[Jpn. J. Parasitol., Vol. 41, No. 6, 519–526, December, 1992]

In vitro Assessment of Morbidity of *Toxocara canis* Larvae Using a Dye Exclusion Assay

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(Accepted for publication; November 20, 1992)

Abstract

A dye exclusion assay was developed for *in vitro* evaluation of larvicidal activity of drugs on the second-stage larvae of *Toxocara canis*. This assay is based on the fact that viable larvae are not stained with 1% gentian violet, while dead larvae are stained with this solution. Using this method, the larvicidal activities of five compounds [chalcone, *n*-capric acid, hexyl 2,4-dihydroxybenzoate (HDB), asarone, and piperamide-C11:O (PA)] were assessed, and the results were compared with those of the relative mobility assay described previously (Kiuchi *et al.*, 1987). For chalcone, HDB and PA, a good correlation was observed between the dye exclusion ability and mobility of the larvae. Both indices decreased simultaneously, which would be expected for the usual mode of larvicidal action of drugs. However, asarone did not affect the dye exclusion ability but strongly inhibited their mobility. This inhibition rapidly recovered when the medium was replaced. On the other hand, with *n*-capric acid, the dye exclusion ability was partially recovered but the mobility was unchanged after replacement of the medium. These results indicate that the dye exclusion assay using 1% gentian violet is an useful tool for the estimation of the nematocidal effect of drugs *in vitro*.

Key words: *Toxocara canis*, anthelmintics, larvicidal activity, *in vitro* assay, dye exclusion assay, relative mobility assay

Introduction

Toxocariasis, a zoonotic parasite infection caused by larvae of the species *Toxocara, T. canis* or *T. cati,* is still difficult to treat, since there are no known effective anthelmintics. To find new drugs which are effective against this parasite, we have been screening nematocidal (or larvicidal) compounds from various sources by means of an *in vitro* assay using the second-stage larvae of *T. canis* (Kiuchi *et al.*, 1987). The assay method is based on assessing changes in larval mobility under the influence of test samples. The larvicidal activity of the sample is evaluated in terms of a relative mobility (RM) index. A small RM index indicates a stronger larvicidal activity, and when all larvae have died, the index becomes zero. The activity of a sample is represented by the minimal larvicidal concentration (MLC), which is the minimum concentration that gives RM = 0 at 24 hrs of incubation.

In the RM assay, usual anthelmintics such as santonin, kainic acid, diethylcarbamazine, milbemycin, and ivermectin were ineffective, and benzimidazole derivatives were only weakly effective, suggesting that they are not larvicidal. However, several extracts of plant materials and spices showed moderate to strong activity (Kiuchi *et al.*, 1989).

There is the question of whether the inhibition of mobility corresponds to the death of the larvae: i.e., whether the decrease of the RM index actually represents the larvicidal activity of the sample. To elucidate this point, we developed a

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new method for estimating larval morbidity using a dye exclusion assay (DE assay), and compared the results with those of the RM assay.

Materials and Methods

Chemicals

Dyes (gentian violet, methylene blue, crystal violet, trypan blue and evans blue) were purchased from Wako Pure Chemical Industries, Ltd., Tokyo, Japan. All dyes were dissolved in phosphate buffered saline (PBS, 50 mM, pH 7.2).

The activities of the following five compounds were tested (Fig. 1): *n*-capric acid, asarone (*Z/E*=7/3), chalcone, hexyl 2,4-dihydroxybenzoate (HDB), and piperamide-C11:0 (PA). *n*-Capric acid, PA and asarone are the larvicidal principles of *Areca catechu* (Kiuchi *et al.*, 1987), *Piper nigrum* (Kiuchi *et al.*, 1988b) and *Acorus calamus* (Ali *et al.*, 1991), respectively. HDB and chalcone are synthetic analogs of active principles of *Usnea purnustri* (Ahad *et al.*, 1991) and *Tephrosia purpurea* (Kiuchi *et al.*, 1988a), respectively. *n*-Capric acid and asarone were purchased from Tokyo-kasei Kogyo Ltd., Japan and Aldrich Chemicals, U.S.A., respectively. Chalcone, PA, and HDB were synthesized in our laboratory. The MLC of these compounds as determined by the RM assay are: *n*-capric acid, 0.17 mM; asarone, 1.2 mM; chalcone, <0.1 mM; PA, 0.02 mM; and HDB, 0.035 mM. These compounds were dissolved in PBS at the following concentrations: *n*-capric acid, 0.05 mg/ml; asarone, 1.0 mg/ml; chalcone, 0.1 mg/ml; PA, 0.2 mg/ml, and HDB, 0.1 mg/ml. At higher concentrations the larvae were killed too rapidly to correctly estimate the mechanisms of larvicidal activity.

