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Original article

Evaluation of larvicidal activity of esters of 4-mercapto-2-butenoic acid against *Aedes albopictus* (Diptera: Culicidae)

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ABSTRACT

Aedes albopictus (Skuse) (Diptera: Culicidae), an aggressive and annoying vector of several arbovirus including Chikungunya and Zika, is a serious health problem worldwide. Control of this mosquito is difficult because of high adaptability, egg resistance to dehydration and ability to exploit many man-made microhabitats. The most effective strategy appears the control of larval population. Based on previous data showing a larvicidal effect of plant extracts containing sulfhydryl and isothiocyanate compounds, we evaluated by bioassays the toxicity of three synthetic esters of 4-mercapto-2-butenoic acid on larvae of *A. albopictus* in comparison to cypermethrin. Among the compounds tested, the most effective was n-octyl 4-mercapto-2-butenoate, about 5 times more effective than ethyl 4-mercaptobut-2-enoate and about 20 times more effective than menthyl 4-mercaptobut-2-enoate. We advance the hypothesis that the larvicidal properties of n-octyl 4-mercapto-2-butenoate are due to its hydrophobic alkyl chain, longer than that of the other two compounds. This chain confers to the molecule the ability to spread on water surface and interfere with larval respiration. The larvicidal activity of n-octyl 4-mercapto-2-butenoate against *A. albopictus* appears interesting and may be developed after toxicological evaluation on vertebrates and humans, and environmental toxicity tests in compliance with WHO and ECDC rules.

1. Introduction

The "Asian tiger mosquito", *Aedes albopictus* (Skuse) (Diptera: Culicidae), native to Southeast Asia (Skuse, 1895), acts as a vector of arboviruses causing dengue, yellow fever, encephalitis and other serious diseases such as dirofilariasis (Mitchell, 1995; Gratz, 2004). Since 1970 the species has spread in all continents (Hawley, 1988) because its eggs, unlike those of other mosquitoes, may survive dehydration and can be transported in different containers, such as used tyres (Knudsen, 1995) and potted ornamental plants (Madon et al., 2002). The first European settling of *A. albopictus* occurred in 1979 in Albania (Adhami and Murati, 1987; Adhami and Reiter, 1998). The species reached Italy in 1990 (Sabatini et al., 1990) and is now well established in all the Italian territory (Dalla Pozza et al., 1992; Romi, 2001; Scholte and Schaffner, 2007). *Aedes albopictus* is a serious problem in all Mediterranean Europe (ECDC, 2016a), causing a significant loss in the quality of life because of its aggressivity and activity in full daytime (Romi, 2001). The species exhibit a high ecological plasticity, exploiting a variety of breeding habitats, natural and man-made: it is able to survive in tropical and temperate conditions, even at temperatures as low as −10 °C (Waldock et al., 2013). The larvae develop from eggs resistant to dehydration after 48 h and at the optimal temperature of 25 °C become pupae after four larval stages; the adult mosquito emerges in about 48 h, ready to reproduce (Estrada-Franco and Craig, 1995).

Aedes albopictus is a vector of viral diseases such as Chikungunya (CHIK) and West Nile (Brustolin et al., 2016). In September 2007 in Castiglione di Cervia (Ravenna, Italy) and nearby towns more than 200 cases of CHIK were registered, the first case of autochthonous spread of a tropical disease in a Western country (ECDC, 2007; Rezza et al., 2007). Recently the species has been reported as a vector of the arbovirus Zika (ZIKV) in Africa, Asia and French Polynesia (Ioos et al., 2014; Musso et al., 2015; ECDC, 2016b; Gatherer and Kohl, 2016; Mlakar et al., 2016) and Brazil (Hennessey et al., 2016), with a transmission potential for ZIKV similar to *A. aegypti* (Chouin-Carneiro et al., 2016). The infection

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by ZIKV has been associated to Guillain-Barré syndrome (microcephaly) in French Polynesia (Cao-Lormeau et al., 2016; Mlakar et al., 2016) and in Brazil (ECDC, 2016b; Gatherer and Kohl, 2016).

The control of this mosquito is difficult because of its adaptability and "domestication" (ECDC, 2012; Waldock et al., 2013): the most effective strategy is to control the larval population (Becker and Margalit, 1993; Flacio et al., 2015). Previous bioassays by our research group showed that plant extracts containing sulfhydryl and isothiocyanate compounds (garlic and horseradish extracts) had a larvicidal effect against *A. albopictus* (Tedeschi et al., 2011). Based on these data, we evaluated the toxicity of synthetic esters of 4-mercapto-2-butenoic acid containing alkyl chains of different length on larvae of *A. albopictus*, with the purpose to obtain new larvicides, environmentally sustainable (Després et al., 2007) and effective to control the spread and diffusion of this species at the larval stage.

