

## LARVICIDAL ACTIVITY OF *TAGETES MINUTA* (MARIGOLD) TOWARD *Aedes Aegypti*

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**ABSTRACT.** The steam distilled oils of 3 species of marigold, *Tagetes patula*, *T. erecta* and *T. minuta*, were tested for larvicidal activity toward third instar *Aedes aegypti*; activity at 10 ppm was demonstrated only for *T. minuta*. The larvicidal property of the whole oil dispersed in water persisted for at least 9 days. The terpene, ocimenone, which is a part of the whole oil, was found to be larvicidal only at a higher concentration than the whole oil and to lose its activity within 24 h after dispersal in water. These results suggest a potential utilization of oil of *T. minuta* or its components for the control of *Ae. aegypti* and other species of mosquitoes.

### INTRODUCTION

In folklore claims (Neher 1968), marigolds are commonly believed to be useful plants for insect control. The scientific literature gives credibility to these beliefs in at least 3 instances: a nematode has been isolated as a root exudate from various species of marigolds (Uhlenbroek and Bijloo 1958, 1959; Downum and Towers 1983); the oil of *Tagetes minuta* Linn. is reported to contain components that act as a juvenile hormone mimic (Saxena and Srivastava 1973); and the oil of *T. minuta* is reported to act as a fly oviposition repellent (Monnig 1936). However, oil of *T. minuta* is not a mosquito repellent (McCullough and Waterhouse 1947).

In an investigation stimulated by folklore claims, Maradufu et al. (1978) reported that the steam distilled oil of *T. minuta* contained a mosquito larvicide active against *Aedes aegypti* (Linn). The active principle was assigned to 5E-ocimenone, a long-known natural component of marigold oils (Jones and Smith 1925, Jones 1926, Naves 1948). Unfortunately, 5E-ocimenone isolated from the plant was not stable in water. According to Maradufu et al. (1978), "introduction of fresh larvae to a 24-h-old mixture of 5E-ocimenone-water did not cause the death of the larvae." However, fresh larvae were not added to a 24-h old mixture of the whole oil. In our work, this important control experiment was carried out. The results of this and related experiments show that 5E-ocimenone is not solely responsible for the mosquito larvicidal activity of the steam distilled oil of *T. minuta* and that other stable components probably are responsible.

### MATERIALS AND METHODS

Seeds of *Tagetes patula* Linn. and *Tagetes erecta* Linn. were obtained from W. Atlee Burpee Co.; seeds of *T. minuta* were provided by J. O. Kokwaro, University of Nairobi, Kenya, and by the Brooklyn Botanic Garden Research Center. The plants were grown in a soilless mix (Redi-Earth Peat-Lite Mix, Grace Horticultural Products) in an automatically ventilated, screened research greenhouse under natural light (6,458–16,146 lumens/m<sup>2</sup>) supplemented by 8 h additional illumination (12,917–16,146 lumens/m<sup>2</sup>, high pressure sodium lamps), 24°C day/16°C night temperature cycle, 50–85% RH. Young plants were fertilized with a 15-30-15 plant food and again at the beginning of flowering. No pesticides were applied to the test plants. Additional marigolds were grown in a fenced field plot in 24-m rows of rototilled Charlton loam soil (pH 6.1). Seedlings were thinned to 2–5 cm apart. The plants were fertilized with a 5-10-5 plant food at 0.45 kg/9.3 m<sup>2</sup> as a side dressing at the beginning of flowering. Supplementary watering was applied during dry periods. No pesticides or herbicides were applied.

The above-ground parts and some roots from marigolds in the greenhouse and field plot were harvested during the period of maximum flowering. The plant material was placed in plastic bags and stored for a few days at 30°C until treatment.

The stems, leaves and flowers were triturated with about 400 ml of distilled water to a medium-fine texture in a commercial stainless steel blender (Scovill, Inc.). The mixture was then suction filtered through a Buchner funnel and Whatman #1 filter paper. The drained, moist mass was transferred to clean, labeled aluminum foil freezer containers. The filtrate was reused throughout this procedure for each batch of plants processed and replenished to original volume as needed. Prior to sealing the containers, this filtrate was apportioned to each container of blended material; all pans were filled to weigh

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approximately 2 kg. The sealed containers were stored at  $-20^{\circ}\text{C}$ .

