

Communication

Not peer-reviewed version

Olive Mill Wastewater Extract as a Potential Mosquito Larvicide

[Alon Silberbush](#)^{*}, Maram Halabi, Nimrod Shteindel, [Yoram Gerchman](#), Hassan Azaizeh, [Ben Shahar](#), [Eyal Kurzbaum](#)

Posted Date: 20 April 2023

doi: 10.20944/preprints202304.0651.v1

Keywords: Olive mill waste water; Culex laticinctus; Aedes albopictus; larvicide; Sub-lethal concentrations



Preprints.org is a free multidiscipline platform providing preprint service that is dedicated to making early versions of research outputs permanently available and citable. Preprints posted at Preprints.org appear in Web of Science, Crossref, Google Scholar, Scilit, Europe PMC.

Copyright: This is an open access article distributed under the Creative Commons Attribution License which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Communication

Olive Mill Wastewater Extract as a Potential Mosquito Larvicide

Alon Silberbush ^{1,*}, Maram Halabi ¹, Nimrod Shteindel ¹, Yoram Gerchman ¹, Hassan Azaizeh ^{2,3,4}, Ben Shahaar ⁵ and Eyal Kurzbaum ^{3,5,6}

¹ Department of Biology and Environment, Faculty of Natural Sciences, University of Haifa – Oranim, Israel.

² Institute of Applied Research, The Galilee Society, P.O. Box 437, Shefa-Amr 20200, Israel;

³ Tel Hai College, Department of Environmental Science, Upper Galilee 12208, Israel.

⁴ Department of Natural Resources & Environmental Management, University of Haifa, Haifa, 3498838, Israel.

⁵ Shamir Research Institute, University of Haifa, P.O.B. 97, Qatzrin, Israel.

⁶ Department of Geography and Environmental Studies, University of Haifa, Mount Carmel, Haifa 3498838, Israel.

* Correspondence: alon-s@sci.haifa.ac.il

Abstract: The larvicidal potential of olive mill waste water (OMWW) extract against mosquito larvae was evaluated. We exposed second instar *Culex laticinctus* (Edwards) and *Aedes albopictus* (Skuse) larvae to increasing OMWW concentrations. In addition, we tested the effects of long term exposure to a low OMWW concentration on larval development. The two mosquito species responded differently to OMWW extracts. Larval *Cx. laticinctus* showed higher vulnerability to increasing OMWW concentrations in comparison to *Ae. albopictus*. By contrast, *Cx. laticinctus* exposed to a low OMWW concentration until pupation did not exhibit survival differences, but rather sex-specific development responses. These trends were not observed in *Ae. albopictus* whose survival was significantly reduced following long exposure to OMWW extraction. These results showed that OMWW, which is known as agricultural byproduct waste, may be reused as a biopesticide.

Keywords: Olive mill waste water; *Culex laticinctus*; *Aedes albopictus*; larvicide; Sub-lethal concentrations

Introduction

The olive oil industry is one of the most important branches of agriculture in the Mediterranean basin, producing over 90% of the global olive oil production [1]. This industry also generates solid and liquid waste byproducts characterized by their dark color and a typical odor, which are not easily degradable. Solid waste can be recycled and used as an ingredient of several products such as fertilizers, ethanol and high value-added biomolecules [2] or lightweight aggregates [3]. On the other hand, the liquid byproduct, olive mill wastewater (OMWW), is significantly more difficult to treat. This liquid is characterized by high concentrations of polyphenols and tannins, in addition to low pH and high chemical and biochemical oxygen demands. These qualities inhibit biological decomposition and place OMWW as one of the most contaminating effluents among those produced by the agrofood industries [4].

