# Assessment of A Simple Compound-Saving Method To Study Insecticidal Activity of Natural Extracts and Pure Compounds Against Mosquito Larvae

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# SCIENTIFIC NOTE

## ASSESSMENT OF A SIMPLE COMPOUND-SAVING METHOD TO STUDY INSECTICIDAL ACTIVITY OF NATURAL EXTRACTS AND PURE COMPOUNDS AGAINST MOSQUITO LARVAE

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ABSTRACT. Research on natural insecticides has intensified with the spread of resistance to chemicals among insects, particularly disease vectors. To evaluate compounds, the World Health Organization (WHO) has published standardized procedures. However, those may be excessively compound-consuming when it comes to assessing the activity of natural extracts and pure compounds isolated in limited amount. As part of our work on the discovery of new mosquito larvicides from Amazonian plants, we developed a compound-saving assay in 5-ml glass tubes instead of WHO larval 100-ml cups. Comparing activity of synthetic and natural chemicals validated the glass tube assay. Raw data, lethal doses that kill 50% ($LD_{50}$) and 90% ($LD_{90}$) at 24 and 48 h, were highly correlated ($0.68 < R^2 < 0.96$, $P < 0.001$, Pearson test) between cups and tubes. It was also established that 10 tubes ($N = 50$ larvae) provided the same level of sensitivity as 20 tubes ($N = 100$). This method proved suitable for rapid screening of natural extracts and molecules, identifying active compounds using 10 times less material than in the WHO protocol.

**KEY WORDS** Mosquitoes, natural insecticides, screening method

Vector-borne diseases account for 70% of the estimated global burden of all infectious diseases. For most of these, vector control remains the sole method to circumvent transmission in the absence of vaccine and/or specific treatments. For this purpose, insecticides have been sprayed for decades, provoking resistance development and resulting in a loss of vector control efficacy (David et al. 2013). As a consequence, research on new insecticides, particularly molecules of natural origin, has intensified in recent years (Isman and Grieneisen 2014).

Screening for compound properties is usually performed according to protocols published by the World Health Organization (WHO) (WHO 2005, 2006). Because identifying active compounds requires testing a large number of candidates, high-throughput systems have been developed to increase capacity, gain time, and laboratory space without losing test sensitivity (Grieco et al. 2005, Pridgeon et al. 2009). The need for developing a specific larval assay arose from issues encountered while the authors were attempting to identify novel larvicides from Amazonian plants. Indeed, the WHO test was excessively compound-consuming to evaluate extracts and molecules from natural origin, which can be difficult to obtain in large amounts. Moreover, as composition and yield of secondary metabolites can be affected by various environmental (collection place or period) or physiological (phenology) parameters (Assad et al. 1997, Figueiredo et al. 2008), it is of significant importance to work on the same batch of extract from bioassay to molecule isolation. Besides Pridgeon et al. (2009), no thorough evaluation of an alternative mosquito larvicide screening method has ever been described that the authors are aware of. In Pridgeon's study, 1st-stage larvae of *Aedes aegypti* (L.) were used, which made it difficult to compare with the reference WHO cup protocol using 3rd- to 4th-stage larvae.

Herein, we present an optimized assay on 11 products of various origin tested against larvae of *Ae. aegypti*. After testing various containers of different sizes and materials, we ended up choosing 5-ml glass tubes, which were convenient in manipulations, easy to store during the assay, and were reusable. Compounds tested covered different chemical families and mode of action, and included synthetic and natural chemicals, plant extracts, and their isolated active constituents.

Assays were conducted with late-3rd or early-4th instars of *Ae. aegypti* susceptible laboratory strain named Paea. The colony has been collected in Paea town, French Polynesia, and maintained in the

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insectary at the Institute Pasteur de la Guyane, Cayenne, French Guiana, under conditions: 28 ± 2°C, 80 ± 10% RH, and 12:12 h ± 20 min of light and dark. *Aedes aegypti* eggs were preserved on dried paper strips at the insectary temperature. Larvae were fed with yeast pellets.

