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ORIGINAL ARTICLE

A study of the larvicidal activity of two *Croton* species from northeastern Brazil against *Aedes aegypti*

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Abstract

The essential oils of *Croton heliotropiifolius* Kunth (Euphorbiaceae) and *Croton pulegiodorus* Baill. were selected for larvicidal evaluation against *Aedes aegypti* L. (Diptera: Culicidae) and studied qualitatively and quantitatively by GC and GC-MS. Sixty-one compounds representing 92.03% (*C. heliotropiifolius*) and 85.68% (*C. pulegiodorus*) of the essential oils, respectively, have been identified. The major components of *C. heliotropiifolius* essential oil were identified as β-caryophyllene (35.82%), bicyclogermacrene (19.98%), and germacrene-D (11.85%). The major components in *C. pulegiodorus* essential oil were identified as β-caryophyllene (20.96%), bicyclogermacrene (16.89%), germacrene-D (10.55%), τ-cadinol (4.56%), and β-copaen-4-α-ol (4.35%). The essential oil of *C. pulegiodorus* (LC_{50} 159 ppm) was more effective against *Ae. aegypt*i than that of *C. heliotropiifolius* (LC_{50} 544 ppm). In order to verify whether the major compound of both essential oils is the active principle responsible for the larvicidal activity, β-caryophyllene was purchased and its larvicidal potential was further evaluated. However, β-caryophyllene (LC_{50} 1038 ppm) showed weak larvicidal potency. Results of larvicidal evaluation suggest the existence of a synergistic effect of minor components in the essential oils.

Keywords: Aedes aegypti*; β-caryophyllene;* Croton *species; bicyclogermacrene; essential oil; larvicidal activity*

Introduction

Aedes aegypti L. (Culicidae) has become a serious public health problem, mainly in tropical and subtropical countries, since it is the vector responsible for the transmission of arboviruses, such as dengue, yellow fever, and dirofilariasis (Forattini, 1998; Serrao et al., 2001).

Since there are no effective treatments for these diseases, the most appropriate way of avoiding virus outbreaks is to manage *Ae. aegypti* propagation by controlling its breeding places. Organophosphates, such as temephos, have been used as larvicides in several countries since the 1960s (Beserra et al., 2007; Kalinga et al., 2007; Kusumawathie et al., 2008; Seccacini et al., 2008). However, resistance to pesticides (Braga et al., 2004) has guided research to find new methods intended to control *Ae. aegypti* propagation (Carvalho et al., 2003). Beserra et al. (2007) evaluated *Ae. aegypti* resistance, monitoring its susceptibility to temephos in the state of Paraiba, Brazil. The results indicated the necessity for changes in strategies for monitoring and controlling the mosquito. Additionally, the synthetic insecticides are toxic and adversely affect the environment by contaminating soil, water, and air (Brown et al., 2000). An alternative conventional chemical control method is the utilization of natural products from plants (Consoli & Oliveira, 1994). The essential oils of plants are outstanding larvicide candidates, since they are readily available in several tropical countries.

Croton (Euphorbiaceae) is an extensive genus composed of about 1200 species (Lima et al., 2008). This genus with a wide range of bioactive compounds has

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been found to exert vasorelaxant activity (Baccelli et al., 2007), and phytochemical studies revealed the presence of proanthocyanidins, alkaloids, and diterpenes (Salatino et al., 2007). Popular uses are found in the treatment of diabetes (Govindarajan et al., 2008), digestive problems (Reyes-Trejo et al., 2008), hypercholesterolemia (Bighetti et al., 2004), intestinal worms, fever, malaria (Noor Rain et al., 2007), and pain (Rao et al., 2007). Species from the genus *Croton* have been evaluated for their larvicidal activities against *Ae. aegypti* (Salatino et al., 2007), including the essential oil of *C. zenhtneri* Pax et Hoffm., which exhibited the highest larvicidal activity with LC_{50} of 28 ppm (Morais et al., 2006).

Croton heliotropiifolius Kunth and *Croton pulegiodorus* Baill. are shrubs locally known as "Velame" and "Velaminho," respectively. Since plants from the *Croton* genus have been found to be active against *Ae. aegypti* (Lima et al., 2006; Morais et al., 2006), the larvicidal activities of the essential oils from two plants belonging to this genus were evaluated by measurement of their LC_{50} and the oils were also studied qualitatively and quantitatively by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS).