Previous studies suggested that the high concentrations of phenols, acids and sugar derivatives associated with OMWW may be applied for crop protection in dry or saline environments [5]. These qualities also make OMWW and its extracts potentially useful biopesticides. Some studies have shown that extracts of OMWW have the potential to inhibit the growth of bacteria, fungi and weeds [6]. However, very few studies explored the potential of OMWW extracts as insecticides or as insect growth inhibitors. Current studies focused on phytophagous insects [7-9].

The main objective of the current study was to examine the biopesticide potential of OMWW extracts against the development and survival of mosquito larvae. We focused on two, very different,

species of mosquitoes that are highly common throughout the Mediterranean basin, specifically in North Israel.

Culex laticinctus (Edwards) is distributed throughout the Middle-east and around the Mediterranean Sea. The larvae of this species are associated with artificial aquatic habitats such as cisterns and concrete pools, containing fresh and brackish water [10]. As such we hypothesize that this species will show a relevant durability to OMWW. The Asian Tiger mosquito, *Aedes albopictus* (Skuse 1894) is one of the world's worst invasive species [11]. This species, native to South-East Asia, was first recorded in the United States in 1985 and spread rapidly into other parts of the world [10]. Currently, it can be found in most areas with warm and temperate climate [12,13]. *Aedes albopictus* were first reported in Israel in 2002 [14]. Since then, they have become highly abundant in the non-arid areas of central and North Israel [15]. Unlike *Cx. laticinctus*, larval *Ae. albopictus* are associated with small, freshwater containers who are difficult to locate and treat with conventional methods such as larvicidal sprays [10,16]. Plant extracts could therefore be potentially excellent bio-larvicides of this species.

Methods

Larval Collection

We collected *Culex laticinctus* (Edwards) egg rafts and *Aedes albopictus* (Skuse 1894) eggs from plastic ovitraps placed at Oranim College Campus, Tivon, Israel. Sampled larvae from each batch were reared to 4th instar and identified to species [10]. First instar larvae were transferred into 400 ml plastic cups containing tap water, aged for 24 hours.

OMWW Extract

We obtained the OMWW for this study from a nearby olive mill press (Iksal, Galilee region, Israel). We treated the OMWW with 20% ethanol (v/v) and stored it at 4°C. We centrifuged one liter OMWW (8000g for 10 min), then filtered the supernatant through a filter paper and evaporated it under vacuum using the rotary evaporator, to 250 ml. We then added 250 ml of 95% ethanol solution to obtain two steps: solid precipitate (cellulose) and liquid layer (dissolved polyphenols). We removed the solid precipitate from the mixture by filtration, and evaporated the liquid fraction at 40 °C, using rotary evaporator to a final volume of 250 ml. We repeated this process until no more solid fraction was obtained from the mixture. The resulting liquid was evaporated under vacuum to form a 10 mg substance (resembling a dark ointment) called an anti-solvent solution. We mixed this solution with distilled water and stirred for 16 hours at 25°C. We then centrifuged and filtered the solution, and finally adjusted it to a final concentration of 145 ppt (parts per thousand).

Larval Toxicity Bioassay

We performed toxicity tests according to standard larvae bioassay [17] to determine LC₅₀ and LC₉₀ values. We placed 25 2nd instar larvae in 100 ml cups containing 5, 10, 15, 20 and 25 ppt OMWW extract. Each concentration repeated 3 times. We recorded larval mortality after 48 hours of incubation, during this time we fed the larvae with a mixture of finely ground fish flakes (42.2% crude protein, Sera-Vipan, Heinsberg, Germany) and rodent chow (17% protein, Ribos, Haifa, Israel). Cups were kept at a temperature of 24.83 ± 0.84 °C (mean ± SD).

