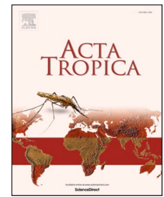
Acta Tropica 255 (2024) 107223

Contents lists available at ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica



Effect of nanoformulation *Azadirachta indica* on some factors associated with the vectorial capacity and competence of *Anopheles aquasalis* experimentally infected with *Plasmodium vivax*

Paula Andrea Urrea Aguirre [a,*], Keillen Monick Martins [b], Campos Duván Dueñas López [c], Fernando Orozco Sánchez [c], Alberto Tobón Castaño [a], Claudia María Ríos Velásquez [b], Adriana Pabón Vidal [a]

[a] *Grupo Malaria, Facultad de Medicina, Universidad de Antioquia, Medellín, Colombia*
[b] *Laboratório de Doenças Transmissíveis na Amazônia, Instituto Leônidas e Maria Deane, FIOCRUZ Amazônia, Brazil*
[c] *Universidad Nacional de Colombia, Medellín, Colombia*

ARTICLE INFO

Keywords:
Malaria
Anti-vector activity
Transmission block anopheles aquasalis
Plasmodium vivax
Azadirachta indica
Neem

ABSTRACT

Malaria remains a highly prevalent infectious disease worldwide, particularly in tropical and subtropical regions. Effectively controlling of mosquitoes transmitting of *Plasmodium* spp. is crucial in to control this disease. A promising strategy involves utilizing plant-derived products, such as the Neem tree (*Azadirachta indica*), known for its secondary metabolites with biological activity against various insect groups of agricultural and public health importance. This study investigated the effects of a nanoformulation prototype Neem on factors linked to the vector competence of *Anopheles aquasalis*, a malaria vector in Latin America. Different concentrations of the nanoformulation were supplied through sugar solution and blood feeding, assessing impacts on longevity, fecundity, fertility, and transgenerational survival from larvae to adults. Additionally, the effects of the Neem nanoformulation and NeemAZAL® formulation on the sporogonic cycle of *P. vivax* were evaluated.

Overall, significant impacts were observed at 100 ppm and 1,000 ppm concentrations on adult survival patterns and on survival of the F1 generation. A trend of reduced oviposition and hatching rates was also noted in nanoformulation-consuming groups, with fertility and fecundity declining proportionally to the concentration. Additionally, a significant decrease in the infection rate and intensity of *P. vivax* was observed in the 1,000 ppm group, with a mean of 3 oocysts per female compared to the control's 27 oocysts per female. In the commercial formulation, the highest tested concentration of 3 ppm yielded 5.36 oocysts per female. Concerning sporozoite numbers, there was a reduction of 52 % and 87 % at the highest concentrations compared to the control group. In conclusion, these findings suggest that the *A. indica* nanoformulation is a potential as a tool for malaria control through reduction in the vector longevity and reproductive capacity, possibly leading to decreased vector population densities. Moreover, the nanoformulation interfered with the sporogonic development of *P. vivax*. However, further basic research on Neem formulations, their effects, and mechanisms of action is imperative to gain a more specific perspective for safe field implementation.

1. Introduction

Malaria is an infectious disease caused by parasites of the genus *Plasmodium*, which remains a global health concern. The main species that infect humans are *Plasmodium vivax* and *Plasmodium falciparum*. Currently, malaria contributes with millions of clinical cases and deaths annually. In 2021, reached 247 million infections and 619,000 deaths. (WHO, 2022) . In South America, malaria transmission is prevalent in countries that share the Amazon rainforest. Specifically, Colombia as for malaria as a persistent public health problem, particularly in rural areas at lower altitudes (Mantilla et al., 2009); together with Venezuela and Brazil, they account for 79 % of cases in this region, with a predominance of *P. vivax* infections (71.5 %) (WHO, 2022).

The life cycle of these parasites involves two hosts: female

* Corresponding author.
E-mail address: andrea.urrea@udea.edu.co (P.A.U. Aguirre).

https://doi.org/10.1016/j.actatropica.2024.107223
Received 22 October 2023; Received in revised form 17 April 2024; Accepted 18 April 2024
Available online 19 April 2024
0001-706X/© 2024 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC license (http://creativecommons.org/licenses/by-nc/4.0/).

mosquitoes of the genus *Anopheles* spp. as invertebrate hosts, and humans or other vertebrates (Antinori et al., 2012). Transmission begins through the bite of mosquitoes infected with sporozoites, which, infect liver cells, where the transform and multiply. Subsequently, these invade red blood cells; in this erythrocytic cycle, sexual dimorphic gametocytes are formed, which are the infective forms for the mosquito vector (Sato, 2021) . The sexual reproduction of the parasite occurs in the midgut of the mosquito, and after several developmental transformations, it gets the sporozoite form, invades the salivary glands, and it is ready to initiate a new transmission cycle (Josling and Llinás, 2015).

Only some species are considered vectors because they possess biological characteristics that confer them with the capacity and competence to transmit a pathogen. The characteristics of vector capacity are related to population density, blood feeding behavior, and longevity. On the other hand, vector competence is related to the mosquito's susceptibility to become infected, enabling parasitic development and ultimately transmitting it to a host (Beier et al., 2008; Cohuet et al., 2010; Shaw and Catteruccia, 2018). In the Americas, there are several species of primary vectors transmitting *Plasmodium: Anopheles darlingi* stands out as the main vector in countries such as Colombia and Brazil. Additionally, *Anopheles aquasalis* is prevalent in Venezuela and Brazil, primarily associated with the transmission of *P. vivax*. In Nicaragua and Panama, the transmission is notably linked to *Anopheles albimanus* (Hiwat and Bretas, 2011).

The continuous use of conventional insecticides for mosquito control has caused resistance and negative impacts on the environment and non-target organisms (Challa et al., 2019). Additionally, drug-resistant strains of *Plasmodium* pose challenges in treatment (Belete, 2020). Therefore, there is a need to develop new tools for disease treatment, surveillance and control; the implementation of methods for integrated vector management remains key to malaria control, and the search for effective alternatives has led to the exploration of bioactive plant compounds. In recent years, plant-derived products are being recognized as potential substitutes for chemical or synthetic insecticides. A large number of secondary metabolites have been identified that are effective, ecologically friendly, biodegradable, cost-effective, and present little to no risk to human health and the environment (Kamaraj et al., 2010; Vivekanandhan et al., 2018). In relation to this, *Azadirachta indica* A. Juss (Neem) is a biologically active plant with repellent and insecticidal properties, which, due to its multiple mechanisms of action on insects, is considered not to generate resistance selection phenomena (Mordue and Nisbet, 2000; Benelli et al., 2017). The biological activity of Neem extracts can be attributed to the presence of many secondary metabolites (e.g., limonoids) (Veitch et al., 2008; Morgan, 2009; Aribi et al., 2020). Some of these metabolites could act as antagonists of the hormones 20-hydroxyecdysone (20E) and juvenile hormone, which regulate metamorphosis in insects and reproductive development. This makes this plant a promising target for the biochemical control of malaria vectors (Liu et al., 2014; Bezzar-bendjazia et al., 2016; Ekoka et al., 2021). Additionally, the effect of Neem compounds on the inhibition of *P. berghei* oocyst development in *An. stephensi* has been demonstrated (Lucantoni et al., 2010), as well as the effect on *P. falciparum* development in *An. coluzzi* (Yerbanga et al., 2014), and the impact on the development of *P. berghei* gametocytes in mice, where infection of this parasite was subsequently inhibited in *An. stephensi* mosquitoes (Dahiya et al., 2016). *A. indica* has shown great potential in the fight against malaria. The effect of nanoformulated Neem tree extracts grown in Colombia on vector mosquitoes and circulating parasites in South America is currently unknown. Therefore, the objective of this study was to evaluate the effects of a nanoformulation *Azadirachta indica* on some factors related to the capacity and vectorial competence of experimentally infected *Anopheles aquasalis* mosquitoes with *Plasmodium vivax*.

2. Material and methods

2.1. Mosquitoes

The *Anopheles aquasalis* were obtained from the colony reared in the insectary of the Laboratory of Transmissible Disease Ecology in the Amazon (EDTA), located at the Leônidas e Maria Deane Institute - Fiocruz Amazônia, in the city of Manaus, Brazil. The mosquitoes were maintained at 26–28 °C, 70–80 % relative humidity, and a photoperiod of 12/12 h. Larvae were reared in water and fed with ground fish food pellets. Pupae were then transferred to small containers of water and placed in protected cages to allow adult emergence. Adult mosquitoes were offered a 10 % sucrose solution on cotton pads ad libitum (Ríos--Velásquez et al., 2013). Three days old adult females of *An. Aquasalis* were used for the experiments.

2.2. Ethical aspects

The project followed the research ethics protocols defined by the Ministry of Health of Brazil and was approved by the Research Ethics Committee (CEP, CAAE: 39,706,514.2.0000.0005). For the experiments were used whole blood from volunteer patients over 18 years old, sick with malaria for *P. vivax*, as diagnosed by optical microscopy using the thick smear method with Giemsa staining (501–10,000 parasites/μL). Diagnosis was performed by trained professionals from the Fundação de Medicina Tropical Dr. Heitor Vieira Dourado (FMT-HVD). For control treatment, concentrations of the nanoformulation were diluted in blood collected from a healthy volunteer member of the research group. Approximately 3 ml of blood were collected for each experimental replicate using a lithium Active Ingredient Vacutainer tube, following a trans-portation and handling protocol established at the EDTA Laboratory (Rios-Velásquez et al., 2013).

2.3. Azadirachta indica formulations

Two formulations of *A. indica* were used for the experiments. The first, a nanotechnology-based formulation, was developed by the Industrial Biotechnology and Natural Product Chemistry, and Food research groups at the Universidad Nacional de Colombia (project code 67540, MinCiencias). This formulation primarily consisted of water and neem oil. The neem oil was extracted from seeds obtained from the Cotové Agricultural Station, located in Santa Fé de Antioquia (6°33′23''N, 75°49′39''W), using a screw press method. Tween 80 (Polysorbate 80), sourced from Protokimica S.A.S., was incorporated as a surfactant.

The nanoemulsion development involved initially mixing deionized water with Tween 80 surfactant at a 1 % concentration. This mixture was then subjected to magnetic stirring at 400 rpm. Gradually, neem oil was added to this stirred mixture at a concentration of 15 %. After thorough mixing, the blend underwent a high-pressure emulsification process using a NanoGenizer 30 K (Genizer LLC, USA) at 800 bars pressure. The emulsification process was performed twice to ensure uniformity and stability. The developed formulation is a nanoemulsion with an average particle size of 370 nm, determined using the Dynamic Light Scattering (DLS) technique with a Zetasizer Nano ZS90 particle analyzer (Malvern Instruments Ltd., Worcester, UK).

For limonoid quantification, 1 mL of neem oil was extracted using methanol, assisted by ultrasound in three steps of 10 mL each. The methanolic extract was then vacuum evaporated and subjected to biphasic separation with dichloromethane-water. The resulting organic phase, after drying, was used for limonoid quantification. A high-performance liquid chromatography (HPLC) Agilent 1200 system equipped with a diode-array detector (DAD) was employed. 10 μL of the diluted organic phase (4 mg/mL) were injected into a LiChroCART® 125–4 column, using an acetonitrile-water mobile phase gradient. The flow rate was set at 1 mL/min, starting with a ratio of 35:65, changing to

45:55 at minute 10, to 70:30 at minute 11, and returning to 35:65 at minute 14 (Orozco-Sánchez, 2009). The analysis was conducted at a wavelength of 213 nm. A calibration curve was constructed using azadirachtin A standard (Sigma-Aldrich, Switzerland) at concentrations ranging from 1 to 200 ppm in methanol ($R^2 = 9.9989$). Peaks with a retention spectrum like azadirachtin and absorption maxima between 210 and 215 nm were considered limonoids related to azadirachtin due to their UV spectrum. The analysis of limonoids in the nanoformulation determined a concentration of 221 µg limonoid/mL formulation.