Materials and methods

Plant material

C. heliotropiifolius and *C. pulegiodorus* were collected in April and May 2005, respectively, at the flowering stage, from plant populations growing wild in Aracaju county, Sergipe State (10°56'S, 37°05'W) and Santana dos Frades village, Pacatuba county (10°25'S, 36°35'W), northeastern Brazil, respectively. Voucher specimens 08217 and 08218 were identified by Dr. Luciano Queiroz Paganucci as *C. heliotropiifolius* and *C. pulegiodorus*, respectively, and deposited in the Federal University of Sergipe Herbarium (Universidade Federal de Sergipe, CCBS, Departamento de Biologia, São Cristovão, Sergipe, 49100-000, Brazil). Prior to hydrodistillation, leaves were dried at 40°C in a forced air oven (Marconi MA 037) for 48 h and pulverized using a mill.

Essential oil extraction

The dried plant powder was submitted to hydrodistillation in a Clevenger-type apparatus consisting of a 500 mL distillation bottle, a 5 mL graduated receiver, and a jacketed-coil condenser. A total of 100 g of dried plant material and 250 mL of H_2O were used, and the distillation was carried out for 4 h. Condensation of the steam followed by accumulation of the essential oil/water system in the graduated receiver resulted on separation of the essential oil from the water, allowing further manual collection of the organic phase. Traces of water were removed by freezing the sample below 0°C followed by transferring unfrozen essential oil to a new vial to yield yellowish volatile oils of *C. heliotropiifolius* (0.2%) and *C. pulegiodorus* (5%). β-Caryophyllene (86%) was purchased from Sigma-Aldrich.

Analytical conditions

GC-MS

The essential oils obtained by hydrodistillation were analyzed by GC-MS using a Shimadzu QP5050A instrument equipped with a DB-5MS fused silica column (30 m × 0.25 mm; film thickness 0.25 μm), under the following conditions: helium as carrier gas at 1.0 mL/min; injector split at 250°C (split ratio 1/20); detector at 280°C; column temperature program 80°C during 1.5 min, with 4°C increase per min to 180°C, then 10°C/min to 300°C, ending with a 10 min isothermal at 300°C. Mass spectra were taken at 70 eV with a scanning speed of 0.85 scan/s from 40 to 550 Da. Peak identification was assigned on the basis of comparison of their retention indices relative to an *n*-alkane homologous series obtained by co-injecting the oil sample with a linear hydrocarbon mixture.

GC-FID

Quantitative analysis of the chemical constituents was performed by flame ionization gas chromatography (FID), using a Shimadzu GC-17A (Shimadzu Corporation, Kyoto, Japan) instrument, under the following operational conditions: capillary ZB-5MS column (5% phenyl-arylene–95%-dimethylpolysiloxane), fused silica capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness) from Phenomenex (Torrance, CA, USA), under the same conditions as reported for GC-MS. Quantification of each constituent was estimated by area normalization (%). Compound concentrations were calculated from the GC peak areas and they were arranged in order of GC elution.

Identification of essential oil constituents

Identification of individual components of the essential oil was performed by computerized matching of the acquired mass spectra with those stored in the NIST21 and NIST107 mass spectral library of the GC-MS data system. Retention indices (RI) for all compounds were determined according to Van den Dool and Kratz (1963) for each constituent, as previously described (Adams, 1995).

Rearing of Ae. aegypti

Eggs of *Ae. aegypti* were provided by the Federal-Rural University of Pernambuco Insectary, attached to paper strips. The paper strips were placed in a rectangular polyethylene container of natural mineral water (1 L). Rat ration (100 mg) was added to allow larvae development. The container was kept at room temperature (28 ± 1°C) for hatching, feeding, and monitoring of larvae development for about 5 days.

Larvicidal assay

The larvicidal assay was performed as described by Thangam and Kathiresan (1991) with some modifications. Third and fourth instar larvae were used in the experiment. The concentration ranges were determined by a previous concentration–response curve with 20 larvae. Tested oil or β-caryophyllene (20 mg) was added to a 1 mL Eppendorf and dispersed in Tween 80 (0.1 mL). Natural mineral water (0.9 mL) was added to make a standard solution (20,000 ppm). The stock solution was used to make 20 mL water solutions ranging from 50 to 2000 ppm for *C. heliotropiifolius*, 30 to 500 ppm for *C. pulegiodorus*, and 300 to 3000 ppm for β-caryophyllene. Twenty larvae were collected with a Pasteur pipette, placed on a filter paper for removal of water, and transferred (20 per test) with a tiny brush into beakers containing 20 mL of test solution. A mortality count was conducted 24 h after the treatment. Controls were prepared with Tween 80 (0.1 mL) and water (19.9 mL) only. Three replicates were used for each concentration and the control. Positive control with the organophosphate temephos, a commonly used insecticide for larvae control, was used under the same conditions as those used by health programs in Brazil (1 ppm). Probit analysis (Finney & Stevens, 1948) was conducted on mortality data collected after 24 h of exposure to different concentrations of testing solutions to determine the lethal concentration for 50% mortality (LC_{50}) and 95% confidence interval values for the respective species and β-caryophyllene (Table 1). The results were further analyzed using one-way analysis of variance (ANOVA), followed by Tukey test. A significance level of 5% was set for all analyses.

