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# Larvicidal Activity of *Artemsia capillaris* and *Setaria palmifolia* Extracts Against *Aedes aegypti* (Diptera: Culicidae)

**Lin-Jie Huang[1], Chen-An Tsai[2], Matan Shelomi[1]***

[1] Department of Entomology, National Taiwan University, Taipei, Taiwan
[2] Division of Biometrics, Department of Agronomy, National Taiwan University, Taipei, Taiwan
* Correspondence author: mshelomi@ntu.edu.tw

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## ABSTRACT

Larvicides are an effective method of vector management to control mosquito-borne diseases such as dengue. Growing awareness of environmental concerns with improperly used synthetic insecticides is driving interest in plant-derived biopesticides, perceived as being safer and to which insects are less likely to evolve resistance. This study examined hexane and ethyl acetate extracts of two plants, *Artemisia capillaris* and *Setaria palmifolia*, previously identified as having repellent abilities against the biting midge *Forcipomyia taiwana*, for any larvicidal ability against the yellow fever mosquito, *Aedes aegypti*. The hexane extract of *Ar. capillaris* was effective, causing almost immediate inactivity of larvae faster than a *Bacillus thuringiensis* subsp. *israelensis* positive control. At doses above 0.03 % by volume, all or nearly all larvae would die within 24 h, while at lower doses, some would recover even if they appeared moribund. The $LC_{50}$ and $LC_{90}$ were estimated as .0188 and .0526 % at 24 h and 0.0128 and 0.0332 % at 48 h, respectively. To summarize, the hexane-extracted essential oil of capillary wormwood has a fast-acting inactivity effect on *Ae. aegypti* larvae with 100% larval mortality at a dose between 0.03 and 0.05 % by volume, classifying it as an effective larvicide.

**Key words:** biopesticide, insecticide, mosquito, vector control, wormwood

## Introduction

Vector management is critical to managing mosquito-vectored diseases like Dengue for which anti-viral medications are unavailable (Lee *et al.*, 2023). The primary Dengue vectors like *Aedes aegypti* and *Ae. albopictus* oviposit in water-filled artificial containers. Targeting these vulnerable larvae with container-based larvicides has higher insecticidal efficacy, better specificity, and smaller contamination areas than aerial spraying of adulticides (Faraji and Unlu, 2016).

Improper use of synthetic insecticides has several consequences, including the evolution of insecticide-resistant mosquito populations (Pai *et al.*, 2023), non-target effects, and long-term environmental pollution (Ullah *et al.*, 2018; Webb, 2022). Plant-derived biopesticides

extracted from plant materials with distillation and/or solvents are a possible alternative (Engdahl *et al.*, 2022), with a long history of human use against mosquitoes (Pavela *et al.*, 2019). Their advantages over synthetic insecticides include reduced likelihood for resistance to develop: plant extracts comprise multiple compounds with multiple modes of action, possibly including synergistic effects, against which multiple mutations would likely be needed to evolve resistance (Rattan, 2010). Plant extracts are also biodegradable, and so under less strict regulation than synthetic insecticides and perceived more favorably by the general public (Isman, 2015; Şengül Demirak and Canpolat, 2022). As *Ae. aegypti* are strongly anthropophilic, the cooperation of local residents is essential for vector control. Drawbacks to biopesticides include unstable quality, short persistence requiring higher application rates, lower chemical stability, and higher industrial production costs (Damalas and Koutroubas, 2020). Simple botanical extracts can be produced at low cost, however, even at the point of use by subsistence farmers with access to local plants; and these have been shown to be effective, albeit variably so (Dougoud *et al.*, 2019).

This study sought to identify never-before-tested plant extracts for larvicidal use against *Ae. aegypti*. In a previous study, several extracts of plants used by Aboriginal Taiwanese as insect repellents showed repellent or inactivity effects against biting midges (*Forcipomyia taiwana*, Ceratopogonidae) (Guo *et al.*, 2023). Two such plants with no prior literature on their effectiveness against mosquitoes are capillary wormwood (*Artemisia capillaris*, Asteraceae), from a genus with several known larvicidal species (Masotti *et al.*, 2012; Ninditya *et al.*, 2020; Luo *et al.*, 2022; Osanloo *et al.*, 2022); and palmgrass (*Setaria palmifolia*, Poaceae), also with a congeneric, larvicidal species (Ashok and Babu, 2021). These locally abundant plants could be readily procured for extract production, even on a small scale by individuals trying to make their own biopesticides (Dougoud *et al.*, 2019). The goal of this study was to test if they are larvicidal against *Ae. aegypti*.

