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ISOLATION OF THE INSECTICIDAL COMPONENTS OF *TAGETES MINUTA* (COMPOSITAE) AGAINST MOSQUITO LARVAE AND ADULTS[1]

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ABSTRACT. Application of *Tagetes minuta* floral extract to silica gel column chromatography produced 2 fractions with the hydrogenate part 20–30 times more toxic to larvae and 12–13 times more toxic to adults of *Aedes aegypti* and *Anopheles stephensi,* respectively, than the oxygenate part. Further fractionation by column chromatography of the hydrogenate fraction produced 4 thiophenes, 5-(but-3-ene-1-ynyl)-2,2′-bithiophene, 5-(but-3-ene-1-ynyl)-5′-methyl-2,2′-bithiophene, 2,2′,5′,2″-terthiophene, and 5-methyl-2,2′,5′,2″-terthiophene. These compounds in *Tagetes minuta* are largely responsible for the toxicity exhibited against the tested mosquitoes.

INTRODUCTION

Mosquito control is becoming increasingly difficult because of several species becoming physiologically resistant to many of the conventional insecticides (World Health Organization 1992) and the public's demand for a decrease in the application of pesticides in the environment. Alternative control methods and materials that are both effective against the target mosquito species and that have minimal detrimental effects on the environment are needed. One potential alternative control method/material is the application of selected botanical derivatives against the target mosquito species.

Several phytochemicals extracted from various botanical sources have been reported to have detrimental effects on mosquitoes (Sukumar et al. 1991), with some of the most promising derived from plants (marigolds) in the genus *Tagetes* (Compositae). In prior research, Perich and colleagues (1994) determined that extracts from the flowers of *Tagetes minuta* exhibited the greatest toxicity against mosquitoes, as compared to other members of the genus *Tagetes.* The purpose of this study was to isolate and evaluate the biocidal components from *T. minuta* floral extracts.

MATERIALS AND METHODS

Plant cultivation and collection procedures: Tagetes minuta seeds were obtained from Richters Seed (Ontario, Canada) and planting and cultivation were done as described by Perich et al. (1994). Plants were harvested after reaching mature height (20–40 cm) and in full bloom. Flowers were separated, placed into 18 × 36-cm plastic zip-lock storage bags and flushed with and stored under nitrogen to slow potential oxidative degradation. They were refrigerated at 10°C until processing.

Extraction and chromatographic procedures: A simultaneous steam distillation and extraction (SSDE) procedure (Perich et al. 1994) was used to obtain the *T. minuta* flower oil extract. Flash column chromatography was carried out on silica gel 60 (Fisher Scientific, Pittsburgh, PA) using cyclohexane, tetrahydrofuran, and their mixture as solvent systems. The eluants were removed with a stream of purified nitrogen. The concentrated fractions were stored at −20°C until biological testing could be carried out. Gas chromatography/mass spectrometry was carried out on an HP5970 MSD (Hewlett Packard, Avondale, PA) using a conventional apolar fused silica capillary column.

Bioassays: Components of the *T. minuta* floral extract were evaluated against adult and 3rd-instar larvae of *Aedes aegypti* (Linn.) and *Anopheles stephensi* Liston from colonies maintained at the U.S. Army Biomedical Research and Development Laboratory for 10 years. Bioassays were done in an environmentally controlled room maintained at 27 ± 1°C and 80 ± 5% RH. A serial dilution range of 5–100 ppm using filtered water was used for the larval bioassays. One hundred milliliters of each dilution were placed into a 100 × 50-mm crystallizing Pyrex™ dish along with 10 3rd-instar larvae of each species. Each concentration together with an untreated control group was replicated 10 times for both test species. Larvae were fed 1 or 2 pellets of catfish chow (Ralston Purina, St. Louis, MO), mortality recorded at 24 h, and dead larvae examined. Mortality data were used

[1] The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or Department of Defense.

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Table 1. Larval mortality of 3rd-instar *Aedes aegypti* and *Anopheles stephensi* to fraction extracts of *Tagetes minuta* (ppm extract).[1]

Extract fraction	*Ae. aegypti*		
	LD_{50} (95% FL)[2]	LD_{90} (95% FL)	Slope ± SE
Fraction 1	9.61 (7.33–16.29)	19.16 (16.18–25.77)	4.20 ± 0.94
Fraction 2	42.48 (65.00–192.78)	386.49 (285.79–403.15)	4.06 ± 0.69
Fraction 1A	152.50 (112.36–231.01)	235.62 (193.18–348.19)	2.69 ± 0.22
Fraction 1B	3.90 (3.10–4.15)	4.56 (4.05–4.79)	1.19 ± 0.68

[1] n = 1,000 for each dosage–mortality response, LD_{50} and LD_{90} in ppm.
[2] 95% confidence limits.

