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Effects of essential oils on *Aedes aegypti* larvae: Alternatives to environmentally safe insecticides

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Abstract

The essential oils from leaves of *Hyptis fruticosa* (Lamiaceae) Salzm., *H. pectinata* (Lamiaceae) Poit., and *Lippia gracilis* (Verbenaceae) HBK were investigated for their larvicidal activity against *Aedes aegypti* and analyzed by GC/MS. Fifty-nine compounds, representing 91.28–98.39% of the essential oils, have been identified. A standard solution was used to make 20 mL solutions ranging from 30 to 2000 ppm. Twenty larvae between third and fourth stages were added to the essential oil solution. A mortality count was conducted 24 h after treatment. Essential oils LC_{50} and their confidence limits at 95% probability were calculated by the methods of Reed-Muench and Pizzi, respectively. The essential oil of *Lippia gracilis* showed potent insecticidal effect against *Aedes aegypti* larvae, the vector of dengue fever. Carvacrol and caryophyllene oxide were the main responsible for the activity of *L. gracilis* and *H. pectinata*. Minor compounds are probably acting synergistically to achieve *H. fruticosa* activity.

Keywords: *Hyptis fruticosa*; *Lippia gracilis*; *Hyptis pectinata*; Larvicidal; Carvacrol

1. Introduction

Within several types of mosquitoes living in tropical and sub-tropical regions, *Aedes aegypti* is the vector known to carry yellow and dengue fever, diseases responsible for a number of morbidity and mortality around the world, due to its severe symptoms (Gubler, 1989).

Although several million dollars are spent attempting to eradicate the vector, no success has been achieved in many parts of the world.

Aedes aegypti is a domiciliary mosquito, which hides in dark and closed places, aggravating its eradication (Gubler, 1989). However, the only efficient way to control dengue resides in controlling *Aedes aegypti* population. Therefore, the ideal method for controlling mosquito infestation is the prevention of mosquito breeding through the use of larvicides. Many synthetic insecticides have been used along the past years. Organophosphates, such as, temephos, have been used as larvicide in several countries since the 1960s (Braga et al., 2004; Gubler, 1989; Romi et al., 2003). However, resistance to pesticides (Macoris et al., 2003; Rodriguez et al., 2002; Wirth and Georghiou, 1999) has guided research to find new methods intended to control *Aedes aegypti*. Additionally, the synthetic insecticides are toxic and adversely affect the environment by contaminating soil, water and air.

The necessity for continued research has been even more apparent in the late years, aiming to find new methods to control the vector and reduce the incidence of dengue. Recent research has focused on natural product alternatives

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for pest control in developing countries (Dharmagadda et al., 2005). The demand for natural and nonpersistent insecticides is gradually increasing. Pyrethrins, the most economically important natural insecticide, comprise a group of six closely related monoterpene esters. The industrial production is based on their extraction from *Chrysanthemum cinerariaefolium*, the first natural product to be used against adult mosquitoes (Edwards, 1948; Godin et al., 1966). The world production of natural pyrethrins still falls short of global market demand (Hitmi et al., 2000). Plant-based insecticides appear to have no ill effect on non-target populations and are biodegradable, in addition to being locally available in many parts of the world most affected by mosquito borne diseases. They act by interfering with the growth and reproduction of the pest and are effective against different stages of their growth (Sukumar et al., 1991).

The search for efficient natural larvicide or pesticide substances with low environmental toxicity has increased (Chantraine et al., 1998; Kabir et al., 2003; Silva et al., 2004). Plants essential oils are outstanding candidates, since they are, in some cases, highly active, readily available in tropical countries, and economically viable. Studies of the essential oil larvicidal activity have been published in the literature. Plants, such as, *Lippia sidoides* (Carvalho et al., 2003), *Atlantia monophylla* (Sivagnaname and Kalyanasundaram, 2004), and *Hyptis martiusii* (Araujo et al., 2003) have been tested and are potent larvicides.

Since the genera *Hyptis* and *Lippia* have been found to be active against *Aedes aegypti*, the larvicidal activities of the essential oils from three plants belonging to these genera were analyzed by measurement of their LC_{50}.

