution was added to each disk. Growth was estimated by measuring root elongation of the emerged seedling. The seeds were observed and photographed every 24 h using an Optika camera 4083B3 coupled to a stereomicroscope. Root size was measured using the software Optika View, Ver 7.1.1.5.

Assay with *Daphnia magna*

This assay evaluated the acute toxicity of the EO on the microcrustacean *Daphnia magna* according to OECD guidelines (OECD 2004). The *D. magna* came from populations kept at the Laboratory of Ecotoxicology and Biosafety at Embrapa Meio Ambiente, bred in aquaria (40 x 20 x 15 cm) containing water reconstituted with nutrients, with pH 7.5, total hardness of 53.58 mg L^{-1} in calcium carbonate ($CaCO_3$) and conductivity of 111.4 µs cm^{-1}. The temperature and light intensity were maintained at 20 ± 1 °C and 1000 lux, respectively. The organisms were fed once daily with the alga *P. subcapitata* (Jonsson and Maia 1999). Neonates less than 24 hours of age were separated from the cultures and used as test organisms.

The EO test concentrations were defined after preliminary testing as 1, 2, 4, 8 and 16 mg L^{-1}. As controls we used reconstituted water and reconstituted water + Tween® 80 (final concentration: 25 mg L^{-1}). For each treatment level and control, one 12-well polystyrene plate was used (seven plates in total), with two *D. magna* neonates per well in a final volume of 5 mL test or control solution.

The daphnids were considered to be immobilized if they were not able to swim within fifteen seconds after gently moving the test plate (even if they still could move their antennae) (OECD 2004). Immobilization was recorded visually every 24 h during 48 h (two counts) using a colony counter equipment. Test values were compared with control values in order to determine the estimated concentration that immobilized 50% of *D. magna* in 48h.

Assay with *Artemia salina*

This assay evaluated the acute toxicity of the EO on the nauplii of the brine shrimp, *Artemia salina*. The test organisms were obtained from the eclosion of viable *Artemia* cysts (Maramar®) at the rate of 3 g cysts per liter, as recommended by the manufacturer. The hatching proceeded with the cysts remaining 24 h in a constantly aerated saline solution (3%)

prepared with distilled water and sea salt to produce nauplii (Castro *et al*. 2018).

Nauplii were exposed to EO test concentrations under controlled light (1000 lux) and temperature (20 ± 2 °C) conditions (USEPA 2002). The EO test concentrations were defined after preliminary tests as 10, 18, 32.4, 58.3 and 105 mg L^{-1}. As controls we used saline solution and saline solution + Tween® 80 (final concentration: 25 mg L^{-1}). For each treatment level and control, one 12-well polystyrene plate was used (seven plates in total), with two *A. salina* nauplii per well in a final volume of 5 mL test or control solution. A nauplius was considered immobilized if its appendages and antennae did not move for 10 seconds of observation (Evans *et al.* 2010). Immobilization was recorded visually every 24 h during 48 h (two counts) using a stereomicroscope (Optika, Italy). Test values were compared with control values in order to determine the estimated concentration that immobilized 50% of *A. salina* in 48h.

Assay with *Panagrolaimus* sp.

This assay evaluated the acute toxicity of the EO on non-parasitic nematodes of the genus *Panagrolaimus*. The assay was carried out according to the methodology by Castro *et al*. 2018. Synchronized nematode cultures were maintained in oat medium (Lara *et al.* 2007) at the Laboratory of Ecotoxicology and Biosafety of Embrapa Meio Ambiente, and the assay was performed for 96 h at 24 ± 1 °C in the dark without renewal of the test solutions. The EO test concentrations were defined as 0.1, 1, 10 and 100 mg L^{-1}. As controls we used K medium (KCl and NaCl; Boyd et al. 2012) and K medium + Tween® 80 (final concentration: 25 mg L^{-1}). For each treatment level and control, one 24-well polystyrene plate was used (six plates in total), with ten nematodes per well in a final volume of 1 mL test or control solution. Immobility was assessed for four days by counting the number of exposed nematodes that appeared immobile under a microscope and did not respond to a stimulus using a small metal wire (Castro *et al.* 2018). Test values were compared with control values in order to determine the estimated concentration that immobilized 50% of *Panagrolaimus sp.* in 96 h.

Determination of the EC_{50}

We determined the average and 95% confidence interval of the effective concentration causing immobility to 50% of the test organisms (EC_{50}) in the acute toxicity assays of the microcrustaceans and nematodes during the respective test periods.