The second formulation used was a plant extract of *A. indica*, commercially available in Colombia by IBICOL S.A.S. and manufactured by E.I.D. PARRY INDIA, known as NeemAZAL1.2EC® emulsifiable concentrate containing 1.20 % azadirachtin A, CAS number 11,141–17–6.

All dilutions used to feed the mosquitoes were prepared from 10,000 ppm and 1000 ppm stock solutions of the nanoformulated A. indica seed oil. The NeemAZAL® commercial stock solutions were prepared at concentrations of 1000 ppm and 100 ppm of the emulsifiable concentrate of azadirachtin A, the active compound of the A. indica plant extract. In both formulations, sterile distilled water was used as the solvent. The stock solutions were prepared at a single time and stored at −20 °C in aliquots used to prepare the feeding solution.

2.4. Membrane feeding assay

For feeding mosquitoes with formulations of *A. indica*, solutions of nanoformulation *A. indica* at 1000 ppm, 100 ppm, 50 ppm and 25 ppm were prepared. Neem AZAL® solutions at 3 ppm, 1.5 ppm and 0.75 ppm, were prepared in a total volume of 1 ml of fresh human blood. Two control groups were used, one uninfected blood and the other contained sunflower oil at 1000 ppm (Solvent Control-SC- of the nanoformulation), with the same stabilizer used in the preparation of the nanoformulation. The blood was offered to the mosquitoes by membrane feeding, following a protocol established by Ríos-Velásquez et al. (2013). The blood solution (infected and non-infected) with the formulations was dispensed into a glass feeder in the form of a cup, coated with a Parafilm® membrane and maintained at a constant temperature of 37 °C. Mosquito feeding took place for approximately 180 min. After this time, only fully engorged females were selected. Subsequently, they were transferred to containers, separated according to their treatment group, and kept in the insectary, fed with 10 % sugar solution ad libitum.

2.5. Survival of An. aquasalis fed with the nanoformulation of A. indica

Each group was fed for three consecutive days with one of the following doses: 25 ppm, 50 ppm, 100 ppm and 1000 ppm of the nanoformulation diluted in 10 % sugar solution. Additionally, two control groups were used, one with sugar solution (CN) and the other with solvent control at 1000 ppm (CS 1000 ppm). On the fifth day, mosquitoes were fed with a solution of blood with A. indica at the concentrations above, using artificial membrane feeding. Female mortality and survival for 26 days were daily recorded.

2.6. oviposition estimation

Three days after blood feeding, 50 % of gravid females from each treatment were randomly selected and transferred in group oviposition containers. After 48 h, surviving females from each group had their ovaries dissected, and the retained eggs were counted. Simultaneously, the oviposited eggs were removed from the oviposition substrate, washed with a 1 % sodium hypochlorite solution using filter paper for retention and quantification. Fecundity (total oviposited eggs), percentage of retained eggs, mean retained and produced eggs per female and percentage of oviposition reduction were calculated.

2.7. Fertility estimation

To assess the effect of different concentrations of nanoformulation *A. indica* on mosquito fertility, the total number of oviposited eggs were incubated in saline water for 72 h. After this period, the first instar larvae (L1) in each group were quantified, and the hatching percentage and the percentage reduction in hatching were calculated. Subsequently, approximately 100 larvae per group were randomly selected.

2.8. Effect on development time and survival of progeny of An. aquasalis females fed with A. indica nanoformulation

Randomly, 100 larvae from each treatment were selected and divided into two trays at a density of 50 larvae/250 ml of water. Mortality and stage change were recorded every 24 h until all specimens completed metamorphosis. The data were analyzed by life tables methodology Rabinovich (1978).

2.9. Extraction and measurement of the hormone 20 hydroxyecdysone (20E)

Seven groups were utilized, consisting of 15 female *A. aquasalis* mosquitoes per group. They were fed with at 25 ppm, 50 ppm, 100 ppm, and 1000 ppm of nanoformulation diluted in sugar solution and uninfected blood. As controls were used, one group of females that never fed with blood (NBM), a group fed with sugar and blood (CN), and a group treated with the solvent of the nanoformulation substance diluted in sugar solution and blood (CS 1000 ppm). After 24hours feeding with blood, female mosquitoes were dissected, their heads and wings were removed, and the remaining bodies were joined to form three pools per group, with 5 females in each pool. For the extraction of ecdysteroid hormones, the samples were macerated in 95 % cold methanol following the centrifugation and final sample acquisition recommendations of McKinney and colleagues (McKinney, Strand, and Brown 2017). Subsequently, the titers of the hormone 20-hydroxyecdysone were measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit specifically designed for 20-hydroxyecdysone competitive detection (Thermo Fisher Scientific), following the manufacturer's instructions. Each sample was measured in duplicate, and a standard curve with a four-parameter logarithmic fit (4LP) was constructed.

2.10. Effect of nanoformulation of A. indica on the sporogonic development of P. vivax

Anopheles aquasalis females were fed with a solution of blood infected with *P. vivax* and the nanoformulation in the concentrations of 50 ppm, 100 ppm, and 1000 ppm, of the commercial formulation, and NeemAZAL® was evaluated at 0,75 ppm, 1,5 ppm, and 3 ppm concentrations. Likewise, two control groups were established: CN, fed with infected blood, and CS, fed with a solvent control (CS 1000 ppm). Mosquitoes were fed by membrane feeding assay, and only fully engorged females were selected. Then, each group was fed with each evaluated concentration diluted in a sugar solution 10 % over three consecutive days. After this period, they were maintained with sugar solution 10 % ad libitum. Seven days after the feeding with infected blood, 15 to 20 females were randomly selected from each group. Each intestine was dissected in phosphate-buffered saline (PBS, 1X), stained with 2 % mercurochrome (Merbromin) for 10 min, and observed under an optical microscope (Leica DM1000, Germany) at 40X magnification. The number of oocysts per intestine was recorded, and the infection rate and infection intensity were calculated (Rios-Velásquez et al., 2013).

On the 14th day after feeding with infected blood, the salivary glands of the survivor females were dissected, joined in pools of up to five of salivary gland pairs, macerated in 50 µl of a 1:10 RPMI solution (Thermo Fisher Scientific), and the sporozoites counted using a Neubauer chamber under light microscopy at 40X magnification. The total number

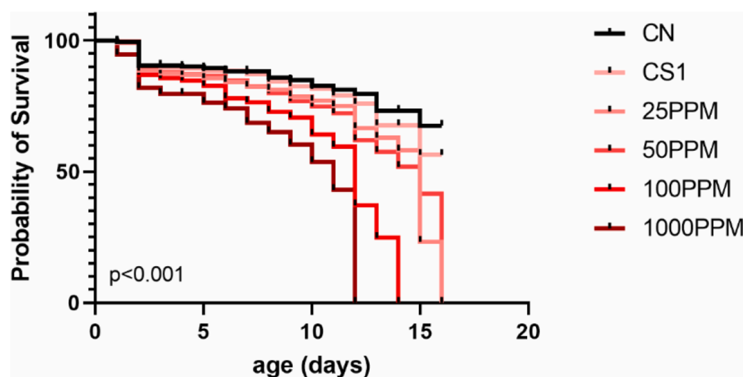


Fig. 1. Kaplan-Meier survival curve of *An. aquasalis* female mosquitoes fed with different concentrations of nanoformulation *A. indica*. (Log-Rank X2 (6) = 80.85, $p < 0.0001$).

Table 1
Survival of An. aquasalis females on day 25 after feeding with the nanoformulation A. indica.

Treatment	Mean survival time (min-max day)	*p* value	Mean survival rate (%min-max.)	*p* value
CN Sugar	25 (0–0)		8.67 (8–9)	
CS 1000ppm	25 (0–0)	>0,9999	6 (5–7)	>0,9999
25ppm	24 (24–25)	>0,9999	1 (1–1)	0.8367
50ppm	22 (22–24)	0.6863	0.00	**0.0398**
100ppm	20 (20–23)	0.0621	0.00	**0.0398**
1000ppm	17 (17–19)	**0.0213**	0.00	**0.0398**

Mean survival time: Kruskal-Wallis test *p*-value 0.0050. Mean survival rate: Kruskal-Wallis test *p* value 0.0033. Dunn's multiple comparisons analysis, *α 0.05. CN: Sugar Control. CS: Solvent Control.

of sporozoites in each treatment was calculated (Prinz et al., 2017) and the mean number of sporozoites per pair of salivary glands was calculated.

2.11. Data analysis

The statistical analyses were conducted using GraphPad Prism V.8 software. For normally distributed data, a one-way Analysis of Variance (ANOVA) was performed with the post hoc test Dunnett's to compare means of each group with the control group (CN). For non-normally distributed data, the non-parametric variant, Kruskal-Wallis test (Kruskal and Wallis, 1952) with the post hoc Dunn's test was used. Survival analysis of the adult mosquitoes was carried out using the Kaplan-Meier estimator (Kaplan and Meier, 1958). A Life Table was constructed for the F1 progeny of exposed females. Regarding the analysis of 20-hydroxyecdysone hormone measurement data, an interpolation fit curve was generated using a four-parameter logistic regression (4PL). Additionally, an ANOVA was performed based on the concentrations, as described earlier. All experiments were conducted in triplicate at different time points, utilizing blood from various patients.

3. Results

3.1. Effect of nanoformulation A. indica on the survival of adult An. Aquasalis mosquitoes

A total of 2160 females fed with different concentrations of the nanoformulation. The survival of females fed with 100 ppm, and 1000 ppm was statistically different compared to the control groups (Log-Rank X2 (6) = 80.85, $p < 0.0001$) with a lower probability of survival

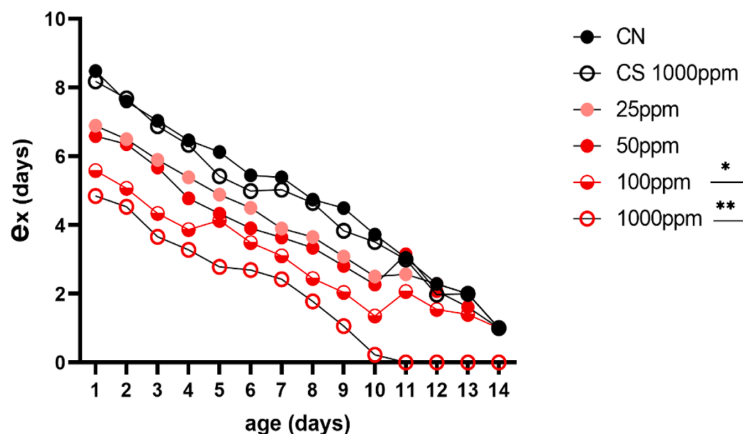


Fig. 2. Life expectancy of An. aquasalis female mosquitoes fed with different concentrations of nanoformulation A. indica. e_x: mean additional lifespan expected if the mortality rate is constant. One-way ANOVA test F (5.78) = 4.573 p-value <0.0010 and Dunnett's multiple comparison analysis, **α 0.01, *α 0.05. CN: Sugar Control. CS: Solvent Control.

