Larvicidal activity of affinin and its derived amides from *Heliopsis longipes* A. Gray Blake against *Anopheles albimanus* and *Aedes aegypti*

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**A B S T R A C T**

*Heliopsis longipes* has been recognized as a potential source of insecticidal compounds called alkamides, which can be used to control populations of insect vector transmitters of several diseases that affect the human health. To determine the insecticidal activity of *H. longipes* against *Anopheles albimanus* and *Aedes aegypti*, crude extract of *H. longipes* roots and affinin were obtained. Catalytic reduction of affinin was performed to obtain two reduced amides, N-isobutil-2E-decanamide and N-isobutil-decanamide. Crude extract, affinin and its reduced amides were evaluated against third instar larvae of *An. albimanus* and *Ae. aegypti*. Results show that crude extract of *H. longipes* possess larvicidal activity against larval stage of *An. albimanus* and *Ae. aegypti*. This effect could be attributed to affinin, on which the conjugated double bonds present in the structure of the molecule are necessary to maintain larvicidal activity. This study demonstrated the potential of *H. longipes* to control larval stage of *An. albimanus* and *Ae. aegypti*, transmitter vectors of malaria and dengue fever, respectively.

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**Introduction**

Mosquitoes are the most important agents in the transmission of several diseases such as encephalitis, dengue fever, filariasis and malaria which compromise the human health (Loumibos, 2002). There are approximately 3500 species of mosquitoes that belong to Culicidae family, among which find the genera *Anopheles* and *Aedes*, act as vectors of important illness worldwide (Reinert, 2001). *Anopheles* includes species that can transmit the four malaria-causing parasites, being *Plasmodium falciparum* the causal agent of the most aggressive malaria form (Bannister and Mitchell, 2003). *Aedes* comprises the species responsible for the transmission of arboviruses which causes both dengue and dengue hemorrhagic fever (San Martin et al., 2010). Malaria and dengue fever cause high levels of morbidity and mortality in poor countries, mainly in tropical regions of Africa, Asia and Latin America, where they inflict great economic losses and social disruption.

Strategies for reducing diseases-transmitted by vectors are focused mainly in preventive measures that control the development of insects. These involve the applications of chemical compounds such as organophosphates, including temephos, fenthion and insect growth regulators in their breeding sites (Roberts and Andre, 1994; San Martin et al., 2010). The mosquito larvae are the main developmental stage selected for the vectors control, as this strategy avoids the dispersion of adult mosquitoes and the diseases spread. Despite their effectiveness, these approaches often cause undesirable side effects that include environmental pollution and development of insecticide resistance (San Martin et al., 2010). This is now a major problem facing vector control programs in most countries where *Anopheles albimanus* and *Aedes aegypti* are showing resistance to one or more of the insecticide classes used in vector control (Cáceres et al., 2011; Lima et al., 2011; Marcombe et al., 2012). For this reason, the development of new strategies for selective mosquito larval control is necessary. Plants are an excellent alternative to search insecticides more efficient, cost effectiveness and eco-friendly. This has stimulated the investigation about natural insecticides as an alternative of control, focused on plant-derived compounds as potentially bioactive substances, some of which have showed to act as insecticides against larval stages and adult mosquitoes (Park et al., 2002; Maharaj et al., 2011).

*Heliopsis longipes* A. Gray Blake is a plant endemic to Central México, whose roots were described as a source of insecticide amide, the affinin (PubMed CID: 6433895). Affinin is the main alkamide present in the *H. longipes* roots and considered responsible of the biological effects...
observed in this plant (Molina-Torres et al., 2004). Crude extracts of *H. longipes* roots exert paralyzing action and toxic effects against houseflies, codling moth larvae, and several leaf-eating insects (Acree et al., 1945; Jacobson, 1971). In respect to insecticidal properties against mosquitoes, it has been demonstrated that a sprayed petroleum ether extract of the *H. longipes* roots cause mortality on adult mosquitoes *Ae. aegypti* in 24 h (Jacobson, 1971). However, it has been reported that the efficiency of insecticidal activity of an extract varies depending on the development stage of the insect (Kalaivani et al., 2012). In this sense, little is known about the larvicidal activity of *H. longipes* and the affinin against the larval stage of mosquitoes: *Ae. aegypti* and *An. albimanus*. Only, the alkamides isolated from *Piper nigrum* fruits have been demonstrated to present insecticidal activity against the third instar larvae of three mosquito species; *Culmens pipens pallens, Ae. aegypti* and *Aedes togoi* (Park et al., 2002). The aim of this study was to evaluate the insecticidal effect of *H. longipes* against two mosquito species, *An. albimanus* and *Ae. aegypti*, in its larval stage, as possible control measure to prevent the incidence of vector-borne diseases such as malaria and dengue.