## Materials and Methods

The essential oils were those from the original study (Guo *et al.*, 2023). Briefly summarizing those methods, *Ar. capillaris* and *S. palmifolia* leaves were collected on a single day in Taipingshan National Forest Recreational Area, oven-dried, and powdered, and 50 grams of powder extracted with 150 mL hexane at 70°C for 24 h and again with 150 mL ethyl acetate at 78°C for 24 h in a Soxhlet extractor. Essential oils were separated from the solvents under a vacuum using a rotary evaporator. Two separate sets of extractions were done for each species, and the resulting oils were combined for long-term storage in amber vials in the dark at room temperature. For this study, these four extracts (one hexane and one ethyl acetate extract per species) were fully dried in their storage vials under room temperature under a fume hood. The dry residues were dissolved to saturation (100% stock solution) with 2-4 mL of dimethyl sulfoxide (DMSO), depending on how much residue was available.

The mosquitoes were from an *Ae. aegypti* colony maintained by the Vector-borne Infectious Diseases Lab, National Taiwan University, and originally sourced in Da-Tong Road, South District, Tainan City, Taiwan (22.9671433 N, 120.2154393 E) in 2005. Larvae were kept in RO water inside an incubator maintained at 28°C, 80% relative humidity, and a 16:8h (light/dark) light cycle, and fed *ad libitum* with crushed goldfish feed.

Following international standard protocols (World Health Organization, 2005), the larvicidal bioassays were conducted in 250 mL beakers containing either 0.1 mL DMSO (negative control, to account for possible larvicidal activity of the DMSO itself), 0.1 mL of DMSO-plant extract solution, or 0.2 mL of 50% v/v DMSO aqueous solution suspended with VectoBac WDG, a commercially available *Bacillus thuringiensis israelensis* (*Bti*) larvicide (positive control); plus enough RO water for a final volume of 100 mL. Each also contained 25 late third instar to early fourth instar *Ae. aegypti* larvae and five grains of fish feed, added prior to the experimental solutions. Beakers were incubated under the same conditions as larva rearing. The status of the larvae was recorded

every hour for the first 6 h, then each beaker was sealed with plastic wrap perforated for gas exchange, and the statuses were recorded every 24 h for 10 days (240 h) or until adults emerged or all larvae had died. The numbers of emerged adults were recorded at each time point, and larvae or pupae were recorded as alive, dead, or inactive [twitching periodically but lacking controlled locomotion or sinking to the bottom]. For mortality rate statistics, inactive larvae were classified accurately as alive. Each bioassay had three replicates.

The initial test concentrations of extracts were 0.05% and 0.1% by volume, the latter corresponding to using 0.1 mL of 100% saturated stock solution in the 100 mL total volume. Exact ppm concentrations of the stock oil solutions, unfortunately, could not be determined due to the nature of the extraction methods used and inability to isolate solid oil to measure its mass, so these concentrations are likely overestimates. Based on these first results, only one extract was used for the toxicity-determining tests. These were carried out as before, but over four days instead of 10. The concentrations used were 0.005, 0.01, 0.015, 0.02, 0.025, and 0.03 % v/v, decided after range testing with a series of hit-and-trial larvicidal assays to find doses that induced mortalities between 0% and 100%.

The effects of dose and time were analyzed using R v 4.4.1 with two-way repeated measures ANOVA, and Tukey's HSD test was used for *post hoc* analyses on the dose-effect on the observation of each day. The recorded mortality rates of each of the concentrations on the 24$^{th}$ and 48$^{th}$ h after the bioassay began were used to calculate median lethal concentration ($LC_{50}$) and $LC_{90}$ using R 4.4.1 and logit dose-response as described in previous publications (Wheeler *et al.*, 2006; Russell *et al.*, 2007). Briefly, the Logit analysis is used to estimate lethal concentration (LC) or time (LT) and the appropriate fiducial confidence limits desired for selected LC's by performing a linear regression of the logits (probability of death) against the log-transformed concentrations. This gives a regression equation of the form: logit(p) = a + b * log10 (concentration), where a is the intercept and b is the slope. Once the two parameters are estimated using the maximum likelihood method, the regression equation provides a sigmoidal dose-response curve and calculates the dose variable values (with fiducial confidence intervals or 95% CI) that correspond to a series of probabilities.