2. Methodology

2.1. Plant material

Hyptis fruticosa (Lamiaceae) Salzm. and *H. pectinata* (Lamiaceae) Poit. leaves were collected in November 2002, at the flowering stage, from plant populations growing wild in Feijão village, São Cristóvão county, Sergipe State, northeastern Brazil (10°56′S, 37°05′W). *Lippia gracilis* (Verbenaceae) HBK was cultivated in the Research farm of the Federal University of Sergipe, Department of Agronomical Engineering, São Cristóvão, Brazil. Voucher specimens (08216, 08215, and 08214, respectively) have been deposited in the Federal University of Sergipe Herbarium, Universidade Federal de Sergipe, CCBS, Departamento de Biologia, São Cristovão, Sergipe, 49100-000, Brazil. Prior to water distillation, leaves were dried at 40 °C in a forced air oven (Marconi MA 037) for 48 h and pulverized using a mill.

2.2. Terpenes

Thymol, 1,8-cineole, γ-terpinene, carvacrol, β-caryophyllene, caryophyllene oxide, and *R*-limonene were purchased from Sigma–Aldrich.

2.3. Essential oil extraction

The dried plant powder was submitted to water distillation for 4 h in a Clevenger-type apparatus to yield a yellowish oil. The essential oils obtained were separated from the aqueous phase and kept in freezer until further analysis.

2.4. Analytical conditions

The essential oils obtained by water distillation were analyzed by GC/MS using a Shimadzu QP5050A 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. The mass spectra were taken at 70 eV with scanning speed of 0.85 scan/s from 40 to 550 Da. Percentage composition was calculated using peak normalization method. Peak identification was assigned on basis of comparison of their retention indices (Aguiar et al., 2003) relative to a *n*-alkane homologous series obtained by co-injecting the oil sample with a linear hydrocarbon mixture. Identification of individual components of the essential oil was performed by computerized matching of the acquired mass spectra with those stored in NIST21 and NIST107 mass spectral library of the GC/MS data system, as well as by visual inspection of published spectral data (Adams, 1995).

2.5. Aedes aegypti hatching

Eggs of *A. aegypti* were provided by the *Federal Rural University of Pernambuco*, (Insetário do Laboratório de Doenças Parasitárias, Departamento de Medicina Veterinária, Av. Dom Manoel de Medeiros S/N, Dois Irmãos Recife, Pernambuco, 52.171-900, Brazil), in dehydrated form, attached to paper strips. The paper strips were placed in a rectangular polyethylene container containing natural mineral water. Rat ration (100 mg) was added to allow larvae development. The container was kept at room temperature for hatching and monitoring of larvae development for about five days.

2.6. Larvicidal activity

The larvae between third and fourth stages were used in the experiment. Testing oil or terpene (100 mg) was placed in a 10 mL beaker and dispersed in tween-80 (0.25 mL). Natural mineral water (4.75 mL) was added to make a standard solution (20.000 ppm). The standard solution was used to make 20 mL solutions ranging from 30 to 2000 ppm. Twenty larvae were added to the essential oil solution. A mortality count was conducted 24 h after treatment. A control solution using tween-80 (0.1 mL) and water (19.9 mL) did not show larvicidal activity. Five

replicates, with 20 larvae in each, were taken for each solution and the control. Positive control with the organophosphorate Temephos (*O*,*O*′-(thio-di-4,1-phenylene)bis(*O*,*O*-dimethylphosphorothioate)), a commonly used insecticide for larvae control, was used under the same conditions as used by health programs in Brazil (100 ppm). LC_{50} and their confidence limits at 95% probability were calculated by the methods of Reed and Muench (1938) and Pizzi (1950), respectively.

3. Results and discussion

The essential oils of *H. fruticosa*, *H. pectinata*, and *L. gracilis* were obtained in 2.0%, 0.5%, and 2.8% yield, respectively. Fifty-nine compounds, representing 91.28–98.39% of the essential oils have been identified; their retention indices and percentage composition, listed in order of elution in the DB-5MS column, are given in Table 1.

The major components of *H. fruticosa* essential oil were identified as 1,8-cineole (15.79%), spathulenol (10.23%), β-caryophyllene (9.79%), bicyclogermacrene (8.57%), and camphor (8.12%). Other minor constituents were found to be caryophyllene oxide (5.21%), *cis*-calamenene (3.86%), and α-pinene (2.99%). The monoterpene fraction amounts 36.58% of the oil, while the sesquiterperne fraction amounts 54.70%.