### Material and methods

**Plant material and extraction**

*H. longipes* A. Gray Blake (Asteraceae) specimens were collected in Puerto de Tablas Xichú, Sierra Gorda in Guanajuato State, Central México. The plant material was authenticated by Jerzy Rzedowsky, and a voucher *H. longipes* JMT-IED was deposited at the Ecology Institute Pátzcuaro Michoacán. Dry roots of *H. longipes* (1 kg) were ground and extracted in 10 L of ethanol at room temperature during one week to obtain the alcoholic extract, which was concentrated to dryness in a rotatory evaporator (Büchi-461). Dry residue (5.4% +/- 0.4 sdv dwt) was dissolved in ethanol to obtain a concentration of 10 g/L quantified on the basis of the major alkamide present in root extract. Extract was stored at 4 °C until needed for use.

**Chromatography separation**

Ethanolic extract was fractionated in an open glass column packed with silica gel mesh 200 (J.T. Baker, Paris, KY). Fractions were eluted with hexane and increasing polarity mixtures of hexane:ethyl acetate with silica gel mesh 200 (J.T. Baker, Paris, KY). Fractions were collected, evaporated and dissolved in 1 mL of ethanol. In order to determine profile compounds of each fractions, the samples were analyzed in a gas chromatograph (Hewlett-Packard GC System model 6890) equipped with a capillary column HP-1MS (30 m × 0.25 mm 0.25 µm) coupled to a quadrupole mass selective detector with electron impact ionization (EI/MSD Hewlett-Packard, model 5973 MSD). The operating conditions were as follows: the injector temperature was maintained at 200 °C. The oven temperature program was: an initial temperature of 150 °C maintained for 3 min, then increased at the rate of 4 °C per min to a final temperature of 300 °C which was maintained for 20 min. Helium was used as the carrier gas, with a constant flow of 1 ml/min. One microliter of the sample was injected with a split ratio of 50:1.

From the glass packed column, fractions eluted with the system 70:30 (v/v) hexane:ethyl acetate showed the highest content of affinin. Those fractions were all mixed and purified by thin layer chromatography (TLC) on 20 cm × 20 cm glass plates covered with a 0.5 mm thick layer of silica (Silica Gel 60 G Merck). The plates were developed with a solvent system consisting of hexane:ethyl acetate (2:1 v/v) during 45 min. The plates were air dried, sprayed with a 0.02% fluorescein ethanolic solution and were observed under ultraviolet light. Affinin (Fig. 1A) was spotted as a dark band on a fluorescent background with Rf = 0.5. This band was scraped and eluted from the silica with ethyl acetate. Solvent was evaporated and the residue resuspended in ethanol to be analyzed by CG/EIMS. The resulting GC chromatogram displayed a peak with retention time (Rt) of 12.03 min; and retention index (RI) of 1,000 in CG. MS: m/z = 221 (10), 192 (4), 141 (100), 126 (39), 98 (26), 81 (94), 68 (14), and 53 (12). At this stage of purification, affinin comprised 99.6% (w/v) of the total extract and methyl affinin (N-2-methylbutyl 2E, 6Z, 8E-decatenamide) comprised the remaining 0.4% (w/v) of the extract. No other compounds were detected in the affinin fraction by CG-MS analysis.