## Results

The initial tests found concentration-dependent responses of *Ae. aegypti* larvae to all of the selected plant extract solutions, with growth delay, feeding inhibition, and mortality observed. On the initial tests using high concentrations, dose-dependent effects of extracts on larval mortality were found for all but the *Ar. capillaris* ethyl acetate extract (Table 1). Pairwise comparisons between treatment groups at each time point performed by means of Tukey's HSD (Honestly Significant Difference) test at an alpha level of 0.05 found significant differences in larval mortality between the 0.05 % containers and the negative control after one day for *Ar. capillaris* hexane extract, one day for *S. palmifolia* hexane extract, and three days for *S. palmifolia* ethyl acetate extract (Supplementary Data S1). The *Ar. capillaris* hexane extract showed the strongest larvicidal effect (Figure 1), inducing inactivity and apparent death of all larvae (100%) within one hour in both concentrations. For all other extracts, some larvae survived and adults emerged even at the highest dose (Figure 2).

Based on these results, the *Ar. capillaris* hexane extract was used for further testing at a range of concentrations (0, 0.05, 0.01, 0.015, 0.02, 0.025, and 0.03 %) determined via hit-and-run trials (World Health Organization, 2005). Significant effects of dose were found (Table 1), and Tukey's HSD pairwise comparisons found significant differences in larval mortality after 24 h between the negative control (0.33±1.16) and 0.01 % (23.56±14.14 % mortality), with nearly total mortality at 0.03 % (93.33±4.62 % mortality) (Supplementary Data S1). From this data, the $LC_{50}$ values for *Ar. capillaris* hexane extract were estimated as follows: 0.018760 % at 24 h with a 95% confidence interval of (0.017214, 0.020307); and 0.012831 % at 48 h, with a 95% confidence interval of (0.011871, 0.013790) (Figure 3). The $LC_{90}$ values were as follows: 0.052624 % at 24 h, with a 95% confidence interval of (0.041015, 0.064233); and 0.033226 %

**Table 1. Results of two-way repeated measures ANOVA**. Results are for the initial tests using 0, 0.05, or .01 % by volume of all four test extracts on a) larval mortality and b) adult emergence; and the follow-up test using different concentrations of *Artemsia capillaris* hexane extract on larva mortality. EtAc = ethyl acetate. Hex. = Hexane. • = $p$<0.1, * = $p$<0.05, ** = $p$<0.01, *** = $p$<0.001

a) ANOVA results for initial larval mortality tests (doses: 0, 0.05, 0.1 % ; times: 0-10 days)

| Extract | *A. capillaris* Hex. | *A. capillaris* EtAc | *S. palmifolia* Hex. | *S. palmifolia* EtAc |
|---|---|---|---|---|
| dose | F2= 3802.367 *** | F2= 0.084 | F2= 15.611 ** | F2= 12.378 ** |
| time | F10= 2037.574 *** | F10= 3.023 ** | F10= 38.979 *** | F10= 31.662 *** |
| dose*time | F20= 466.526 *** | F20= 0.602 • | F20= 8.265 *** | F20= 9.726 *** |

b) ANOVA results for initial adult emergence tests (doses: 0, 0.05, 0.1 %; times: 0-10 days)

| Extract | *A. capillaris* Hex. | *A. capillaris* EtAc | *S. palmifolia* Hex. | *S. palmifolia* EtAc |
|---|---|---|---|---|
| dose | F2= 424.277 *** | F2= 8.034 * | F2= 11.266 ** | F2= 30.891 *** |
| time | F10= 194.378 *** | F10= 2632.655 *** | F10= 60.220 *** | F10= 133.357 *** |
| dose*time | F20= 194.378 *** | F20= 16.739 *** | F20= 8.726 *** | F20= 17.021 *** |

c) ANOVA results for larval mortality (doses: 0, 0.005, 0.01, 0.015, 0.02, 0.025, 0.03 % ; times: 1-2 days)

| | |
|---|---|
| dose | F6= 73.307 *** |
| time | F1= 202.410 *** |
| dose*time | F6= 28.484 *** |

at 48 h, with a 95% confidence interval of (0.028190, 0.038262) (Figure 4).

At doses ≥ 0.025 % *Ar. capillaris* hexane extract, all of the *Ae. aegypti* larvae (100%) turned inactive within 2 h. At lower concentrations (≤ 0.01 %), larvae were not necessarily inactive, but all larvae showed visible lethargy and sometimes behavioral abnormalities like repeatedly touching their rear end with their mouthparts, being trapped on the surface, and taking a dorsal side-down position with the breathing tube pointing downwards. The rapid onset of inactivity was faster than in the *Bti*-positive control groups, where approximately 60% of larvae remained active at the 2hr mark. However, with *Bti* nearly all larvae (> 98%) were dead within 24 h, while most (> 90%) larvae treated with *Ar. capillaris* hexane extract doses ≤ 0.01 % fully recovered 24 h after treatment, including some that originally appeared inactive.