Sublethal Effects of OMWW on Larvae

We transferred 30 larvae into 400 ml plastic cups within 24 hours of hatching. The experiment consisted of paired cups (blocks), each containing either 1 ppt OMWW extract solution or aged water control (4 blocks in total). This concentration was assumed to be sublethal based on preliminary toxicity results showing it was 10% of *Cx. laticinctus* LC₅₀. The larvae in each block were sibling *Cx. laticinctus* (from the same egg raft) or *Ae. albopictus* larvae originated from the same ovitrap. We fed the larvae every three days with 0.05 ± 0.003 (mean ± SD) grams of the mixture used in the previous

experiment. We removed the pupae and identified emerging adults by sex. We recorded the number of days to pupation and the number of emerging adults

Statistical Analysis

We calculated LC₅₀ and LC₉₀ values at 95% confidence intervals using Probit analysis [18]. We used a Pearson goodness of fit test in order to evaluate the observed distribution. We used a heterogeneity factor to calculate confidence limits when model assumptions were not met ($p < 0.15$). We analyzed the effects of sublethal concentrations on larval time to pupation using Linear Mixed Models (LMM), with the variables "Sex" and "Treatment" (OMWW and control) as fix factors and "Block" as a random factor. These analyses considered random sampling (Block effect), and the fact that male mosquitoes usually pupate faster than females (Sex effect). All analyses used SPSS statistics for windows version 24 with Type III sums of squares [19].

Results

The toxicity of OMWW extract for both species are summarized in Table 1. We found 100% mortality for *Cx. laticinctus* larvae that were exposed to 25 ppt OMWW for 48 hours. *Aedes albopictus* larvae expressed a stronger resistance to the extract with only ~60% mortality at the same concentration. The LC₅₀ value for *Ae. albopictus* was 2.27 times higher than that of *Cx. laticinctus* and 1.15 times higher than its LC₉₀ value.

Table 1. Probit analyses results for LC₅₀ and LC₉₀ values, slopes of the linear models and heterogeneity test results for second instar larvae exposed to increasing OMWW extraction concentrations for 48 hours.

Species	LC50 (ppt)	Upper; Lower LC50 limits (95% CI)	LC90 (ppt)	Upper; Lower LC90 limits (95% CI)	Slope ±SE	χ^2 (df)	Sig.
<i>Culex laticinctus</i>	10.08	8.47;11.74	19.89	17.5;23.3	0.13±0.09	40.86 (19)	>0.01
<i>Aedes albopictus</i>	22.91	21.19;25.44	31.1	28.8;37.9	0.14±0.015	33.13(19)	0.02

The number of larval *Cx. laticinctus* surviving to adulthood in sublethal OMWW treatment did not differ in comparison to control (paired t-test: $t_3=0.52$; $p=0.64$ and $t_3=0.42$; $p=0.7$ for males and females, respectively), (Figure 1). We found a strong sex effect on time to pupation, i.e. males pupated faster than females ($F_{1,147.13}=27.9$; $p < 0.001$). Female pupation time did not vary among the treatments but males exposed to OMWW prolonged time to pupation (Figure 2). This resulted in a significant "Sex × Treatment" interaction ($F_{1,147.08}=6.4$; $p < 0.001$). The overall effect of OMWW by itself was not statistically significant ($F_{1,147.04}=1.5$; $p=0.23$).

Less *Ae. albopictus* larvae metamorphosed in sublethal the treatment in comparison to control. This trend was shown in males ($t_8=2.5$; $p=0.02$) and females ($t_8=1.9$; $p=0.05$), (Figure 1). Males pupated faster than females (Sex effect: $F_{1,70.27}=12.3$; $p < 0.001$). We did not find a significant "Sex × Treatment" interaction ($F_{1,70.27}=0.4$; $p=0.52$), or a significant effect of the overall treatment on time to pupation ($F_{1,5.03}=0.03$; $p=0.88$), (Figure 2).