Interestingly, unlike other studies, (Malan et al., 1988; Pietschmann et al., 1998) only sesquiterpenes were found in the *H. pectinata* essential oil. Two major compounds (β-caryophyllene and caryophyllene oxide) accounted for 78.95% of its composition.

The major components in *L. gracilis* essential oil were identified as carvacrol (44.43%), *o*-cymene (9.42%), γ-terpinene (9.16%), and β-caryophyllene (8.83%), which accounted for 18.14% of sesquiterpenes and 80.25% of monoterpenes.

In searching for new forms to control *A. aegypti* propagation, the essential oil of *H. fruticosa*, *H. pectinata*, and *L. gracilis* were tested and exhibited larvicidal effect compared to other plants essential oils (Chantraine et al., 1998). At higher essential oil concentrations, the larvae showed restless movement for some time and then settled at the bottom of the beakers with abnormal wagging and died slowly. The rate of mortality was directly proportional to the concentration.

Results on mortality of larvae of *A. aegypti* with increase in oil concentration are shown in Table 2. The volatile constituents of leaves induced 100% mortality of *A. aegypti* larvae after 24 h at 2000 mg/L (*H. fruticosa*), 1000 mg/L (*H. pectinata*), and 300 mg/L (*L. gracilis*). Positive control Temephos exhibited 100% mortality after 24 h. Both, *H. fruticosa* and *H. pectinata* exhibited LC_{50} of 502 ± 2.70 ppm and 366 ± 2.56 ppm, respectively.

Among all the three plant species tested, *L. gracilis* was the most potent larvicidal (LC_{50} 98 ± 1.99 ppm).

Selected compounds of the essential oils were purchased and their larvicidal potential was further evaluated (Table

Table 1
Essential oil composition from the leaves of *Hyptis fruticosa*, *H. pectinata*, and *Lippia gracilis* characterized by GC/MS

RI	Compound	*H. fruticosa* (%)	*H. pectinata* (%)	*L. gracilis* (%)
923	Tricyclene	–	–	0.82
935	α-Pinene	2.99	–	0.21
947	α-Fenchene	–	–	0.06
951	Camphene	0.71	–	–
976	Sabinene	0.39	–	–
977	1-Octen-3-ol	–	–	0.03
980	β-Pinene	2.39	–	–
989	β-Myrcene	0.30	–	1.67
1005	α-Phelandrene	–	–	0.11
1007	Isosylvestrene	–	–	0.06
1015	δ(3)-Carene	–	–	1.66
1023	*o*-Cymene	–	–	9.42
1024	*p*-Cymene	0.55	–	–
1030	Limonene	1.51	–	0.24
1033	1,8-Cineole	15.79	–	0.96
1045	(*Z*)-β-Ocimene	–	–	0.07
1057	γ-Terpinene	0.29	–	9.16
1090	*p*-Cymenene	0.30	–	–
1099	Linalool	0.52	–	0.41
1140	Ipsdienol	–	–	0.28
1146	Camphor	8.12	–	0.21
1167	Borneol	2.34	–	–
1179	Terpin-4-ol	–	–	0.59
1192	α-Terpineol	0.38	–	–
1228	2-Isopropyl-5-methylanisole	–	–	5.85
1237	5-Isopropyl-2-methylanisole	–	–	0.18
1352	α-Cubebene	1.67	–	–
1379	α-Copaene	2.71	3.62	–
1288	Thymol	–	–	3.83
1297	Carvacrol	–	–	44.43
1393	β-Cubebene	0.67	–	–
1393	β-Elemene	–	1.87	–
1422	β-Caryophyllene	9.79	40.90	8.83
1431	α-*trans*-Bergamotene	–	–	0.77
1437	Aromadendrene	–	–	1.17
1445	α-Himachalene	–	–	0.09
1457	α-Humulene	1.81	1.12	1.30
1466	*cis*-Muurola-4(14),5-diene	0.52	–	–
1479	γ-Muurolene	0.43	–	–
1484	Germacrene-D	2.31	–	–
1487	β-Selinene	–	–	0.20
1489	Viridifloreno	–	–	1.49
1495	Epizonarene	0.45	–	–
1498	α-Selinene	–	1.21	–
1500	Bicyclogermacrene	8.57	–	2.88
1505	β-Bisabolene	–	–	0.35
1517	γ-Cadinene	0.58	–	0.10
1524	*cis*-Calamenene	3.86	4.16	–
1535	Cadine-1,4-diene	0.47	–	–
1540	α-Cadinene	2.04	–	–
1581	Spathulenol	10.23	–	0.61
1585	Globulol	–	–	0.08
1587	Caryophyllene oxide	5.21	38.05	0.27
1591	β-Copaen-4-α-ol	0.45	–	–
1634	γ-Eudesmol	0.35	–	–
1652	α-Eudesmol	0.81	–	–
1656	α-Cadinol	1.07	–	–
1672	α-Kusinol	0.70	–	–

