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Research

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Azadirachta indica (Sapindales: Meliaceae) Neem Oil as a Repellent Against *Aedes albopictus* (Diptera: Culicidae) Mosquitoes

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Abstract

Increased concerns regarding the safety of many chemicals used as insecticides or repellents, together with the development of resistance by insects to various insecticides, have led to the search for new active natural ingredients. For what we believe is the first time, that is monitored the efficiency of neem oil (*Azadirachta indica*) as a repellent against adult *Aedes albopictus* directly in the field. To test the efficacy of repellency against adults, treatments were performed (5 ml/l) in six different locations. For each location, two closed gardens distance between them 200 m were chosen (one where the treatment was performed, consisting in spraying a mixture of water and Neem oil 5 g/l and one which was used as a control where only water with emulsifier was used). To monitor the treatment efficacy, six ovitraps were used (for each location three for the treated garden and three for the control garden). Data (n° mosquito eggs trapped in each ovitrap) were collected weekly for 6 mo (from May to October) for a total of 900 samplings. Daily temperature and daily rainfall were monitored throughout the trial. The treatment was most effective in June and July. Very high temperatures and high rainfall reduced the efficacy of neem oil over time.

Key words: neem oil, azadirachtin, adult, *Aedes albopictus*, repellent activity

The presence of mosquitoes in urban environments has always been a cause of annoyance to citizens. This has been aggravated by the introduction into Italy of exotic species such as *Aedes albopictus*. The enjoyment of many green areas such as parks, public and private gardens has become marred during the summer months.

There is also a real health and hygiene hazard due to the possible transmission of pathogenic microorganisms such as viruses responsible for dengue and chickungunya as well as other arboviruses (Moore and Mitchell 1997, Leta et al. 2018) and nematodes *Dirofilaria* spp. (Cancrini et al. 2007).

Aedes albopictus, the so-called 'tiger mosquito' is an insect of Asian origin, which has spread rapidly to other continents thanks to its notable ability to adapt quickly (Paupy et al. 2009). The infested areas are generally limited since this mosquito generally moves a few hundred meters from the larval breeding, especially with wind (Ferrarese et al. 2012). The current methods used to combat and control adult of mosquitoes, family Culicidae involve the use of both antilarval products applied to water containers and insecticides which are spatially distributed in the atmosphere and in green areas. These substances have a negative impact on ecosystems, they are toxic for not target insects, warm-blooded animals and for humans, and the insects involved have often developed insecticide resistance (Macchioni et al. 2008, Nicoletti et al. 2012).

On the other hand, natural compounds generally have a lower residual property, more rapidly biodegraded and a lower environmental impact than synthetic insecticides. Consequently, scientific studies are increasingly investigating plant-based products for insect control and infestation management in urban environments. The biocidal and repellent activity of neem oil extracted from the seeds of *Azadirachta indica*, against Culicidae has been confirmed by many studies in laboratory, but no studies are carried out in the field.

A solution of 5 ml consisting of coconut oil and neem oil was tested to all the exposed parts of the baits (volunteers) during the night to prevent mosquito bites. The results showed that the repellent property was greater in closed environments (50% and 40.9%, respectively) than in open environments (17.4% and 5.6%, respectively) probably due to the effect of absorption, abrasion, and component evaporation (Ravindran et al. 2002).

Vatandoost & Vaziri in 2004 tested neem oil (Neemarin) in the laboratory on larvae at the third and fourth stages of *Anopheles stephensi* and *Culex quinquefasciatus*, and in the field in artificial ponds against larvae, pupae, and adults belonging to *Anopheles* and *Culex*. The use of this product showed a mortality of 50% and 90% for concentrations of 0.35 and 1.81 mg/l for *An. stephensi* and 0.69 and 3.18 mg/l for *Cx. quinquefasciatus*, while in the field it had a maximum effectiveness time of 7 d using the maximum recommended quantity (2 liters/hectare).