## Discussion

To summarize, this study found that a hexane extract of essential oils from capillary wormwood, when applied to water containing *Ae. aegypti* larvae, would induce behavioral abnormalities and inactivity within one hour of application. Experiments recorded 90% larval mortality at doses of 0.03 % by volume within one day, and 100% mortality at doses above 0.05 % within 1 h, with an estimated $LC_{50}$ for 24 h calculated as 0.018760 %. While there are no international standards for evaluating the effectiveness of botanical larvicides, using previously published definitions (Komalamisra *et al.*, 2005), the *Ar. capillaris* hexane extract falls in the category of effective plant extracts ($LC_{50}$ between 0.01 and 0.075 %). A more accurate measurement of the mass-to-volume ppm concentration for this oil would facilitate future analysis.

Prior GC-MS identification of the composition of the very extracts used in this study (Guo *et al.*, 2023) found multiple terpenes and aromatic compounds in the *Ar. capillaris* hexane extract like β-eudesmol, caryophyllene, β-turmerone, γ-muurolene, γ-terpinene, trans-β-ocimene, linalool, humulene, nonacosane, chavibetol, nerolidol, and D-limonene. All of those have been found in other biopesticides (Kiran *et al.*, 2006; Di Campli *et al.*, 2012; Lawal *et al.*, 2014; Acharya *et al.*, 2021; Andrin'iranto *et al.*, 2021; França *et al.*, 2021; Reyes-Ávila *et al.*, 2023; Silva *et al.*, 2023). The oil thus likely has

