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Chemical composition, cytotoxicity, larvicidal, antimicrobial, adulticidal activities against Aedes aegypti of essential oils from leaves of *Piper cytorphodon* and *Piper krukoffii Yunck*

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Abstract

Plants of the piper genus have shown great potential in antimicrobial and insecticidal activities. This work analyzed the chemical composition and biological activities of Piper cyrtopodon and Piper krukoffii essentials oils, collected in the Amazon. These oils were

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obtained by hydrodistillation and the chemical compounds were characterized by gas chromatography/mass spectroscopy (GC-MS), showing limonene and selinene were the majority compounds of Piper cyrtopodon and Piper krukoffii, respectively. The essential oils of both species presented toxicity against Artemia salina and the antimicrobial activity showed greater responses to gram-positive bacteria. The insecticidal activity of essential oils had a mean activity against larvae and mosquitoes of Aedes aegypti, being possible agents for the control of vectors.

Keywords: Piper, essential oil, biological activities, *Aedes aegypti*

INTRODUCTION

Mosquito-borne diseases are a major concern for global public health. The *A. aegypti* (Diptera; Culicidae) mosquito is not native to the Americas and was inserted in Brazil from Africa, possibly in the beginning of the 19th century, where it found a satisfactory environment for its survival and reproduction, being eradicated from the country in 1957 and reintroduced in 1967 and again eliminated in 1973 (França et al., 2017). It has a wide geographical distribution and is considered a vector of epidemiological importance due to its ability to transmit the viruses which cause dengue, chikungunya, zika and yellow fever diseases (Martins et al., 2010; Vasantha-Srinivasan et al., 2017).

Among the control strategies, the use of chemical products is widely used, mainly using organophosphates, although the constant use of synthetic insecticides has attributed resistance to mosquitoes, in addition to the issue of environmental pollution (Huong et al., 2019; Zara et al., 2016; Benelli, 2015; Braga & Valle, 2007; Nunes et al., 2018). The use of natural products has been considered a promising alternative to current treatments, due to the great interest in seeking new environmentally safe alternatives, potentially suitable and more effective for use in programs to combat vectors (Huong et al., 2019; Bae et al., 2017; Correa et al., 2019).

Piper is the largest genus of the Piperacea family, composed of more than 2000 species distributed throughout the tropics. The species belonging to this genus have economic, ecological, and medicinal importance (Gogosz et al., 2012). In folk medicine, they have been used for the treatment of pathologies of the respiratory tract and digestive system, such as anti-inflammatory, antimicrobial, and anti-leukemic (Salehi et al., 2019).

It is known that plants are important natural sources of chemical compounds with numerous biological activities, including insecticidal and larvicidal activities, among others. (Mendes et al., 2017; Mitić et al., 2018). Thus, currently one of the main goals of recent research is the creation of efficient bio-insecticides and that are eco-friendly, avoiding adverse effects on the environment (Pinto et al., 2016; Hari & Mathew, 2018).

In Brazil, *Piper cyrtopodon* is found in the states of Amazonas, Amapá, Pará, Roraima, Tocantins and Mato Grosso and can be recognized by the presence of villous trichomes along the branches and veins of the abaxial surface, oblong-elliptical leaves with an asymmetric cord base. It also occurs in Guyana and Peru (Monteiro, 2018; Guimarães. et al., 2015). On the other hand, *Piper krukoffii* is endemic in Brazil, in the states of Acre, Amazonas, Amapá, Pará and Rondônia, and for its identification, the more windy branches of *P. krukoffii* are sufficient to grant specific recognition (Monteiro, 2018; Guimarães et al., 2015)

The antimicrobial, antiparasitic and antifungal activity of the chemical constituents of *Piper* essential oil has been confirmed in some studies (Salehi et al., 2019; Cysne et al., 2005). In addition, insecticidal activity has shown to be a promising agent against many vector species (Bae et al., 2017; Vasantha-Srinivasan et al., 2017; Hematpoor et al., 2016; Huong et al., 2019). However, in the literature, studies related to the species *Piper krukoffii Yunck* and *Piper cyrtopodon* are rare, both in terms of their morphologies and essential oils. In the present work, an investigation of the chemical composition of the essential oil of these Piper specie, collected in the Amazon. From there, initially we studied the mortality of saline artemia in relation to essential oils (cytotoxic assays), verifying its

efficiency and, besides that, their potential antimicrobial, larvicidal and adulticidal activity against *A. aegypti*, are also presented.