Larvae were exposed to technical-grade insecticides (Sigma-Aldrich, St. Louis, MO). Synthetic compounds tested were deltamethrin (CAS no. 52918-63-5), propoxur (CAS no. 114-26-1), dichlorvos (CAS no. 62-73-7), and malathion (CAS no. 121-75-5), whereas pyrethrum (CAS no. 8003-34-7) and spinosad (CAS no. 168316-95-8) were selected as commercial compounds of natural origin. Pyrethrum, isolated from the flowers of *Chrysanthemum* (L.) sp. (Asteraceae) (WHO 2009), is composed of several active esters. Spinosad is a mixture of spinosyn A and D originally produced by the bacterium *Saccharopolyspora spinosa* (Mertz and Yao) (WHO 2014). A number of natural insecticides were also available from our laboratory, following previously described procedures (Rodrigues et al. 2010, Nirma et al. 2012). These were *Lonchocarpus monilis* (L.) A.M.G. Azevedo (syn. *Muellera frutescens* (Aubl.) Standl.) (Fabaceae) leaf ethyl acetate extract, *Sextonia rubra* (Mez) van der Werff (Lauraceae) wood ethyl acetate extract, as well as pure rotenone, rubrenolide, and rubrynolide, respectively, obtained from the above cited extracts.

The WHO cup assay (WHO 2005) was performed by exposing 100 larvae to insecticides into plastic cups containing 99 ml of distilled water and 1 ml of the insecticide solution at the desired concentration. Four cups per concentration (4 × 25 larvae) and at least 5 concentrations of each compound diluted in ethanol were used to obtain from 0% to 100% mortality. Control treatments were performed for each test with 1 ml of ethanol only. Larval mortality was recorded at 24 and 48 h after exposure. Tube assay was performed in 5-ml glass test tubes (75 mm × 12 mm × 0.8–1.0 mm rimless, catalog no. 212-0013; VWR International, Radnor, PA) containing 2.97 ml of distilled water and 30 µl of the insecticide at the desired concentration. Twenty tubes per concentration, each containing 5 larvae (20 × 5 larvae), and at least 5 concentrations of each compound diluted in ethanol were used to obtain 0% to 100% mortality. Control treatments were performed for each test with 30 µL of ethanol. Larval mortality was recorded 24 and 48 h after exposure. To determine if a 50-larvae test in tube could be robust enough, 3 sets of 10 tubes for each insecticide and dose from the original data sets (20 tubes) were randomly sampled by using a stratified function in R software version 3.2.0 (http://news.mrdwab.com/2011/05/20/stratified-random-sampling-in-r-from-a-data-frame). Abbott's formula (Abbott 1925) was applied to records. Test was invalidated if mortality in control was >20%. Mean percent mortality and standard error were then calculated for each compound and dose. Correlations between mean mortality percentages from cups and either 100-larvae or 50-larvae tubes were evaluated by Pearson correlation test. A linear regression was performed on this data set. Parameters of the linear equation, as well the adjusted $R^2$ coefficient between the observed and predicted data values were computed. In addition, lethal doses per compound and assay were obtained by a Probit regression under a general linearized model and processed through the same analysis. All analyses were computed using the MASS package in R software version 3.2.0 (https://www.r-project.org/).

Pearson correlation tests demonstrated a strong correlation between data from cups and tubes assays for the complete data set of 100 larvae as well as for each random data set of 50 larvae, with $R^2$ of 0.93 (95% confidence interval: 0.89–0.95) ($P < 0.001$) at both 24 and 48 h. The linear model led to identical conclusion, suggesting that the tube assay was robust. A predictive model of cup results was obtained using the tube assay independently from the number of larvae and time of exposure (Fig. 1).

