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A quick insecticide bioassay with mosquitoes【Scientific note】

以蚊子幼蟲作為篩選殺蟲效力之生物檢定法【科學短訊】

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Abstract

In order to facilitate the high-throughput insecticide screen, a simple bioassay is developed using mosquito larvae Aedes aegypti as an indicator. The minimum inhibition concentration (MIC-24h) approach, instead of the traditional half-lethal concentration (LC50-24h) approach, is used to evaluate the compounds insecticidal effects. The MIC-24h of carbaryl, trichlorfon, permethrin, spinosad and Bacillus thuringiensis israelensis matched well with their respective LC50-24h data. This new bioassay is developed in a 96-well microtiter plate format. This mini scale setup can handle thousands of samples per day and requires only trace amount of sample volume. Several natural products have been tested in this new system with promising results.

摘要

為了能快速篩選具有殺蟲成分的化學物質，本文敘述以埃及斑蚊 (Aedes aegypti) 幼蟲做為指標所開發出的一種簡單、有效率的生物檢定法，以提供未來殺蟲劑開發研究之用。主要的方法是以最低抑制濃度 (MIC-24h) 法來取代傳統半致死濃度 (LC50-24h) 方式作為評估化學物質的殺蟲效力。五種殺蟲劑，包括：加保利、三氯松、百滅寧、賜諾殺及蘇力菌，所有的MIC-24h皆與其所對應藥劑之LC50-24h數據相符。此新發展的生物檢定法是在96孔的微量滴定盤上所進行，利用此微量滴定設備可以在一天之內處理數千個樣本。本文中亦就數種天然產物進行殺蟲效力評估，並呈現出具體的結果。

Key words: MIC, LC50, quick insecticide bioassay, Aedes aegypti, natural products

關鍵詞: 最低抑制濃度、半致死濃度、快速殺蟲劑生物檢定法、埃及斑蚊、天然產物。

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A quick insecticide bioassay with mosquitoes

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ABSTRACT

In order to facilitate the high-throughput insecticide screen, a simple bioassay is developed using mosquito larvae *Aedes aegypti* as an indicator. The minimum inhibition concentration (MIC-24h) approach, instead of the traditional half-lethal concentration (LC_{50}-24h) approach, is used to evaluate the compounds insecticidal effects. The MIC-24h of carbaryl, trichlorfon, permethrin, spinosad and *Bacillus thuringiensis israelensis* matched well with their respective LC_{50}-24h data. This new bioassay is developed in a 96-well microtiter plate format. This mini scale setup can handle thousands of samples per day and requires only trace amount of sample volume. Several natural products have been tested in this new system with promising results.

Key words: MIC, LC_{50}, quick insecticide bioassay, *Aedes aegypti*, natural products

Introduction

Simple technique and fast turn-around time are two key considerations in any high throughput insecticide screen. We used yellow-fever mosquito, *Aedes aegypti,* larvae as a preliminary indicator. This insect has been widely used as a test organism for insecticide research due to its high sensitivity, easy maintenance, and excellent hatchability (Mulla *et al*., 1982). In addition, *Aedes aegypti* has a huge data base as an insecticidal indicator. Its rich data base enables scientists to compare their research results with previous publications. We have decided to set up a bioassay screen with this mosquito in the Department of Entomology, National Taiwan University, to screen for insecticides from natural products. Traditionally, almost all mosquito larvicide activities are reported in the LC_{50} format; the concentration of test compounds that provide 50% control of the population. This LC_{50} format however, creates a big challenge in our high-throughput screen as 20 insects per treatment will need to be counted out. Exact counting is not practical when thousands of compounds are scheduled to be assayed per day. A different approach is therefore needed. We report here a Minimum Inhibition Concentration (MIC) approach for insecticide screen. The MIC approach is commonly used in antimicrobial studies where organisms are checked against a series of test concentrations. The lowest concentration that kills off all test organisms is defined as MIC. This

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approach does not require exact counting as a compound's efficacies are recorded as total kill (+), partial kill (±), and no kill (-). To further facilitate this high output screen, we developed this assay in the 96-well microtiter plate format. Therefore, 96 samples can be screened in one batch, and up to 20 batches can be assayed in one day. This mini scale setup also enables us to test samples as small as 100 μl.

To verify the functional diversity of this bioassay, several commercial mosquito larvicides, representing different mode of actions, are included in the bioassay development. As one of the major natural products is from fermentation, and the fermentation broth is a very complex matrix, we also conducted a matrix effects study as part of the assay development.