**Catalytic reduction of affinin: obtaining N-isobutil-2E-decenamide and N-isobutil-decanamide**

A catalytic reduction of affinin was performed: briefly ten milligrams of platinum oxide was added to 10 mg of affinin resuspended in 2 mL of ethanol. A hydrogen gas stream was bubbled through the mixture maintained at 80 °C. After 3 min of reduction, the N-isobutil-2E-decanamide (Fig. 1B) was collected as major component and was purified by preparative argented TLC using hexane:ethyl acetate (2:1 v/v) as a solvent system. The N-isobutil-2E-decanamide was detected as a fluorescent band on a dark background with Rf = 0.7. The TLC was repeated three times and the fraction containing the intermediary was submitted to GC/EIMS detection, giving only one peak. The N-isobutil-decanamide (Fig. 1C) was obtained after a 7 min reduction as the only product (Molina-Torres et al., 2004). The platinum oxide was centrifuged at 14,000 rpm, and the supernatant was decanted, evaporated to dryness, dissolved in ethanol and submitted to GC/EIMS detection, giving only one peak. Compounds were identified as N-isobutil-2E-decanamide and N-isobutil-decanamide by their mass spectra. The partially reduced amide N-isobutil-2E-decanamide: Rt = 11.46 min; and Rf = 0.953 in CG. UV (MeOH): λmax 211 nm. MS: m/z (%) 225 (15.6), 210 (12.5), 170 (27.7), 153 (100), 126 (21.3), and 55 (26.4). Totally reduced amide N-isobutil-decanamide: Rt = 10.01 min; and Rf = 0.832 in CG. MS: m/z (%) 227 (6), 172 (30.1), 155 (31.1), 128 (25), 115 (100), and 57 (24.6). The spectroscopic data for the reduced alkamides for 1H NMR (200 MHz) and 13C NMR were shown in Table 1.
**Table 1**

<table>
<thead>
<tr>
<th>N-isobutyl-2E-decanamide</th>
<th>N-isobutyl-decanamide</th>
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<tr>
<td>( \delta_c ) (mg/L)</td>
<td>( \delta_h ) (mg/L)</td>
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<tr>
<td>4′</td>
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*Recorded in CDCl3; TMS was used as the internal standard. Coupling constants (J) are in parentheses and are stated in Hz.

Determination of insecticidal activity

**Insects**

Dr. Humberto Lanz-Mendoza of the National Institute of Public Health, Cuernavaca Morelos, Mexico, provided *An. albimanus* eggs. Furthermore *Ae. aegypti* eggs were kindly donated by Dr. Jorge E. Ibarra of Cinvestav Unidad Irapuato. The eggs were immersed in chlorine-free water to benefit emergence of larvae, which were maintained at 28 ± 2 °C, RH > 75% and 12-h light:12-h dark cycle, without exposure to any insecticides.

**Larvicidal assay**

To evaluate toxicity, the ethanolic extract, affin and its derived molecules were dissolved in chlorine-free water with Triton X-100 (0.01%) to obtain several concentrations of each test compound, ranging from 0 to 50 mg/L. Ten larvae, either *An. albimanus* or *Ae. aegypti*, were put into plastic cups containing 50 mL of each test solution with the corresponding compound dosage. Positive controls received Thermephos®, while negative controls received ethanol. Treated and control larvae were incubated under the same conditions used for the colony maintenance. Larvicidal activity was evaluated 48 h after treatment. The larvae were considered dead if appendages did not move when prodded with a plastic pipette. All treatments were replicated three times. Data analysis was performed using STATISTICA 8 software. The percentages of larval mortality were determined and transformed to arc sine square-root values for analysis of variance (ANOVA). Differences between the treatments were determined by Tukey’s multiple range test (\( P = 0.05 \)). Means (±SD) of untransformed data are reported. The lethal concentrations, LC50 values were calculated by Probit Analysis.

**Results and discussions**

**Ethanolic extract of *H. longipes* roots shows larvicidal activity against *An. albimanus* and *Ae. aegypti***

To determine whether *H. longipes* possess larvicidal effect against *An. albimanus* and *Ae. aegypti*, third instar larvae of each mosquito species were exposed to increasing concentrations of ethanolic extract of *H. longipes* roots during 48 h. The results showed that ethanolic extract causes larval mortality, whose effect is dependent of extract concentration. Concentrations from 7 mg/L of ethanolic extract of *H. longipes* roots cause approximately 100% of larval mortality for *An. albimanus*, and the same effect on *Ae. aegypti* larvae. The data analysis showed a lethal concentration LC50 of 2.48 mg/L and 4.07 mg/L to *An. albimanus* and *Ae. aegypti*, respectively. These results indicate that larvicidal activity of the extract differs according to the mosquito species tested, in where *An. albimanus* appears to be the more sensitive since less concentration was required to the LC50.