The question of whether or not lethal doses (LD) obtained by Probit analysis and used to normalize and compare results in screening processes were also correlated between cups and tubes, and would eventually allow prediction of cup assay results from the tube assay. In the present assays, $LD_{50}$ varied from $5.964 \times 10^{-5}$ ppm to 8.611 ppm across compounds and methods. Deltamethrin was the most active compound, with $LD_{50}$ at a scale of $10^{-5}$ ppm (Table 1). This value is consistent with previous results (Dusfour et al. 2011). The $LD_{90}$ values varied from $1.335 \times 10^{-4}$ ppm to 37.868 ppm. Results were consistent with those obtained for $LD_{50}$ (Table 1). It should also be mentioned that although *Sextonia rubra* wood ethyl acetate extract as well as rubrynolide had been previously identified as termiticidal (Rodrigues et al. 2011), it was the first time that this extract and its compounds, more particularly rubrenolide, were described as larvicidal products (Falkowski et al. 2014).

The $R^2$ computed by the Pearson correlation test between $LD_{50}$s and $LD_{90}$s at 24 and 48 h varied from 0.77 to 0.98, demonstrating a significant correlation between $LD_{50}$s measured in cups and tubes. However, significance of the correlation between tube and cup assay results was weaker for $LD_{90}$s, with lower $R^2$ and larger confidence intervals, in particular at 24 h. Results were similar whatever the number of larvae and subsets used to compute the correlation tests. According to the general linear model, equations obtained with $LD_{50}$ from cup and tube assays were similar whether the complete data set or subsets were used, with adjusted $R^2$ varying from 0.89 to 0.96 to 0.93 ($P < 0.001$).

In conclusion, the experiments presented herein confirm the appropriateness of the 5-ml glass tube

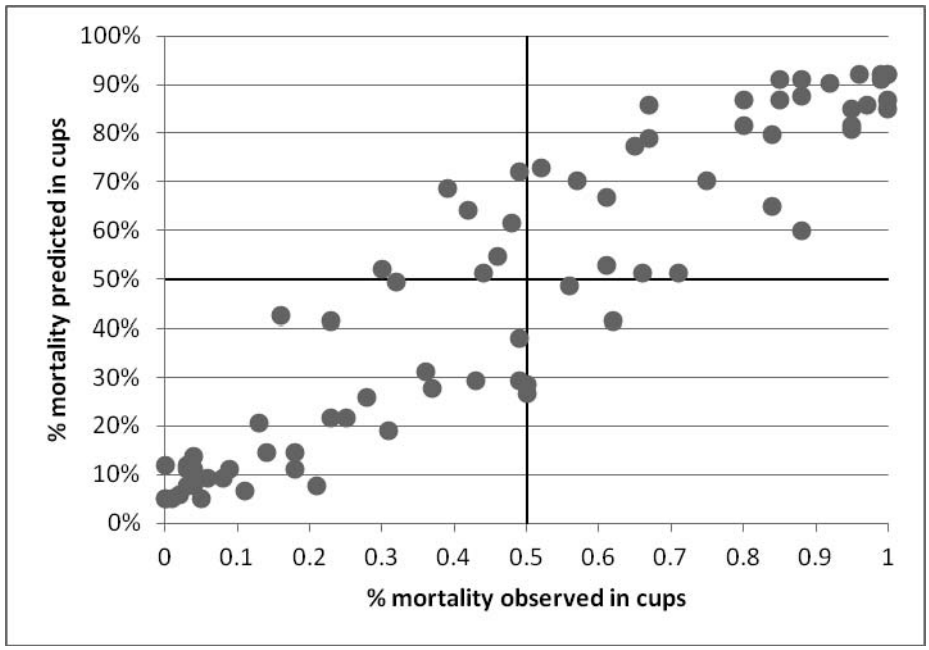


Fig. 1. Plot of percentage of mortalities in cup (abcissas) to the predictive values (ordinates) using the equation $y = 0.87x + 0.05$ obtained by general linear model regression of mortalities in cup and tube assays. Fifty percent of lines are drawn.

assay to screen insecticidal activity of a variety of compounds, purified or in complex mixtures, with different chemical structures and mode of action. The assay is equally sensitive to the WHO assay with a lower number of larvae and a smaller amount of product. In fact, to test a compound or extract at a concentration of 10 ppm, the WHO protocol requires 4 mg of material for 4 × 100-ml cups containing 25