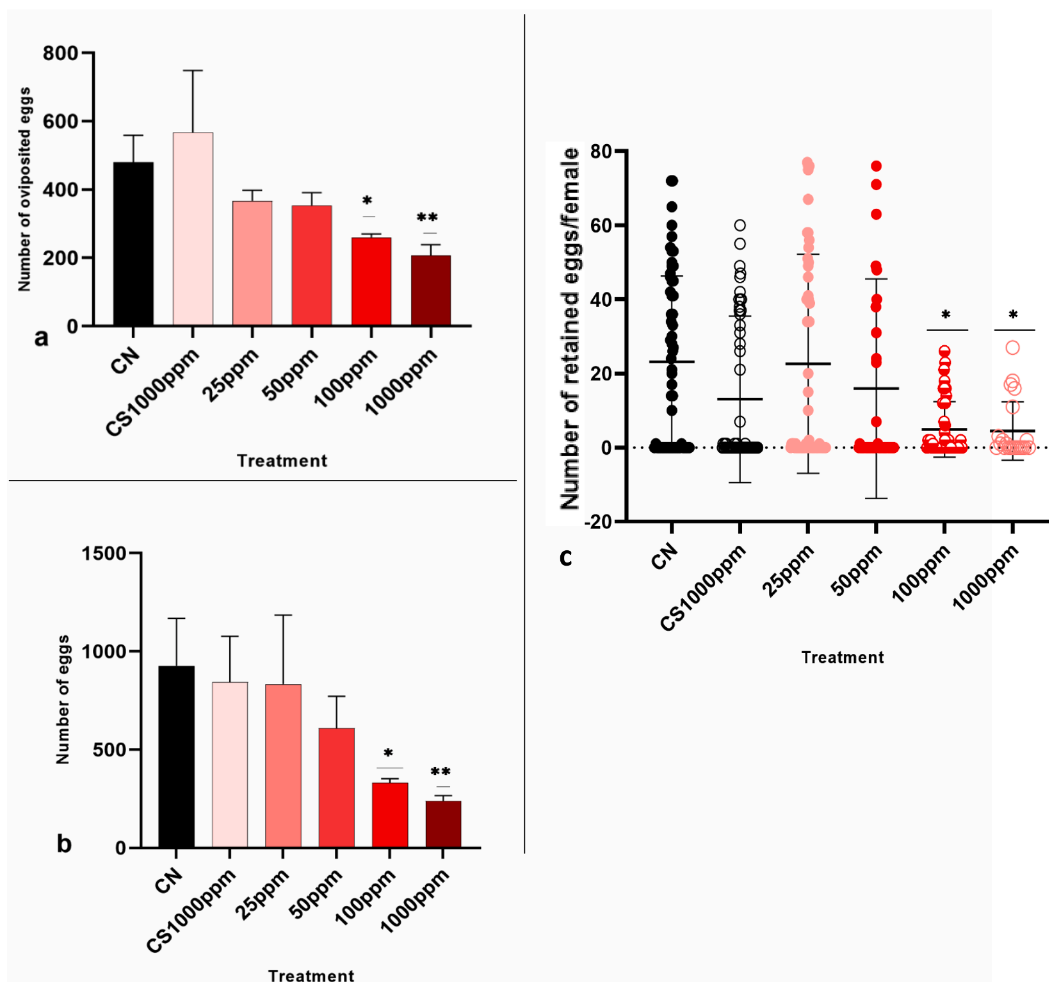


Fig. 3. Fecundity of *An. aquasalis* females fed with nanoformulation *A. indica*. (2a) mean number of oviposited eggs per group, one-way ANOVA test, F (6,14) = 7.179, *p*-value = 0.0012. (2b). Total number of eggs per group (oviposited +retained), one-way ANOVA test, F (6,14) = 4.507, *p*-value = 0.0095, and Dunnett's multiple comparisons analysis. (2c) number eggs retained per female, Kruskal-Wallis test *p*-value < 0.008 and Dunn's multiple comparisons analysis **α = 0.01, *α = 0.05. CN: Sugar Control. CS: Solvent Control.

over follow-up time, compared to the groups fed with 25 ppm and 50 ppm (Fig. 1). The mean survival for groups fed with 25 ppm and 50 ppm was 24 and 22 days, respectively, while for the 100 ppm and 1000 ppm groups, it was 20 and 17 days. In comparison to the control groups, where about 7 % of each group as still alive on day 25 after the start of the follow-up (Table 1). Each group was monitored for survival from the initial feeding with the nanoformulation. However, the survival curve and life expectancy were constructed using the population that remained after blood feeding and oviposition which corresponds to day 9 post-treatment. This approach was adopted due to the identification, in these analyses, of a decline in survival that was associated with withdrawal of individuals, but not mortality, and the inability to track them over time to collect survival data.

The life expectancy in days (e_x) of females in each group exhibited a progressive decline as age increased, which corresponds to an expected population behavior. However, it was observed that the groups exposed to 100 ppm and 1000 ppm, had on mean an additional time of life less than the other groups. This difference became evident from day 1, with a life expectancy of 5 days (e_x) for the mentioned groups, as opposed to the control groups which displayed 8 additional days of life expectancy (e_x). Additionally, it was observed that at day the life expectancy was 2 days for the 100 ppm group and 0 for the 1000 ppm group (Fig. 2)

Table 2
Number of eggs developed and retained from colonized *An. Aquasalis* females fed with nanoformulation *A. indica*.

Treatment	Mosquitoes for oviposition	Mean developed eggs (min-max.)	Value *p*	Dissected mosquitoes	% Retained eggs (min-max.)	*p* Value
CN sugar	72	926 (693–1117)	–	58	47 (43–53)	–
CS 1000ppm	81	844 (604–1068)	0.9940	64	33.6 (25–41)	0.9566
25ppm	73	833.7 (612–627)	0.9893	62	52 (42–68)	0.9810
50ppm	66	608.7 (484–793)	0.3592	48	39.3 (21–54)	0.8854
100ppm	59	332 (315–355)	0.0260	44	21.6 (18–24)	**0.0529**
1000ppm	21	239.3 (219–270)	0.0099	17	13.8 (10–17)	**0.0098**

One-way ANOVA test, F (6.14) = 4.507, *p* = 0.0095, followed by Dunnett's multiple comparisons analysis, **α = 0.01, *α = 0.05. CN: Sugar Control. CS: Solvent Control.

Table 3
Percentage of hatching of colonized An. aquasalis females fed with nanoformulation of A. indica.

Treatment	Mean of oviposited eggs per group (min-max.)	Value *p*	% hatched eggs per group	*p* Value
CN sugar	479.7 (393–549)	–	79.67(70–87)	–
CS 1000ppm	566 (391–494)	0.6273	81,33(67–90)	0.9997
25ppm	366.7(331–391)	0.3820	70.33(65–75)	0.6184
50ppm	353.7 (311–382)	0.2874	69.67(62–78)	0.5569
100ppm	260.3 (251–271)	**0.0243**	50.33(46–58)	**0.0051**
1000ppm	206.7(181–243)	**0.0052**	49(44–52)	**0.0036**

One-way ANOVA test F (6,10) = 7.428, p = 0.010, and Dunnett's post hoc multiple comparisons analysis, **α 0.01. CN: Sugar Control. CS: Solvent Control.

3.2. Effect of nanoformulation A. indica on the reproductive capacity of An. aquasalis

Feeding with the nanoformulation of *A. indica* through sugar solution 10 % and blood, led to a reduction in oviposition, affecting the fecundity of *An. Aquasalis* significantly at concentrations of 100 ppm and 1000 ppm. The mean oviposition in the control groups (CN) was 479.7 eggs and 566 eggs for the group fed with a solvent solution of the nanoformulation (CS 1000 ppm). In contrast, for the 100 ppm and 1000 ppm groups, it was 260.3 and 206.7 eggs respectively (Fig. 3a). Similarly, the total number of developed eggs was evaluated adding the number of oviposited and retained eggs. These results align with fecundity analyses, where a lower number of eggs laid was observed as the nanoformulation concentration increased (Fig. 3b). These data indicate that in the 100 ppm and 1000 ppm groups, the number of produced eggs was 2.8 and 3.9 times lower than the control group, respectively. The control groups retained between 47 % (CN sugar) and 33 % (CS 1000 ppm) of the developed eggs (Table 2), and it was observed that, in the two higher concentrations evaluated, the mean retained eggs per female were lower. This outcome may be linked to the effect of the nanoformulation on fecundity, where lower oogenesis results in reduced retention of these eggs (Fig. 3c).

When evaluating the viability of oviposited eggs, it was observed that it was also significantly affected by the consumption of the nanoformulation *A. indica*. As a result, a lower hatching percentage was evident in the groups that consumed the higher concentration of the nanoformulation, impacting the viability of at least 50 % of the oviposited eggs in the 100 ppm and 1000 ppm groups (Table 3). The inhibition of oviposition had a greater impact in these same groups at 100 ppm and 1000 ppm. Conversely, in the inhibition of hatching, a higher impact was observed, with a significant difference between the control group of solvent CS 1000 ppm and all nanoformulation groups (one-way ANOVA F (4,10) = 19.75, p < 0.0001), the specific differences were between the group at 25 ppm and 1000 ppm (p 0.045), the group at 50 ppm and 1000 ppm (p 0.0336), while no difference was observed in the group at 100 ppm and 1000 ppm (p 0.9150) (Fig. 4). A dose-response behavior was also observed in the inhibition of oviposition and

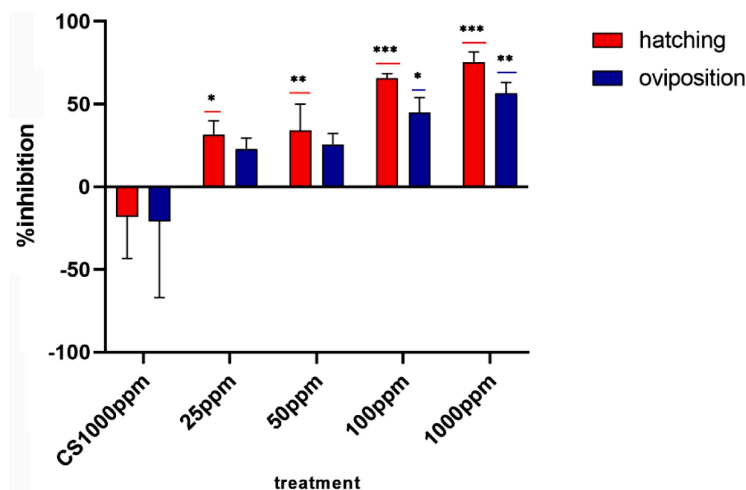


Fig. 4. Percentage of inhibition of hatching and oviposition of colonized *An. aquasalis* females fed with sugar and nanoformulation of *A. indica*. One-way ANOVA test F (4,10) = 5.541, p < 0.0129 and multiple comparisons analysis Tukey`s, *α 0.05, ***α 0.001. CS: Solvent Control.