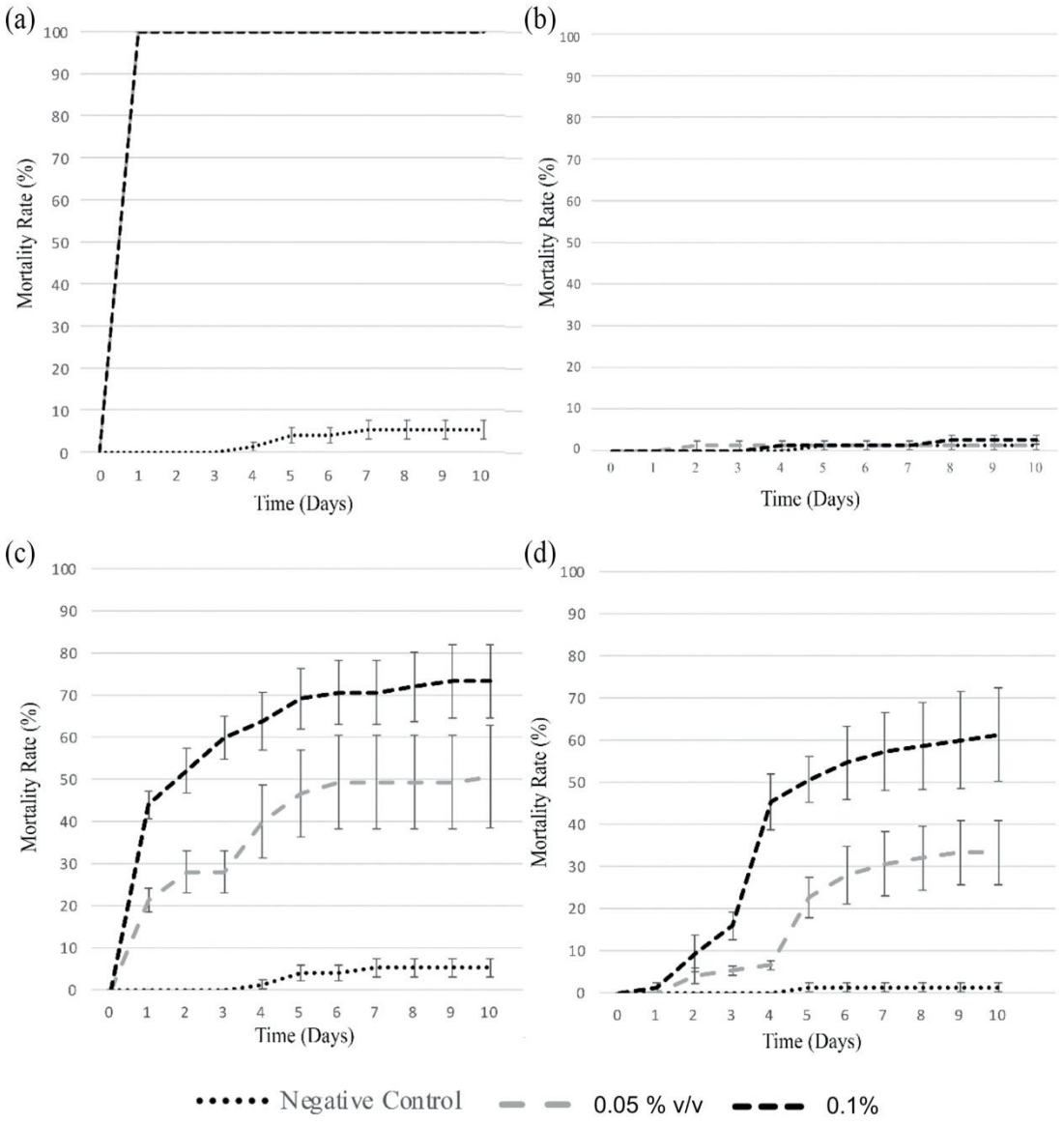


Fig. 1. **Average daily larval/pupal mortality rate of *Aedes aegypti* in the pretests.** (a) *Artemisia capillaris* hexane extract (b) *Ar. capillaris* ethyl acetate extract (c) *Setaria palmifolia* hexane extract (d) *S. palmifolia* ethyl acetate extract. Mortality with a positive control (VectoBac WDG *Bti* granules) was 100% within the first day. Mean values are in Supplementary Data S1.

multiple modes of action that may work synergistically or antagonistically, as has been noted in other cases (Sarma *et al.*, 2019). Future studies could test partitioned fractions of *Ar. capillaris* hexane extract to identify which of its active components causes inactivity and-or lethality and check for synergistic effects. Because the concentration for 100% mortality is a relatively high 0.05 %, future work would also need to test for toxicity against non-target aquatic organisms, including fish, and explore the persistence rate of this extract in the environment and its potential toxicity to mammals.

The observed phenomenon of larvae recovering from inactivity at doses below 0.05 % suggests a low extract persistence or stability of only a few hours. In the field, an inactive larva may be vulnerable to other causes of mortality, but that remains to be demonstrated, as does this extract's effect on adult mosquitoes (Yohana *et al.*, 2022). Doses as high as 0.05 % would also pose a higher risk to non-target organisms, so follow-up studies would be needed before any field testing or commercial application. Degradation or efficacy tests over a 48-h period could help assess the persistence of the larvicidal effect. Formulations can be designed that