2. Materials and methods

2.1. Synthetic esters

Three esters of 4-mercapto-2-butenoic acid containing alkyl chains of different length, ethyl 4-mercaptobut-2-enoate (E-MB), menthyl 4-mercaptobut-2-enoate (M-MB) and octyl 4-mercaptobut-2-enoate (O-MB) were synthesized and employed for the bioassays. The compounds were structurally similar to allicin (Fig. 1) because of the presence of a double bond on the β-carbon to the sulfur atom and a sulfur-containing group: in 4-mercapto-2-butenoic acid the thiol was in a terminal position and the double bond was conjugated to an ester, while in allicin there was a thiosulfinate functional group, R-S(O)-S-R. The compounds were obtained by a Wittig reaction (Fig. 2) between a thioaldehyde and triphenyl phosphonium ylide with different ester groups (Pommer, 1977; Robiette et al., 2006; Favre and Powell, 2014).

The synthetic esters were separated by thin layer chromatography (TLC) and analyzed by nuclear magnetic resonance (NMR). The TLC was performed on 0.20 mm Poligram SIL G/UV254 layers (Macherey-Nagel, Duren, Germany), with 1% (w/v) $KMnO_4$ solution to visualize spots. Chromatographic purifications were performed by a 60-M silica gel, 0.04–00.063 mm, 230–400 mesh (Macherey-Nagel). The ^{1}H NMR spectra were registered on Varian spectrometers (Varian Inc., Palo Alto, California) at 300 MHz and 400 MHz at room temperature. Spectral data of chemical shifts (δ) (multiplicity, coupling constants and integer value) were reported in relation to deuterated chloroform ($CDCl_3$). Signal multiplicity was indicated as follows: s for singlet; d for doublet; t for triplet; q for quartet; m for multiplet; dd for double doublet; dt for double triplet.

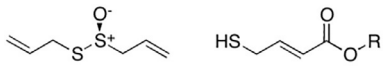

Fig. 1. Comparison between structures of allicin (left) and the synthetic esters of 4-mercapto-2-butenoic acid (right). R = ethyl (E-MB), menthyl (M-MB) or n-octyl (O-MB).

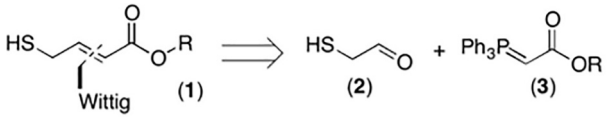

Fig. 2. Example of a Wittig reaction between a thioaldehyde and triphenyl phosphonium ylide carrying different R groups (see Fig. 1).

E-MB: ^{1}H NMR ($CDCl_3$) δ: 1.3 (t, 3H, J = 7 Hz), 3.3 (m, 2H), 4.2 (q, 2H, J = 7 Hz), 5.9 (dt, 1H, J = 14 Hz, J = 2 Hz), 7.0 (dt, 1H, J = 14 Hz, J = 7 Hz).
M-MB: ^{1}H NMR ($CDCl_3$) δ: 0.55 (d, 3H), 0.75 (d, 3H), 0.85 (d, 3H,), 0.9–1.1 (m, 2H), 1.15–1.4 (m, 2H), 1.4–1.7 (m, 4H), 4.4–4.6 (dt, 1H), 5.9 (dt, 1H, J = 14 Hz, J = 2 Hz), 7.0 (dt, 1H, J = 14 Hz, J = 7 Hz).
O-MB: ^{1}H NMR ($CDCl_3$) δ: 0.9 (t, 3H, J = 7 Hz), 1.21–1.42 (m, 12H), 1.7 (m, 2H, J = 7 Hz), 3.3 (m, 2H), 4.15 (q, 2H, J = 7 Hz), 5.9 (dt, 1H, J = 14 Hz, J = 2 Hz), 7.0 (dt, 1H, J = 14 Hz, J = 7 Hz).