Pseudokirchneriella subcapitata cell growth-rate and *L. sativa* root growth-rate were calculated at the end of the exposure period by calculating the angular coefficients of the linear regression curves (from algae suspension absorbance and root size) as a function of time (Basu and Pal 2011; Becaro *et al.* 2015). The EC_{50} and its 95% confidence intervals was determined for the parameter concerning the phytotoxic effect on the growth rate. The analyses were made using the "Probit Analysis" and "Simple Regression" modules of the Statgraphics Centurion XVII program, version 1.17.04 (StatPoint Technologies 2014).

Estimation of the EO hazardous concentration (HC5)

NOEC was estimated for the five test organisms by considering $EC_{50/10}$ (Elmeggard and Akkerhuis 2000). The NOEC values estimated for each test species were used to construct the SSD curve. Based on the SSD, a hypothetical environmental concentration at which only 5% of the species in the aquatic environment would be affected (HC5) was determined by the log-logistic distribution of the NOEC values (OECD 1995; Jonsson *et al.* 2015) using simple logistic regression in the "Logistic" module of the Statgraphics Centurion XVII program (Statpoint Technologies 2014; Zolezzi *et al.* 2005). The fit of the distribution of the NOEC values was assessed with a Kolmogorov-Smirnov test. The HC5 was calculated with a confidence level of 50%, which corresponds to the "most likely" estimate of the 5th percentile (Traas and Van Leeuwen 2007; Liu *et al.* 2016).

An estimate of the exposure level at which organisms in the ecosystem would not suffer any damage was calculated by applying safety factors of "1" and "5" to the HC5 value to determine the predicted no-effect concentration (PNEC), which is an ecological safety threshold that represents the critical concentration at which no effects are expected in a given ecosystem (Liu *et al.* 2016; Castro *et al.* 2018). The respective safety factor values represent the minimum and maximum limits of a scale from 1 to 5 in a risk assessment (Traas and Van Leeuwen 2007; Mertens 2018).

We also used data for the fish *Arapaima gigas* Schinz from Queiroz (2012) to calculate the NOEC for this species and added it to the log-logistic distribution. Queiroz (2012) reported that the immersion of *A. gigas* in a bath of a solution of aqueous extract of *P. aduncum* at 80 ml L^{-1} over 24 h did not affect the viability of the fish. As Queiroz (2012) used the aqueous extract, not the EO, we applied an arbitrary safety factor of 1000 to the concentration of 80 mL L^{-1} and thus, we estimated an EC50-24h > 80 mg L^{-1} and, consequently, a NOEC = 8 mg L^{-1}.

RESULTS

The GC-MS chromatogram (Figure 1) allowed the identification of 18 compounds (Table 1). The main peak in the GC-MS chromatogram corresponded to dillapiole, which accounted for 75.5% of the EO composition, followed by *(E)*-caryophyllene (4.7%) and myristicin (4.2%).

The most sensitive organism to the EO was *D. magna*, with an $EC_{50\text{-}48\,h}$ of 6.8 mg L^{-1}, followed by *A. salina*, *Panagrolaimus* sp., *P. subcapitata* and *L. sativa* (Table 2). The latter two

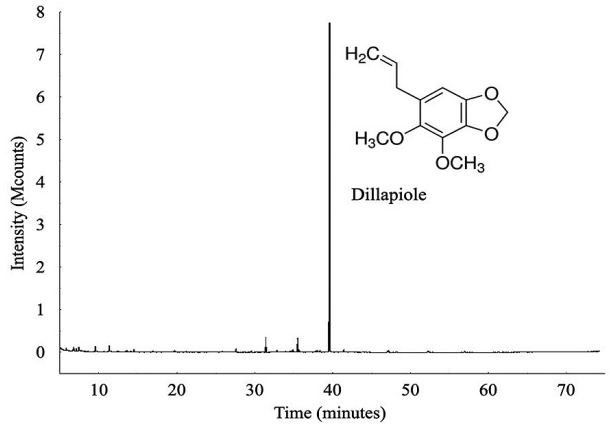

Figure 1. Chromatographic profile of the essential oil of *Piper aduncum* performed by gas chromatography coupled to mass spectrometry.

species showed values >100 mg L^{-1}, indicating that the EO was "practically nontoxic" to both organisms according to the USEPA (2019) classification.

