ACTA ZOOLOGICA BULGARICA

Applied Zoology
Research Article

Acta Zool. Bulg., 72 (3), September 2020: 479-485

Published online 30 September 2020
http://www.acta-zoologica-bulgarica.eu/002404

Larvicidal and Pupicidal Activity of Neem Oil (*Azadirachta indica*) Formulation against Mosquitoes *Aedes albopictus* (Skuse, 1894) (Diptera: Culicidae)

Fabio Macchioni[1*], Marta Sfingi[1], Daniele Chiavacci[2] & Francesca Cecchi[1]

[1] Department of Veterinary Science, University of Pisa, Italy
[2] Entomox Srl (Disinfestation and Rat Extermination), Pisa, Italy

Abstract: The control against mosquitoes should be specifically directed at the larvae. Combating the adults is temporary, ineffective and polluting the environment, while larval treatment is more localised in time and space and is thus less dangerous. The aim of our work is to evaluate the insecticidal activity under laboratory conditions of a new natural anti-mosquito formulation (neem oil, a.i azadirachtin A 0.3%) against *Aedes albopictus*. The neem oil (azadirachtin A 0.3%) was tested in water at different dosages: 5 mg; 10 mg; 25 mg; 50 mg and 75 mg on larvae at the stages L1–L2, L3–L4 and pupae. Four replicas were conducted. The LD50 and LD90 levels were 14 mg (95%; CI = 5–19 mg) and 70 mg (95%; CI = 27.9–98.1 mg), respectively, for the larvae at the stages L1–L2 and 20 mg (95%; CI = 4.4–24.4 mg) and 30 mg (95%; CI = 3.6–50.4 mg), respectively, for the larvae at the stages L3–L4. Regarding the treatment at the pupal stage, after the first 24h, there is a higher mortality rate compared to the control, showing its effectiveness as pupicide.

Key words: azadirachtin; larvae; pupae; Asian tiger mosquito; antimosquito activity

Introduction

The Asian tiger mosquito *Aedes albopictus* (Skuse, 1894) is native to Southeast Asia but has spread to all continents except Antarctica. It has been recorded in Italy since 1990 (Sabatini et al. 1990, Romi 2001, Romi et al. 2009a). Aside from their importance as potential vectors of the dengue virus, tiger mosquitoes can pass the encephalitis virus to humans and animals and several arboviruses (Boromisa et al. 1987, Benelli 2015, Benelli & Mehlhorn 2016) and carry a host of filarial species such as *Dirofilaria immitis* (Leydi, 1856), *D. repens* (Railliet & Henry, 1911), and *Setaria labiatopapillosa* (Alesandrini, 1848) (Cancrini et al. 1995, 2003a, 2003b, 2007). Mosquitoes are also a cause of irritation, blood loss and allergic reactions, and can disrupt the normal behaviour of livestock. Mosquito bites can result in decreased weight gain and milk production in cattle (Foster et al. 2019).

Mosquito control should be specifically directed against the larvae. Combating the adults is temporary, ineffective, and polluting for the environment, while larval treatment is more localised in time and space and is thus less dangerous. Common antilarval products are synthetic molecules (methoprene, piryproxifen, diflubenzuron) or natural compounds such as a crystal protoxin of *Bacillus thuringiensis* var. *israelensis* (Bti) produced during the sporulation process. This product is the most commonly used because it is considered to be harmless to humans, fish, and other non-target insects. However, the antilarval activity of Bti is very short (Romi et al. 2009b).

[*]Corresponding author: fabio.macchioni@unipi.it

Substances with insecticidal activity are usually synthesised, and these present problems concerning the level of insecticide-resistance, the environmental impact on ecosystems and their toxicity for warm-blooded animals and humans (Macchioni et al. 2008; 2019, Nicoletti et al. 2012a). In general, natural compounds have a lower residual capacity and environmental impact than synthetic drugs; recent studies have evaluated and confirmed the activity of numerous plant-derived natural products as valuable sources of novel bioactive substances against fungi, protozoa, helminths, and even arthropods (Nicoletti et al. 2012a, 2012b). Numerous plant products have been reported for killing larvae of mosquitoes and provide excellent alternatives for mosquito control (Brown 1986).