(*continued on next page*)

Table 1 (*continued*)

RI	Compound	*H. fruticosa* (%)	*H. pectinata* (%)	*L. gracilis* (%)
Monoterpenes		36.58	0.00	80.25
Sesquiterpenes		54.70	90.93	18.14
Total		91.28	90.93	98.39

RI: Relative retention index calculated against *n*-alkanes applying the Van den Dool equation.
%: Compound percentage.

Table 2
Larvicidal activities (LC_{50}) of the essential oil of *Hyptis fruticosa*, *Hyptis pectinata*, *Lippia gracillis*, and their main constituents

Essential oil/ terpene	$LC_{50} \pm CL$ (ppm)
Hyptis fruticosa	502 ± 2.70
Hyptis pectinata	366 ± 2.56
Lippia gracillis	98 ± 1.99
Thymol	79 ± 2.20
1,8-Cineole	1381 ± 2.10
γ-Terpinene	95 ± 2.10
Carvacrol	70 ± 2.10
β-Caryophyllene	1202 ± 2.00
Caryophyllene oxide	125 ± 2.05
R-limonene	37 ± 2.08

LC_{50} = Lethal concentration (ppm) at which 50% of the larvae showed mortality.
CL = confidence limits at 95% probability.

2). The major compound in the essential oil of *L. gracilis*, carvacrol, exhibited LC_{50} of 70 ± 2.10 ppm. Thus, carvacrol is probably the active principle responsible for *L. gracilis* larvicidal action, causing 100% larval mortality at 150 ppm. Regardless of the fact that thymol and γ-terpinene are found in the essential oil of *L. gracilis* in small amounts (3.83% and 9.16%, respectively), their larvicidal activities suggest the existence of synergistic activity in the essential oil.

Similarly, Carvalho et al. (2003) found that the essential oil of *Lippia sidoides* was toxic against *A. aegypti* larvae. The predominance of thymol in *L. sidoides* essential oil, with concentrations ranging from 50.57% to 22.37% (Sousa et al., 2002), was believed to be determinant for its activity.

Although thymol was present in the essential oil of *L. gracilis*, its proportion was considerably lower than *L. sidoides* (3.83%). However, the most abundant compound in the essential oil of *L. gracilis*, carvacrol, was found to be active against our strain of *A. aegypti*.

Although β-caryophyllene (LC_{50} 1202 ± 2.00 ppm) is the major compound in the essential oil of *H. pectinata*, caryophyllene oxide exhibited higher larvicidal activity (LC_{50} 125 ± 2.05 ppm). The later is most likely the major responsible for *H. pectinata* essential oil activity.

The larvicidal bioassays showed 1,8-cineole is not the active principle of the essential oil of *H. fruticosa*, since the results of bioassays with this component revealed CL_{50} of 1381 ± 2.10. However, the synergistical action of other minor constituents, such as caryophyllene oxide, *R*-limonene (LC_{50} 37 ± 2.08 ppm), and γ-terpinene (LC_{50} 95 ± 2.10 ppm) cannot be disregarded.

4. Conclusions

The larvae of *A. aegypti* are susceptible to the composition of the essential oil herein evaluated, particularly to the essential oil of *L. gracilis*.

The use of natural products may be considered as an important alternative insecticide for the control of *A. aegypti* larvae, since they constitute a rich source of bioactive compounds that are biodegradable, nontoxic, and potentially suitable for use in integrated larvae management programs. However, the cost of the essential oil may also be an important factor for its implementation, which depends on the availability of the plant and its yield/ha. Such studies are currently being conducted by our research group. In accordance with the present conclusions, *L. gracilis* essential oil may be used as an ecologically safe alternative larvicide. Carvacrol, the major compound in the essential oil of *L. gracilis*, was found to be the active principle responsible for the larvicidal action.

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