Second-stage larvae of T. canis

Newly-hatched second-stage larvae were collected by the modified procedure of de Savigny (1975), maintained in a culture medium (Eagle's MEM, Nissui, Tokyo, Japan) until use (Kondo *et al.*, 1984). Larvae which had been cultured for less than 6 months were used for the following assay.

To examine the stainability of dead larvae, the larvae were killed either by contact with hot PBS (approximately 70°C) for a few seconds, or by incubation with HDB (1 mg/ml) for 3 hrs. The dead larvae were then mixed with a dye solution and the stainability was observed as described below.

Assay

The larvae were incubated with the test compounds in a 96-well plate (Corning, U.S.A.) and the RM assay was performed as previously described (Kiuchi *et al.*, 1987). The total volume of the medium was 200 μl per well.

In the dye exclusion assay (DE assay), 10 μl

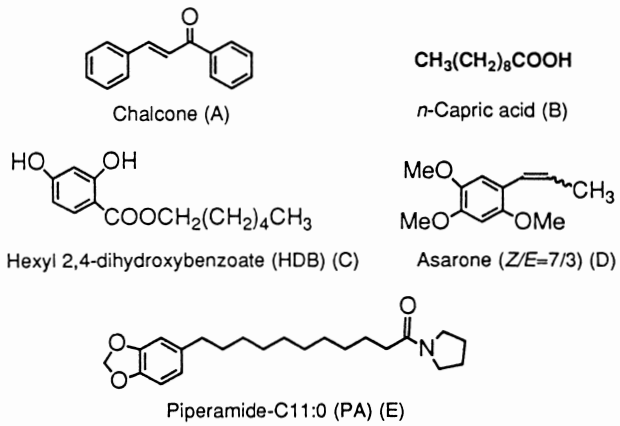

Fig. 1 Chemical structures of larvicidal compounds used in this investigation.

of appropriately diluted dye solution was dropped on a slide glass and mixed with an equal volume of the test medium containing approximately 20 larvae. The mixture was covered with a 22×22 mm glass. After being maintained at room temperature for 10 minutes, the stainability of the larvae was observed under an inverted microscope. The drug-induced stainability of the larvae was expressed by the dye exclusion index (DE index) as follows:

$$\text{DE index} = \frac{\Sigma nN_n}{3N} \times 100$$

where N is the total number of larvae observed and N_n is the number of larvae with a score of n, where

score 3: no staining was observed on and in the body except in the mouth,

score 2: partially (less than one third of the whole body) stained with light purple color,

score 1: partially stained with deep purple color, and

score 0: more than half of the body was stained with deep purple color.

The RM assay was performed concurrently. Note that a decrease of the RM index indicates a reduction of larval mobility, while an decrease of the DE index means cellular death of the larvae.

In addition to the above assays, we examined whether or not the larvae were able to recover their RM or DE index when the incubation medium was replaced with the fresh medium. When either the RM or DE index reached zero, the larvae were transferred to a 10-ml tube which was filled with 8 ml of fresh MEM, and centrifuged at 1500 rpm for 5 min. The supernatant was discarded. Two hundred μl of fresh MEM was then added to the tube. The fresh larvae-containing medium was incubated in a 96-well plate for 2 consecutive days, and then RM and DE indices were evaluated.

Results

Stainability of the larvae with dye solutions

Table 1 summarizes the effects of five dyes on living and dead larvae of *T. canis*. All of the larvae killed by either hot PBS or HDB were stained deep purple with dye solutions (Figs. 2B, 2D and 2E), except for 0.1% trypan blue and 0.1% evans blue, which stained dead larvae only faintly.