2.2. Bioassays

Larvae of *A. albopictus* belonging to the Rimini F36 strain, kindly provided by the Centro Agricoltura Ambiente "Giorgio Nicoli" in Crevalcore (Bologna, Italy) were used for bioassays. The eggs, arranged on filter paper strips, were deposited in a glass jar containing about 700 mL of decanted tapwater and 0.25 g of bacterial broth (Nutrient Broth, Oxoid LTD, Basingstoke, United Kingdom) and maintained in an incubator (ISCO, Milan, Italy) at 27 °C under a 16:8 h light:dark photoperiod until hatching. The first instar larvae were transferred to a plastic tray containing 2L of decanted tapwater and were fed daily with a pinch of fish food (Tetra Spectrum Brands, Blacksburg, Virginia). Fourth instar larvae were used for bioassays five days after hatching. Preliminary tests were performed to identify the range of mortality (from a minimum of 1% to a maximum of 98%) and to choose the concentrations of compounds to be tested. The sequential solutions (each with a final volume of 20 ml) of the compounds were prepared from a standard solution and placed in 50-ml plastic cups. A number of 20 randomly chosen larvae were placed in the plastic cups at room temperature. The following solutions were prepared: E-MB 7.81, 15.63, 31.25, 62.50, 125, 250, 500 ppm; M-MB 15.63, 31.25, 62.50, 125, 250, 500, 1000, 2000, 4000 ppm; O-MB, 1.95, 3.90, 7.81, 15.63, 31.25, 62.50, 125, 250 ppm. The positive control was cypermethrin (α-Cyano-(3-phenoxy-phenyl)-methyl 3-(2,2-dichloro-vinyl)-2,2 dimethyl-cyclopropane-carboxylate) (Sigma-Aldrich, Milan, Italy) at the following solutions: 0.24, 0.49, 0.98, 1.95, 3.90, 7.81, 15.63, 31.25 ppm. Five replicas were set up for each concentration of the compounds and of the positive control, together with five replicas of a negative control made only by decanted tapwater. After 24 h of exposure, larvae were considered dead when unable to move autonomously. No larval mortality was observed in the negative control after 24 h.

2.3. Statistical analysis

The data were analyzed by the POLO-PC program (LeOra Software, 2002) using the Abbott's formula (Abbott, 1925) to obtain the relationship between the applied dose and mortality for each compound tested. Based on the program output, the LC_{50} values and the related confidence intervals (95% CI) were obtained for each compound (Finney, 1971). The regression equation and R^2 were obtained by STATISTICA7.1 program (StatSoft, Tulsa, Okhlahoma). The data of mortality transformed in percentage were verified for normality and homoscedasticity by the Levene and Kolmogorov-Smirnov tests. The differences were evaluated by one-way ANOVA test using STATISTICA7.1, followed by a post hoc Tukey's test. The F-values were considered significant when $P < 0.05$.

3. Results

The three compounds tested had different mortality effects on the larvae of *A. albopictus* (Table 1). For compound E-MB, no mortality was observed before a concentration of 7.81ppm: a

Table 1
Percentages of larval mortality of *Aedes albopictus* at different concentrations (ppm) of the synthetic esters of 4-mercapto-2-butenoic acid and cypermethrin (positive control). E-MB: ethyl 4-mercaptobut-2-enoate; M-MB: menthyl 4-mercaptobut-2-enoate, O-MB: octyl 4-mercaptobut-2-enoate. Percentages are expressed as means ± DS. Percentages in the same column followed by the same letter are not significantly different (P > 0.05). Percentages in the same row followed by the same symbol (*, **, ◆) are not significantly different (P > 0.05).

Concentration (ppm)	E-MB	M-MB	O-MB	Cypermethrin
0.24	–	–	–	3.00 ± 4.47[a]
0.49	–	–	–	9.00 ± 4.18[a]
0.98	–	–	–	37.00 ± 8.37[b]
1.95	–	–	3.00 ± 2.70[a, ◆]	52.00 ± 5.70[c, *]
3.90	–	–	8.00 ± 4.47[a, ◆]	71.00 ± 12.94[d,*]
7.81	3.00 ± 2.74[a, **]	–	21.00 ± 6.52[b, ◆]	92.00 ± 4.47[e, *]
15.63	9.00 ± 4.18[b, **]	2.00 ± 2.74[a, **]	66.00 ± 8.22[c, ◆]	97.00 ± 4.47[f, *]
31.25	11.00 ± 4.18[b, ◆]	6.00 ± 2.24[b, ◆]	89.00 ± 6.52[d, *]	100.00 ± 0.00[f, *]
62.50	28.00 ± 9.08[c, ◆]	8.00 ± 2.74[b, c, **]	92.00 ± 4.47[d, *]	–
125.00	80.00 ± 11.18[d, ◆]	16.00 ± 7.42[c, **]	99.00 ± 2.24[e, *]	–
250.00	97.00 ± 2.50[e, *]	48.00 ± 10.37[d, ◆]	100.00 ± 0.00[e, *]	–
500.00	100.00 ± 0.00[f, *]	76.00 ± 9.62[e, ◆]	–	–
1000.00	–	91.00 ± 4.18[f]	–	–
2000.00	–	93.00 ± 2.74[f]	–	–
4000.00	–	100.00 ± 0.00 [g]	–	–