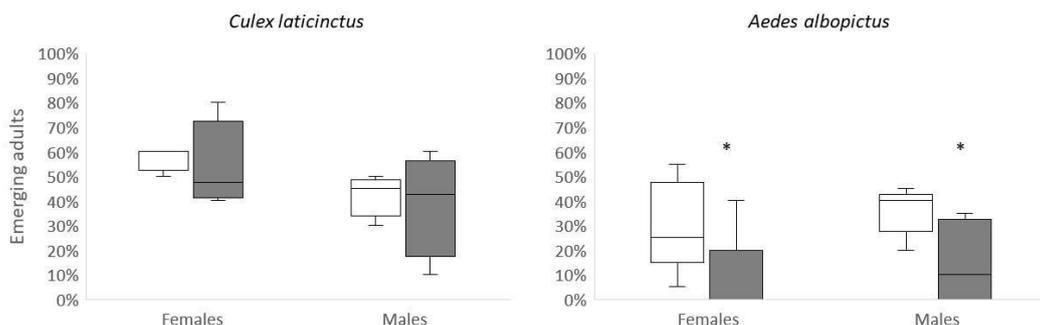


Figure 1. Larvae surviving to adulthood from control (light) and OMWW (dark) cups from the original number of first instars. Asterisks mark significant ($p < 0.05$) effect of treatment. Box limits are upper and lower quartiles in addition to the median. Whiskers are min and max values.

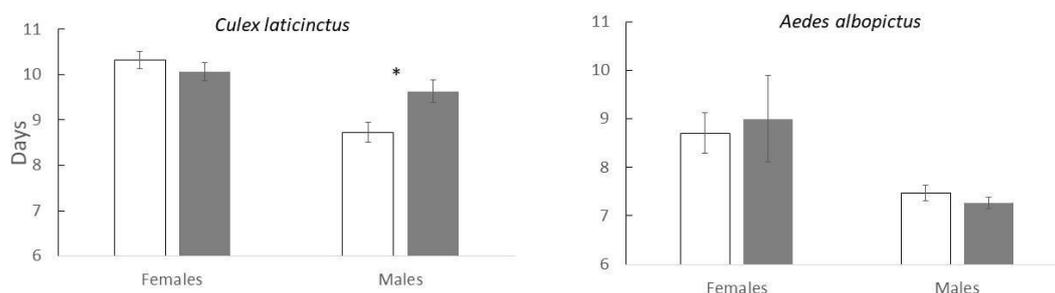


Figure 2. Number of days to pupation for male and female larvae treated with 1 ppt OMWW solution and control. Asterisks mark significant ($p < 0.05$) effect of treatment. Error bars denote ± 1 SE.

Discussion

Results showed that OMWW extract is potentially toxic to both mosquito species. Extracts from numerous plant species are known to have potential larvicide qualities [20]. The uniqueness in the use of OMWW extracts, is that it could prove as a method for the reuse of agricultural waste products as eco-friendly larvicides. We tested the lethal effect of OMWW on early instar larvae who are more sensitive to insecticides than late instars [21], hence efficiency is usually measured for late instars. *Culex laticinctus* is one of the most common mosquito species in the Mediterranean region, and their breeding sites often consist of small, artificial water bodies [10]. Hence, we may assume that larval *Cx. laticinctus* are often exposed to olive foliage and are relatively resistant to OMWW. Therefore, we expect that OMWW extracts should be more lethal for larval mosquito species that are not associated with that region.

By contrast to our original hypothesis, the LC_{50} and LC_{90} levels of OMWW were significantly lower for *Cx. laticinctus* in comparison to *Ae. albopictus* (Table 1). We can expect that the lethal effect of OMWW extracts will increase further by removal of some of the compounds by different types of fractionation, [22]. For example, fractions containing mainly polyphenols from OMWW cause mortality to *Euphyllura olivina* and *Aphis citricola* by direct spraying of liquid solutions. The LC_{50} in these studies were recorded at 0.36 and 2.12 ppt for the two Hemipterans respectively [7].