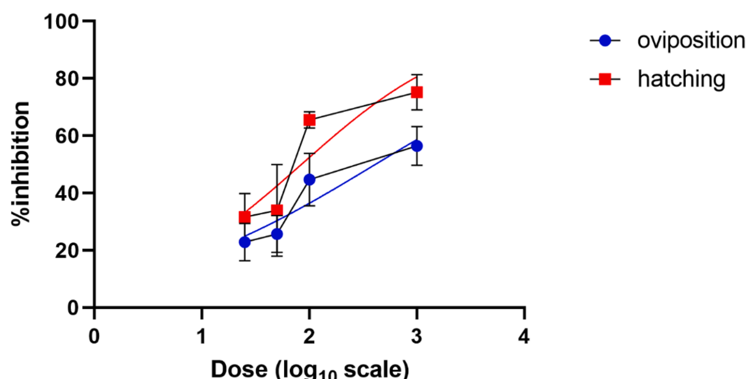


Fig. 5. Dose-response curve of inhibition of hatching and oviposition of colonized An. aquasalis females fed with the nanoformulation of A. indica.

Table 4
Mean development time in days (minimum-maximum) of immature stages, pupae and adults of An. aquasalis progeny fed with nanoformulation A. indica.

Treatment	stages	Sample size	Mean development time (days)	*p* Value
CN sugar	Instar I	300	12 (8–19)	–
	Instar II	287	6.33 (3–9)	–
	Instar III	281	9.66 (7–12)	–
	Instar IV	277	18.67 (12–23)	–
	Pupa	257	11.67 (9–17)	–
	Adults	257	8.66 (7–11)	–
CS 1000ppm	Instar I	300	11.33 (9–16)	>0.9999
	Instar II	253	5.66 (3–7)	>0.9999
	Instar III	251	7.66 (7–9)	>0.9999
	Instar IV	242	16.67 (11–23)	>0.9999
	Pupa	237	11 (7–17)	>0.9999
	Adults	237	11.33 (8–16)	>0.9999
25ppm	Instar I	270	10.33 (7–16)	>0.9999
	Instar II	246	5.33 (3–7)	>0.9999
	Instar III	238	9.33 (7–11)	>0.9999
	Instar IV	195	18.67 (12–23)	>0.9999
	Pupa	187	10 (7–16)	>0.9999
	Adults	186	11 (8–17)	>0.9999
50ppm	Instar I	245	12.67 (11–15)	>0.9999
	Instar II	228	6.33 (2–10)	>0.9999
	Instar III	214	7.66 (4–10)	>0.9999
	Instar IV	163	16.67 (11–23)	>0.9999
	Pupa	141	11.67 (7–18)	>0.9999
	Adults	141	8.33 (5–14)	>0.9999
100ppm	Instar I	238	11.67 (9–14)	>0.9999
	Instar II	224	8.33 (5–2)	>0.9999
	Instar III	195	10,67 (6–14)	>0.9999
	Instar IV	160	14.67 (9–21)	>0.9999
	Pupa	132	7.67 (6–9)	>0.9999
	Adults	132	8(7–10)	>0.9999
1000ppm	Instar I	236	10.33 (8–13)	>0.9999
	Instar II	176	8 (2–12)	>0.9999
	Instar III	167	11 (7–14)	>0.9999
	Instar IV	108	15 (7–19)	>0.9999
	Pupa	94	5.66 (2–9)	0.3421
	Adults	92	7.33 (5–9)	>0.9999

Kruskal-Wallis test and Dunn's multiple comparison analysis. CN: Sugar Control. CS: Solvent Control.

hatching, with a higher concentration of the nanoformulation resulting in a greater percentage of inhibition in both reproductive parameters evaluated in *An. aquasalis*. According to the probabilistic estimation of the 50 % inhibition concentration (IC_{50}), the IC_{50} for oviposition was calculated as 410.7 ppm (R^2 = 0.7321), and for hatching, the IC_{50} was determined as 84.32 ppm (R^2 = 0.6942) (Fig. 5).

3.3. Effect of nanoformulation A. indica on the developmental time of progeny from An. aquasalis

Analyses were performed considering the total population of the three conducted replicates. In the developmental time of immature stages of the F1 generation, no differences were observed between the control groups and the groups fed with the nanoformulation. Overall, in all evaluated groups, the mean time from egg to adult was approximately 30.6 days (±3.7). A tendency of shorter time was observed in the L2 stage with an average duration of 6.2 days (±3.2), while a longer time was seen in the L4 stage with 16.6 days (±6.1). Table 4 presents the developmental time data for each group.

3.4. Life tables of immature stage, pupae and adults of the progeny from An. aquasalis females, fed with nanoformulation A. indica

From these data, survival curves were obtained with the proportion of survivors by age (lx), the life expectancy in days (ex), the probability of surviving (px), of dying (qx), and the probability of surviving between consecutive stages (Lx) (Table 5). The survival attribute (lx) concerning the stage exhibited a significant difference in the groups treated with 50 ppm, 100 ppm, and 1000 ppm. There was observed a constant mortality rate (qx) across all age groups without predominance in a specific stage (Fig. 6a). This result suggests that it could be a type II curve, which represents a population in which a constant number of individuals die per unit of time, both at young and advanced ages (Fig. 5b) (Rabinovich 1978). The life expectancy trend was similar across all groups, being higher in the LI and LII stages, and then decreasing towards the pupal stage. However, in the early stages, there is a difference of over two days between the control group with 5.55 days in LI, compared to the 1000 ppm group with 2.98 days (Table 5). Overall, the survival of the control groups CN (sugar control) and CS (1000 ppm control) was over 80 % (lx), while for the 50 ppm, 100 ppm, and 1000 ppm groups, the survival upon completing the development cycle to adulthood was 35 %, 32 %, and 19 %, respectively. From these data, it can also be observed that there is a higher probability of dying in the LIV stage for specimens that consumed the nanoformulation in all evaluated concentrations, which aligns with a lower probability of survival between LIV and pupa in the 50 ppm, 100 ppm, and 1000 ppm groups (Fig. 6c). In addition to the great impact on the progeny survival, some body malformations related to the structural formation of the exoskeleton were observed (Fig. 7)

3.5. Effect of nanoformulation A. indica on hormone 20 hydroxyecdisone (20E)

The peak of the 20E hormone occurs approximately 24 h after blood feeding (post-blood meal, pBM) (Redfern, 1982; Werling et al., 2019). As expected, a lower level was observed in the group that did not have blood meal, averaging 125.7 pg/ml per female. However, this difference was not statistically significant, likely due to intragroup variation across the three replicates (±125.4). The overall results indicated that ecdysteroid levels, specifically hormone 20E, did not reveal significant differences among the evaluated groups. Though, a trend of increased 20E levels with higher nanoformulation concentrations was observed (Table 6, Fig. 8). It is possible that this non-significant increase is due to the cross-detection with fictoecdysone, which could be present in the nanoformulation. Fictoecdysone compounds are analogous to insect ecdysteroids (Vasil et al., 1984) and have been reported in *A. indica* by Nakanishi (1971). This suggests that the observed 20E levels in the mosquito groups might not be a result of increased synthesis.

3.6. Effect of nanoformulation A. indica on the sporogonic development of P. vivax in An. aquasalis

A total of 2880 female mosquitoes were used, fed with blood from three *P. vivax* malaria patients (one patient per replicate, three replicates were done). All groups fed with nanoformulation *A. indica* and the commercial formulation NeemAZAL® developed *P. vivax* infection. However, the infection rate (IR) of the mosquitoes was significantly different between the sugar control group (CN) with an IR of 73 % and the group fed at 1000 ppm with 22 %. The other groups exhibited infection rates ranging between 38 % and 69 % (Table 7).

When analyzing the intensity of infection per infected mosquito, significant variation was observed among the evaluated groups. Specifically, in the group fed with the highest concentration of the nanoformulation, at 1000 ppm, there was a mean of 3,42 oocysts per midgut.

Table 5
Life table attributes for the immature stages, pupae, and adults of An. aquasalis progeny fed with nanoformulation A. indica.

Treatment	X	P_x	D_x	q_x	p_x	l_x	L_X	e_x
CN sugar	LI	287	13	0.0443	0.956	0.956	0.935	5.55
	LII	287	0	0.0000	1.000	0.914	0.914	4.78
	LIII	281	6	0.0211	0.979	0.914	0.904	3.78
	LIV	277	4	0.0143	0.986	0.894	0.888	2.85
	Pupa	257	20	0.0749	0.925	0.882	0.849	1.89
	Adult	257	0	0.0000	1.000	0.815	0.815	1.00
CS1.000ppm	LI	256	44	0.1583	0.842	0.860	0.792	4.96
	LII	253	3	0.0118	0.988	0.724	0.720	4.80
	LIII	251	2	0.0079	0.992	0.715	0.713	3.85
	LIV	242	9	0.0365	0.963	0.710	0.697	2.88
	Pupa	237	5	0.0209	0.979	0.684	0.677	1.97
	Adult	237	0	0.0000	1.000	0.669	0.669	1.00
25ppm	LI	270	30	0.1053	0.895	0.9	0.853	4.52
	LII	246	24	0.0930	0.907	0.805	0.768	4.00
	LIII	238	8	0.0331	0.967	0.730	0.718	3.36
	LIV	195	43	0.1986	0.801	0.706	0.636	2.45
	Pupa	187	8	0.0419	0.958	0.566	0.554	1.94
	Adult	186	1	0.0054	0.995	0.542	0.542	1.00
50ppm	LI	245	55	0.2018	0.798	0.816	0.734	3.89
	LII	228	17	0.0719	0.928	0.651	0.628	3.75
	LIII	214	14	0.0633	0.937	0.604	0.585	3.00
	LIV	163	51	0.2706	0.729	0.566	0.490	2.17
	Pupa	141	22	0.1447	0.855	0.413	0.383	1.78
	Adult	141	0	0.0000	1.000	0.353	0.353	1.00
100ppm	LI	238	62	0.2305	0.770	0.793	0.702	3.72
	LII	224	14	0.0606	0.939	0.610	0.592	3.69
	LIII	195	29	0.1384	0.862	0.573	0.534	2.89
	LIV	160	35	0.1972	0.803	0.494	0.445	2.28
	Pupa	132	28	0.1918	0.808	0.397	0.358	1.71
	Adult	132	0	0.0000	1.000	0.320	0.320	1.00
1000ppm	LI	236	64	0.2388	0.761	0.786	0.692	2.98
	LII	176	60	0.2913	0.709	0.598	0.511	2.76
	LIII	167	9	0.0525	0.948	0.424	0.413	2.69
	LIV	108	59	0.4291	0.571	0.402	0.316	1.81
	Pupa	94	14	0.1386	0.861	0.229	0.213	1.79
	Adult	92	2	0.0215	0.978	0.198	0.198	1.00

X= age/stage.
P_x= live specimens in age X.
D_x= specimens dead in age X.
q_x= probability of dying in age X.
p_x= probability of surviving in age X.
l_x= proportion of survivors by age.
L_X= probability of survival between two successive ages.
e_x= Life expectancy in days.
CS= Solvent Control.

Meanwhile, the three groups fed with NeemAZAL (NA)® showed a similar infection intensity among them. In the highest evaluated NeemAZAL® concentration of 3 ppm, there was a mean of 5.36 oocysts per midgut. It is also noteworthy that the concentration of 100 ppm exhibited a similarly low average number of oocysts per midgut at 8291, while the 50 ppm concentration was comparable to the control groups (Table 7, Fig. 9). In Fig. 10, a representative image of gut infection intensity is shown, where 78 % of dissected mosquitoes at the 1000 ppm concentration did not exhibit the presence of *P. vivax* oocysts.

The red arrow indicates oocysts developed after seven days post-infected blood meal. Each photograph corresponds to a specimen from the evaluated group. (A) and (B) correspond to the control group with sugar (CN) and 1000 ppm CS (Solvent Control), respectively. (C) nanoformulation at 50 ppm, (D) nanoformulation at 100 ppm, (E) nanoformulation at 1000 ppm, (F) NeemAZAL® at 0.7 ppm, (G) NeemAZAL® at 1.5 ppm, (H) NeemAZAL® at 3 ppm. Leica DM1000 optical microscope, Germany, visualization at 40X.