Neem trees *Azadirachta indica* (Juss, 1830) are fast-growing evergreen trees that are native to India and belong to the family Meliaceae. For over 2000 years, the tree has been known as one of the most versatile medicinal plants, with a wide spectrum of biological activity. All parts of the tree have been used as traditional medications for household remedies against various human ailments, dating from antiquity (Ogbuewu et al. 2011). Neem seeds contain approximately 99 biologically active compounds, of which azadirachtin, nimbin, nimbidin and nimbolides are the main molecules (Dua et al. 2009). The neem oil is extracted from the seeds of *A. indica*, whose active ingredient is azadirachtin, a natural organic substance that acts primarily through ingestion and secondarily by contact. As reported by EPA (2012), the effective properties of 100% Cold Pressed Neem oil against a huge range of pests of medical and veterinary importance led to consider this oil the most interesting active ingredients that can be used in biotechnical processes, and in organic farming against certain species of insects including mosquitoes, whereas, useful insects, like bees, are not affected. Furthermore, its low impact on both vertebrate health and the environment as reported by which declared that there is no concerns for any non-target organisms increased his consideration (Nicoletti et al. 2012b). Its toxicity is extremely low for humans and for vertebrates including fish. The confirmed oral LC50 in rats is higher than 5000 mg/kg and the confirmed dermal LC50 in rabbits is above 2000 mg/kg) (Raizada et al. 2001). The environmental impact is also very low: it shows remarkable selectivity towards useful entomofauna including bees and therefore it is also compatible with biological approaches. In the soil it degrades in few days. In addition, the insect growth regulatory activity of neem weakens the cuticle defence system of the larvae causing easy penetration of pathogenic organisms into the insect system (Dua 2009). Some of experiments and important veterinary applications against parasites and bloodsucking arthropods are reported, evidencing that the research on neem activities is far to be completed (Akihisa et al. 2009, Al-Samarrai et al. 2012, Chava et al. 2012, Montezano et al. 2014, Del Serrone et al. 2015, Ramachandramohan & Mamatha 2015, Shanmugapriyan & Dhanalakshmi 2015, Alzohairy 2016).

The aim of our work is to assay new natural anti-mosquito formulation based on neem oil (azadirachtin A) under laboratory conditions.

Materials and Methods

Experimental design and collection of insects

The larvae and pupae used in the experiment were derived from *Ae. albopictus* eggs captured through ovitraps (Entomox Srl, Pisa) placed in selected areas of Pisa (Italy) (latitude 43°42'42"48 N, Longitude 10°24'52"92 E) where the presence of mosquitoes was observed during previous inspections. Masonite strips (3x15 cm) were suspended vertically in black pots filled with 350 ml of water to provide a suitable surface for eggs deposition. Every week, the pots were rinsed and filled and the strips changed and checked for eggs presence. The test containers were held at 25–28 °C and preferably a photoperiod of 12 h light followed by 12 h dark.

To obtain both larvae and pupae, the strips were left to dry at room temperature for three days, then placed individually into plastic trays containing dechlorinated tap water, after which first-stage larvae were obtained and the strips were again left to dry. This alternating wet and dry procedure was repeated twice (Toma et al. 2008). In the experiment three groups, larvae at the stages L1–L2, L3–L4 and pupal stage of *Ae. albopictus* were tested with the following dosages of neem oil: 5 mg, 10 mg, 25 mg, 50 mg and 75 mg. The Neem oil used in the experiment is cold-extracted to maintain the integrity of the active substance (azadirachtin), and is soluble in water (0.3% azadirachtin A).

Insecticidal bioassays

The experimental unit consisted of plastic cup containing 500 ml of solution composed of tap water at room temperature and neem oil (Azadirachtin A 0.3% in 1 liter, containing the emulsifier Sorbitan, tri-9-octadecenoate, poly(oxy-1,2-ethanediyl) (Macchioni et al. 2019) and 25 larvae at the stages L1–L2 and L3–L4, or at the pupal stage for each cup. Each bioassay included a control where no

neem oil but water with emulsifier was applied. The treatments were repeated four times.

During the experiment the larvae were fed with dry cat food consisting of chicken protein (38%), fat (16%), fiber (3%), and inorganic substance (8%), for a larva quantity of 2.5 g. To prevent the formation of a film caused by an excess of provided food, which is harmful to the larvae as it prevents breathing on the surface of the water, in the first six days food was given according to larvae age: first and second day 10%, third day 15%, fourth day 21%, and fifth and sixth days 22% (Carrieri et al. 2003). If food was still observed in the cups, no more was added to avoid the film formation.