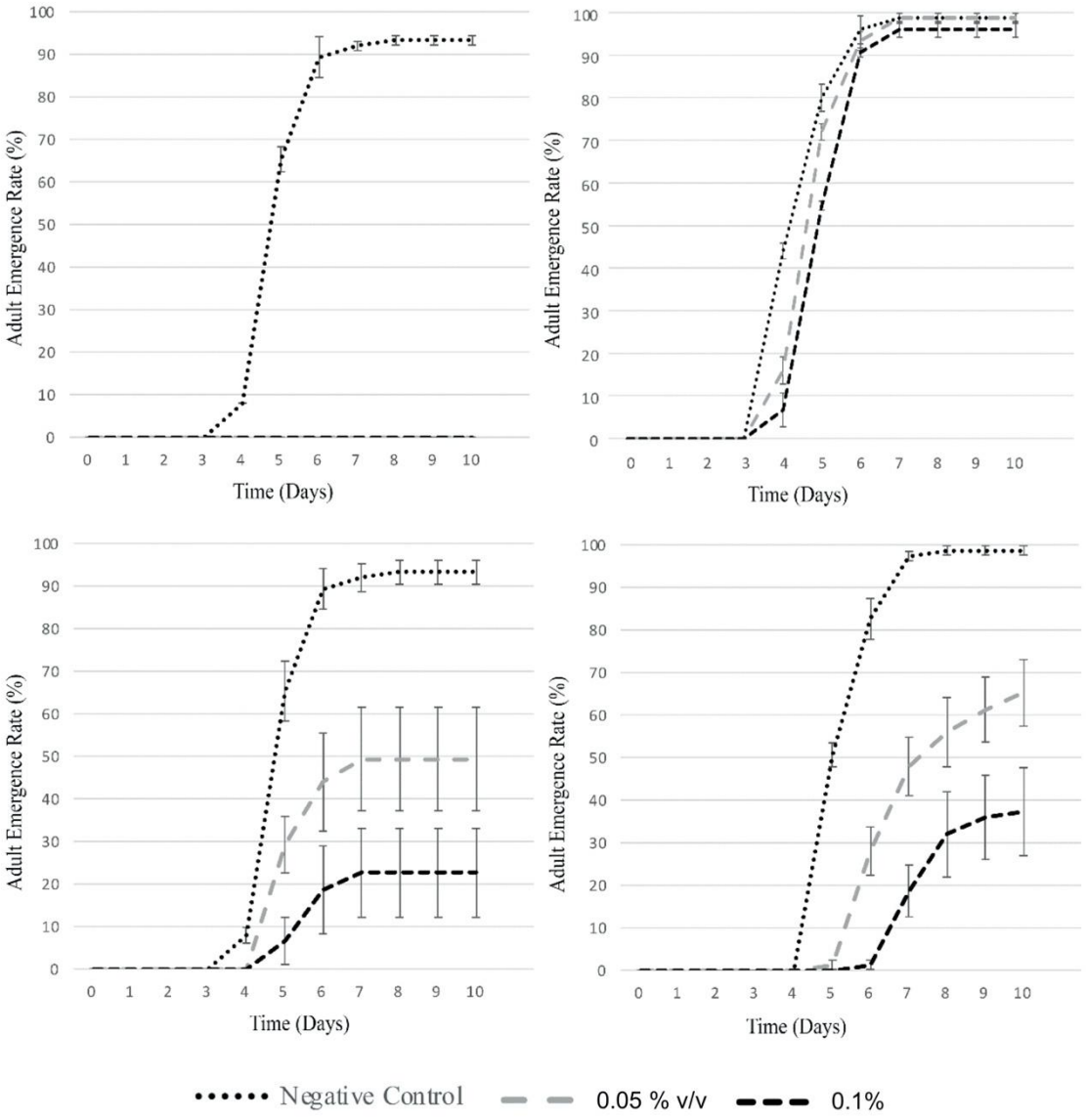


Fig. 2. **Average daily adult emergence rate of *Aedes aegypti* in the pretests.** (a) *Artemisia capillaris* hexane extract (b) *Ar. capillaris* ethyl acetate extract (c) *Setaria palmifolia* hexane extract (d) *S. palmifolia* ethyl acetate extract. The positive control (VectoBac WDG *Bti* granules) induced larval 100% mortality, and so no pupae existed for adult emergence. Mean values are in Supplementary Data S1.

improve the extract's efficiency and persistence, such as encapsulation with polymer materials (Kala *et al.*, 2020; Kamari *et al.*, 2021).

For the ethyl acetate extract of *Ar. capillaris* or either extract of *S. palmifolia*, some larvicidal effects were noted at the highest doses tested, but none approaching the efficacy of the hexane extract of *Ar. capillaris* (Figure 1). For those extracts, many larvae survived and developed into adults (Figure 2). These extracts would be classified as ineffective (Komalamisra *et al.*, 2005). In the initial study using these plant extracts as repellents against *F. taiwana* adults (Guo *et al.*, 2023), the ethyl acetate extracts of both plants had significant repellency effects, but not the hexane extracts, suggesting different chemical compounds within the oils are responsible for the different effects. That study also noted inactivity effects on the adult *F. taiwana* from several plant essential oils, including a commercial *Artemesia absinthium* oil, but not from either *Ar. capillaris* or *S. palmifolia* extracts (Guo *et al.*, 2023). While repellency against one insect was never and could never be expected to perfectly predict larvicidal activity against another, the *Forcipomyia* repellency study did generate hypotheses that ended up identifying an