Fourteen days after feeding on infected blood, the salivary glands of the surviving mosquitoes were analyzed. The presence of *P. vivax* sporozoites was observed in all evaluated groups. However, the number of sporozoites varied significantly compared to the control group with sugar (CN). In the groups that consumed the nanoformulation, a reduction in sporozoite count was observed. In the 50 ppm group, an average of 703 sporozoites per female was quantified; in the 100 ppm group, 874.8 sporozoites per female; and in the 1000 ppm group, 250 sporozoites per female were observed, with absence of this parasitic form in some specimens (range=0–375). The number of sporozoites was reduced by 7.24 times compared to the control group. Regarding the commercial formulation, a significant decrease in sporozoite count was also observed at concentrations of 1.5 ppm and 3 ppm, with averages of 705.9 and 656.3 sporozoites per female, respectively. However, the reduction was around 2.6 times less than the control group (Table 7, Fig. 11).

3.6.1. Principal observations

In summary, a total of 5865 female mosquitoes were used for all experiments. We observed a significant impact on survival at concentrations of 100 ppm and 1000 ppm of the formulation. The effect was particularly pronounced on the survival and development of the F1 generation, with survival rates of 35 % at 50 ppm, 32 % at 100 ppm, and 19 % at 1000 ppm, compared to the near 80 % survival in the control groups. Overall, there was a trend of reduced oviposition and lower hatching percentages in groups that consumed the nanoformulation, affecting both fecundity and fertility with a directly proportional relationship to concentration. Finally, a significant decrease in the infection rate of *P. vivax* was observed when examining parasitic forms in the

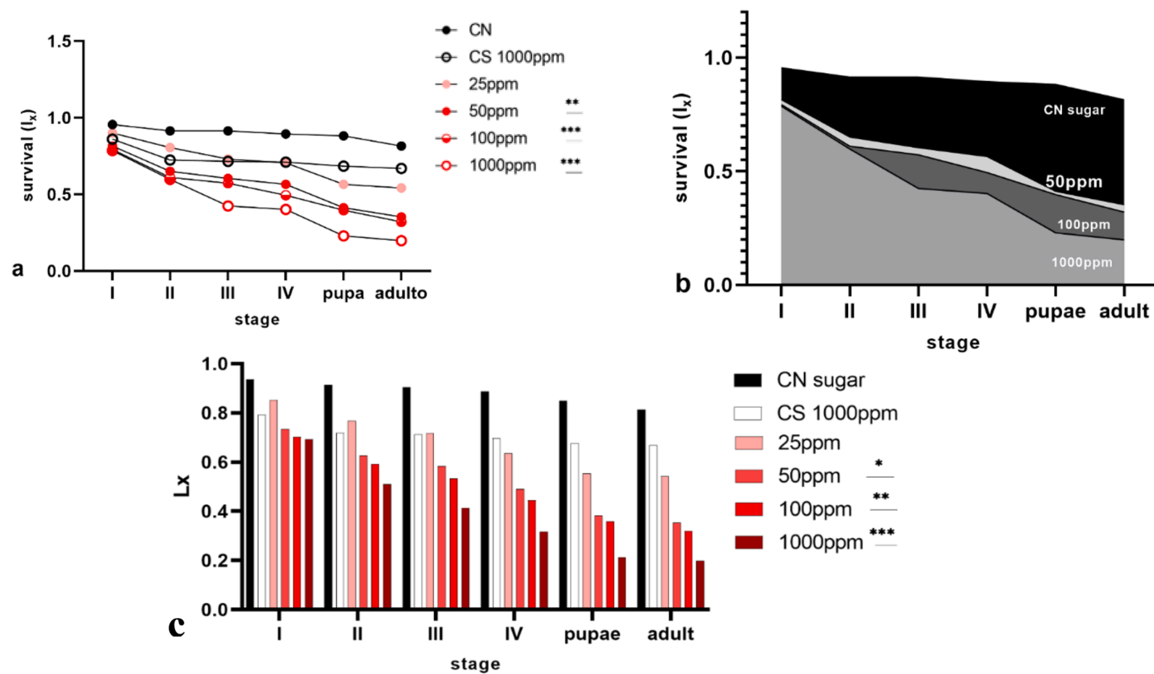


Fig. 6. Survival parameters of the progeny of *An. aquasalis* fed with nanoformulation *A. indica* (a) Survival curve of the immature stages, pupae, and adults of *An. aquasalis*. (b) graph of survival (lx) of groups with statistical difference. One-way ANOVA test F (5,30) = 7371, p = 0,0001. (c) Probably of survival between successive ages (L_x) of immature stage, pupae and adults. One-way ANOVA test F (6,30) = 5.798, p = 0,0007, and Dunnett's multiple comparisons analysis. * α 0,05, ** α 0,01, *** α 0.001. CN: Sugar Control. CS: Solvent Control.

midgut of the group exposed to 1000 ppm, with an average of approximately 3 oocysts per female, compared to the control group which had 27 oocysts per female. In terms of the number of sporozoites, the 100 ppm group had 874.8, while the 1000 ppm group had 250 sporozoites per mosquito, compared to the control group which had 1809 sporozoites per female. The nanoformulation significantly impacted the attributes of vector capacity and competence.

4. Discussion

Vector-borne diseases (VBD) remain a global concern, despite being preventable. The World Health Organization (WHO) underscores that 17 % of global burden of communicable diseases is due VBD. At the same time, more than 80 % of the global population resides in regions at risk for at least two types of VBD (WHO, 2017). In response to this issue, the WHO has prioritized enhancing vector control, encompassing heightened research and development in this area. In this context, the synergy of *Azadirachta indica* and nanotechnology emerges as a promising strategy to effectively address vector control. In line with analogous previous investigations, our study also indicates that secondary metabolites of Neem present in the nanoformulation at higher concentrations had an impact on the longevity and lifespan of fed females. In our overall results, the groups where a significant effect was observed were those fed with 1000 ppm and 100 ppm seed oil, considering that the concentration of limonoids (predominantly azadirachtin A) amounted to 221 µg/ml in the concentrated formulation (150,000 ppm seed oil). In the 1000 ppm group, there was 1.47 µg/ml, and in the 100 ppm group, 0.147 µg/ml of azadirachtin A. On mean, a mosquito consumes 6 µl in a complete feeding. Assuming that the mosquitoes in each group fed only once a day, the 1000 ppm group ingested approximately ≈ 0.035 µg, and the 100 ppm group ≈ 0.0035 µg in the 4-day treatment period. These results are consistent with the sublethal effect of azadirachtin A reported in the literature, where close concentrations to 1 µg/ml in feeding solution have an inhibitory impact on the 20E hormone signaling pathway (Lucantoni et al., 2006; Dembo et al., 2015). In *Drosophila melanogaster*, a well-studied insect model, the topical application of 0.63 µg of a commercial Neem formulation (Neem Azal 1 %, Lahunau Germany) has been described to affect survival by up to 46.87 % and reduce offspring by 80 % (Oulhaci et al., 2018). Conversely, in a study employing a feeding methodology with NeemAzal® at an azadirachtin A concentration of 150 mg/kg of mouse body weight, it was reported that the survival of *Anopheles stephensi* was not affected. However, the oral administration was conducted with a four-day interval between each feeding, which might have contributed to the survival of the evaluated groups (Dembo et al., 2015).

The limonoids present in Neem seed oil, specifically the azadirachtins, belong to the class of molecules known as 'Insect Growth Regulators' (IGRs). IGRs are a type of insecticides that interfere with the signaling pathway of the hormone 20E, disrupting the process of metamorphosis, insect growth, reproductive capacity, and survival (Viñuela et al., 1991; Dinan et al., 1997; Zou et al., 2018; Kapitskaya et al., 2000; Chen et al., 2004; Gabrieli et al., 2014). In mosquitoes, the 20E signaling pathway is activated when 20E binds to the nuclear receptor known as the 'ecdysone receptor complex.' This complex is a heterodimer formed by the ultraspiracle protein (USP) and the ecdysone receptor protein (EcR). The 20E hormone only binds to EcR but requires heterodimerization with USP to successfully bind (Kumar et al. 2002; Yao et al. 1992). The EcR-USP complex acts as a transcription factor for various genes referred to as 'early genes,' such as E75, E74, HR3, and Broad-Complex (Kapitskaya et al., 2000; Sun et al., 2004; Margam et al., 2006; Zhu et al., 2007; Mane-Padros et al., 2012). In turn, these genes function as transcription factors, inducing or repressing the expression of several genes involved in reproduction, immunity, and development (Johnson, 2018). The molecular details of the 20E signaling pathway is so far not fully elucidate (Ekoka et al., 2021), but the effects of interfering with this pathway are extensively documented. In *D. melanogaster*, Hervé Tricoire et al. (2009) demonstrated that by inactivating the EcR receptor, both longevity and lifespan were reduced (Tricoire et al., 2009; Gáliková et al., 2011), suggesting a direct relationship between these population indicators and the 20E hormone signaling pathway.

In mosquitoes, blood consumption stimulates the release of two types of peptidic hormones from the brain: ovarian ecdysteroidogenic