After 24 hr contact at room temperature, the number of dead larvae in each cup was counted and reported as the average of the four replicates for each dosage. The larvae were considered dead if they were immobile and unable to reach the water surface (Macêdo et al. 1997). The observations were carried out until all the larvae either had died or developed adults.

Statistical analysis

The mortality percentage was calculated based on the formula of mean number of deceased larvae/ mean number of initial larvae.

The experiments were established under a completely randomized design. The data were tested to verify the normality of errors (Shapiro-Wilks test) and the homogeneity of variances (Bartlett test).

Data were subjected to analysis of variance ANOVA, in which different dosage levels (5 mg, 10 mg, 25 mg, 50 mg, 75 mg; control) and the day of the survey along with their interactions were considered as fixed effects, with mortality percentage of larvae at the stages L1–L2 or L3–L4 or pupae as the dependent variable, using the statistical package JMP (SAS 2007). Tukey-Kramer HSD (honestly significant difference) test was used to test differences in group means.

The dosages leading to 50% and 90% mortality (LD50 and LD90) were determined for larvae at the stages L1–L2 and L3–L4 by probit analysis. Statistical analysis was undertaken using the statistical package Graph Pad Prism 8 ®.

Results

Larvicidal activity of neem oil against of larva at the stages L1–L2

Dosages with 75 mg, 50 mg, and 25 mg (Table 1) caused almost 100% of the larvae mortality. At the 75 mg dosage, all larvae died within one week, while for the other two dosages, 50 mg and 25 mg, they died within 19 days. At the dosage of 10 mg, the percentage of larvae mortality was 71% within 22 days, while at the dosage of 5 mg it was 49% within 18 days. At 25 mg, 10 mg and 5 mg dosages, the larvae lived longer than those in the control. In fact on the eighth day of the control all of the larvae had either developed into adults (48%) and successively killed or were dead for natural causes (52%). At the dosages of 5 mg and 10 mg, the difference between the mortality of larvae in the experiment and in the control for the entire period was not statistically significant.

The dosages 25 mg, 50 mg and 75 mg were, however, all equally effective (there are not statistically significant differences between them), and eliminated all the larvae in the majority of cases. Highly significant differences (df=5; F=3.443; P=0.0067) were found between each of these dosages and the control (Table 1). We could observe that at a dosage of 25 mg neem oil had good larvicidal activity comparable with the highest dosages tested. However, when analysing the results up to the eighth day of treatment (Table 2), significant differences in the timing of elimination of larvae between the different dosages can be observed.

In the first 24 hours, there were no dead larvae in the control but there was a decrease in the number of larvae presented in all neem oil dosages. In particular, the resulting mortality value (57%) at the 75 mg of dosage was significantly (df=35; F=3.836; P=0.0042) higher than the other dosages. Lethal dosage at 50% and 90% (LD_{50} and LD_{90}) of larvae at the stages L1-L2. In Table 3 the results obtained using the statistical package Graph Pad are reported. The LD_{50} can be seen to correspond to a dosage of 14 mg while the LD_{90} corresponds to a dosage of 70 mg of neem oil.

Larvicidal activity of Neem oil against larvae at the stages L3-L4

As shown in Table 2, in the control all larvae were developed into adults, while all dosages of neem oil tested had good larvicidal activity, resulting in a significant statistical difference from the control (df=5; F=4.243; P=0.0056). Dosages of 75 mg and 50 mg 100% larval mortality in one week, while at dosages of 25 mg, 10 mg, and 5 mg the mortality was 94% in 16 days, 71% in 18 days, and 41% in 25 days, respectively, highlighting a delay in larval development compared to the control.

Larvae at the stages L3–L4, similarly to those in the previous stages, live longer than those of the control in dosages of 25 mg, 10 mg and 5 mg. From

Table 1. Number of dead larvae at L1-L2 and L3-L4 larval stages with the relative percentages per dosage (Means± SE).

Dosage	L1 and L2 larval stages		L3 and L4 larval stages	
	No of dead larvae Means ± SE	Mortality rate	No of dead larvae Means ± SE	Mortality rate
5 mg	12.25±1.109^{B}	49%	10.25±0.478^{D}	41%
10 mg	17.75±3.614^{ABb}	71%	17.75±0.750^{C}	71%
25 mg	25.00±0.00^{Aa}	100%	23.50±0.288^{B}	94%
50 mg	23.25±0.436^{Aa}	93%	25.00±0.00^{A}	00%
75 mg	25.00±0.00^{Aa}	100%	25.00±0.00^{A}	100%
Control	13.00±2.380^{B}	52%	0.00±0.00^{E}	0%

Means within the column followed by different letters show significant differences (A–E: P<0.01; a, b: P<0.05).