The essential oil was obtained in one stage of the work, by classical steam distillation of 500 g portions of chopped, frozen plant material for 4–6 h. The distillate was extracted twice with pentane, and the pentane solution stored under nitrogen in amber bottles, with Teflon capliners at  $-20^{\circ}\text{C}$  until needed for analysis. Alternatively, 200–300 g of chopped, frozen plant material was steam distilled for 4 h, with pentane as the organic solvent, in a simultaneous steam distillation apparatus (J&W Scientific Corporation<sup>4</sup>). Loss of volatiles was minimized by use of a cold-finger containing a dry ice/methanol slurry, installed on top of a condenser. The pentane containing oil was stored in the 50-ml conical flask used with the extractor and kept under nitrogen at  $-20^{\circ}\text{C}$  until needed. Prior to analysis the oil was obtained by removal of the pentane on a rotary thin film evaporator without heat. All oils were stored in 0.3-ml ReactiVials (Pierce Chemical Co.) with Teflon-faced capliners under nitrogen at  $-20^{\circ}\text{C}$  until analysis.

Ocimenone, 2,6-dimethylocta-2,5,7-trien-4-one, was prepared as a mixture of isomers according to the literature (deVilliers et al. 1971, Adams et al. 1975).

Mosquito larvae from the Rockefeller strain of *Ae. aegypti*, which has been in continuous culture at Rutgers University since 1935, were used in these tests. Between 250–500 larvae were hatched 3 days prior to the bioassay in enameled pans ( $33 \times 21.6 \times 5$  cm) containing 1 liter of distilled water; approximately 6–8 pans were required for each bioassay. The larvae developed at  $27^{\circ}\text{C}$  and were provided daily approximately 300 mg/pan/day of ground hog supplement (Agway). The water was skimmed daily to remove surface scum. The pans were transferred to an incubator at  $30^{\circ}\text{C}$  for the final 12 h prior to the test. At the time of the bioassay the larvae were at mid-3rd instar. Larvae from several pans were washed in a cloth strainer with 1 liter of distilled water and transferred to clean pans containing 1 liter of distilled water.

The test substances were volumetrically diluted in absolute ethanol (USP grade) at a stock concentration of  $2.5 \mu\text{l/ml}$  (v/v) such that upon

dilution a final concentration of the specified ppm (v/v) of oil was achieved.

Aliquots of 224 ml of distilled water were added to 250-ml test beakers. One ml of the test solution was added to each beaker, followed by 25 larvae in 25 ml of distilled water. The beakers were arranged on a tray, covered loosely with a sheet of aluminum foil and placed in an incubator at  $30^{\circ}\text{C}$ . At intervals thereafter, the beakers were removed and the number of dead and living individuals and their respective stages of development were recorded. Mortality was established as the lack of head-to-tail flexion in response to tapping the beaker with the end of a pencil. "Sluggishness" was characterized by the larvae appearing at the surface, pointed downward, without consistent head-to-tail flexion response to beaker tapping; failure of sluggish larvae to respond was confirmed by prodding the larvae with a pencil tip.

## RESULTS AND DISCUSSION

A mixture of Z and E ocimenone stereoisomers (Weyerstahl et al. 1986) was synthesized according to the literature (deVilliers et al. 1971, Adams et al. 1975); these authors suggest that the isomers are isolated in a ratio of 9:1. The isomer mixture we obtained was subjected to gas chromatography/mass spectrometry (GC/MS), and it was discovered that the major chromatographic peak previously assigned to one of the isomers is actually a mixture of 2 ocimenone isomers in the approximate ratio of 3:2 and that the minor chromatographic peak is not an ocimenone. The fragmentation patterns closely match those reported by Hethelyi et al. (1987), deVilliers et al. (1971) and Adams et al. (1975). Extensive chromatographic experiments were in agreement with these conclusions (Singer 1987<sup>5</sup>).