Table 1. Larvicidal activities of tested agents on third and fourth instar larvae of *Ae. aegypti*.

Oil/compound	LC_{50} (95% confidence interval) (ppm)
C. heliotropiifolius	544 (435–668)*
C. pulegiodorus	159 (128–200)*
β-Caryophyllene	1038 (754–1421)*

*$p < 0.05$, one-way ANOVA followed by Tukey.

Results and discussion

The essential oils of *C. heliotropiifolius* and *C. pulegiodorus* were obtained in 0.2 and 5% yield (w/v), respectively. Sixty-one compounds, representing 92.03 and 85.68% of the total oils, have been respectively identified (Table 2). The major components of *C. heliotropiifolius* oil were identified as β-caryophyllene (35.82%), bicyclogermacrene (19.98%), and germacrene-D (11.85%). The monoterpene fraction amounted to 7.58% of the oil, while the sesquiterperne fraction was 84.45%. Major components in *C. pulegiodorus* essential oil were β-caryophyllene (20.96%), bicyclogermacrene (16.89%), germacrene-D (10.55%), τ-cadinol (4.56%), and β-copaen-4-α-ol (4.35%); representing the monoterpenes were α-pinene, sabinene, β-myrcene, δ(3)-carene, *o*-cymene, limonene, β-phellandrene, γ-terpinene, linalool, borneol, terpin-4-ol, bornyl acetate, and 2-undecanone (2.19%); and sesquiterpenes amounted to 83.49%. The essential oil compositions of the plants analyzed herein were different from other *Croton* species previously characterized, in which the main components were identified as methyleugenol (*C. nepetafolius* Baill.) (Lima et al., 2006), *E*-anethole (*C. zehntneri* Pax et Hoffm.) (Santos et al., 2007), α-pinene (*C. argyrophylloides* Mull. Arg.), and β-phellandrene (*C. sonderianus* Mull. Arg.) (Morais et al., 2006).

In searching for new control measures against *Ae. aegypti*, oils of *C. heliotropiifolius* and *C. pulegiodorus* were tested and found to exhibit a larvicidal effect. At higher concentrations, the larvae showed restless movement for some time, then settled at the bottom of the beakers with abnormal wagging, and died. The rate of mortality was directly proportional to the concentration. Test results were acceptable if the mortality in the controls did not exceed 10%. Mortality data of *Ae. aegypti* larvae when treated with essential oils or β-caryophyllene are shown in Table 3. Both oils induced 100% mortality of *Ae. aegypti* larvae after 24 h at 2000 ppm (*C. heliotropiifolius*, LC_{50} 544 ppm) and 500 ppm (*C. pulegiodorus*, LC_{50} 159 ppm), while the positive control, temephos, also exhibited 100% mortality after 24 h. *C. pulegiodorus* was a more potent larvicide.

The major compound of both essential oils, β-caryophyllene, showed significantly weaker larvicidal potency (LC_{50} 1038 ppm), and may not be the principle responsible for the observed larvicidal actions. Additionally, Kiran et al. (2006) reported the toxicity of germacrene-D against *Ae. aegypti* (LC_{50} 63.3 ppm), which may be one of the terpenes responsible for the observed activity. Consequently, minor components are probably acting synergistically to achieve the experimental larvicidal action.

Table 2. Essential oil composition of *C. heliotropiifolius* and *C. pulegiodorus* leaves.