Table 2. Percentage of mortality for day of treatment of larvae at L1 and L2 larval stages.

Day	Neem oil dosage					Control tests
	5 mg	10 mg	25 mg	50 mg	75 mg	
	% Dead larvae	% Dead larvae	% Dead larvae	% Dead larvae	% Dead larvae	% Dead larvae
1	17^{BC}	23^{B}	25^{B}	27^{B}	57^{A}	0^{C}
2	32^{BC}	35^{BC}	60^{AB}	68^{AB}	97^{A}	25^{CD}
3	37^{BC}	40^{ABC}	72^{AB}	73^{A}	99^{A}	29^{CD}
4	40^{B}	40^{B}	76^{A}	77^{A}	99^{A}	39^{B}
5	42^{B}	40^{B}	80^{A}	88^{A}	99^{A}	45^{B}
6	45^{B}	43^{B}	80^{A}	90^{A}	99^{A}	50^{B}
7	47^{B}	59^{B}	82^{A}	94^{A}	100^{A}	52^{B}
8	48^{B}	61^{B}	85^{A}	94^{A}		52^{B}

Means within the same row followed by different letters show significant differences (A–D: P<0.01).

Table 3. Lethal dosage at 50% and 90% on larvae at L1–L2 and L3–L4 larval stages, confidence intervals and Slope ± Standard Error (SE).

Stage	LD_{50}	Confidence intervals		LD_{90}	Confidence intervals		Slope ± SE
		lower bound	upper bound		lower bound	upper bound	
L1-L2	14	5	19	70	28	98	2.86±0.282
L3-L4	20	4.4	24.4	30	4	50	4.46±0.611

Table 4. Percentage of mortality for day of treatment of larvae at L3 and L4 larval stages .

Day	Neem oil dosage					Control tests
	5 mg	10 mg	25 mg	50 mg	75 mg	
	% Dead larvae	% Dead larvae	% Dead larvae	% Dead larvae	% Dead larvae	% Dead larvae
1	13^{C}	23^{BC}	43^{B}	70^{A}	83^{A}	0^{BC}
2	13^{D}	23^{C}	52^{B}	77^{A}	81^{A}	0^{D}
3	13^{D}	25^{B}	60^{B}	82^{A}	88^{A}	0^{D}
4	13^{C}	25^{B}	72^{A}	83^{A}	88^{A}	0^{C}
5	13^{C}	28^{B}	75^{A}	89^{A}	88^{A}	0^{C}
6	13^{C}	34^{B}	84^{A}	92^{A}	100^{A}	0^{C}
7	21^{C}	41^{B}	97^{A}	100^{A}		0^{C}
8	22^{C}	42^{B}	98^{A}			0^{D}
9	23^{C}	43^{B}	98^{A}			0^{D}
10	24^{C}	43^{B}	98^{A}			0^{D}
11	26^{C}	44%^{B}	98%^{A}			0%^{D}

Means within the same row followed by different letters show significantly differences (A–D: P<0.01).

analysing the results up to the eleventh day of treatment (Table 4), significant differences in the timing of elimination of larvae in the different dosages were found. In the first three days the dosages of 50 mg and 75 mg of neem oil caused a significantly higher number of deaths compared to the control and to the other dosages (df=50; F=5.864; P=0.0038).

Up to the fourth day, the dosage of 25 mg had the same effect as the two previous dosages.

The lower dosages (5 mg and 10 mg) had a lower significant larvicidal effect in comparison to the higher dosages, but the results were effective with respect to the control.

Lethal dosage at 50% and 90% (LD_{50} and LD_{90}) of larvae at the stages L3–L4

The percentages of larvae at the stages L3–L4 that died in 72 h for each replica (A, B, C, D) was performed and these data were used to calculate the lethal dosages at 50% and 90% (Table 3). The results show that the LD_{50} corresponds to a dosage of 20 mg of neem oil while the LD_{90} corresponds to a dosage of 30 mg.

Pupicidal activity of neem oil

The results on the mortality of pupae treated at different dosages of neem oil (Table 5) show that overall, after the first 24 h, the number of dead pupae was significantly higher compared to the control variant.