On the other hand, stainability of living larvae varied depending on the nature of the dye solution. In 1% and 0.1% gentian violet solutions, the buccal cavities and esophagi of the larvae were stained (Fig. 2A), suggesting that living larvae took the dye solution into their bodies either actively or passively. Other body parts were not stained. When the dye concentration rose to 5%, the entire bodies of all larvae was stained deep purple, and some burst. In 1% methylene blue and crystal violet solutions, all larvae were partially stained blue. Their bodies were swollen

Table 1 Stainability of living and dead larvae of *Toxocara canis* treated with dyes

Dye	Concentration (%)	DE index†	
		Living larvae	Dead larvae
Crystal violet	1	43	0
Evans blue	0.1	100*	33
Gentian violet	0.1	100	0
	1	100	0
	5	60	0
Methylene blue	1	60	0
Trypan blue	0.1	98	67

*: some larvae were shrunken but not stained (see Fig. 2F)

†: Larval stainability index (see text)

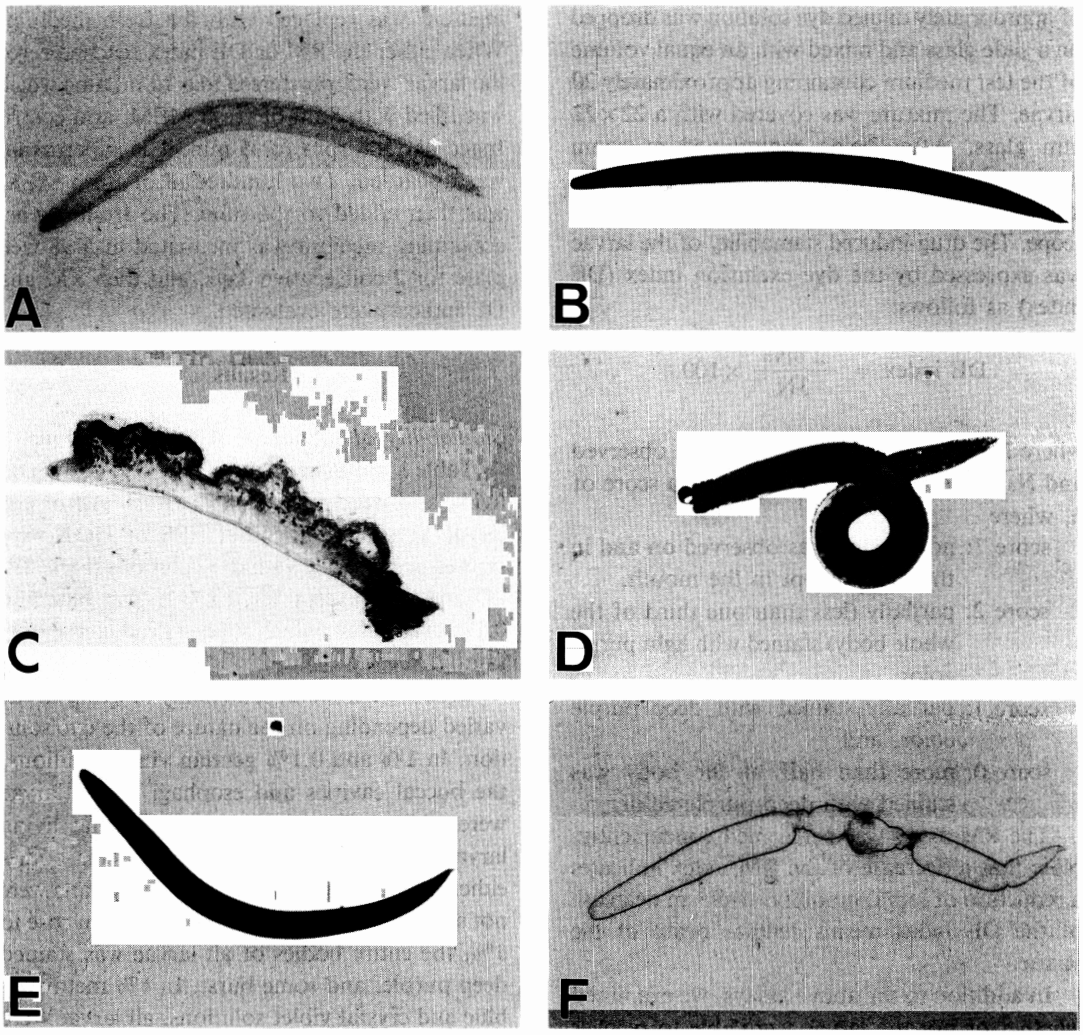

Fig. 2 Stainability of the second-stage larvae of *Toxocara canis* with dye solutions. (A) A living larva stained with 1% gentian violet. Only the buccal cavity was stained. (B) A larva killed with HDB and stained with 1% gentian violet. (C) A living larva stained with 1% crystal violet. The cuticle appeared to be melting. (D) A larva killed with HDB and stained with 1% methylene blue. (E) A larva killed with heat and stained wih 1% crystal violet. (F) A living larva stained with 0.1% evans blue. The larva is not stained, but the entire body was shrunken.