To the best of our knowledge, neem oil repellency studies on Culicidae adults have not been performed in the field. Repellence studies involving neem oil have been performed using the oil on people's skin (Mandal 2011, Mukesh et al. 2014, Abiy et al. 2015, Nasir et al. 2015) or by mixing it in water as a repellent for ovo-deposition (Benelli et al. 2015). Its efficacy as an insecticide is due to the limonoid, called azadiractin, contained in the seeds (Schmutterer 1990). A high concentration of azadiractin can act as an insect repellent and can potentially also interrupt its reproduction. Neem extracts have minimal toxicity to nontarget organisms, such as parasitoids, predators, and pollinators (Naumann 1996) and rapidly degrade into the environment (Barrek et al. 2004). In contemporary paper (F. Macchioni, M. Sfingi, D. Chiavacci, and F. Cecchi F. 2020; unpublished data), we tested the efficacy of neem oil in the laboratory demonstrating it was highly effective as a larvicide in all larval stages of the insect.

The aims of the work were to test the efficacy of neem oil as a repellent against *Ae. albopictus* adults in the field, and to verify the influence of temperature and rainfall on neem oil used as a repellent.

Materials and Methods

Study Area

The study was carried out from May to October 2018 in six locations in Tuscany (central Italy): Cenaia, Ghezzano, La Vettola, S. Lorenzo alle Corti in the provinces of Pisa and Lucca with similar environmental conditions (light, temperature, humidity, and air quality). In each location, two private gardens with a hedge with a total perimeter of about 40 m were chosen. One garden was treated with neem oil in a spray and the other was used as a control, where only water with an emulsifier was used. The treated and control gardens of each location had similar characteristics, and the distance between them was at least 200 m in order to rule out possible interference during the experiment.

Treatment

The treatment consisted of spraying a mixture of water and neem oil (5 g/l; 0.3% Azadiractin in 1 liter containing the emulsifier Sorbitan, tri-9-octadecenoate, poly(oxy-1,2-ethanediyl) with a shoulder pump on the perimeter of each hedge. Each garden was treated with 1.6 liters of solution. A dosage of 5 g/l was selected following insecticide manufacturers' recommendations and by that 5 g/l is the concentration used by many pest companies for other products. The treatment was carried out once a week. After thunderstorms, the treatment was repeated as rain causes run-off from the surface of vegetation. Control gardens were treated with water and emulsifier without Azadiractin.

No pesticides were used during the experimental trial by the owners of the gardens and by the neighbors to avoid drift effects from wind carrying insecticides.

To monitor the effect of Neem oil as a repellent, and to check the number of *Aedes albopictus* in the treated and control gardens, a total of six ovitraps, three for the treated garden and three for the control garden were used for each location, arranged along the perimeter at a distance of about 5 m apart. The ovitraps were used specifically to catch *Aedes* spp. eggs, and were positioned close to vegetation, where the greatest concentration of adults can be found.

All ovitraps were checked weekly to count and specifically identify the eggs of *Ae. albopictus*, for a total of 900 samplings. Treatment began only when the control and treated areas reached equilibrium conditions, in which a similar number of eggs in the masonite strips contained in each ovitrap were found in the control and treated areas. In addition, in order to check the species of mosquitoes present in the considered location, Biogents mosquito traps (BGS and MOSQ, Biogents AG, Regensburg, the Biogents sentinel - BGS trap) were equipped with standard BG-lure (lactic acid, ammonia, and hexanoid acid). However, it was not possible to place adult traps in all the gardens, thus six Biogents mosquito traps were set up, one for the treated garden and one for the control garden in three locations (Ghezzano, La Vettola and Pontedera). Daily temperature (minimum, maximum, and average) and daily rainfall were also monitored throughout the trial.

Statistical Analysis

The whole sample, consisting of all the eggs collected each week for each month in each location, was used to verify the normality of errors (Shapiro-Wilks test: W = 0,95; $P < 0,05$) and the homogeneity of variances (Bartlett test). If the dataset was not normally distributed, it was log-trasformed. We analyzed data as repeated-measures ANOVA. In the model, the different months (from May to October), the treatments, and the locations along with all their interactions (treatment per month, treatment per location, month per location, and treatment per location per month) were considered as fixed effects and the different weekly collection within each month was considered as the random effect while the number of eggs was considered as the dependent variable (Table 1). Tukey–Kramer HSD (honestly significant difference) test was used to test differences in group means.

The average temperature and rainfall were calculated for each month. Analysis of variance (ANOVA) was used to test the differences between treatments in different months. Tukey–Kramer HSD test was used to test differences in group means. All analyses were performed using the statistical package JMP (SAS 2007).