Table 5. Average mortality of pupae treated with different dosages of neem oil.

Day	Neem Dosage					
	5 mg	**10 mg**	**25 mg**	**50 mg**	**75 mg**	**Control**
1	23.00	23.25	25.00	25.00	24.50	0.00
2	24.00	24.50	-	-	24.50	0.00
3	24.75	-	-	-	25.00	0.25
4	25.00	-	-	-	-	0.50

Discussion

The results have shown that with the increase in neem oil dosage, there are a greater number of deaths of the larvae in all stages. The effect of the treatment is therefore dosage-dependent as also found by Benelli et al. (2015) and by Kempraj & Bhat (2008).

The larvae at the stages L1–L2 treated with 5 mg of neem oil had a higher mortality level than the larvae at the stages L3–L4 (subjected to the same dosage). Conversely, for all the other dosages there is no difference in mortality between the two groups L1–L2 and L3–L4. By analysing the two control groups, we can observe that the first group had a mortality rate of 52% while in the second group the larvae were all alive. This may be due to the different larval ages. Those in the early stages are physiologically and naturally more vulnerable than the larvae in the later stages. It has been also observed that the average life span of larvae at all stages at various dosages of neem oil, before flicking or dying, is generally longer than that of the control. In fact, in the control the larvae conclude their cycle by flicking within a week, while with the various dosages of neem oil the time is in general longer. This observation is reported in other studies, which show that neem oil prolongs the larval and pupal stages (Sagar & Sehgal 1997).

The effect of neem oil on the extension of life span is particularly evident for the larvae at the stages L1–L2. At these stages, the LD_{90} is particularly high, showing that a high dose of the substance is required to eliminate 90% of the larvae within 72 hours. The effect of neem oil on the extension of larvae life span is therefore higher than the mortality effect. However, in the larvae at the stages L3–L4 the LD_{90} is very low, confirming the greater effect of the oil on the mortality of the larvae.

Azadirachtin, the main active substance in neem oil, interferes with the synthesis and release of the moult hormone, causing an inhibition of larval development (Isman 2006). Champagne et al. (1998) reported that the active substance azadirachtin influences the growth and metamorphosis of various insects, interfering with the production of ecdysone and the juvenile hormone.

In our experiments, treatment with neem oil at the pupal stage has been very effective in the first 24 h at a lower dosage (5 mg) and a high mortality rate has been recorded compared to the control. This result is interesting because it is typically reported that treatments with insect growth regulators (IGRs) do not kill the pupae in a short time (Romi et al. 2009a).

As far as we know, no experiments have been conducted in which natural substances are tested on larvae at the first stage or on pupae (Anjali et al. 2012, Benelli et al. 2015, Dua et al. 2009). Our experiment included all the larval stages (L1, L2, L3 and L4) and the pupal stage at the same time.

Conclusion

This experiment using neem oil on larvae at all stages can be considered a preliminary study that can lead to further investigations of the effective-

ness of neem oil on *Ae. albopictus* and others culicids. Additional tests with a larger number of replicas will be necessary to obtain more accurate data. Based on our preliminary results we conclude that neem oil showed strong insecticide action against larvae and pupae of the Asian tiger mosquito and it is a promising alternative control method for mosquito larvae.

Acknowledgements: This study has been supported by Fondi di Ateneo University of Pisa (Dr. Fabio Macchioni) and by "Entomox Srl, Company of Disinfestation and Rat Extermination, Pisa Italy".

Disclosure statement: No potential conflict of interest was reported by the authors.

References

Akihisa T., Noto T., Takahashi A., Fujita Y., Banno N., Tokuda H., Koike K., Suzuki T. & Yasukawa K. 2009. Melanogenesis inhibitory, anti-inflammatory, and chemopreventive effects of limonoids from the seeds of *Azadirachta indica* A. Juss. (neem). Journal of Oleo Science 58: 581–594.

Anjali C., Sharma H. Y., Mukherjee A. & Chandrasekaran N. 2012. Neem oil (*Azadirachta indica*) nanoemulsion – a potent larvicidal agent against *Culex quinquefasciatus*. Pest Management Science 68(2): 158–163.

Al-Samarrai G., Singh H. & Syarhabil M. 2012. Evaluating eco-friendly botanicals (natural plant extracts) as alternatives to synthetic fungicides. Annals of Agricultural Environmental Medicine 19: 673–676.