Table 2. Mortality of adult male and female *Aedes aegypti* and *Anopheles stephensi* to fraction extracts of *Tagetes minuta.*[1]

Extract fraction	Males		
	LD_{50} (95% FL)[2]	LD_{90} (95% FL)	Slope ± SE
	Ae. aegypti		
Fraction 1	0.15 (0.13–0.18)	0.57 (0.43–0.86)	1.15 ± 0.15
Fraction 2	1.48 (0.86–1.79)	8.53 (4.38–10.56)	2.25 ± 0.33
	An. stephensi		
Fraction 1	0.16 (0.14–0.19)	0.46 (0.37–0.63)	1.38 ± 0.17
Fraction 2	1.25 (0.80–5.73)	5.64 (2.34–10.53)	2.21 ± 0.18

[1] n = 500 for each sex and dosage–mortality response, LD_{50} and LD_{90} in percent.
[2] 95% confidence limits.

Table 3. Percent of each thiophene compound isolated from *Tagetes minuta* flower oil.[1]

Thiophene compound	% of total flower oil
5-(but-3-ene-1-ynyl)-2,2′-bithiophene	0.15 ± 0.01
5-(but-3-ene-1-ynyl)-5′-methyl-2,2′-bithiopene	0.03 ± 0.01
2,2′,5′,2″-terthiophene	0.36 ± 0.01
5-methyl-2,2′,5′,2″-terthiophene	0.20 ± 0.01

[1] Extract obtained by flash column chromatography and quantitated by gas chromatography/mass spectrometry.

to determine LD_{50} and LD_{90} values using probit analysis (SAS/STAT 1985).

Adult bioassays were conducted with 3–6-day-old male and female *Ae. aegypti* and *An. stephensi.* Mosquitoes were subjected to knockdown with CO_2, then immobilized on a chill table while applications were administered. A serial dilution range of 0.05–10%, using insecticide-grade acetone as the diluent, was applied at 5 µl to the thorax of 10 males and 10 females using a Drummond digital micropipetter. Each concentration and an untreated control group (acetone only) was replicated 5 times for each sex and mosquito species. Mosquitoes were then placed into 30.5 × 30.5 × 30.5-cm screened cages in an environmentally controlled room maintained at 27 ± 1°C and 80 ± 5% RH and provided with 10% sucrose solution. Adults were observed for any behavioral changes immediately and at 15, 30, and 60 min after application. Mortality was recorded at 24 h and analyzed similar to the larval bioassay data.

RESULTS AND DISCUSSION

The fractionation of *T. minuta* flower oil by flash column chromatography resulted in 2 major fractions. Because the separation was done on silica gel, Fractions 1 and 2 represent the hydrogenates and oxygenates of *T. minuta* flower oil, respectively. The biocidal activity of the 2 fractions differed significantly among larvae and adults of both mosquito species tested (Tables 1 and 2.) Fraction 1, the hydrogenate part of *T. minuta* flower oil, was the more toxic fraction, giving LD_{90}s of 19–21 ppm against larvae and 0.41–0.73% against adults. These LD_{90}s were 20–30 times higher against larvae and 12–15 times higher against adults compared to Fraction 2. This indicates that the compounds re-

Table 1. Extended.

Extract fraction	*An. stephensi*		
	LD_{50} (95% FL)	LD_{90} (95% FL)	Slope ± SE
Fraction 1	10.12 (8.78–17.25)	20.80 (17.18–23.41)	3.68 ± 0.76
Fraction 2	150.12 (129.16–209.89)	648.61 (532.45–811.56)	3.15 ± 0.68
Fraction 1A	148.83 (99.48–201.83)	229.57 (153.26–289.65)	3.52 ± 0.47
Fraction 1B	3.89 (3.32–4.14)	4.55 (4.31–4.70)	1.09 ± 0.44

Table 2. Extended.

Extract fraction	Females		
	LD_{50} (95% FL)	LD_{90} (95% FL)	Slope ± SE
	Ae. aegypti		
Fraction 1	0.29 (0.15–0.33)	0.73 (0.57–1.08)	0.77 ± 0.15
Fraction 2	2.78 (1.08–4.32)	9.12 (4.68–11.21)	1.00 ± 0.31
	An. stephensi		
Fraction 1	0.23 (0.20–0.27)	0.62 (0.50–0.86)	1.00 ± 0.15
Fraction 2	2.11 (0.98–3.42)	7.98 (5.18–9.32)	2.01 ± 0.46

sponsible for most of the biocidal activity of *T. minuta* flower oil are concentrated in Fraction 1. The lethal levels found for *Ae. aegypti* and *An. stephensi* with either extract fraction were only slightly different, indicating negative specificity among mosquito species.