The steam-distilled oil of *T. minuta* was also subjected to GC/MS analysis, and the chromatographic peak of identical retention time to the major peak from the synthetic ocimenone was shown to contain an identical isomeric mixture. It is unlikely that the identity of the ratio of the mixtures arises from an identical production of isomeric ocimenones in the plant and in the *in vitro* synthesis, but rather that isomerization (Boehme et al. 1963) has led both systems to the same point of equilibrium. For the bioassay work the ocimenone was used as this mixture of isomers.

<sup>4</sup> The activity of *T. patula* oil in the bioassays was strongly dependent on the method of steam distillation. Classical steam distillation led, irreproducibly, to high larvicidal activities compared with the simultaneous apparatus. This difference could be due to the decreased potential of carryover of the still pot contents into the distillate in the simultaneous system.

<sup>5</sup> Singer, J. M. 1987. Mosquito larvicidal investigation of oil of marigold. Doctoral Thesis, Polytechnic University, Brooklyn, NY.

Table 1. Larvicidal activities of steam distilled *Tagetes* oils and control substances to *Aedes aegypti*.

Substance	Concentration (ppm)	% mortality $\pm$ SD at indicated contact time			
		24 h	48 h	72 h	96 h
<i>T. minuta</i> oil	10	69 $\pm$ 26	96 $\pm$ 9	99 $\pm$ 5	99 $\pm$ 3
	4 <sup>c</sup>	20, 24	88, 88	92, 96	100, 96
	0.4		No mortality observed <sup>d</sup>		
<i>T. patula</i> oil	10	3 $\pm$ 3	1 $\pm$ 2	7 $\pm$ 3	11 $\pm$ 6
<i>T. erecta</i> oil	10		No mortality observed <sup>d</sup>		
C <sub>2</sub> H <sub>5</sub> OH <sup>a</sup>	—		No mortality observed <sup>d</sup>		
Ocimenones <sup>b</sup>	10	18 $\pm$ 3	68 $\pm$ 23	77 $\pm$ 24	78 $\pm$ 22
	40	96 $\pm$ 5	100	100	100

<sup>a</sup> 1 ml of C<sub>2</sub>H<sub>5</sub>OH to 224 ml in test beaker.

<sup>b</sup> Synthetic mixture of isomers. See text.

<sup>c</sup> Two replicates.

<sup>d</sup> In various replicate observations the standard deviation could not distinguish mortalities differing from zero.

Table 2. Time stability of the larvicidal activity of *Tagetes minuta* whole oil and ocimenones against *Aedes aegypti*.

Substance	Concentration (ppm)	Time (h) <sup>a</sup>	% mortality $\pm$ SD <sup>b</sup> at indicated contact time		
			24 h	48 h	72 h
Ocimenones	40	24	0	7 $\pm$ 11	19 $\pm$ 1
	10	24	0	28 $\pm$ 34	36 $\pm$ 28
<i>T. minuta</i> oil	10	24	100	100 <sup>c</sup>	100 <sup>c</sup>
	10	72	95 $\pm$ 5	100	—
	10	216	98 $\pm$ 1	100	—
C <sub>2</sub> H <sub>5</sub> OH	— <sup>d</sup>	72	0	16 $\pm$ 8	—
		216	0	10 $\pm$ 2	—

<sup>a</sup> Time between preparation of test solution and addition of larvae.

<sup>b</sup> Time after addition of larvae to test solution.

<sup>c</sup> Removed dead larvae and added 25 fresh larvae in 25 ml of distilled water.

<sup>d</sup> 1 ml of C<sub>2</sub>H<sub>5</sub>OH to 224 ml of water.

Table 1 shows the larvicidal activity for the oils of 3 species of marigolds, an ethanol control and the ocimenone isomers described above, as a function of time. These results generally agree with the previous findings of Maradufu et al. (1978) who tested only the oil of *T. minuta*. From these data, *T. minuta* is highly larvicidal compared with the other species *T. patula*, *T. erecta* and with the control. We observed that the larvae were sluggish before death. This sluggish behavior affected proportionately larger percentages of larvae at one of the lower concentrations used (4 ppm). However, by 72 h both concentration levels (4 and 10 ppm) produced about the same mortality. At the 0.4 ppm level of *T. minuta* oil there was essentially no mortality; however, many failed to pupate and emerge as adults in contrast to ethanol controls. Results indicated that the oil of *T. minuta* acts as a larvicide at a lower concentration than does the synthetic ocimenone; only at 40 ppm did ocimenone produce high initial mortality. The greater toxicity of the *T. minuta* oil does not

support the claim of Maradufu et al. (1978) that ocimenone is the sole active principle in the oil.