EXPERIMENTAL SECTION

Plant material

Leaves of *Piper krukoffii Yunck* were collected at Embrapa Amazônia Ocidental, located on Highway AM-010 of the Manaus-Itacoatiara highway at km 30 (2 ° 53'18.5 "S and 59 ° 58'08.9" W), and leaves of *Piper cyrtopodon* were obtained from the Adolpho Duck reserve, located at Km 26 of the Manaus-Itacoatiara Road (AM-010). To confirm the authenticity of the species collected, two specimens of each plant were submitted to identification and deposited in the herbarium of the National Institute for Research in the Amazon under the numbers 278782 (*P. cyrtopodon*) and 278768 (*P. krukoffii Yunck*).

Isolation of Essential oil

To obtain the essential oil of *P. cyrtopodon* and *P. krukoffii*, 200 and 300 g, respectively, of dried and crushed leaves were subjected to the method of hydrodistillation in a modified Clevenger-type apparatus, under the heating blanket for 4 hours. At the end of the process, a total of 1.2 and 4.42 ml of oil from *P. cyrtopodon* and *P. krukoffii* were obtained, respectively.

GC/MS analysis

The analysis of the chemical composition of the essential oils was performed by HP6890 gas chromatograph interfaced with an HP5873, Mass Selective Detector (ionization energy 70 eV), equipped with a DB-5MS capillary column (30 m×0.25 mm thickness; 0.25 μm film coating). The column temperature was programmed to 70-305 ºC at 5 °C/min using helium as the carrier gas (1.0 mL/min). The injector and detector temperatures were 230 °C and 290 °C respectively. The determination of the chemical composition of the essential oils was carried out based on the retention time data and mass spectra

compared with the information from the library (WILEY 7.0) and according to (Adams, 2011).

Bioassays

Toxicity front *Artemia salina Leach*

Cytotoxic assays were performed at the Chromatography Laboratory (LABCRO) at UFAM, using the method of (Meyer et al., 1982)with modifications. *A. salina* eggs were purchased commercially and placed in a glass aquarium containing 35% saline solution and left under artificial lighting at 28° C for 48 hours to hatch the larvae. For each tested species (*P. cyrtopodon and P. krukoffii*), concentrations of 1000, 750, 500, 250, 100 and 50 µg/mL were prepared, diluted in DMSO. For each concentration tested, 10 larvae of *A. salina* in metanauplios stage were used, which were kept under lighting at 28° C for 24 h. Lapachol was used for the positive control and the 1% DMSO negative control was used. After 24h, individuals were counted, and Median Lethal Concentration (CL50) and Final Lethal Concentration (CL90) determined by probit analysis using the POLO PLUS® program (LeOra Software Berkeley, CA). Tests were performed in triplicate.

Antimicrobial activity

The antimicrobial activity of *P. cyrtopodon* and *P. krukoffii* was tested using the disk-diffusion technique. Strains of Escherichia coli (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 29245), *Staphylococcus aureus* (ATCC 25923) and *Bacillos cereus* (ATCC 12228) were used for antimicrobial assays. The bacteria were inoculated in tubes containing Muller-Hinton agar until reaching the 0.5 turbidity of the Mac-Farland scale (1.5 x 108 CFU / mL), where the suspensions were sown (in triplicate) with the aid of a drigalski loop, across the surface of the plate containing the Muller-Hinton agar medium. Then, 6 mm diameter filter paper discs were added, impregnated with 10 µL of the oils at a concentration of 500 µg/mL, diluted in DMSO. For the positive control, antibiotic methicillin was used and for the negative control DMSO. Subsequently, the plates were incubated in an oven at 35° C and after 24 hours the reading of the halos size was performed

with the aid of a caliper using a reflected light source to illuminate the inverted plate on a black and opaque background.