Alzohairy M.A. 2016. Therapeutics role of *Azadirachta indica* (Neem) and their active constituents in diseases prevention and treatment. Evidence-Based Complementary and Alternative Medicine, Article ID 7382506. doi: 10.1155/2016/7382506.

Benelli G., Bedini S., Cosci F., Toniolo C., Conti B. & Nicoletti M. 2015. Larvicidal and ovideterrent properties of neem oil and fractions against the filariasis vector *Aedes albopictus* (Diptera: Culicidae): a bioactivity survey across production sites. Parasitology Research 114(1): 227–236.

Benelli G. & Mehlhorn H. 2016. Declining malaria, rising of dengue and Zika virus: insights for mosquito vector control. Parasitology Research 115: 1747-1754.

Boromisa R.D., Rai K.S. & Grimstad P. R. 1987. Variation in the vector competence of geographic strains of *Aedes albopictus* for dengue l virus. Journal of America Mosquito Control Association 3: 378–386.

Brown A.W.A. 1986. Insecticide resistance in mosquitoes: a pragmatic review. Journal of American Mosquito Control Association 2: 123–140.

Cancrini G., Pietrobelli M., Frangipane di Regalbono A., Tampieri M.P. & Della Torre A. 1995. Development of *Dirofilaria* and *Setaria* nematodes in *Aedes albopictus*. Parassitologia 37: 141–145.

Cancrini G., Frangipane di Regalbono A., Ricci I., Tessarin C., Gabrielli S. & Pietrobelli M. 2003a. *Aedes albopictus* is a natural vector of *Dirofilaria immitis* in Italy. Veterinary Parasitology 118: 195–202.

Cancrini G., Romi R., Gabrielli S., Toma L., Di Paolo M. & Scaramozzino P. 2003b. First finding of *Dirofilaria repens* in a natural population of *Aedes albopictus*. Medical and Veterinary Entomology 17: 448–451.

Cancrini G., Scaramozzino P., Gabrielli S., Paolo M.D, Toma L. & Romi R. 2007. *Aedes albopictus* and *Culex pipiens* implicated as natural vectors of *Dirofilaria repens* in central Italy. Journal of Medical Entomology 44(6): 1064–1066.

Carrieri M., Bacchi M., Bellini R. & Maini S. 2003. On the competition occurring between *Aedes albopictus* and *Culex pipiens* (Diptera: Culicidae) in Italy. Environmental Entomology 32(6): 1313–1321.

Champagne D.E., Isman M.B. & Towers G.H.N. 1998. Insecticidal activity of phytochemicals and extracts of Meliaceae. In: Amason J.T., Phylogene B.J.R. & Morand P. (Eds.) Insecticides of plant origin. ACS Symposium Series 387: 95–109.

Chava V. R., Manjunath S. M., Rajanikanth A.V. & Sridevi N. 2012. The efficacy of neem extract on four microorganisms responsible for causing dental caries viz *Streptococcus mutans*, *Streptococcussalivarius*, *Streptococcus mitis* and *Streptococcus sanguis*: An in vitro study. The Journal of Contemporary. Dental Practice 13: 769–772.

Del Serrone P., Failla S. & Nicoletti M. 2015. Natural control of bacteria affecting meat quality by a neem (*Azadirachta indica* A. Juss) cake extract. Natural Product Research 29: 985–987.

Dua V.K., Pandey A.C. Raghavendra K., Gupta A., Sharma T. & Dash A. P. 2009. Larvicidal activity of neem oil (*Azadirachta indica*) formulation against mosquitoes. Malaria Journal 8: 124.

EPA. 2012. Biopesticide Registration Action Document. Cold Pressed Neem Oil. PC Code 025006. Washington, DC: Office of Pesticide Programs.

Foster W.A. & Walker E.D. 2019. Mosquitoes (Culicidae). In: Mullen G.R. & Durden L.A. (Eds.): Medical and Veterinary Entomology (Third Edition). London: Academic Press, pp. 261-325.

Isman M. B. 2006. Botanical insecticides, deterrents, and repellents in modern agriculture and an increasingly regulated world. Annual Review of Entomology 51: 45–66.

Kempra J.V. & Bhat S.K. 2008. Ovicidal and larvicidal activities of *Cyperus giganteus* Vahl and *Cyperus rotundus* Linn. Essential oils against *Aedes albopictus* (Skuse). Natural Product Radiance 7(5): 416–419.