One of the key findings of Maradufu et al. (1978) was the loss of activity of the ocimenone isolated from the plant in the aqueous test mixtures with time. If found for the whole oil, this instability would preclude usage of marigold oil or its derivatives for controlling mosquito larvae. To reexamine this aspect, we mixed the appropriate concentrations of *T. minuta* whole oil or ocimenone with water, incubated the mixture at 30°C for various periods of time, and added the *Ae. aegypti* larvae. Table 2 indicates that ocimenone, as reported by Maradufu et al. (1978), in aqueous suspension for 24 h substantially loses its larvicidal activity (compare with Table 1) at 10 ppm and 40 ppm. In contrast, in our studies the whole oil maintains its activity in mixtures of whole oil and water for as long as 9 days (216 h). Parallel chromatographic experiments (Singer 1987<sup>5</sup>) on both the whole oil and the synthetic ocimenone in aqueous mixtures indicate that the ocimenone is almost entirely

decomposed after 24 h in both the synthetic samples and in the oil.

We have shown that synthetic ocimenone is less toxic to the mosquito larvae than the plant whole oil and that the plant whole oil on standing in water maintains its larvicidal activity long after ocimenone loses its activity. Therefore, we conclude that the larvicidal activity of the steam distilled oil of *T. minuta* against *Ae. aegypti* cannot be attributed only to ocimenone (Maradufu et al. 1978).

The results in Table 2, demonstrating in a laboratory environment that the whole oil in water retains larvicidal activity for at least 9 days, suggest that field evaluations are warranted. In preliminary plastic barrel tests (48 liter) at Rutgers University using 40 ppm of whole oil, *Culex pipiens* Linn. was controlled. It is likely that the material active both against *Ae. aegypti* and *Culex* would also be active against *Anopheles* mosquitoes. This likelihood, in combination with the ease of obtaining the active oil with steam and the wide geographic range of this common plant (Neher 1968, Cloudsley-Thompson 1976, Bailey and Bailey 1976, Gray 1887), suggests that this plant could play a role in the formulation of pest management strategies in some areas (Brattsen et al. 1986, Smith 1976, Casida 1976, Dame et al. 1976).

As yet there is insufficient information to identify the exact chemical or chemicals responsible for the larvicidal activity or its mode of action in the larva. In larvae exposed to lethal levels of whole oil, the anal papillae become highly swollen, suggesting that interruption of osmotic and ionic regulation may be at least part of the mechanism (Clements 1963).

#### ACKNOWLEDGMENTS

We are grateful to Carl Djerassi (Stanford University) and David Schooley (University of Nevada at Reno) for advice during the course of this work and to Charles Swanson (deceased) of Potsdam, NY, and the Adirondack Museum (Blue Mountain Lake, NY) for first suggesting to one of us (M. M. G.) to look into the folklore of the marigold-insect interaction. We also thank Revlon Corporation for allowing use of equipment necessary for this work, David Taylor, for carrying out preliminary experiments on the plant material treatment and Justin Skoble for preparing samples for the barrel tests. This is New Jersey Agricultural Experiment Station Publication No. D-40100-01-90, supported by Hatch Funds.