Larvicidal bioassay

Essential oils were placed in beakers and diluted in a 2% DMSO solution in concentrations of 250, 150, 100 and 50 µg/mL. For each concentration tested, 10 3rd stage larvae were separated and placed in plastic cups (50 mL), containing 30 mL of the dilutions. After 24 hours of exposure of the larvae to treatments, the number of dead larvae was counted and those which did not show movement or did not respond to stimuli were considered dead. The experiments were carried out in triplicate and as a negative control, water and DMSO were used and, for the positive control, a solution of the larvicide temefos.

Adulticidal

For the tests, 250 ml glass bottles impregnated with essential oil samples in concentrations of 500, 250, 150, 100, 50 µg/mL were dissolved in acetone. After evaporation of the solvent (acetone), 15 females of *Aedes aegypti* aged 3-5 days and previously fed with mice's blood meal were placed in each bottle. Mosquito mortality was recorded at regular intervals of 15 minutes up to a total time of 90 minutes. The mosquitoes were transferred to recovery cages, where the reading was performed 24 h after the start of the test. The experiments were carried out in triplicate and for the control, bottles impregnated with acetone were used.

RESULTS AND DISCUSSION

The analysis of the composition of the essential oil of the leaves of *P. cytorphodon* by GC/MS resulted in the identification of 31 compounds, of which 5 are monoterpenes (16.1%) and 23 (74.2%) are sesquiterpenes, corresponding to a total 95% of the identified constituents (Table 1). Among the compounds Limonene (14.47%) was majority, followed by ß-elemene (11.74%), α-copaene (6.11%), Pinene (5.41%) and sphatulenol (4.79%). In case of the *P. krukoffii Y.*

essential oil, about 92% of the composition was identified, being found, in its majority, sesquiterpenes compounds, the main being alpha selinene (25.56%), followed by naphthalene (9.80%) and ß-element (8.93%). All these results are shown in table 1.

Table (1): Chemical composition of essential oils from leaves of *P. cytorphodon* and *P. krukoffii Y.*

P. cytorphodon			***P. krukoffi***		
Compound	**Area%**	**I.R.**	**Compound**	**Area%**	**I.R**
α-pinene	2.82	12.560	Bicycloelemene	0.58	27.952
β-pinene	5.41	14.439	α-cubebene	0.34	28.465
β-Myrcene	0.46	15.049	Pentadiynylbenzeno	0.29	29.208
Limonene	14.47	16.676	α-copaene	8.04	29.445
trans-Carveol	0.24	24.141	ß-bourbonene	2.46	29.705
Cyclohexenone	0.27	25.048	ß-elemene	8.93	29.913
Bicycloelemene	0.40	27.945	calarene	8.48	30.857
δ-Elemene	0.29	28.056	ß-cubebene	2.71	31.199
α-Cubebene	0.61	28.457	α-Guaiene	0.30	31.340
Ylangene	0.47	29.208	α-Humulene	1.01	32.016
α-Copaene	6.11	29.445	*Epi*-bicyclosesquiphellandrene	0.91	32.209
β-Bourbonene	0.39	29.698	α-Muurolene	1.18	32.469
β-elemene	11.74	29.943	ß-Selinene	1.86	32.565
trans-Caryophyllene	3.79	30.879	Germacrene D	8.10	32.848
γ-Elemene	1.26	31.161	Naphthalene	9.80	33.063
Aromadendrene	0.10	31.466	α-selinene	25.56	33.397
δ-Cadinene	0.14	31.793	α-Amorphene	0.49	33.799
α-Humulene	0.93	32.008	δ-Cadinene	1.86	33.932
Germacrene D	0.80	32.810	α-cadinene	0.20	34.504
β-Selinene	2.41	33.071	Elemol	2.38	34.861
α-selinene	2.55	33.286	Guaiol	4.89	36.302
α-Amorphene	0.45	33.791	Heptadiene	0.76	37.327
δ-Cadinene	2.37	33.925	Muurolol	0.33	37.654
CIS-Calamenene	0.72	34.051	N.I.	2.29	37.988
Nerolidol	0.31	35.210	Neointermedeol	1.66	38.092