significant increase (P < 0.05) of mortality percentage was observed at all concentrations, except between 15.63 and 31.25 ppm. A mortality percentage of 100% was observed at 500 ppm. For compound M-MB, no mortality was observed before a concentration of 15.63 ppm and a significant increase (P < 0.05) of mortality percentage was observed with the increase of concentration, except between 31.25 and 62.50 ppm, between 65.50 and 125.00 ppm, and between 1000 and 2000 ppm. The 100% mortality percentage was obtained at 4000 ppm. For compound O-MB, no mortality was observed before 1.95 ppm: a significant increase (P < 0.05) in mortality percentage was observed from 3.90 ppm onwards, except between 31.25 and 62.50 ppm, and between 125.00 and 250.00 ppm. The 100% mortality percentage was obtained at 250.00 ppm. The mortality percentages of the positive control, cypermethrin, at the comparable concentrations, were always significantly higher (P < 0.05) than those of the three compounds tested. The only result of mortality percentage approaching that of cypermethrin (although significantly different, P < 0.05) was that of O-MB at 31.25 ppm. In the range of 7.81 to 250 ppm O-MB showed values of mortality percentage always significantly higher (P < 0.05) than the other three compounds. The two compounds E-MB and M-MB did not show significantly different values of mortality percentage for all concentrations except 15.63 and 31.25 ppm.

Concerning the lethal concentrations (Table 2), the lowest LC_{50} value was that of O-MB (13.27 ppm), in comparison to E-MB and M-MB (respectively 68.92 and 260.51 ppm). Comparing the LC_{50} values of the three compounds with cypermethrin, the highest RP value was that of O-MB. Based on these data, the compound with the most effective larvicidal activity among the three ones tested was O-MB.

4. Discussion

Aedes albopictus, an aggressive and annoying mosquito vector of several viral diseases including ZIKV (Waldock et al., 2013; Mlakar et al., 2016), is characterized by a high ecological plasticity and its eggs may be laid in a large array of man-made microhabitats (Romi, 2001; Waldock et al., 2013). The increasing diffusion of this species in Mediterranean Europe has encouraged research to identify new active ingredients, chemically modified from natural compounds and effective as larvicides. Previous studies by our research group showed larvicidal effects of extracts of *Armoracia rusticana* Gaertn., Mey. & Scherb. (Capparales: Brassicaceae) and *Allium sativum* L. (Liliales: Amaryllidaceae) against *A. albopictus* (Tedeschi et al., 2011). In this study we tested against *A. albopictus* larvae three synthesized esters of 4-mercapto-2-butenoic acid with structure similar to allicin.

Among the three compounds, the most effective as larvicide was n-octyl 4-mercapto-2-butenoate (O-MB): it was about 5 times more effective than E-MB and about 20 times more effective than M-MB. Although no data are yet available about its toxicity mechanism, we may advance the hypothesis that the larvicidal properties of O-MB are due to a hydrophobic alkyl chain in the ester longer than that of the other two compounds: this chain bestows to the molecule a higher ability to spread as a thin layer on the water surface.

According to the literature, natural compounds with larvicidal effects against mosquitoes are often phenolic terpenes. Among the terpenes recently tested as larvicides against Culicidae there are α-phellandrene, limonene, p-cymene and α-terpinene, isolated from leaves of *Eucalyptus camaldulensis* Dehnhardt and *Eucalyptus urophylla* Blake (Myrtales: Malvaceae), and tested on *A. albopictus* and *Aedes aegypti* L. (Diptera: Culicidae). These compounds were effective as larvicides against *A. aegypti* with LC_{50} values similar to that of O-MB (respectively 16.6, 18.1, 19.2 and 14.7 ppm), but about twice less effective against *A. albopictus* (Cheng et al., 2009a).