Results

During the trial, 73,473 eggs were collected (38,699 in the control gardens and 34,774 in the treated gardens). In the trial, the total number of eggs counted in the control gardens was greater than 3,925 eggs compared to those revealed in the treated gardens. Despite the higher number of the eggs in the control gardens, the pattern of results from ANOVA for repeated-measures analysis shows that the treatment had not significant effect (Table 1), on the contrary location and month resulted significant (P = 0.0055 and $P < 0.0001$, respectively). Significant interactions were observed between treatment with month (P = 0.0036), treatment with location (P = 0.0055), and location with month ($P < 0.0001$).

Analyzing the data in detail, in all the gardens, with the exception of the gardens in S. Lorenzo alle Corti, there was a smaller overall number of collected eggs in the treated gardens than in the control. The differences in eggs between treated and control gardens were statistically significant only in the locations of Ghezzano and Lucca (Table 2).

Table 1. Repeated measures analysis of variance results

Source	df	*F*	*P*
Location	5	3.3319	**0.0055**
Treatment	1	3.0767	0.0798
Month	5	35.2558	**<0.0001**
Treatment × Month	5	3.9261	**0.0036**
Treatment × Location	5	5.2380	**0.0055**
Location × Month	28	3.5002	**<0.0001**
Treatment × Location × Month	28	1.2392	0.1839

Statistically significant *P*-value (<0.05) are shown in bold.

Table 2. Average numbers of weekly collected eggs for each month in the treated and control gardens of each location

Location	Cenaia		Ghezzano		La Vettola		Lucca		Pontedera		S. Lorenzo	
	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control
Month												
May	28.41	25.33	26.21	42.98	57.86	46.31	17.45	30.29	27.89	31.89	48.34	36.30
June	52.66	60.99	64.72	64.38	111.17	109.45	32.56	65.09	56.38	63.30	64.69	61.94
July	98.48	90.94	83.91^B	102.95^A	152.67	143.77	53.31*B*	120.10*A*	111.20	84.24	109.91	92.48
Aug.	81.00	71.17	64.77^B	102.84^A	130.46	128.73	51.14	85.03	79.79	78.41	90.58	74.58
Sept.	103.49	100.67	90.50	129.10	157.50	153.68	82.65	105.50	100.67	109.52	118.90	98.24
Oct.	44.76	50.59	31.75	79.05	105.00	97.37	39.46	39.89	54.71	46.68	51.61	56.73
Total monthly eggs for each location	4,720	5,333	5,234	6,534	10,106	10,340	3,220	5,139	6,083	6,086	5,413	5,269

Means within the same row and in the same location followed by different letters show significant differences (A,B:$P < 0.001$).

Table 3. Average number of eggs collected weekly in each month in the treated and control gardens

Month		Control		Treated	
		Mean	SE	Mean	SE
May	N°	42.15	9.189	32.46	8.182
June	“	76.03^A	6.637	58.54^B	6.145
July	“	119.68^A	19.492	90.36^B	6.498
Aug.	“	85.38	6.126	87.70	12.131
Sept.	“	106.86	12.885	118.27	12.648
Oct.	“	58.83	8.333	57.44	6.497

Means within the same line followed by different letters show significant differences (A,B:$P < 0.01$).

SE, standard error.

Table 4. Weather data (total monthly rainfalls and temperatures for each month)

	Total monthly rainfall (mm)	Average Monthly T (°C)	Monthly T min (°C)	Monthly T max (°C)
May	19.66^A	16.46^C	13.33^D	23.58^C
June	3.35^B	21.88^B	16.08^C	27.66^B
July	4.62^B	24.79^A	19.47^A	30.09^A
Aug.	18.19^A	24.22^A	18.62^B	29.82^A
Sept.	0.90^B	22.20^B	16.41^C	27.99^B
Oct.	2.00^B	19.25^C	14.33^D	24.17^C

Means within the same column followed by different letters show significant differences (A,B,C,D:$P < 0.001$).