Spathulenol	4.79	35.767	N.I.	1.31	38.248
Caryophyllene oxide	3.35	35.901			
α-Cadinol	0.69	37.647			
Caryophylla	1.43	38.003			
Heptadecane	0.39	39.125			
Cyclododecatriene	4.36	45.736			

	Chemical Groups	
16,1	Monoterpenes	0
74,2	Sesquiterpenes	84,60
9,7	Others	7,70
93,5	Total identified	92,30

Terpenes comprise a wide variety of substances of plant origin and their ecological importance as pesticides for plants is well established (Maffei et al., 2011). Chemical identification studies of essential oil in plants of the genus Piper have shown monoterpenes and sesquiterpenes as major constituents (Araujo et al., 2018; Machado et al., 1994; Perigo et al., 2016; Salleh et al., 2012). However, a great variability in chemical composition has also been observed in several studies. Analyzes performed on the essential oil of *P. cytorphodon* showed variability in the chromatographic profiles between the samples, mainly in the percentage of the compounds (Andrade et al., 2006). Studies carried out on *P. krukoffii* showed major compounds different from our results, but with similarity to other species of *Piper* sp. (Santana et al., 2019). The variability in the chemical composition of essential oils is mainly related to the species and geographic location (Huong et al., 2019), as well as the environmental conditions in which the plant is found (Prins et al., 2010).

Essential oils of both species showed larvicidal activity against *A. salina* (Table 2). According to Meyer *et al*, the substances can be classified as toxic (CL50 <1000 □g/ml) and non-toxic (CL50> 1000 □g/ml). Studies carried out with oil of *P. krukoffii* have shown greater toxicity than in extracts (da Silva et al., 2011). Lethality analyzes showed that substances classified as toxic (Meyer et al.,

1982) have presented a good correlation with antitumor, antiparasitic and insecticidal activity (Setzer et al., 2008; Alves et al., 2000; Assis et al., 2013).

Table (2): Essential oil toxicity from leaves of *P. cytorphodon* and *P. krukoffi* front to *A. salina*.

	CL_{50} ± SD(μg/ml)
Piper cytorphodon	117.7 ± 0.4 (108.64 – 215.39)
Piper krukoffii	130.6 ± 0.5 (129.45 – 239.82)
Lapachol	54.51 ± 0.5 (35.99 – 83.67)

Numbers in parentheses are 95% fiducial limit values.

Analyzes of antimicrobial activity in Gram-positive and Gram-negative bacteria showed halos in the size of 4 to 1 mm (Table 3), respectively. According to the results, *P. cytorphodon* and *P. krukoffii* essential oils showed activity against *S. aureus* and *B. cereus*, with inhibition halos of 3 and 4 mm, respectively. Studies of antimicrobial activity in Piper species showed that the oil was effective against gram-positive bacteria (da Silva et al., 2017; Perigo et al., 2016) and, among monoterpenes, limonene showed a high correlation with growth bacteria inhibitor, mainly from *Staphylococcus* sp (Perigo et al., 2016), a majority component in our results for *P. cytorphodon*.

Table (1): Antimicrobial activity oil from leaves of *P. cytorphodon* and *P. krukoffi*

	Inhibition halo (mm) ± SD			
	Microorganism			
	Gram-Positive		Gram-Negative	
	S. aureus	***B. cereus***	***E. coli***	***P. aeruginosa***
P. cytorphodon	3 ± 0.3	3 ± 0.2	2 ± 0.1	2 ± 0.1
P. krukoffii	4 ± 0.3	4 ± 0.2	1 ± 0.1	1 ± 0.1
DMSO	-	-	-	-
Meticicline	8 ± 0.3	6 ± 0.3	3 ±0.2	3 ±0.2

The results of the larvicidal bioassays on *A. aegypti* larvae showed high mortality after 24h of exposure (Table 4). The genus Piper presents a diversity of studies showing the effect against *A. aegypti* larvae, mainly in Brazil (Marques & Kaplan, 2015). In our results, the LC50 value illustrates that the essential oils from leaves of *P. cytorphodon* and *P. krukoffii* are potential larvicidal agents, because they have an LC50 below 1000 µg/mL (Meyer et al., 1982).