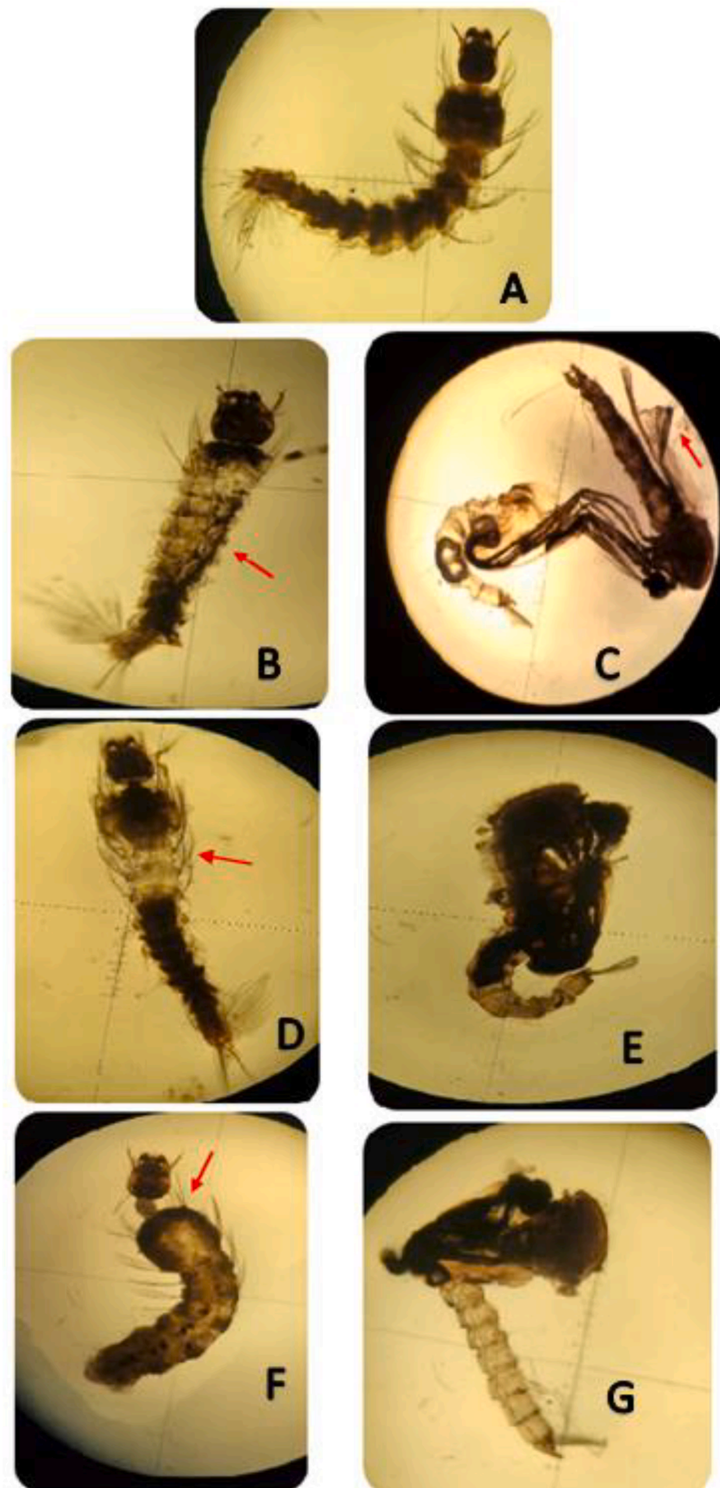


Fig. 7. Optical microscopy images. Transgenerational malformation effects of nanoformulation *A. indica* on F1 progeny of *An. aquasalis*. Red arrows indicate malformations. (A) Normal Larva IV of group CN sugar. (B) Larva IV from the 50 ppm group, with undefined thorax and abdomen with diffuse brown pigment. (C) Adult from the 50 ppm group with failed emergence and wrinkled wings. (D) Larva IV from the 100 ppm group with disrupted intestine. (F) Larva IV from the 1000 ppm group with undefined thorax and abdominal segments with enlarged size. (G) Intermediate stage between adult and pupa from the 1000 ppm group, undeveloped wings are observed.

hormone (OEH) and Active Ingredient-like peptides (ILP) (Brown et al., 2008). These hormones bind to receptors on the ovarian membrane, where OEH stimulates follicular cells to produce ecdysteroids, mainly ecdysone (Hagedorn et al., 1975; Dhara et al., 2013; Valzania et al., 2019). While ILP required for the transcription of trypsin-like enzymes, used in the digestion of blood in the midgut and in the utilization of nutrients for egg maturation (Brackney et al., 2010; Gulia-Nuss et al., 2011). Thus, ecdysone is released from the follicular cells of the ovary and transported to the fat body where it is converted into 20E. As mentioned above, 20E functions as a transcription factor, with some of its most

Table 6
Concentration of the hormone 20E in An. aquasalis fed with nanoformulation A. indica.

Treatment	Mean 20E pg/ml per female (min-max.)	p Value
CN sugar	326.9 (238.2–491.9)	–
NBM	125.7(0–250.7)	0.2952
CS 1000ppm	201(124.9–305.3)	0.7096
25ppm	339.7(253.9–410.9)	0.9999
50ppm	300.5(106.5–484.4)	0.9996
100ppm	365.9(263.5–559–3)	0.9979
1000ppm	463.3(394.1–545)	0.645

NBM: No blood meal. One-way ANOVA test F (6.14) = 2.126, p = 0.1149, Dunnett's multiple comparisons analysis. CS: Solvent Control.

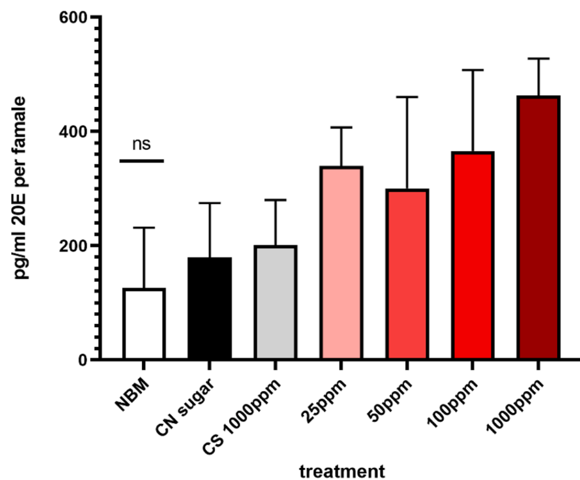


Fig. 8. Detection of the hormone 20-hydroxyecdysone by the enzyme-linked immunosorbent assay (EIA) in *An. aquasalis* fed with the nanoformulation *A. indica*. One-way ANOVA test F (6,14) = 2126, p = 0,1149. Tukey's multiple comparisons analysis. CS: Solvent Control.

relevant target genes in reproduction is the yolk precursor protein (YPP) gene and vitellogenin (Hansen et al., 2014). Developing oocytes capture these yolk proteins through endocytosis to provide nutrients and complete the process of oogenesis (Sappington et al., 1996; Attardo et al., 2005; Baldini et al., 2013). Consequently, it can be inferred that the nanoformulation had the capacity to interfere with this signaling pathway, leading to a dose-dependent reduction in fertility and fecundity. Females fed with 100 ppm and 1000 ppm doses laid 50 % fewer eggs than the control groups, and the egg viability also decreased by the same percentage. Interestingly, the solvent control group (CS) produced a higher number of eggs than the untreated control group (CN). Though not statistically significant, a negative inhibition in the oviposition and hatching percentage was observed in the CS group. This might have been influenced by an additional nutritional component, possibly lipids, as this control group contained sunflower seed oil. In these groups of 100 ppm and 1000 ppm, despite the evidence of blood feeding, it was observed that some females did not develop any eggs (supplement 1). In a similar study, researchers examined the detailed effect of a Neem seed extract on the ultrastructure of *An. stephensi* oocytes, reporting a complete blockage of oogenesis, partial or complete impairment of vitellogenesis and formation of the vitelline envelope, along with marked degeneration of follicular cells (Lucantoni et al., 2006). This leads to a significant impact on the fecundity and egg viability of *Anopheles* spp. mosquitoes and other insect groups (Dembo et al., 2015; Bezzar-bendjazia et al., 2016; Amaral et al., 2018; Oulhaci et al., 2018).

In addition to affecting the survival, fecundity, and egg viability of directly exposed adult specimens, a significant transgenerational effect was observed in the F1 generation. The life tables used allowed tracking the life cycle from egg to adult, revealing an impact on survival at the end of the development cycle. Specimens that completed metamorphosis were only 35 % in 50 ppm, 32 % in 100 ppm, and 19 % in 1000 ppm, compared to the near 80 % survival in the control groups. Additionally, the most critical stage change was from larva IV to pupa, where there was higher mortality. In *D. melanogaster*, M. Ferdenache et al. (2019) reported transgenerational effects of a Neem formulation (1 % azadirachtin A) at a dose of 0.67 μg, inducing survival rates of 27 % in exposed males and 16 % in exposed females. Furthermore, the effect on the F1 generation showed a survival rate of 81 % for males and 64 % for females. They also reported developmental abnormalities in exposed immature stages, including larval melanization, death in the larva-pupa and pupa-adult transitions, deformed wings, smaller body size, and deformed abdomen (Ferdenache et al., 2019). In our study, some of these malformations were also identified in the F1 generation groups of females exposed to 50 ppm, 100 ppm, and 1000 ppm. The most common abnormalities included abdominal deformations with regions of thinner and darker cuticle, curved and shortened abdomens with apparent disruption, and undefined thoraxes. Less frequently observed were adults trapped within the pupal casing. These effects are related to the interference in the hormonal regulation of JH and 20E, affecting chitin formation during the molting process (Agrell and Lundquist, 1973; Rattan and Summt, 2014; Boulahbel et al., 2015; Bezzar-bendjazia et al., 2016; Chinnamma et al., 2017; Schneider et al., 2017; Aribi et al., 2020). Recently, another interesting effect related to insect exoskeleton formation was discovered, where azadirachtin regulates the growth of *Spodoptera frugiperda* by affecting the chitin synthesis pathway through negative regulation of 31 chitin-related proteins (Shu et al., 2021). The transgenerational effects of Neem on vector mosquitoes have been poorly studied, and the long-term effects remain unknown. This knowledge would be highly useful in determining the frequency of application and administered dosage, ultimately optimizing costs and ecological safety for other insect groups, such as pollinators (Challa et al., 2019; Kilani-Morakchi et al., 2021).

There are several studies that have used different formulations of

Table 7
Susceptibility of An. aquasalis fed with nanoformulation A. indica and NeemAZAL® experimentally infected with to Plasmodium vivax, infected by membrane feeding assay. CS: Solvent Control.

Treatment	Dissected mosquitoes	% infection rate	*p* value	Mean per mosquito oocysts (min-max)	Value *p*	Dissected mosquitoes	Mean per mosquitos sporozoites (min-max)	Value *p*
CN sugar	55	73	–	27.40 (0–169)	–	17	1809 (1500–3375)	–
CS 1000ppm	45	69	>0.9999	22.56 (0–160)	>0.9999	12	1250 (750–1650)	>0.9999
50ppm	55	68	>0.9999	23.73 (0–250)	>0.9999	16	703 (375–1.050)	**<0.0001**
100ppm	55	56	>0.9999	8.291 (0–63)	0.1555	12	874.8 (150–2062)	**0.0002**
1000ppm	50	22	**0.0147**	3.42 (0–64)	<0.0001	9	250 (0–375)	**<0.0001**
0,7 ppm NA	50	44	0.8857	4.86 (0–91)	0.0007	17	1235 (750–3000)	0.1645
1,5 ppm NA	55	51	>0.9999	7.055 (0–86)	0.0402	17	705.9 (375–1050)	**<0.0001**
3 ppm NA	55	38	0.2539	5.36 (0–78)	0.0011	16	656.3 (375–1500)	**<0.0001**

min = minimum number, max = maximum number Kruskal-Wallis test and analysis of multiple Dunn's comparisons.