Macchioni F., Siciliano T., Magi M., Cecchi F., Cioni P.L. & Braca A. 2008. Activity of aqueous extract of *Panax notoginseng* flowers buds against *Aedes albopictus* larvae and pupae. Bulletin of Insectology 61(1): 31–35.

Macchioni F., Sfinfi M., Chiavacci D. & Cecchi F. 2019. *Azadirachta indica* (Sapindales: Meliaceae) neem oil as a repellent against *Aedes albopictus* (Diptera: Culicidae) mosquitoes. Journal of Insect Science 19(6): 12 (1-4).

Macêdo M.E., Consoli R.A.G.B., Grandi T.S.M., Anjos A.M.D., Oliveira A.B.D., Mendes N.M., Queiròz R.O. & Zani C. L. 1997. Screening of Asteraceae (Compositae) plant extracts for larvicidal activity against *Aedes fluviatilis* (Diptera: Culicidae). Memórias do Instituto Oswaldo Cruz 92: 565–570.

Montezano D.G., Specht A., Sosa-Gomez D.R., Roque-Specht V.F. & Barros N.M. 2014. Immature stages of *Spodoptera eridania* (Lepidoptera: Noctuidae): developmental parameters and host plants. Journal of Insect Science 14: 1–11.

Nicoletti M., Maccioni O., Coccioletti T., Mariani S. & Vitali F. 2012a. Neem tree (*Azadiractha indica* A. Juss) as source of bioinsecticides. In: Perveen F. (Ed.): Insecticides – advances in integrated pest management. Rijeka: InTech; pp. 411–428.

Nicoletti M., Mariani S., Maccioni O., Coccioletti T. & Murugan K. 2012b. Neem cake: chemical composition and larvicidal activity on Asian tiger mosquito. Parasitology Research 111: 205–213.

Ogbuewu I.P., Odoemenam V.U., Obikaonu H.O., Opara M.N., Emenalom O.O., Uchegbu M.C., Okoli I.C., Esonu B.O. & Iloeje M.U. 2011. The Growing Importance of Neem (*Azadirachta indica* A. Juss) in Agriculture, Industry, Medicine and Environment: A Review. Journal of Medical Plants Research 5(3): 230–245.

Ramachandramohan M. & Mamatha P. 2015. Impact of Biopesticide Neem oil for beneficial to fisheries resources – studies on skin with neem oil exposure to fresh water fish *G. giuris*. Journal of Fisheries and Livestock Production 3: 2.

Raizada R., Srivastava M., Kaushal R.A. & Singh R. 2001. Azadirachtin, a neem biopesticide: Subchronic toxicity assessment in rats. Food and chemical toxicology: an international journal published for the British Industrial Biological Research Association 39: 477–483.

Romi R. 2001. *Aedes albopictus* in Italia: un problema sanitario sottovalutato. Annali dell'Istituto Superiore di Sanità 37(2): 241–247.

Romi R., Toma L., Severini F. & Di Luca M. 2009a. 20 years of presence of *Aedes albopictus* in Italy: from the annoying pest mosquito to the real diseases vector. European Infectious Diseases 2: 98–101.

Romi R., Toma L., Severini F., Di Luca M., Boccolini D., Ciufolini M.G., Nicoletti L. & Majori G. 2009b. Linee guida per il controllo di Culicidi potenziali vettori di arbovirus in Italia. Rapporti ISTISAN 9: 3–12, 24–31.

Sabatini A., Ranieri V., Trovato G. & Coluzzi M. 1990. *Aedes albopictus* in Italia e possibile diffusione della specie nell'area mediterranea. Parassitologia 32: 301–304.

Sagar S.K. & Sehgal S.S. 1997. Toxicity of neem seed coat extract against mosquitoes. Indian Journal of Entomology 59(2): 215–223.

SAS, JMP. 2007. User's Guide ver. 7.0 SAS Inst. Cary, NC, U.S.A.

Shanmugapriyan R. & Dhanalakshmi V. 2015. Biological effects of neem on the pupation of *Henosepilachna vigintioctopunctata* Fab. on bittergourd. Journal of Entomology and Zoology Studies 3(1): 78–81.

Toma L., Cipriani M., Goffredo M., Romi R. & Lelli R. 2008. Primo report sull'attività entomologica condotta nell'ambito del piano nazionale per la sorveglianza della West Nile Disease in Italia. Veterinaria Italiana 44(3): 483–497.

Received: 16.01.2020
Accepted: 13.05.2020