Table 3 shows the average values of the eggs collected for each month in the total treated gardens (df = 5) and in the total control gardens (df = 5). Significant differences between treated and control gardens were detected in June and July. Table 4 reports the weather data (rainfall and temperature for each month; df = 29), with July and August having the highest significant temperatures ($F = 26.159$; $P < 0.001$), while the significant lowest temperatures ($F = 40.168$; $P < 0.0001$) were detected in May and October. The maximum rainfall was in May and August ($F = 14.473$; $P < 0.001$).

Considering Tables 3 and 4 that analyzed data month by month, we can see that in May, which was characterized by high frequency rainfall, low temperatures, and few mosquitoes, the treatment showed no effect. In June, an increase in temperature and a drastic drop in rainfall together with a significant increase in the number of eggs produced were observed. The treatment in June was effective (Table 3), showing statistically significant differences between the treated and control gardens (58.54 eggs vs 76.03 eggs, respectively). The maximum effect of the treatment was observed in July (90.36 eggs vs 119.68 eggs in the control area) in relation to high temperatures, low rainfall, and a peak in the number of mosquitoes.

In August, there was a combination of high temperatures and high rainfall which may have affected negatively the efficacy of the active principle despite repeated postrain treatments. In September, there was a significant increase in mosquitoes similar to July but the treatment had no effect.

As mentioned in the introduction, it was not possible to place adult traps in all the gardens used in the study. For this reason, only the numbers of the different species, divided according to sex, captured with adult traps in the various gardens, are reported here (Table 5).

Discussion

Although the rainfall was similar for amount in May and August, there were differences in the efficacy of Neem oil tested in the two following months. In May, the high amount of rainfall did not influence the efficacy of the treatment in the following months (June and July).

In August, the high rainfall influenced the efficacy of the Neem oil tested in the following months (October and November) as there was a great intensity of mosquitoes. Probably we suggest that when the number of mosquitoes is high, the Neem oil should have a higher percentage of azadirachtina.

Interestingly, although the repellent treatment was statistically effective only in two locations (Ghezzano and Lucca), in some months, there was an overall reduction of number of mosquitoes in the treated areas with respect to the control areas in all locations. Neem oil seems to have the higher effect in June and July, i.e., in periods with the highest temperatures and lowest rainfall, highlighting the effectiveness of the treatment in these months.

It is possible that its scarce and nonhomogeneous efficacy as a repellent during the trial period may be due to a low dosage or also to the interaction of many other climatic factors, such as high summer temperatures, humidity, rain, which may have accelerated the degradation of the active principle (azadiractina), as hypothesized by Nicoletti et al. (2012), or may have influenced the insect biology. It

Table 5. Adult individuals caught in three locations in both treated and control gardens by species and sex

	Ghezzano		La Vettola		Pontedera	
	Treated	Control	Treated	Control	Treated	Control
	N°	N°	N°	N°	N°	N°
Ae. albopictus species	880	1,019	857	752	1,126	1,179
Females	810	902	780	683	1,092	974
Males	70	117	77	69	34	205
Cx. pipiens species	835	730	1,119	621	1,590	566
Females	779	634	1,089	599	1,134	530
Males	36	96	30	22	256	36

is however interesting to note that in total approximately 4,000 eggs less, corresponding to 5% of the total, were produced throughout the entire test in the treated gardens which could lead to a reduction in mosquitoes in the long run.

During the monitoring phase, only two species, *Cx. pipiens* and *Ae. albopictus,* were captured using adult traps. This indicates that these two species are common in this area, as reported by Severini et al. (2009), thus confirming that over the years the invasive species *Ae. albopictus* has spread widely in this area. Both female and male specimens were found inside the traps. The number of females was clearly greater than males, since the substance, which is located inside the trap, attracts female specimens. The presence of male specimens was probably due to accidental entry, sexual attraction based on acoustic stimuli of the female's wing beat, and the noise caused by the trap (Ikeshoji 1981). In order to understand whether neem oil could become a valid repellent against the tiger mosquito, further experiments are necessary. For example, in future researches, neem oil could be used with higher percentages of azadiractin than 0.3%/l, or/and also more than 5 ml/l of neem oil could be diluted in water. Considering the costs/benefits, a liter of Neem oil costs as the best commercial synthetic product that contain coformulants dangerous for vegetation. Moreover, it has the advantage aspects of being a natural oil product.

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