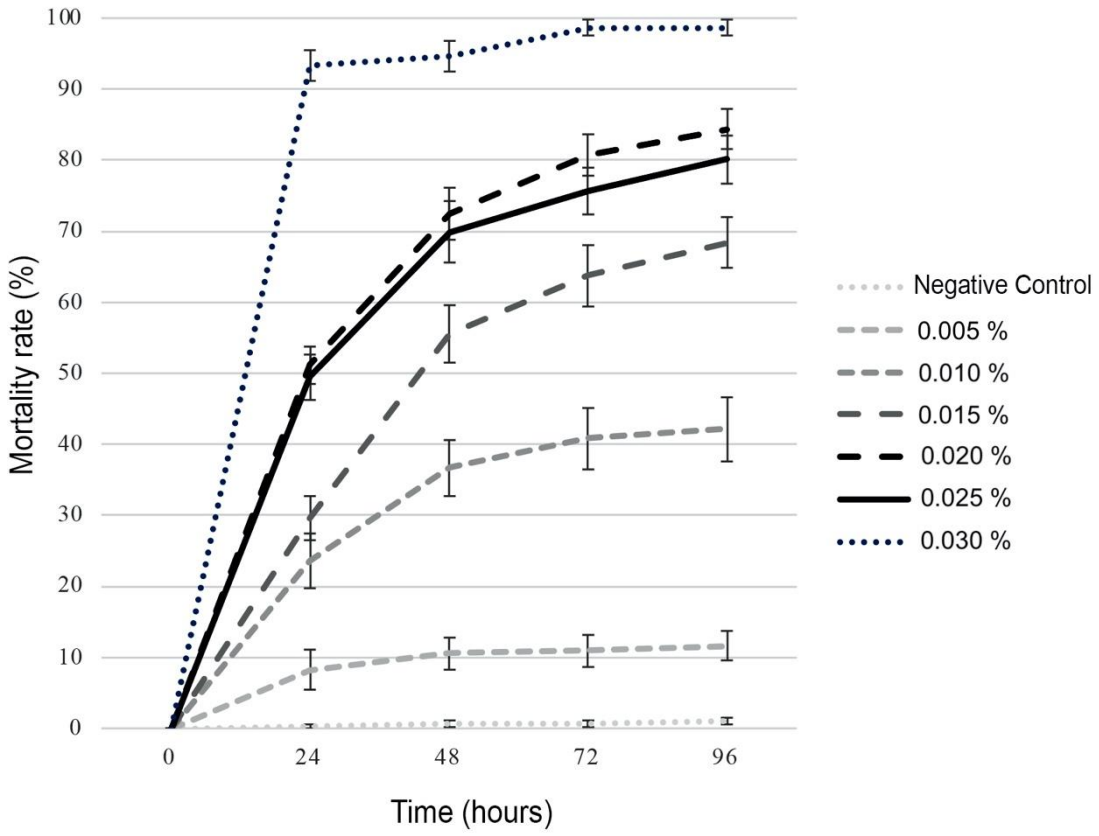


Fig. 3. **Average larval/pupal mortality rate of *Ae. aegypti* exposed to *Ar. capillaris* hexane extract in the toxicity determining tests.** Mean values are in Supplementary Data S1.