Table 1. Theoretical lethal doses (ppm) that kill 50% ($LD_{50}$) and 90% ($LD_{90}$) of the mosquitoes, with associated standard error (SE), for synthetic compounds, natural extracts, and associated molecules in tube and cup assays after 24 and 48 h of exposure.

| | | 24 h | | 48 h | |
|---|---|---|---|---|---|
| Compound | Assay | $LD_{50}$ (SE) | $LD_{90}$ (SE) | $LD_{50}$ (SE) | $LD_{90}$ (SE) |
| Deltamethrin | Cup | 5.96e-05 (0.020) | 1.39e-04 (0.026) | 5.14e-05 (0.023) | 1.33e-04 (0.028) |
| | Tube | 7.65e-05 (0.016) | 1.69e-04 (0.029) | 7.01e-05 (0.017) | 1.55e-04 (0.027) |
| Dichlorvos | Cup | 0.039 (0.012) | 0.064 (0.026) | 0.029 (0.012) | 0.049 (0.020) |
| | Tube | 0.041 (0.008) | 0.054 (0.014) | 0.030 (0.012) | 0.049 (0.019) |
| Malathion | Cup | 0.082 (0.014) | 0.145 (0.025) | 0.046 (0.024) | 0.078 (0.020) |
| | Tube | 0.087 (0.012) | 0.141 (0.023) | 0.049 (0.028) | 0.099 (0.026) |
| *Lonchocarpus monilis* | Cup | 7.441 (0.020) | 17.203 (0.038) | 5.845 (0.020) | 11.902 (0.034) |
| | Tube | 8.611 (0.020) | 20.869 (0.039) | 4.374 (0.041) | 12.271 (0.040) |
| Propoxur | Cup | 0.384 (0.017) | 1.068 (0.033) | 0.341 (0.016) | 0.898 (0.028) |
| | Tube | 0.588 (0.027) | 2.572 (0.072) | 0.298 (0.624) | 2.248 (0.066) |
| Pyrethrum | Cup | 0.029 (0.017) | 0.069 (0.030) | 0.026 (0.016) | 0.059 (0.026) |
| | Tube | 0.024 (0.017) | 0.054 (0.024) | 0.023 (0.018) | 0.054 (0.026) |
| Rotenone | Cup | 2.689 (0.043) | 37.868 (0.125) | 1.243 (0.032) | 7.829 (0.054) |
| | Tube | 2.535 (0.030) | 15.125 (0.067) | 1.861 (0.026) | 8.648 (0.048) |
| Rubrenolide | Cup | 0.605 (0.023) | 2.110 (0.041) | 0.300 (0.024) | 0.788 (0.027) |
| | Tube | 0.503 (0.022) | 1.567 (0.034) | 0.300 (0.026) | 0.898 (0.030) |
| Rubrynolide | Cup | 3.840 (0.018) | 8.902 (0.031) | 2.105 (0.023) | 8.200 (0.043) |
| | Tube | 2.720 (0.016) | 5.780 (0.025) | 1.617 (0.017) | 3.894 (0.028) |
| *Sextonia rubra* | Cup | 3.150 (0.018) | 8.442 (0.029) | 2.062 (0.018) | 4.513 (0.022) |
| | Tube | 2.168 (0.017) | 4.444 (0.021) | 1.591 (0.022) | 3.405 (0.022) |
| Spinosad | Cup | 0.387 (0.025) | 1.118 (0.065) | 0.193 (0.020) | 0.444 (0.028) |
| | Tube | 0.277 (0.017) | 0.631 (0.034) | 0.118 (0.024) | 0.242 (0.024) |

larvae each, whereas our assay requires 300 μg of material only distributed in 10 tubes each containing 5 larvae. Using less of each compound allows investigations to be pushed further, measuring for example adulticidal toxicity, excito-repellency action, or ecotoxicity, thus gaining a wider glimpse into active product activity and mode of action before taking active compounds and/or extracts to development.

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