The biocide effect of crude extract of *H. longipes* roots has been demonstrated against insects of agronomic importance as well as houseflies; causing toxicity and paralyzing effects (Acree et al., 1945; Jacobson, 1971). On the other hand, it was observed that concentrations of 100 mg/L of crude extract of *P. nigrum* fruits are required to cause 100% of mortality on third instar larvae of *Ae. aegypti* at 48 h, a concentration approximately ten-fold to those required in our work to obtain the same result (Park et al., 2002). This suggests the significant effectiveness of ethanolic extract of *H. longipes* roots to the control of, at least, this vector.

Larvicidal activity observed with the crude extract of *H. longipes* roots can be attributed to affin, the main alkalide present in the roots and responsible for the biological effects observed in this plant (Jacobson, 1971; Molina-Torres et al., 2004). Ethanolic extract from *H. longipes* roots contain nearly of 80% of affin and other minor alkalides that exert synergic insecticidal effects (Molina-Torres et al., 2004). Analysis by GC/EIMS demonstrated this composition in tested extract (data not shown).

**Role of affin in larvicidal activity**

To determine the contribution of affin in the insecticidal activity observed in the ethanolic extract; the molecule was isolated, purified and tested against the larvae of both mosquitoes. The results showed that affin possess biocide effects against *An. albimanus* and *Ae. aegypti*, causing 100% of larval mortality at 10 mg/L and 14 mg/L concentrations, respectively. These results show again that larvicidal activity is dependent of mosquito species evaluated and dependent on the manner of concentration. However unlike those obtained with crude extract, higher concentrations of affin were required to cause 100% mortality. This could be due to the synergistic effect of minority alkalides present in the crude extract, whose presence was confirmed by GC/EIMS (data not shown). Moreover the results show that *Ae. aegypti* is less susceptible to affin in comparison to *An. albimanus*. The LC50 data were estimated at 4.24 mg/L and 7.38 mg/L for *An. albimanus* and *Ae. aegypti*, respectively, showing again higher susceptibility of *An. albimanus* to the test compound (Table 2).

**Relationship of the affin structure activity to larvicidal effect**

Affin is a molecule that has an aliphatic chain of 10 carbon atoms bound to amide moiety, whose structure has three conjugated double bonds at positions 2E, 6Z and 8E, which has been suggested, plays an important role in the biological activities (Greger, 1984). To determine the importance of the conjugated double bonds; 2E, 6Z and 8E; on larvicidal activity of the affin and establish a relationship structure-activity, the molecule was modified by catalytic hydrogenation to obtain the partially reduced amide (N-isobutyl-2E-decanamide) and totally reduced amide (N-isobutyl-decanamide). The results show that partially reduced amide, N-isobutyl-2E-decanamide, show larvicidal activity against *An. albimanus* with a LC50 of 7.47 mg/L and 100% of larval mortality at 16 mg/L; while the totally reduced amide, N-isobutyl-decanamide, show the same effect at LC50 of 18.33 mg/L and 100% of larval mortality at 30 mg/L (Figs. 2B, C). Comparison of LC50 data and relative toxicity based on affin activity indicated that N-isobutyl-2E-decanamide exerted 60% of the activity obtained with affin, while totally reduced amide N-isobutyl-decanamide retained only 23% of the activity (Table 2). Despite that both derived amides exhibited larvicidal activity against *An. albimanus*, this activity diminished in relation to the lack of unsaturated bonds. The order of larvicidal activity was: N-isobutyl 2E, 6Z, 8E-decatrienamide > N-isobutyl-2E-decanamide > N-isobutyl-decanamide (Fig. 2A, B, C). These results suggest that the conjugation of the carbonyl moiety to the double bond in position 2E, is sufficient...
to exert larvicidal activity. There are no other reports on the unsaturated alkamides biocide activity, but the nematicidal activity of aliphatic compounds from C8 to C14 were: C9 to C11 alkanols; C8 and C9 \( \varepsilon \)-alkenals; C10 and C11 \( \varepsilon \)-alkenols; and the C9 and C10 alkanoic acids showed the higher nematicidal activity when tested against pine wood nematode \textit{Bursaphelenchus xylophilus} as reported by Seo et al. (2010). However, alkamides contain alpha unsaturation to the carbonyl group, in the case of affinin, a C10 alkamide, the conjugation with the...
unsaturation in either positions 6Z, 8E, or both are necessary to maintain the highest larvicidal activity against An. albimanus, suggesting that they play an important role for biological activity.