NOEC and HC5 values calculated for each organism are shown in Table 2 and the curve fitted to the NOEC values is shown in Figure 2. Derived from the fitted curve, a value of p = 0.8073 was obtained with the Kolmogorov-Smirnov test, which means that the data correspond to a logistic function. The application of safety factors (5 and 1) to the HC5 value (0.47 mg L^{-1}; Table 2) allowed us to obtain a PNEC concentration range of 0.09 to 0.47 mg L^{-1}.

DISCUSSION

Variability in the chemical composition of an essential oil may influence its intrinsic ecotoxicity (Almeida *et al.* 2017). While we obtained a 75.5% dillapiole composition in our *P. aduncum*, Silva *et al.* (2013) reported a 85.6% dillapiole composition in *P. aduncum* EO from the same locality

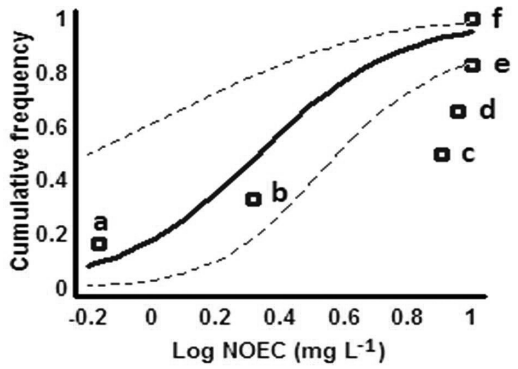

Figure 2. Log-logistic function of the cumulative sensitivity according to NOEC values of *Piper aduncum* essential oil for the test organisms: a – *Daphnia magna*; b – *Artemia salina*; c – *Arapaima gigas*; d – *Panagrolaimus* sp.; e – *Pseudokirchneriella subcapitata;* f – *Lactuca sativa*. Dotted lines correspond to the 50% confidence interval of the curve.

Table 1. Chemical composition of *Piper aduncum* essential oil. Plants sampled at Embrapa Amazônia Ocidental (Manaus, Amazonas, Brazil). RI calc = retention index values calculated in this study; RI liter = retention index values retrived from literature.

Nr.	RI calc	RI liter	Compound	Area %
1	935	932	α-pinene	2.1
2	977	974	β-pinene	4.0
3	1024	1022	o-cymene	0.3
4	1029	1024	limonene	1.3
5	1038	1032	β-(*Z*)-ocimene	0.6
6	1048	1044	β-(*E*)-ocimene	1.3
7	1058	1054	γ-terpinene	0.1
8	1100	1095	linalool	0.1
9	1165	1165	borneol	0.2
10	1177	1174	terpinen-4-ol	0.1
11	1190	1186	α-terpineol	0.2
12	1421	1417	(*E*)- caryophyllene	4.7
13	1455	1452	α-humulene	0.7
14	1521	1517	myristicin	4.2
15	1579	1577	spathulenol	0.4
16	1585	1582	caryophyllene oxide	1.2
17	1625	1620	dillapiole	75.5
18	1682	1677	apiol	0.2
Total identified				97.0
Total not identified				3.0
Total				100.0

Table 2. Ecotoxicity parameters of *Piper aduncum* essential oil against test organisms. NOEC = No Observed Effect Concentration; HC5 = hazardous concentration for 5% of biological species; PNEC = Predicted No Effect Concentration .

Test organism	EC_{50} (mg L^{-1})	NOEC (mg L^{-1})	NOEC calculation
Daphnia magna	6.80 (5.77 - 8.29)[a]	0.68	$EC_{50\text{-}48h/10}$
Artemia salina	20.80 (19.08 - 27.40)[a]	2.08	$EC_{50\text{-}48h/10}$
*Arapaima gigas**	> 80.00	8.00	$EC_{50\text{-}24h/10}$
Panagrolaimus sp	89.78 (51.87 - 326.66)[a]	8.98	$EC_{50\text{-}96h/10}$
Pseudokirchneriella subcapitata	>100.00	10.00	$EC_{50\text{-}7d/10}$
Lactuca sativa	>100.00	10.00	$EC_{50\text{-}7d/10}$

HC_5 = 0.47 (0.028 - 1.19)[b] mg L^{-1}
PNEC = 0.09 - 0.47 mg L^{-1}

[a]Values are the average followed by the 95% confidence interval in parentheses.
[b]Values are the average followed by the 50% confidence interval in parentheses.
*EC_{50} value from Queiroz (2012) (see Material and Methods for details).