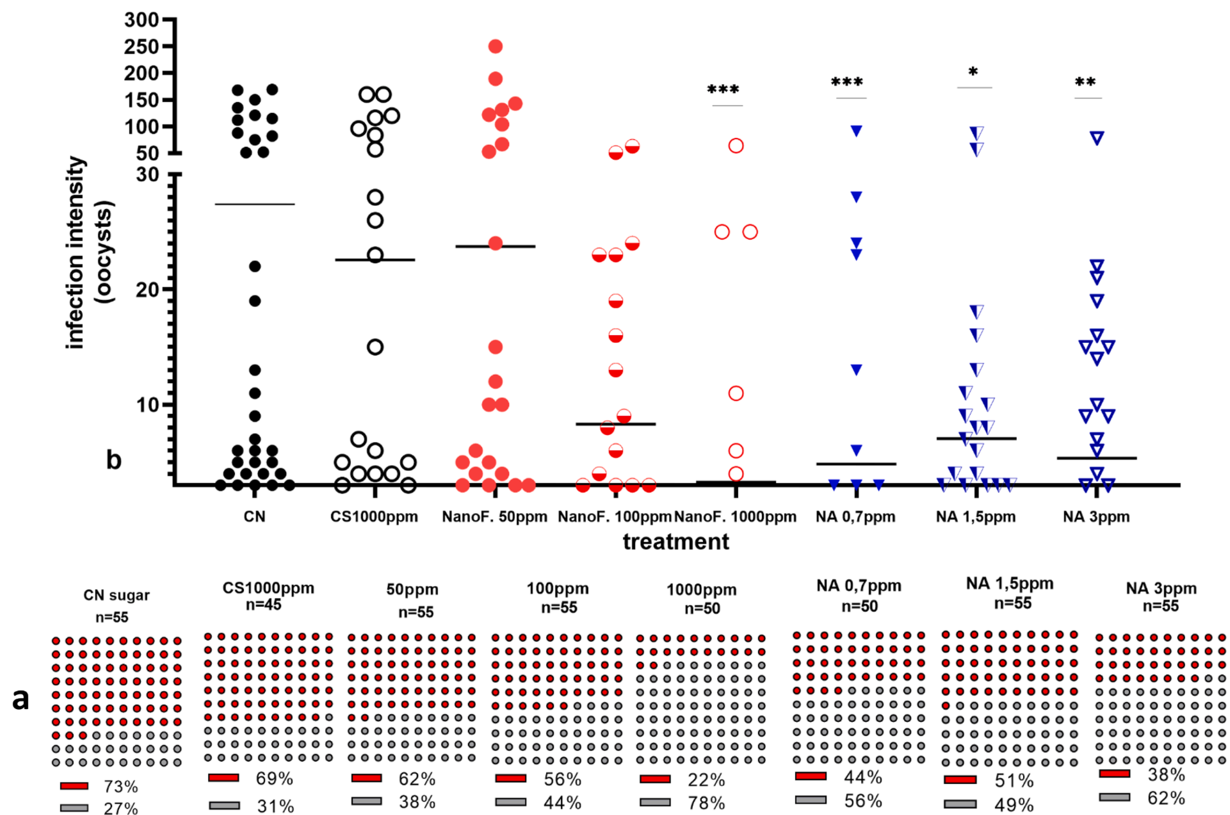


Fig. 9. a) Infection rates are represented as red= percentage of infected, light gray = percentage of non-infected. b) Number of P. vivax oocysts in the gut of female An. aquasalis fed with nanoformulation A. indica and the commercial formulation NeemAzal (NA). Infection rate Kruskal-Wallis test p-value = 0.0387, * α 0.05, and Dunn's multiple comparisons analysis. Infection intensity Kruskal-Wallis test p-value < 0.0001, and Dunn's multiple comparisons analysis. * α 0.05, ** α 0.01, *** α 0.001. CN: Sugar Control. CS: Solvent Control.

Neem to evaluate the effects on the development of *Plasmodium* spp. However, these studies have been conducted using murine *Plasmodium* models like *P. berghei* (Chianese et al. 2010a; Lucantoni et al., 2010; Tapanelli et al., 2016; Dahiya et al., 2016; Habluetzel et al., 2019) and in vitro models of *P. falciparum* (Udeinya et al., 2006; Ashok Yadav et al., 2017). This study is the first to report the bioactivity of a Colombian Neem seed nanoformulation on oocysts and sporozoites of *P. vivax*. The most significant finding was the impact on oocyst prevalence and intensity at concentrations of 100 ppm and 1000 ppm, where oocyst formation decreased by 3.3 times and 8 times, respectively, compared to the control. The mechanism of action of Neem on *Plasmodium* parasites is not fully described, but in vitro studies have revealed that it inhibits the exflagellation process of microgametes in *P. falciparum* and *P. berghei* (Jones et al.,. 1994). Oliver Billker et al. (2002) used 50 μM of pure azadirachtin on activated *P. berghei* microgametocytes. Through scanning electron microscopy and fluorescence microscopy, they confirmed that azadirachtin interfered with the formation of the mitotic spindle and the reorganization of microtubules in the cytoskeleton. At this concentration, no nucleated and motile microgametes were released (Billker et al., 2002). Another study used a commercial formulation of NeemAzal® containing 57.6 % limonoids, administered to mice infected with gametocytes. In the group of mice given 50 mg/kg, the presence of *P. berghei* oocysts in *An. stephensi* mosquitoes was inhibited by 100 %. Additionally, none of the healthy mice exposed to mosquito bites from these infected mosquitoes developed parasitemia. Furthermore, in infected mosquitoes that fed on mice treated with the same concentration, there was a reduced number of zygotes in the midgut and an absence of mature ookinetes. Microscopic observations identified morphological alterations consistent with the hypothesis that azadirachtin interfered with the functionality of microtubule organizing centers and cytoskeleton assembly (Lucantoni et al., 2010). Similar to these findings, the oral administration of a crude Neem seed extract to mice (200 mg/kg) for 9 days reduced parasitemia levels by 45 % (95 % CI 40 %−50 % $p < 0.001$) compared to a control group (Chianese et al., 2010b). This result could be related to the effect that azadirachtin seems to have on the mobile forms of the parasite, in this case, sporozoites, as sliding motility depends on actin (Kumpula et al., 2017). Related to this, Anuradha et al. (2007) studied the effect of azadirachtin A on different tissues of *D. melanogaster* and reported that it induces actin depolymerization at the cellular level, leading to cell immobilization and subsequent caspase-independent apoptosis. They proposed actin as a target for the activity of azadirachtin A (Anuradha et al., 2007). Furthermore, the bioactivity of Neem on an important vector of Chagas disease has also been reported. In *Rhodnius prolixus* fed with a blood solution containing 1 μg/ml of azadirachtin, the formation of tetracyclic promastigotes of *Trypanosoma cruzi* was inhibited due to an alteration in rectal structure caused by the antagonism of azadirachtin A on the 20E hormone (Mendonça Lopes et al., 2022).

Few studies addressing *Plasmodium* development within the vector achieve to report an effect up to the sporozoite stage, as it is complex to maintain survival and monitor the infection up to 14 day post-infection. While the number of surviving mosquitoes was low in all groups in our study, it provided an insight into the number of sporozoites that reached the salivary glands in each concentration of the nanoformulation and NeemAzal®. These numbers were significantly lower in all groups, except for the lowest NeemAzal® concentration (0.75 ppm). However, this result should be interpreted cautiously due to the small number of mosquitoes analyzed and our inability to assess the motility and infectivity of these sporozoites in hepatocytes. Nonetheless, Lucantoni *et al.* (2010) evaluated the effect of NeemAzal® on the maturation of *P. berghei* oocysts in *An. stephensi*. They offered infected mosquitoes a second blood meal from mice treated with 50 mg/kg of NeemAzal® on

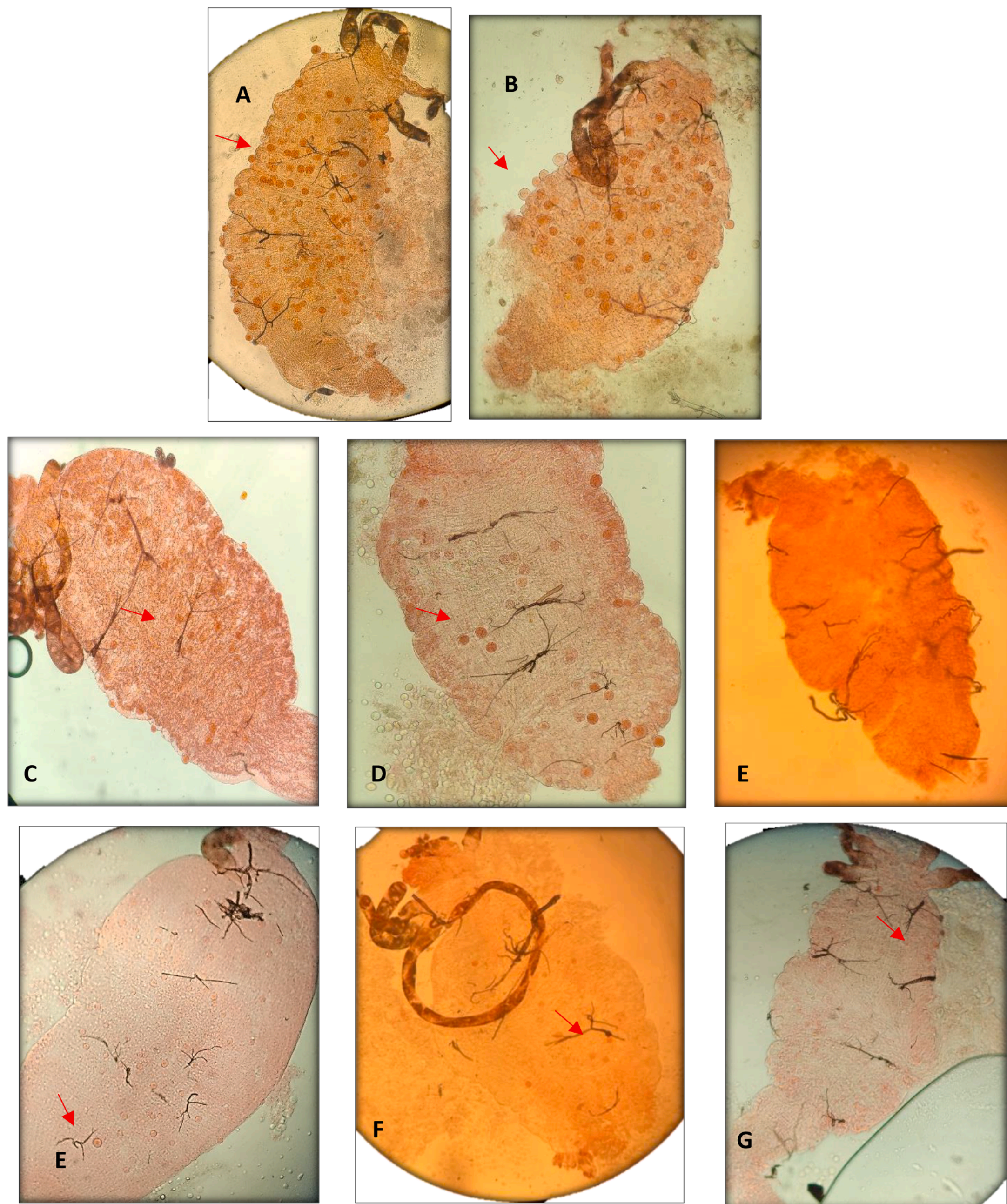


Fig. 10. Midgut of *An. aquasalis* experimentally infected with *P. vivax*.

day 7 post-infection and did not report any effect on oocyst development. Salivary glands were infected with sporozoites (Lucantoni et al., 2010), supporting the hypothesis of azadirachtin's effect on the mobile forms of the parasite. Finally, our results regarding the decrease in sporozoites could be related to the lower number of oocysts that reached to establish and complete their development in the treated groups, suggesting a possible direct effect on sporozoites. It's also probable that on days 10–14, the nanoformulation and NeemAzal® might not have been present in the mosquitoes' hemolymph, as mechanisms of detoxification have been described in *D. melanogaster* in response to Neem compounds (Zhang et al., 2018). The concentrations of NeemAzal® that had a similar effect to the nanoformulation on the survival of female *An. aquasalis* mosquitoes allowed the evaluation of *P. vivax* development. These concentrations were 3 ppm, 1.5 ppm, and 0.75 ppm, and in turn, the higher NeemAzal® concentration groups consumed 0.072 µg (3 ppm) and 0.036 µg (1.5 ppm) of azadirachtin A, a similar amount to the consumption in the 1000 ppm group of the nanoformulation. This provides us with an approximate value of the concentration of azadirachtin required to cause a sublethal effect on mosquitoes, resulting in a significant decrease in reproductive capacity, transgenerational survival, and *P. vivax* infection intensity. By simultaneously intervening in these characteristics related to vector competence and capacity, the use of Neem-derived products for the control of malaria vector mosquitoes becomes a necessary proposition.