Another plant-derived compound recently tested as larvicide is β-sitosterol, isolated from *Abutilon indicum* (L.) (Malvales: Malvaceae) and experimented on *A. aegypti*, *Anopheles stephensi* Liston (Diptera: Culicidae) and *Culex quinquefasciatus* Say (Diptera: Culicidae). This compound, very effective against *A. stephensi* (LC_{50} 3.58 ppm) showed an efficacy against *A. aegypti* (LC_{50} 11.49 ppm) (Abdul Rahuman et al., 2008) similar to that of O-MB against *A.*

Table 2
Larvicidal efficacy of synthetic esters of 4-mercapto-2-butenoic acid and of cypermethrin (positive control) against *A. albopictus*. LC_{50} = concentration required to kill 50% of insects, expressed in ppm. 95%CI = 95% Confidence Interval. RP = Relative Potency (LC_{50} positive control/LC_{50} test substance). Other abbreviations as in Table 1.

Active ingredient	Regression equation	R^2	LC_{50} [95%CI]	RP
E-MB	y = 2.3083x + 0.7566	0.8369	68.92 [58.50–83.51]	0.026
M-MB	y = 1.8493x – 0.5324	0.9128	260.51 [226.76–299.78]	0.007
O-MB	y = 2.2529x + 2.4701	0.8831	13.27 [11.74–14.99]	0.136
Cypermethrin	y = 1.9438x + 4.4995	0.9148	1.81 [1.58–2.07]	1

albopictus. An efficacy similar to that of O-MB against *A. albopictus* was shown by dodecyl acetate, one of the main components of the essential oil of *Blumea eriantha* DC. (Asterales: Asteraceae), tested against six Culicidae species, *A. albopictus*, *A. aegypti*, *A. stephensi*, *Anopheles subpictus* (Grassi), *C. quinquefasciatus and Culex tritaeniorhynchus* Giles (LC_{50} respectively 13.45, 11.18, 10.22, 12.31, 12.16 and 14.68 ppm) (Benelli et al., 2017). Concerning E-MB and M-MB, they were less effective than O-MB as larvicides against *A. albopictus* (LC_{50} respectively 68.92 and 260.51 ppm). Values of LC_{50} comparable to that of E-MB were obtained with phenolic terpenes isolated from essential oil of *Cinnamomum osmophloeum* Kanehira (Laurales: Lauraceae), an endemic forest tree from Taiwan. The compounds (caryophyllene oxide, eugenol, and citral) were tested against larvae of *A. albopictus* and other Culicidae, yielding against *A. albopictus* LC_{50} values respectively of 65.6, 67.4 and 70.7 ppm (Cheng et al., 2009b).

Other natural compounds with larvicidal effects similar to that of E-MB were germacrene D, a sesquiterpene extracted form leaves and stem of *Chloroxylon swietenia* de Candolle (Sapindales: Rutaceae), tested on *A. aegypti* and *A. stephensi* (LC_{50} respectively 63.6 and 59.5 ppm) (Ravi Kiran et al., 2006), and carvacrol and thymol, components of essential oils of Lamiaceae and Verbenaceae, tested against *A. aegypti* (LC_{50} respectively 70 and 79 ppm) (Silva et al., 2008).

In our study the compound (L)-menthyl 4-mercapto-2-butenoate (M-MB) resulted the least toxic as a larvicide. Probably the larvicide may be less effective when the molecule is more soluble in water (as in E-MB) and more effective when the hydrocarbon chain of the ester moiety is longer. The higher hydrophobicity allows the molecule to float and spread on the water surface, possibly interfering with the larval respiration and/or its metabolic activity. The low efficacy as a larvicide of M-MB in comparison to O-MB and E-MB could be ascribed to the steric bulk of the menthyl chain that could interfere with the uptake of the molecule by the larva. Further studies are required to verify whether the mechanism of action affects the larval cuticles or other effects are involved. Tests for a time longer than 24 h would be required to verify whether the larvicidal efficacy of O-MB could be compared to that of a biological larvicide containing *Bacillus thuringiensis* Berliner (Bacillales: Bacillaceae), or to a chemical one such as diflubenzuron. Field studies would also be required to verify the dispersion of the compounds in favoured egg-laying sites of *A. albopictus*, such as drains, manholes, flowerpot saucers and other small water reservoirs. If O-MB acts as larvicide within 24 h on *A. albopictus*, it would also be interesting to test whether the association of O-MB with currently employed larvicides may result in a quicker action. The n-octyl ester of 4-mercapto-2-butenoate (O-MB) may be developed as new larvicide after toxicological evaluation for vertebrates, including humans, and environmental toxicity studies in compliance with WHO and ECDC rules. This study is also an example of a collaboration between entomologists and chemists aimed to obtain a synthetic compound with the ability to control the diffusion of the aggressive and harmful tiger mosquito at an early larval stage.

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