Regarding larvicidal activity of reduced amides against Ae. aegypti, the results indicated that only the partially reduced amide, N-isobutyl-2E-decanamide, shows moderate effect with a \( LC_{50} \) of 36.97 mg/L and 93% larval mortality at 50 mg/L (Fig. 2E). While that N-isobutyl-decanamide did not show larvicidal activity at any of the tested concentrations. The data indicated that partially reduced amide; N-isobutyl-2E-decanamide conserved only 20% of afnin activity, which suggests the importance of 2E unsaturation on larvicidal activity of afnin; and the requirement of the unsaturated bonds in positions 6Z and 8E to maintain the optimum larvicidal activity (Table 2). This is in agreement with the fact that totally reduced amide did not show activity, confirming the importance of the double bonds 2E, 6Z, 8E to observe the highest larvicidal activity against Ae. aegypti.

The importance of double bonds on the afnin biocidal activity has been tested only against bacteria and fungi showing that their role varies depending on the organism evaluated (Molina-Torres et al., 2004). Antimicrobial activity of the reduced amides N-isobutyl-2E-decanamide and N-isobutyl-decanamide against bacteria and fungi was demonstrated, showing that only partially reduced amide (unsaturated 2E) increases ten-fold the bactericide activity against Escherichia coli, Erwinia carotovora and Bacillus subtilis, while the totally reduced amide, N-isobutyl-decanamide, displayed a bacteriostatic activity against B. subtilis but was not inhibitory against E. coli and E. carotovora (Molina-Torres et al., 2004).

On the other hand, when these reduced amides were tested against fungi, they did not show fungicidal activity, suggesting that the three 2E, 6Z, and 8E unsaturated bonds are required for this activity (Molina-Torres et al., 2004).

However the hormone like activity of afnin on plant roots development has been studied (Ramirez-Chavez et al., 2004) where in vitro Arabidopsis thaliana displays an enhanced primary root growth and root hair elongation in the presence of afnin at low concentration (7 × 10^{-6} M), but the homologous N-isobutyl-decanamide displays hair root elongation at lower concentrations but lesser primary root elongation.

In general, the results of structure-activity of afnin show that the 2E unsaturated bond in the partially reduced amide is sufficient to exert larvicidal activity, unlike to other studies on nematodes where bioactive aliphatic C10 alkanols and alkanals and their homologues with double bond at 2E position showed similar nematicidal activity (Seo et al., 2010). Nevertheless in afnin the double bonds at positions 6Z, and 8E are necessary to maintain the optimum larvicidal activity against both mosquitoes An. albimanus and Ae. aegypti.

Conclusions

This work demonstrates that H. longipes could be a good candidate for its use in programs for control of vector transmitters of malaria and dengue because of its biocide effect against the larval stage of these mosquitoes and to the low concentrations required to carry out this effect, as this species is non-toxic to mammals as showed the use as traditional flavoring component by inhabitants of the Sierra Gorda in Guanajuato State, Central México. Ethanolic extract showed better larvicidal activity in comparison with pure afnin, due to the possible synergistic effect of alkanamides. Furthermore, the results show that the double bonds present in afnin are important in order to maintain the insecticidal properties against An. albimanus and Ae. aegypti. This is the first report that demonstrates the biocide capacity of H. longipes against mosquito in larval stage and the importance of the double bonds for this larvicidal activity.

Acknowledgments

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