Low concentrations of pesticide may reduce pest population fecundity over time without causing immediate mortality. For example, inhalation of OMWW extracts by pupae of the Mediterranean flour moth, *Ephesia kuehniella* (Lepidoptera: Pyralidae) resulted in extended pupal duration period. In addition, emerging adults delayed time to oviposition and oviposited less eggs over fewer days [8]. Crude extract of OMWW caused weight loss for larvae of the palm tree pest *Potosia opaca* (Coleoptera: Scarabeidae). Increased larval mortality was recorded well over a week following multiple treatments [9].

The two mosquito species in this study also varied in their response to what we originally assumed to be a sublethal OMWW concentration. This concentration, was indeed not lethal to larval

Cx. laticinctus (Fig 1). The same concentration significantly reduced the survival of *Ae. albopictus* larvae whose LC₅₀ concentration was ~23 times higher (Fig 1; table 1). This response to relatively low OMWW concentration may point to the possibility that OMWW extracts can be applied by spraying urban areas that may contain *Ae. albopictus* breeding sites with limited danger to nontarget species. Although *Cx. laticinctus* survival was not reduced after a long exposure to low concentration extract, we did find a sex-specific effect on pupation time. Time for pupation was extended for males by almost a full day whereas females were not significantly affected. This resulted in an almost simultaneous emergence of males and females from OMWW treated cups (Fig 2). In most mosquito species, males have a shorter larval stage, emerge 1-2 days before the females and are significantly smaller than them [10]. However, males become sexually mature only a day after emergence, at the same time as later emerging female of the same cohort [10]. A situation where males and females emerge simultaneously following exposure to OMWW may result in delayed mating and a reduction of the overall number of mating couples.

Overall, our results support the possibility that OMWW, or its fractions, may be a potential source for the development of mosquito larvicide. Future research should focus on fractionation, isolation and identification of compounds from different fractions of OMWW with strong insecticidal activity. It is important to emphasize that this approach suits well the circular bio-economy and green chemistry models that concern the importance of valorization of agro-wastes and by-products generated by agricultural and agro-industries.

Acknowledgments: Avi Bar-Massada, and Elad Chiel helped with several aspects of this study. This work was supported by the program for University of Haifa and Shamir Research Institute joined research, and the Margolin grant awarded to Maram Halabi.