and cuticles appeared to be melting (Fig. 2C). In 0.1% evans blue solution, living larvae were not stained, but every body area was shrunken (Fig. 2F). In the 1% solution, the larvae were stained deep blue and broken-down, with their contents exposed. These larvae were stained deep blue.

Based on these observations, we chose 1% gentian violet as the best reagent for the DE assay, because the dye at this concentration stained dead larvae deep purple, but did not stain living larvae.

Comparison of RM assay and DE assay

Fig. 3 shows the changes in the RM and DE indices after incubation of larvae with the five test compounds. The RM and DE indices of chalcone decreased concurrently, suggesting that the reduced mobility and cellular death of the

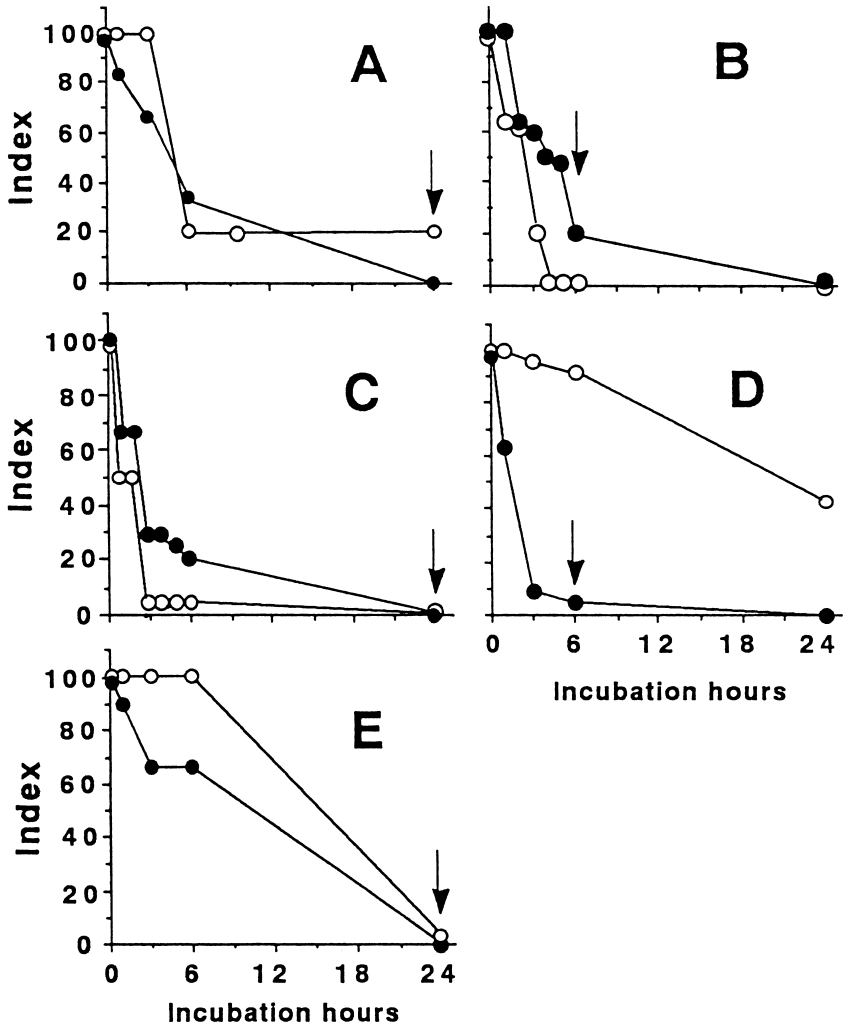

Fig. 3 Comparison of relative mobility index (RM, –•–) and dye exclusion index (DE, –o–) of the second-stage larvae of *T. canis* under the action of chalcone (A; 0.1 mg/ml), *n*-capric acid (B; 0.05 mg/ml), HDB (C; 0.1 mg/ml), asarone (D; 1.0 mg/ml), and PA (E; 0.2 mg/ml). Arrow indicates the time when the medium was replaced (see Fig. 4).