#### REFERENCES CITED

- Adams, D. R., S. P. Bhatnager, R. C. Cookson and R. M. Tuddenham. 1975. Simple synthesis of the atlantones, the ocimenones, the tagenones, and filifolone from isoprene. *J. Chem. Soc. Perkin I.* 1975:1741-1743.
- Bailey, L. H. and E. Z. Bailey. 1976. *Hortus* third. A complete dictionary of plants cultivated in the United States and Canada, Revised. Macmillan, New York.
- Boehme, E. E., V. Thaler and M. C. Whiting. 1963. Synthetical studies on terpenoids. Part II. The structure of tagetone. *J. Chem. Soc.* 1963:2535-2540.
- Brattsen, L. B., C. W. Holyoke, J. R. Leeper and K. F. Raffa. 1986. Insecticide resistance: challenge to pest management and basic research. *Science.* 231:1255-1260.
- Casida, J. E. 1976. Prospects for new types of insecticides. pp. 349-366. *In:* R. L. Metcalf and J. J. McKelvey (eds.). *The future for insecticides: needs and prospects.* Wiley, New York.
- Clements, A. N. 1963. *The physiology of mosquitoes.* Macmillan, New York.
- Cloudsley-Thompson, J. L. 1976. *Insects and history.* St. Martins Press, New York.
- Dame, D. A., R. E. Lowe, G. J. Wichterman, A. L. Cameron, K. R. Baldwin and T. W. Miller. 1976. Laboratory and field assessment of insect growth regulators for mosquito control. *Mosq. News* 36:462-472.
- Downum, K. R. and G. H. N. Towers. 1983. Analysis of thiophenes in the Tageteae (Asteraceae) by HPLC. *J. Nat. Prod.* 46:98-103.
- Gray, A. 1887. *Gray's school and field book of botany.* American Book Co., New York.
- Hethelyi, E., P. Tetenyi, E. Dabi and B. Danos. 1987. The role of mass spectrometry in medicinal plant research. *Biomed. Environ. Mass. Spectrom.* 14:627-632.
- Jones, T. G. H. 1926. Olefinic terpene ketones from the volatile oil of flowering *Tagetes glandulifera*, Part II. *J. Chem. Soc.* 129:2767-2770.
- Jones, T. G. H. and F. B. Smith. 1925. Olefinic terpene ketones from the volatile oil of flowering *Tagetes glandulifera*, Part I. *J. Chem. Soc.* 127:2530-2539.
- Maradufu, A., R. Lubega and F. Dorn. 1978. Isolation of (5E)-ocimenone, a mosquito larvicide from *Tagetes minuta* L. *Lloydia* 41:181-183.
- McCulloch, R. N. and D. F. Waterhouse. 1947. Laboratory and field tests of mosquito repellents. *Austral. Commonwealth Sci. Ind. Pes. Organ. Bull.* 21:3.
- Monnig, H. O. 1936. A new fly repellent made from oil of *Tagetes*. *Onderstepoort J. Vet. Sci. Animal Ind.* 7:419-430.
- Naves, Y.-R. 1948. Etudes sur les matieres vegetales volatiles LVI. Sur de nouvelles cetones, les tagetenones, isolees de l'huile essentielle de *Lippia asperifolia* Rich. *Helv. Chim. Acta* 31:29-32.
- Neher, R. T. 1968. The ethnobotany of *Tagetes*. *Econ. Bot.* 22:317-325.
- Saxena, B. P. and J. B. Srivastava. 1973. *Tagetes minuta* L. oil—a new source of juvenile hormone mimicking substance. *Ind. J. Exp. Biol.* 11:56-58.
- Smith, R. F. 1976. Insecticides and integrated pest

- management. pp. 489-506. In: R. L. Metcalf and J. J. McKelvey (eds.). Future for insecticides: needs and prospects. Wiley, New York.
- Uhlenbroek, J. H. and J. D. Bijloo. 1958. Investigations on nematocides I. Isolation and structure of a nematocidal principle occurring in *Tagetes* roots. Recueil 77:1004-1009.
- Uhlenbroek, J. H. and J. D. Bijloo. 1959. Investigations on nematocides II. Structure of a second nematocidal principle isolated from *Tagetes* roots. Recueil 78:382-390.
- deVilliers, D. J. J., C. F. Garbers and R. N. Laurie. 1971. Synthesis of tagetenones and their occurrence in oil of *Tagetes minuta*. Phytochem. 10:1359-1361.
- Weyerstahl, P., W. Zambik and C. Gansau. 1986. Terpene und terpen-derivate, XVIII thermische und  $AlCl_3$ -katalysierte reaction von ocimenon. Liebigs Ann. Chem. 1986:422-427.