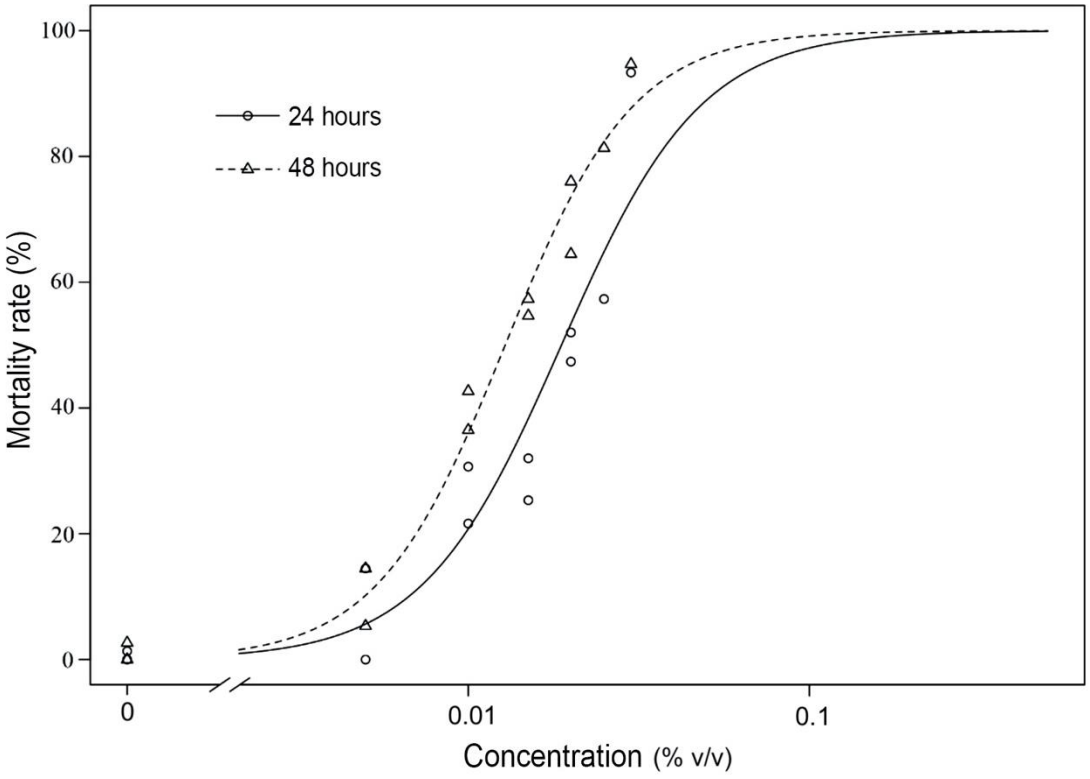


Fig. 4. The dose-response curves of larval mortality of *Aedes aegypti* exposed to *Artemisia capillaris* hexane extract using logit dose-response analysis.

effective botanical larvicide against *Aedes*.

Bioprospecting and botanical pesticide researchers thus need not limit the search for, say, new mosquito larvicides to plants with known larvicidal effects in the literature or from ethnobotanical records. A broader net can be cast in the search for new biopesticides, as one can reasonably hypothesize that any plant oil with physiological effects on one insect could have similar or different effects on another insect, even if the validity of the hypothesis or nature of the effects cannot be perfectly predicted. Humanity has not yet exhausted the bioprospecting potential of plants, so identifying novel plants associated with different geographical areas to test for various pesticidal applications outside their known or traditional ones could be a fruitful endeavor.

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## Supplementary Data S1

Mean values and results of pairwise comparisons between treatment groups at each time point performed by means of Tukey's HSD (Honestly Significant Difference) test at an alpha level of 0.05 for: a) Larval mortality among the four initially tested extracts. b) adult emergence rate among the four initially tested extracts. c) larval mortality for the follow-up *Artemsia capillaris* hexane extract tests. Treatments with the same letter are not significantly different.

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# 茵陳蒿（*Artemisia capillaris*）與棕葉狗尾草（*Setaria palmifolia*）萃取物對埃及斑蚊（*Aedes aegypti*，雙翅目：蚊科）的殺幼蟲活性

**黃麟傑[1]、蔡政安[2]、薛馬坦[1*]**

[1] 國立臺灣大學昆蟲學系　106 臺北市大安區羅斯福路四段 1 號
[2] 國立臺灣大學農藝學系生物統計組　106 臺北市大安區羅斯福路四段 1 號
* 通訊作者 email: mshelomi@ntu.edu.tw

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## 摘　要

幼蟲殺蟲劑是一種有效的病媒管理方法，可用於控制登革熱等蚊媒疾病。由於對不當使用合成殺蟲劑所帶來的環境問題日益關注，人們對植物來源的生物農藥的興趣逐漸增加，因其被認為較安全，且昆蟲較不易產生抗藥性。本研究探討了兩種植物——茵陳蒿（*Artemisia capillaris*）和棕葉狗尾草（*Setaria palmifolia*）的己烷與乙酸乙酯萃取物，這兩種植物先前已被確認對小黑蚊（*Forcipomyia taiwana*）具有驅避作用，本研究則進一步評估其對埃及斑蚊(*Aedes aegypti*）幼蟲的殺幼蟲能力。研究結果顯示，茵陳蒿的己烷萃取物具有顯著效果，能夠迅速使幼蟲失去活性，作用速度甚至快於蘇利菌以色列亞種（*Bacillus thuringiensis subsp. israelensis*）的陽性對照組。在濃度高於 0.03 %時，所有或幾乎所有幼蟲在 24 h 內死亡，而在較低濃度時，即使幼蟲表現出瀕死狀態，仍有部分可恢復。根據試驗結果，24 h 的半數致死濃度（median lethal concentration, $LC_{50}$）與 90%致死濃度（$LC_{90}$）分別為 0.0188 %和 0.0526 %，而 48 h 的 $LC_{50}$ 和 $LC_{90}$ 則分別為 0.0128 %和 0.0332 %。總結而言，茵陳蒿的己烷萃取精油對埃及斑蚊幼蟲具有快速的失活作用，在 0.03 至 0.05 %之間的劑量可達 100%致死率，顯示其為一種有效的幼蟲殺蟲劑。

**關鍵詞：**生物農藥、殺蟲劑、蚊子、病媒防治、艾草