Compound	*C. heliotropiifolius* (%)[a]	*C. pulegiodorus* (%)[a]	RI[b]
α-Pinene	0.22	0.03	935
Sabinene	0.40	0.05	974
β-Myrcene	1.02	0.02	991
α-Phellandrene	0.20	–	1007
δ(3)-Carene	–	0.04	1008
o-Cymene	–	0.03	1009
p-Cymene	0.17	–	1024
Limonene	3.82	0.44	1029
β-Phellandrene	–	0.04	1030
1,8-Cineole	0.28	–	1032
γ-Terpinene	0.50	0.10	1057
Linalool	0.33	0.22	1098
Borneol	–	0.23	1171
Terpin-4-ol	–	0.05	1180
α-Terpineol	–	0.02	1194
Bornyl acetate	0.64	0.90	1285
2-Undecanone	–	0.02	1290
γ-Elemene	–	0.08	1335
α-Cubebene	–	0.03	1346
Cyclosativene	–	0.53	1367
α-Copaene	0.19	0.49	1378
β-Bourbonene	–	0.10	1382
β-Elemene	–	0.77	1388
(*Z*)-Caryophyllene	–	0.05	1403
α-Gurjunene	–	0.12	1406
β-Caryophyllene	**35.82**	**20.96**	1423
β-Gurjunene	–	0.19	1429
Geranyl acetone	–	0.21	1445
α-*neo*-Clovene	–	0.13	1448
β-Farnesene	–	0.22	1451
α-Humulene	3.92	3.95	1456
Allo-aromadendrene	–	0.88	1459
γ-Muurolene	–	0.40	1474
Germacrene-D	**11.85**	**10.55**	1483
Valencene	–	0.36	1491
Bicyclogermacrene	**19.98**	**16.89**	1499
β-Curcumene	–	0.27	1502
Germacrene-A	–	0.89	1506
Cubebol	–	0.77	1513
Sesquicineole	3.85	1.89	1515
cis-Calamenene	–	0.23	1520
δ-Cadinene	1.76	1.55	1525
Cadine-1,4-diene	–	0.10	1531
α-Cadinene	–	0.06	1535
α-Calacorene	–	0.06	1539
β-Germacrene	–	1.79	1557
Germacrene-B	0.45	–	1560
β-Copaen-4-α-ol	–	4.35	1576
Spathulenol	2.12	–	1579
Globulol	–	0.77	1584
Caryophyllene oxide	1.82	1.66	1586
Guaiol	–	1.08	1592
1,10-di-*epi*-Cubenol	–	1.17	1613

Table 2. continues on next page.

Table 2. Continued.

Compound	*C. heliotropiifolius* (%)[a]	*C. pulegiodorus* (%)[a]	RI[b]
10-epi-γ-Eudesmol	–	0.22	1619
1-*epi*-Cubenol	–	0.42	1626
epi-α-Cadinol	–	0.88	1640
epi-α-Muurolol	–	1.15	1642
τ-Cadinol	–	4.56	1645
α-Muurolol	–	2.23	1654
β-Bisabolol	0.90	0.48	1676
α-Bisabolol	1.79	–	1679
Monoterpenes	7.58	2.19	
Sesquiterpenes	84.45	83.49	
Total	92.03	85.68	

[a]Compound percentage.
[b]Relative retention index calculated against *n*-alkanes applying the Van den Dool equation (Van den Dool & Kratz, 1963).

Table 3. Toxicity of *C. heliotropiifolius*, *C. pulegiodorus*, and β-caryophyllene against *Ae. aegypti* larvae.

	Mortality rate ± SE (%)[a]		
Concentration (ppm)	*C. heliotropiifolius*	*C. pulegiodorus*	β-Caryophyllene
30	–	0.0	–
50	0.0	–	–
70	–	6.7 ± 1.66	–
100	–	46.7 ± 1.66	–
300	25.0 ± 5.0	86.7 ± 4.40	1.6 ± 1.66
500	43.3 ± 6.0	100	–
900	–	–	26.6 ± 1.66
1000	76.6 ± 8.33	–	–
1500	–	–	63.3 ± 4.40
2000	100	–	86.6 ± 1.66
3000	–	–	95.0 ± 2.88

[a]Average of triplicate.

Croton species have been reported as larvicidal against *Ae. aegypti* (Lima et al., 2006; Morais et al., 2006), and are locally used as insect repellents. Reports about the larvicidal action of *C. nepetaefolius* (LC_{50} 84 ppm), *C. zehntneri* (LC_{50} 28 ppm), *C. argyrophyloides* (LC_{50} 102 ppm), and *C. sonderianus* (LC_{50} 104 ppm) were found in the literature (Morais et al., 2006). Santos et al. (2007) demonstrated the larvicidal activity of *C. zehntneri* leaves, stalks, and inflorescences, as well as its major compound, *E*-anethole. Similarly, *E*-anethole showed relatively weaker larvicidal potency compared to the oil, and may not have been responsible for the observed larvicidal actions.

Conclusions

This study illustrated the efficacy of two *Croton* species against *Ae. aegypti* larvae. Furthermore, it may represent a contribution to alternative methods of mosquito control. The essential oil of *C. pulegiodorus* exhibited higher larvicidal potential against *Ae. aegypti* larvae than that of *C. heliotropiifolius*, while the major compound, β-caryophyllene, exhibited relatively lower larvicidal potential than the oils. Results indicate the existence of a synergistic effect of the minor components in the essential oils.

Declaration of interest

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