(Manaus), and Maia *et al.* (1998) reported a proportion of dillapiole from 31.5% to 97.3% in *P. aduncum* samples from eight different locations in the Amazon region. This variability in EO composition data indicates that the safety parameters calculated in here are likely specific to the composition of our EO samples, and probably need to be estimated for a wider range of the EO composition spectrum in order to better establish its overall safety parameters.

The higher sensitivity of the microcrustaceans *D. magna* and *A. salina* to the EO of *P. aduncum* found in here is supported by another study. Quignard *et al.* (2003) evaluated the lethality for *Artemia franciscana* Kellogg of 74 species of Amazonian plant extracts at a concentration of 500 mg L^{-1} for 24 h, and *P. aduncum* was among the most active plants, with lethality above 90%. Microcrustaceans also have high sensitivity to a variety of chemical compounds (USEPA 2002). The short duration (40 -50 days) of the life cycle of *Daphnia magna* (USEPA 2002) may be related to higher sensitivity, as the usual experimental exposure periods to stressor agents of one or two days represent a higher exposure relative to their life span. The sensitivity level also depends on the development phase, tested concentrations, exposure time and species used (Rand and Petrocelli 1985).

Other studies have also observed the deleterious effect of Piper sp. compounds against arthropods. The EO of *P. aduncum* had lethal effects on larvae of the coleopteran *Tenebrio molitor* at concentrations above 2.5% $v.v^{-1}$ (Fazolin *et al.* 2007). *Piper aduncum* extract at a concentration of 0.1 mg L^{-1} showed the highest insecticidal activity among three Piper species, promoting 92% mortality on larvae of the lepidopteran *Ostrinia nubitalis* (Bernard *et al.* 1995). These studies suggest the insecticidal action of dillapiole, as it is the main compound in the chemical composition of *P. aduncum* oil.

No adverse effect of the EO was observed on *L. sativa* seeds, in contrast to the study by Alves *et al.* (2004), who identified the allelopathy of different EOs on *L. sativa* germination and root length, and demonstrated the inhibitory potential of volatile extracts of cinnamon (*Cinnamomum zeylanicum*), pepper-rosmarin (*Lippia sidoides*), lemon grass (*Cymbopogum citratus*) and clove basil (*Ocimum gratissimum*) for *L. sativa* seeds. There is no obvious explanation for the lack of phytotoxicity of *P. aduncum* EO on plant seeds and algae in our study, even at the highest concentration of 100 mg L^{-1}. It may be related to the inhibitory cholinesterase effect that was described for compounds of several *Piper* species (Silva *et al.* 2017), which can have appreciable effects on arthropods, in contrast to plants and algae.

No toxicity data and NOEC estimates of *P. aduncum* EO exist for fish. However, Queiroz (2012) reported that the immersion bath in a solution of aqueous extract of *P. aduncum* (80 mL L^{-1} for 24 h) did not affect the viability of *Arapaima gigas*. Due to the fact that Queiroz (2012) used aqueous extract, not the EO, we applied an arbitrary safety factor to the concentration of 80 mL L^{-1} of aquous extract for the estimation of the NOEC. The attribution of non-toxicity of *P. aduncum* EO to fish could also be reinforced by the absence of adverse effects in *A. gigas* treated at a high oral dose (80 mL kg^{-1} bw) for 15 days (Corral *et al.* 2018).

The PNEC concentration range obtained (0.09 – 0.47 mg L^{-1}) justifies the application of a factor in the range of 1 to 5 to the HC5 on a case-by-case basis for practical purposes in establishing a maximum permissible concentration for a water body. The factor depends on environmental evidence supported by data on the presence of nonnative species, the number of species involved and any gaps between laboratory and field data (Traas and Van Leeuwen 2007; Liu *et al.* 2016).

CONCLUSIONS

The essential oil of *Piper aduncum* differentially affected the growth and mortality of target organisms of different trophic levels, with microcrustaceans showing higher sensitivity than plant seeds, algae and nematodes. The safest concentration estimated indicated for use of this oil in aquaculture production without compromising the co-existing biota was 0.09 mg L^{-1}. This concentration may be considered a preliminary result, pending on further ecotoxicological evaluations of other target organisms and a wider range of chemical composition of *P. aduncum* essential oil to improve the risk estimate.

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