Table (4): Larval mortality (%) of *Aedes aegypti* and Lethal Concentrations (LC_{50} and LC_{90}) of the essential oils from leaves of *P. cytorphodon* and *P. krukoffi*

	Mortality after 24h (%)	LC_{50} ± SD (µg/ml)	LC_{90} ± SD (µg/ml)
P. cytorphodon	100	118.5 ± 0.4 (110.6 –255.4)	232.9 ± 0.4 (229.2 –390.7)
P. krukoffii	100	120.4 ± 0.5 (109.5 –232.4)	236.4 ± 0.5 (231.6 - 319.4)
Temephos	100	12.2± 0.5 (10.3 – 32.7)	30.6± 0.5 (28.7 – 42.2)
DMSO	0	-	-

Numbers in parentheses are 95% fiducial limit values.

Data referring to the mortality rate of mosquitoes, when exposed to different concentrations in a time of 90 min, are shown in table 5. These results indicate that the rate was dependent on the dose and time of exposure as shown in Figure 1.

Table (5): Lethal Concentrations (LC_{50} and LC_{90}) of the essential oils from leaves of *P. cytorphodon* and *P. krukoffi* against adult mosquitoes *A. aegypti* after 90 min exposure period.

	CL_{50} ± SD (µg/ml)	CL_{90} ± SD (µg/ml)
P. cytorphodon	218.5 ± 0.4 (210.5 – 355.4)	432.9 ± 0.4 (349.2 – 590.7)
P. krukoffii	185.4 ± 0.5 (169.4 – 323.4)	363.4 ± 0.5 (231.6 – 519.3)
Temephos	22.2 ± 0.5 (13.3 – 32.7)	34.6 ± 0.5 (28.7 – 42.2)

Numbers in parenthesis are 95% fiducial limit values.

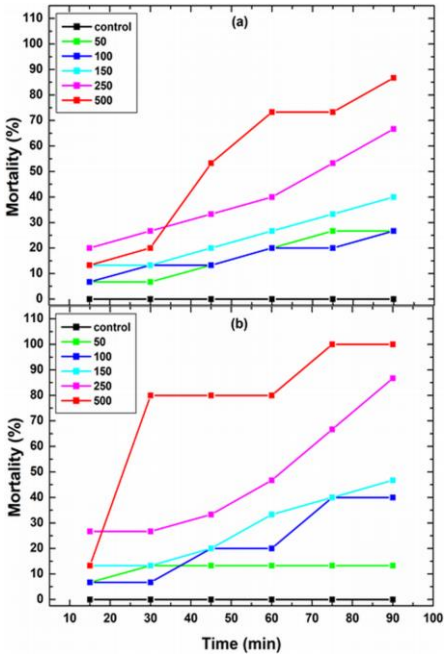

Figure (1): Concentration-mortality curves for the larvae of the Aedes aegypti of essential oils of (a) *P. cytorphodon* and (b) *P. krukoffii*.

The biological test showed strong adulticidal activity against *A. aegypti* females was observed after 90 min of exposure with values of CL50 185.4 ± 0.5 and CL90 363.4 ± 0.5 for *P. krukoffii* and, CL50 218.5 ± 0.4 and CL90 432.9 ± 0.4 for *P. cytorphodon*. In other species of the genus Piper, such as *P. hispidum*, *P. callosum*, *P. marginatum* and *P. divaricatum*, studies showed the effectiveness of their oils as insecticides that can act by contact, synergism, repellent and anti-food (Marques & Kaplan, 2015; Scott et al., 2007). Our results are the first report of larvicidal activity of *P. cytorphodon* and *P. krukoffii* against larvae of A. aegypti.

CONCLUSIONS

As found in this work, the chemical constituents of the essential oils extracted from the leaves of *P. cyrtopodon* and *P. krukoffii* were mainly limonene and selinene, respectively, showing high toxicity for

artemia tests, inhibition for gram-positive bacteria - due to greater susceptibility to the action of essential oils - and, promising biological activities against larvae and mean toxicity to *A. aegypti* mosquitoes, being possible agents for the control of vectors.

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