worm occurred simultaneously (Fig. 3A). For *n*-capric acid and HDB, the DE indices decreased more rapidly than the RM indices, suggesting that, with these compounds, cellular death occurred before depression of mobility (Figs. 3B and C). On the other hand, the RM indices for asarone and PA decreased faster than the decrease of their DE indices, suggesting that these drugs suppressed larval mobility before cellular death began (Figs. 3D and E).

Recovery of RM or DE indices after replacement of the medium

For all compounds, both RM and DE indices were zero or very low levels at 24 hrs of incubation, and both were unchanged after replacement of the medium (Fig. 4A, 4C and 4E). However, after a shorter incubation period, the RM and

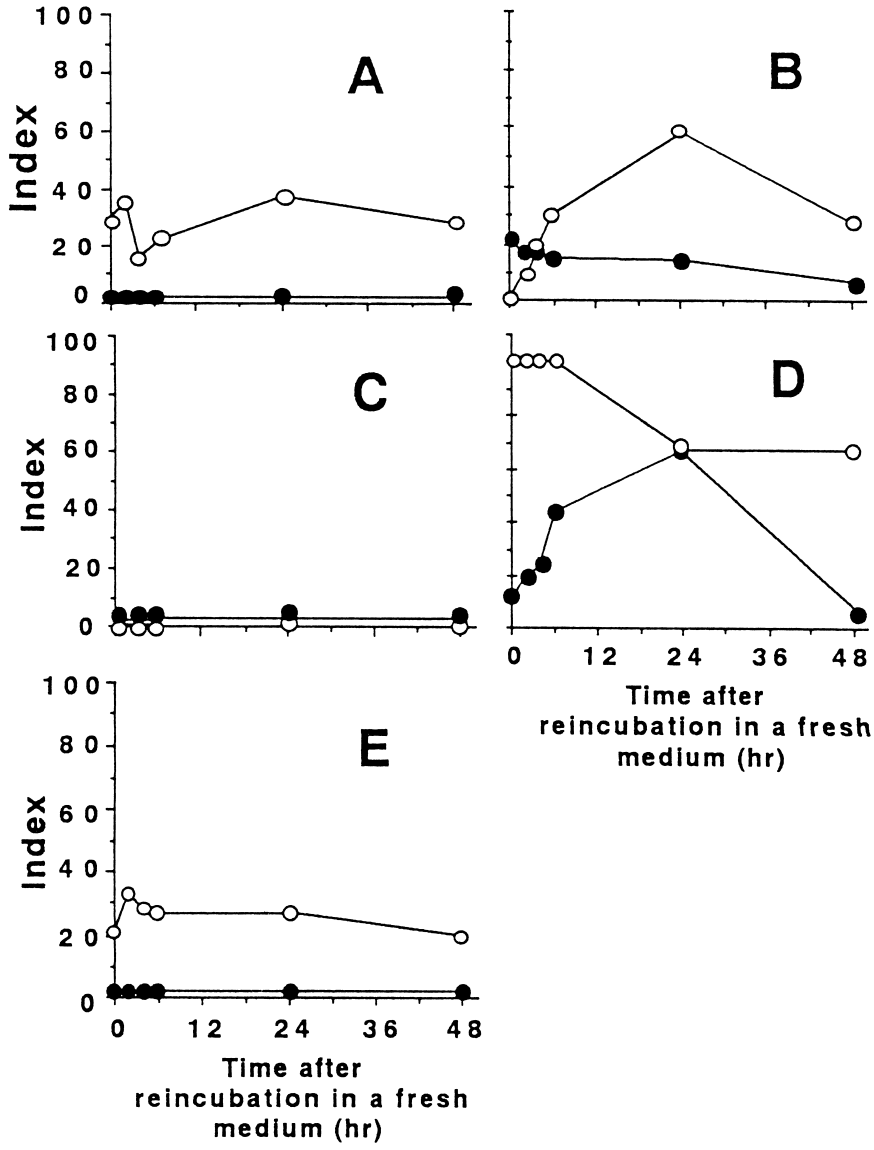

Fig. 4 Recovery of relative mobility index (RM, –•–) and dye exclusion index (DE, –o–) of *T. canis* larvae after replacement of the medium at 24 hrs for chalcone (A), HDB (C) and PA (E), and at 6 hrs for *n*-capric acid (B) and asarone (D).