We are here to report the development of this new assay system along with some preliminary test results from several natural products.

Materials and Methods

Test insects: Yellow fever mosquito eggs (*Aedes aegypti* Bora, a susceptible strain from Prof. E. L. Hsu, National Taiwan University).

General-purpose culture media (LB broth, lot 6F1262) from Bioshop (tryptone 10 g/l, yeast extract 5 g/l, sodium chloride 10 g/l).

Test chemicals: carbary, trichlorfon, permethrin, *Bacillus thuringiensis israelensis* (Bti) (above-mentioned are from Prof. E. L. Hsu) and spinosad (aka Conserve is from DowAgro Sciences Company, Taiwan).

Natural product samples: Essential oil from leaves of indigenous cinnamon (from GetLowers Company), extracts of sea lilies and Gracilaria (from Prof. T. M. Lee, National Sun Yat-sen University), plant extract fractions (from Prof. S. S. Cheng, National Taiwan University) and fermentation broth samples (from Dr. H. H. Chiu, National Taiwan University).

Insect preparation: Hatch out yellow fever mosquito eggs in 1 liter distilled water with 5 mg fine-ground Purina Laboratory Chow and 5 mg yeast extracts. Maintain mosquito larvae in this solution at room temperature for 4-5 days until they reach the 3rd instar stage. At the end of each study, kill off all larvae with boiling water or by sonication.

Bioassay (4-6 replicates): Prepare 1% stock solution for all tested compounds with methanol. Make 11 serial dilutions (5 X) from the stocks with methanol. Transfer 100 μl each of these 11 dilutions to a 96-well microtiter plate. Evaporate test solutions to complete dryness by a heat block. Store plate at 4°C until assay. At assay, reconstitute samples with mild antibiotic solution to prevent contamination from air-born microorganisms. The 0.01% Keflin solution at 100 μl per well rate is able to inhibit any air-born microorganisms without hurting the larvae. To improve the sample homogeneity within the cells, place the whole microtiter plate in a sonicator for approximately 1 minute. Then transfer 100 μl distilled water with 3-10 3rd instar mosquito larvae with the pipetman repeat pipette to each well with sample being reconstituted with Keflin solution earlier. Record larvae mobility and mortality at 24 hrs post treatment with "+" for no movement, "±" for some movement, "-" for normal. Place microtiter plate on the light box for easier observation. Then determine minimum inhibition concentrations (MIC) of each tested compounds. Natural products (essential oil, marine extracts and fermentation broths) were also diluted with methanol and tested following the same procedure.

Matrix effects study (4-6 replicates): Make 5 serial dilutions (10 X) from the 0.01% carbaryl solution with LB fermentation broth and with methanol. Then transfer 100 μl of those mixtures to a microtiter plate. Followed the same test procedure and recorded insect mortality at 24 hr post treatment. Used LB fermentation broth as control in this study.

Table 1. Responses* of *Aedes aegypti* larvae to 5 common insecticides

	ng/ml											
Compounds	10,000,000	2,000,000	400,000	80,000	16,000	3,200	640	128	25.6	5.1	1.0	0.0
Bti	+	+	+	+	+	+	+	+	+	±	-	-
Carbaryl	+	+	+	+	+	+	+	+	±	-	-	-
Permethrin	+	+	+	+	+	+	+	+	+	+	±	-
Spinosad	+	+	+	+	+	+	+	+	+	±	-	-
Trichlorfon	+	+	+	+	+	+	+	+	±	-	-	-

* "+" means total kill, "±" means partial kill, "-" means no kill at 24 hr.

Table 2. LC_{50} and MIC values of five insecticides against mosquito larvae

Compounds	Test species	24 hr LC_{50} (ng/ml)	Selected-References	Test species	24 hr MIC (ng/ml)
Permethrin	*Aedes triseriatus*	8.4	Cilek *et al*., 1995	*Aedes aegypti*	5.1
Spinosad	*Aedes aegypti*	55.0	Dariet and Corbel, 2006	*Aedes aegypti*	25.6
Bti	*Aedes aegypti*	60.0	Mittal *et al*., 2001	*Aedes aegypti*	25.6
Carbaryl	*Aedes aegypti*	167.0	Shamann *et al*., 1993	*Aedes aegypti*	128.0
Trichlorfon	*Aedes punctor*	178.0	Rettich, 1977	*Aedes aegypti*	128.0

Results and Discussion

The responses of the yellow fever mosquito larvae to different tested compounds are listed on Table 1.