Further workup of Fraction 1 was carried out to isolate the biocidal compounds. Two fractions were obtained by flash chromatography. Fractions 1A and 1B have minimal overlap between the separated components. Fraction 1B consisted of fewer components (7) compared to Fraction 1A (23). Again there was a significant difference between the 2 fractions in the toxicity against larvae of both mosquito species tested (Table 1). Only larval bioassays were done, due to the very small quantity of Fractions 1A and 1B obtained from *T. minuta* flower oil extract. Fraction 1B gave an LD_{90} of 4.6 ppm for both mosquito species tested, which was approximately 4–5 times more toxic than Fraction 1 and 50–52 times more than Fraction 1A (Table 1).

In adult bioassays, mosquitoes were observed to show typical symptoms of nerve poisoning at initial excitation, followed by paralysis and finally death similar to that caused by pyrethrins (Quraishi 1977). However, *Tagetes* species do not contain pyrethrins, but rather closely related monoterpenoid esters known as thiophenes (Hogstad et al. 1984). Through the use of capillary column gas chromatography and mass spectral analysis it was confirmed that thiophenes were present in Fraction 1B.

Four thiophenes were identified in Fraction 1B, 5-(but-3-ene-1-ynyl)-2,2′-bithiophene, 5-(but-3-ene-1-ynyl)-5′-methyl-2,2′-bithiophene, 2,2′,5′,2″-terthiophene, and 5-methyl-2,2′,5′,2″-terthiophene. Quantitative analysis of Fraction 1B indicates that each thiophene makes up less than 1% of the total flower oil (Table 3). This indicates that even such a small amount of the 4 identified thiophenes causes significant mortality of the mosquitoes tested. Maradufu et al. (1978) reported that the larvicidal activity of *T. minuta* extracts was due to an ocimenone component, but in a subsequent study Green et al. (1991) reported other unidentified components in the whole-oil extract of *T. minuta* to be responsible for larval toxicity. Our results indicate that the unidentified components reported by Green et al. (1991) are probably the 4 thiophenes described above. Swelling of the anal papillae was seen in the larval bodies of both mosquito species, suggesting possible interruption of osmotic and ionic regulation (Clements 1992) by the thiophenes in *T. minuta* floral extract.

We conclude that the hydrogenate fraction of *T. minuta* floral extract has the greatest biocidal effect on the larvae and adults of *Ae. aegypti* and *An. stephensi*. In addition, further fractionation and analysis of determined that 4 thiophenes are the compounds potentially responsi-

ble for the toxicity exhibited by *T. minuta* against mosquitoes. Thiophenes from *T. minuta* floral extracts offer potential for development as a new biorational insecticide against target mosquito species. Further evaluation of thiophenes from *T. minuta* floral extracts against natural populations of target species and their impact on nontarget fauna is warranted.

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REFERENCES CITED

Clements, A. N. 1992. Biology of mosquitoes, Volume 1. Chapman and Hall, New York.

Green, M. M., J. M. Singer, D. J. Sutherland and C. R. Hibben. 1991. Larvicidal activity of *Tagetes minuta* (marigold) toward *Aedes aegypti*. J. Am. Mosq. Control Assoc. 7:282–286.

Hogstad, S., G. L. Johansen and T. Anthonsen. 1984. Possible confusion of pyrethrins with thiophenes in *Tagetes* species. Acta Chem. Scand. 38:902–904.

Maradufu, A., R. Lubeya and F. Dorn. 1978. Isolation of ocimenone, a mosquito larvicide from *Tagetes minuta*. Lloydia 41:181–183.

Perich, M. J., C. Wells, W. Bertsch and K. E. Tredway. 1994. Toxicity of extracts from three *Tagetes* against adults and larvae of yellow fever mosquito and *Anopheles stephensi* (Diptera: Culicidae). J. Med. Entomol. 31:833–837.

Quraishi, M. S. 1977. Biochemical insect control. Wiley, New York.

SAS/STAT. 1985. Guide for personal computers, 6th ed. SAS Institute Inc., Cary, NC.

Sukumar, K., M. J. Perich and L. R. Boobar. 1991. Botanical derivatives in mosquito control: a review. J. Am. Mosq. Control Assoc. 7:210–257.

World Health Organization. 1992. Vector resistance for pesticides. W.H.O. Tech. Rep. Ser. 818.