References

1. International Olive council- Olive oil & table olive figures.
2. Muscolo, A.; Romeo, F.; Marra, F.; Mallamaci, C. Recycling agricultural, municipal and industrial pollutant wastes into fertilizers for a sustainable healthy food production. *J Environ Manage* **2021**, *300*, 113771.
3. Moreno-Maroto, J.M.; Uceda-Rodríguez, M.; Cobo-Ceacero, C.J.; de Hoces, M.C.; MartínLara, M.Á; Cotes-Palomino, T.; García, A.B.L.; Martínez-García, C. Recycling of 'alperujo' (olive pomace) as a key component in the sintering of lightweight aggregates. *J Clean Prod* **2019**, *239*, 118041.
4. Messineo, A.; Maniscalco, M.P.; Volpe, R. Biomethane recovery from olive mill residues through anaerobic digestion: A review of the state of the art technology. *Sci Total Environ* **2020**, *703*, 135508.
5. Azzaz, A.A.; Jeguirim, M.; Kinigopoulou, V.; Doulgeris, C.; Goddard, M.; Jellali, S.; Ghimbeu, C.M. Olive mill wastewater: From a pollutant to green fuels, agricultural and water source and bio-fertilizer-Hydrothermal carbonization. *Sci Total Environ* **2020**, *733*, 139314.
6. El-Abbassi, A.; Saadaoui, N.; Kiai, H.; Raiti, J.; Hafidi, A. Potential applications of olive mill wastewater as biopesticide for crops protection. *Sci Total Environ* **2017**, *576*, 10-21.
7. Larif, M.; Zarrouk, A.; Soulaymani, A.; Elmidaoui, A. New innovation in order to recover the polyphenols of olive mill wastewater extracts for use as a biopesticide against the *Euphyllura olivina* and *Aphis citricola*. *Research on Chemical Intermediates* **2013**, *39*, 4303-4313.
8. Lahcene, S.; Taibi, F.; Mestar, N.; Ahmed, S.A.; Boumendjel, M.; Ouafi, S.; Houali, K. Insecticidal effects of the *Olea europaea* subsp. *laperrinei* extracts on the flour Pyralid *Ephesia kuehniella*. *Cell Mol Biol* **2018**, *64*, 6-12.
9. Boutaj, H.; Boutasknit, A.; Anli, M.; Ahmed, M.A.; El Abbassi, A.; Meddich, A. Insecticidal Effect of Olive Mill Wastewaters on *Potosia opaca* (Coleoptera: Scarabeidae) Larva. *Waste and Biomass Valorization* **2020**, *11*, 3397-3405.
10. Becker, N.; Petric, D.; Zgomba, M.; Boase, C.; Madon, M.; Dahl, C.; Kaiser, A.; SpringerLink *Mosquitoes and Their Control*, 2nd ed.; Springer: Berlin, 2010;.
11. Lowe, S.; Browne, M.; Boudjelas, S.; De Poorter, M. *100 of the world's worst invasive alien species: a selection from the global invasive species database*, Invasive Species Specialist Group: Auckland, New Zealand, 2000;.
12. Bonizzoni, M.; Gasperi, G.; Chen, X.; James, A.A. The invasive mosquito species *Aedes albopictus*: current knowledge and future perspectives. *Trends Parasitol* **2013**, *29*, 460-468.
13. Kamal, M.; Kenawy, M.A.; Rady, M.H.; Khaled, A.S.; Samy, A.M. Mapping the global potential distributions of two arboviral vectors *Aedes aegypti* and *Ae. albopictus* under changing climate. *PloS one* **2018**, *13*, e0210122.

14. Pener, H.; Wilamowski, A.; Schnur, H.; Orshan, L.; Shalom, U.; Bear, A. *Aedes albopictus* in Israel. *European Mosquito Bulletin* **2003**, *14*, 32.
15. Müller, G.C.; Kravchenko, V.D.; Junnila, A.; Schlein, Y. Tree-hole breeding mosquitoes in Israel. *Journal of Vector Ecology* **2012**, *37*, 102-109.
16. Chandel, K.; Suman, D.S.; Wang, Y.; Unlu, I.; Williges, E.; Williams, G.M.; Gaugler, R. Targeting a hidden enemy: pyriproxyfen autodissemination strategy for the control of the container mosquito *Aedes albopictus* in cryptic habitats. *PLoS neglected tropical diseases* **2016**, *10*, e0005235.
17. World Health Organization Guidelines for laboratory and field testing of mosquito larvicides. *WHO/CDS/WHOPES/GCDPP/2005.13* **2005**.
18. Finney, D.J. *Probit analysis: a statistical treatment of the sigmoid response curve*, Cambridge university press, Cambridge: Cambridge, England, 1952;.
19. IBM, C. IBM SPSS Statistics for Windows. **2016**, *version 24*, Armonk, NY-IBM Corp.
20. Pavela, R.; Maggi, F.; Iannarelli, R.; Benelli, G. Plant extracts for developing mosquito larvicides: From laboratory to the field, with insights on the modes of action. *Acta Trop* **2019**, *193*, 236-271.
21. Mulla, M.S. Susceptibility of various larval instars of *Culex p. quinquefasciatus* Say to insecticides. *Mosquito News* **1961**, *21*, 320-323.
22. Tafesh, A.; Najami, N.; Jadoun, J.; Halahlih, F.; Riepl, H.; Azaizeh, H. Synergistic antibacterial effects of polyphenolic compounds from olive mill wastewater. *Evidence-Based Complementary and Alternative Medicine* **2011**, *2011*, 1-9.

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.