DE indices of *n*-capric acid and asarone, respectively, changed after replacement of the medium. For *n*-capric acid, the RM index was about 20 and the DE index was 0 at 6 hrs of incubation (Fig. 3B). Replacement of the medium at this stage resulted in an appreciable recovery of the DE index, to a maximum of 59, without producing a significant change in the RM index (Fig. 4B). When incubated with asarone, the RM index of the larvae decreased rapidly and reached zero within 6 hrs, but the DE index remained relatively unchanged at a high level (Fig. 3D). When the medium was replaced at this stage, the RM index increased rapidly and reached a

maximum of 67 after reincubation, indicating that more than half of the larvae recovered their mobility (Fig. 4D).

Discussion

Although several *in vivo* studies on anthelmintics in mice infected with *T. canis* have been reported, the results are indefinite (Abdel, 1984; Abo and Herbert, 1984; Carrillo and Barriga, 1987; Delgard *et al.*, 1989; Cuellar *et al.*, 1990). Moreover, an *in vivo* assay is time-consuming and requires tremendous effort to assess the effectiveness of samples (Parsons and Grieve, 1990). On the other hand, if the proper conditions is chosen, an *in vitro* assay is easy to perform and gives consistent, reproducible results. Therefore, it is reasonable to use an *in vitro* method to select effective drugs from several samples. However, the question of whether the *in vitro* result is transferable to the *in vivo* assay still remains.

In addition to the RM assay previously reported (Kiuchi *et al.*, 1987), we now have the new *in vitro* assay (DE assay). This assay is based on the fact that viable cells in a physiological salt solution exclude a variety of dyes, while dead cells do not exclude the dyes and would be stained (Shrek, 1936; Phillips and Terryberry, 1957; Kaltenbach *et al.*, 1958). In the present study, 1% gentian violet solution gave the best results for this purpose.

All of the samples used in this study strongly inhibited larval mobility in the RM assay, though their structural characteristics are different. These five compounds were applied at concentrations slightly higher than their MLCs, since at higher concentrations the larvae were killed too rapidly to correctly observe the mechanisms of larvicidal activity.

Compared to the RM assay, the DE assay described here may have a unique advantage. While the RM assay describes the depression of larval mobility under the influence of drugs, the DE assay estimates the degree of cellular death of the larvae.

The RM and DE indices for chalcone, HDB and PA decreased concurrently and neither index recovered after replacement of the medium, suggesting that inhibition of mobility and cellular death were closely related for these compounds. However, these results were sometimes different after only a short-term contact with the drugs. For asarone, the DE index remained at a high level when the RM index was zero (6 hrs after incubation), and the RM value recovered rapidly after replacement of the medium. For *n*-capric acid, the DE index rapidly reached zero before the RM decreased to a low level (6 hrs after incubation), and the DE index recovered rapidly, while the RM index remained unchanged below 20 after replacement of the medium. These discrepancies may be attributable to differences between the larvicidal mechanisms of these two compounds. Recovery of the RM index after a short-term incubation may indicate that asarone acts as a temporal paralytic against the larvae without causing cellular death. Recovery of the DE index in *n*-capric acid may be explained by assuming that *n*-capric acid reversibly interacts with larval cell membrane. Since *n*-capric acid has a structure similar to fatty acids which constitute the cell membrane, it should cause changes in permeability (Sŭbík *et al.*, 1977) and surface death, as well as a depression of mobility. Therefore, removing *n*-capric acid should produce partial recovery of the DE index, while larval mobility is still inhibited, because the larvae may have been irreversibly damaged. Further study is necessary to clarify the actual mechanisms of these compounds.

The above examples indicate that there are several mechanisms of mobility inhibition and that a decrease of the RM index does not always signify death of the larvae. Therefore, in the case of asarone, the two methods (RM and DE assay) discussed in this paper are complement each other to clarify the mechanisms of larvicidal drugs, and the dye exclusion assay using 1% gentian violet is an useful tool for the estimation of the nematocidal effects of drugs.

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