The minimum inhibition concentrations (MIC) of compounds used in descending order were permethrin, spinosad, Bti, carbaryl, and trichlorofon. With no exception, the MIC values were slightly less than the LC_{50} values from the respective compounds (Table 2). This was troubling at first as the MIC values in theory, should be higher, not lower than the LC_{50} values providing all testing conditions were the same. But later, it was recognized that all previous LC_{50} reports were generated by testing against the 4^{th} instar of mosquito larvae while we used the 3^{rd} instar larvae in this MIC study. Older larvae are well documented to be more tolerable to insecticides than their younger counterparts (Pantuwatana and Youngvanitsed, 1984; Waldstein and Reissig, 2001). In the serendipity term, younger instar of *Aedes aegypti*, played a key role in the spinosad discovery due to its higher sensitivity than their older counterparts (Chio, 2007). Therefore, in my opinion, the respective MIC values matched reasonable well with the reported LC_{50} values from the same or closely related species.

The spiking study results in Table 3 showed that the MIC value generated in the LB broth matrix was 100 ng/ml which exactly matched results from its methanol counterpart. This suggested that the LB broth did not cause any matrix effects on this assay. Data also suggested that the common LB fermentation broth was nontoxic to the mosquito.

Several natural products showed decent insecticidal activities (Table 4). The sea lilies extract was the best and killed all mosquitoes at 10 parts per million level (ppm). Prof. T. M. Lee informed me recently that more marine samples would come in the near future. The essential oil from cinnamon killed mosquito at 50 ppm level. A separated study showed that this essential oil also showed

Table 3. Responses* of *Aedes aegypti* larvae to carbaryl solutions made up in methanol or in LB broth

Carbaryl concentration (ng/ml)	In methanol	in LB broth
10,000	+	+
1,000	+	+
100	+	+
10	±	±
1	-	-
0	-	-
Methanol	-	-
Broth	-	-

* "+" means total kill, "±" means partial kill, "-" means no kill at 24 hr.

Table 4. MIC values of 4 natural products against the *Aedes aegypti* larvae

Natural products	Source	24 hr MIC (ppm)
Sea Lilies	Marine	10
Cinnamyl acetate+ Cinnamaldehyde	Plant	50
Gracilaria-2	Marine	1,000
Gracilaria-1	Marine	10,000
Plant extract fractions	Plant	?
Fermentation broths	Actinomycetes	›100,000

strong repellent effects upon *Aedes aegypti* adults (personal communication with Prof. E. L. Hsu). Further studies are warranted for this essential oil.

The result of the plant extract fractions study was not listed here. That study was incomplete as samples were discarded prematurely by mistake. However, its 2 hrs evaluation suggested that the positive control (Pine) and 2 other fractions (H2 and H4) started showing insecticidal activities at 2 hrs. We plan to reevaluate the insecticidal activities of those plant extract fractions in the near future. None of the 27 fermentation broth samples showed any activity at 100,000 ppm level in this bioassay. Those samples did not show any antimicrobial activity either. We speculated that there were no or very low secondary metabolites in the samples. Dr. Hsiu-Hui Chiu promised to look into the fermentation conditions closely. Keep in mind that those natural products are crude extracts. We are calculating their potency assuming they are 100% pure which is most likely overestimated. Therefore, their true potencies are unknown but should be better than that reported here.

Conclusion

This bioassay is able to detect insecticidal activity from different classes of insecticides and several natural products with reasonable accuracy. It requires only trace amount of materials and can handle high volume of sample submission. Data suggests that it a useful tool for finding novel insecticides from natural products.

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以蚊子幼蟲作爲篩選殺蟲效力之生物檢定法

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摘　要

爲了能快速篩選具有殺蟲成分的化學物質，本文敘述以埃及斑蚊 (*Aedes aegypti*) 幼蟲做爲指標所開發出的一種簡單、有效率的生物檢定法，以提供未來殺蟲劑開發研究之用。主要的方法是以最低抑制濃度 (MIC-24h) 法來取代傳統半致死濃度 (LC_{50}-24h) 方式作爲評估化學物質的殺蟲效力。五種殺蟲劑，包括：加保利、三氯松、百滅寧、賜諾殺及蘇力菌，所有的 MIC-24h 皆與其所對應藥劑之 LC_{50}-24h 數據相符。此新發展的生物檢定法是在 96 孔的微量滴定盤上所進行，利用此微量滴定設備可以在一天之內處理數千個樣本。本文中亦就數種天然產物進行殺蟲效力評估，並呈現出具體的結果。

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