5. Conclusions

The nanoformulation of *A. indica* seed oil had a negative impact on the longevity, reproductive capacity, and metamorphic process of the F1 generation of *An. aquasalis*, possibly through disruption in endocrine signaling pathways. Specifically, there was a significant reduction in the survival of adult mosquitoes that consumed the nanoformulation at

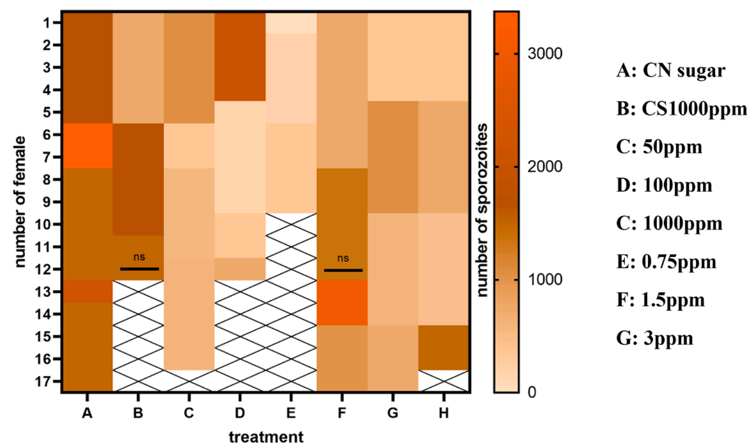


Fig. 11. Heat map showing the relative amount of P. vivax sporozoites in the salivary glands of An. aquasalis fed with nanoformulation An. indica and NeemAZAL®. Each column represents the evaluated group, each row corresponds to an analyzed mosquito, with color intensity relative to the quantified number of sporozoites. CS: Solvent Control.

higher concentrations, with a decrease of up to 8 days in longevity, a critical time period directly impacting the probability of survival necessary for a vector to transmit a pathogen. Furthermore, this impact on survival extended to the F1 generation, which did not have direct exposure to the nanoformulation.

Regarding reproductive capacity, the nanoformulation had an inhibitory effect on oviposition of 44 % and 56 % at concentrations of 100 ppm and 1000 ppm, respectively. Additionally, it affected the viability of at least 50 % of the eggs at both concentrations. In terms of vector control, it could reduce population density and decrease the likelihood of a human being bitten by a vector.

On the other hand, the nanoformulation had a significant impact on the development of experimental *P. vivax* infection in An. aquasalis, reducing the intensity of oocysts by 8 times and the number of sporozoites by 7.24 times compared to the control group. This, in turn, decreased the vector competence of *An. aquasalis*, where fewer parasitic forms were able to develop and establish themselves in the intestine and salivary glands, respectively.

It's important to mention that these results are related to the oral administration of a Neem nanoformulation to female *An. aquasalis* mosquitoes through feeding on sugar solution and blood. Therefore, direct extrapolation to real field conditions may not be applicable. But, this is an important approach to understanding the potential effects of this nanoformulation. It explores alternatives proposed by the WHO's Global Vector Control Strategy 2017–2030, which aims to enhance vector control capacity. The use of *Azadirachta indica* combined with nanotechnology holds great applicability in achieving this purpose.

CRediT authorship contribution statement

Paula Andrea Urrea Aguirre: Conceptualization, Investigation, Writing – original draft. **Keillen Monick Martins:** Methodology. **Campos Duván Dueñas López:** Resources. **Fernando Orozco Sánchez:** Resources, Funding acquisition. **Alberto Tobón Castaño:** Writing – review & editing. **Claudia María Ríos Velásquez:** Conceptualization, Methodology, Validation, Writing – review & editing. **Adriana Pabón Vidal:** Conceptualization, Supervision, Project administration.

Declaration of competing interest

The authors declare that they have no competing interests associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Data availability

Data will be made available on request.

Acknowledgments

This work was supported by project "Nanoformulados de neem (*Azadirachta indica*) en la lucha antivectorial para la prevención de la malaria y dengue" code 110184467540, contract 876-2019 de MinCiencias and the Universidad de Antioquia. We thank doctors Guillermo León Rúa Uribe and Omar Cantillo Barraza for intellectual support; Elen Sabrina dos Reis Martins, Anny Caroline Araujo, João Victor Nobre and Jordam William Pereira Silva for technical support.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actatropica.2024.107223.

References

Agrell, I.P.S., Lundquist, A.M., 1973. Physiological and biochemical changes during insect development. The Physiology of Insecta. Elsevier, pp. 159–247. https://doi.org/10.1016/b978-0-12-591601-1.50011-9.

Amaral, K.D., Luis, C.M., Augusta Pereira Lima, M., Eduardo Serrão, J., Maria Castro Della Lucia, T., 2018. Azadirachtin impairs egg production in atta sexdens leaf-cutting ant queens. Environ. Pollut. (Barking, Essex : 1987) 243 (Pt B), 809–814. https://doi.org/10.1016/J.ENVPOL.2018.09.066.

Antinori, S., Galimberti, L., Milazzo, L., Corbellino, M., 2012. Biology of human malaria plasmodia including plasmodium knowlesi. Mediterr. J. Hematol. Infect. Dis. 4 (1), 201. https://doi.org/10.4084/MJHID.2012.013.

Anuradha, A., Annadurai, R.S., Shashidhara, L.S., 2007. Actin cytoskeleton as a putative target of the neem limonoid azadirachtin A. Insect. Biochem. Mol. Biol. 37 (6), 627–634. https://doi.org/10.1016/J.IBMB.2007.03.009.

Aribi, N., Denis, B., Kilani-Morakchi, S., Joly, D., 2020. Azadirachtin, a natural pesticide with multiple effects. Med./Sciences 36 (1), 44–49. https://doi.org/10.1051/medsci/2019268.

Ashok Yadav, P., Kumar, C.P., Siva, B., Suresh Babu, K., Allanki, A.D., Sijwali, P.S., Jain, N., Veerabhadra Rao, A., 2017. Synthesis and evaluation of anti-plasmodial and cytotoxic activities of epoxyazadiradione derivatives. Eur. J. Med. Chem. 134, 242–257. https://doi.org/10.1016/j.ejmech.2017.04.016.

Attardo, G.M., Hansen, I.A., Raikhel, A.S., 2005. Nutritional regulation of vitellogenesis in mosquitoes: implications for anautogeny. Insect. Biochem. Mol. Biol. 35 (7), 661–675. https://doi.org/10.1016/J.IBMB.2005.02.013.

Baldini, F., Gabrieli, P., South, A., Valim, C., Mancini, F., Catteruccia, F., 2013. The interaction between a sexually transferred steroid hormone and a female protein regulates oogenesis in the malaria mosquito anopheles gambiae. PLoS Biol. 11 (10), e1001695 https://doi.org/10.1371/JOURNAL.PBIO.1001695.

Beier, J.C., Keating, J., Githure, J.I., MacDonald, M.B., Impoinvil, D.E., Novak, R.J., 2008. Integrated vector management for malaria control. Malar. J. 7 (SUPPL. 1) https://doi.org/10.1186/1475-2875-7-S1-S4.

Belete, T.M., 2020. Recent progress in the development of new antimalarial drugs with novel targets. Drug Des. Develop. Ther. 14, 3875–3889. https://doi.org/10.2147/DDDT.S265602.

Benelli, G., Canale, A., Toniolo, C., Higuchi, A., Murugan, K., Pavela, R., Nicoletti, M., 2017. Neem (Azadirachta Indica): towards the ideal insecticide? Nat. Prod. Res. 31 (4), 369–386. https://doi.org/10.1080/14786419.2016.1214834.

Bezzar-bendjazia, R., Kilani-morakchi, S., Aribi, N., 2016. Growth and molting disruption effects of azadirachtin against drosophila melanogaster (diptera : drosophilidae). J. Entomol. Zool. Stud. 4 (1), 363–368.

Billker, O., Shaw, M.K., Jones, I.W., Ley, S.V., Jennifer Mordue, A., Sinden, R.E., 2002. Azadirachtin disrupts formation of organised microtubule arrays during microgametogenesis of plasmodium berghei. J. Eukaryot. Microbiol. 49 (6), 489–497. https://doi.org/10.1111/J.1550-7408.2002.TB00234.X.

Boulahbel, B., Aribi, N., Kilani-Morakchi, S., Soltani, N., 2015. Insecticidal activity of azadirachtin on drosophila melanogaster and recovery of normal status by exogenous 20-hydroxyecdysone. Afr. Entomol. 23 (1), 224–233. https://doi.org/10.4001/003.023.0104.

Brackney, D.E., Isoe, J., Black, W.C., Zamora, J., Foy, B.D., Miesfeld, R.L., Olson, K.E., 2010. Expression profiling and comparative analyses of seven midgut serine proteases from the yellow fever mosquito, Aedes Aegypti. J. Insect Physiol. 56 (7), 736–744. https://doi.org/10.1016/J.JINSPHYS.2010.01.003.

Brown, M.R., Clark, K.D., Gulia, M., Zhao, Z., Garczynski, S.F., Crim, J.W., Suderman, R. J., Strand, M.R., 2008. An Active Ingredient-like peptide regulates egg maturation and metabolism in the mosquito Aedes Aegypti. Proc. Natl. Acad. Sci. U.S.A. 105 (15), 5716–5721. https://doi.org/10.1073/PNAS.0800478105.

Challa, G.K., Firake, D.M., Behere, G.T., 2019. Bio-pesticide applications may impair the pollination services and survival of foragers of honey bee, apis cerana fabricius in oilseed brassica. Environ. Pollut. (Barking, Essex : 1987) 249 (June), 598–609. https://doi.org/10.1016/J.ENVPOL.2019.03.048.

Chen, L., Zhu, J., Sun, G., Raikhel, A.S., 2004. The Early Gene Broad Is Involved in the Ecdysteroid Hierarchy Governing Vitellogenesis of the Mosquito Aedes Aegypti. J. Mol. Endocrinol. 33 (3), 743–761. https://doi.org/10.1677/jme.1.01531.

Chianese, G., Yerbanga, S.R., Lucantoni, L., Habluetzel, A., Basilico, N., Taramelli, D., Fattorusso, E., Taglialatela-Scafati, O., 2010a. Antiplasmodial triterpenoids from the fruits of neem, azadirachta indica. J. Nat. Prod. 73 (8), 1448–1452. https://doi.org/10.1021/NP100325q.

Chianese, G., Yerbanga, S.R., Lucantoni, L., Habluetzel, A., Basilico, N., Taramelli, D., Fattorusso, E., Taglialatela-Scafati, O., 2010b. Antiplasmodial triterpenoids from the fruits of neem, azadirachta indica. J. Nat. Prod. 73 (8), 1448–1452. https://doi.org/10.1021/NP100325Q/SUPPL_FILE/NP100325Q_SI_001.PDF.

Chinnamma, C.A., Govt Degree College, and Anantapur Dist, 2017. Influence of neem oil on the morphogenesis of uzifly exorista bombysis. Int. J. Innov. Sci. Res. Technol. 2 (5). ISSN No: 2456-2165.

Cohuet, A., Harris, C., Robert, V., Fontenille, D., 2010. Evolutionary forces on anopheles: what makes a malaria vector? Trend. Parasitol. 26 (3), 130–136. https://doi.org/10.1016/J.PT.2009.12.001.

Dahiya, N., Chianese, G., Abay, S.M., Taglialatela-Scafati, O., Esposito, F., Lupidi, G., Bramucci, M., et al., 2016. In vitro and ex vivo activity of an azadirachta indica A. Juss. seed kernel extract on early sporogonic development of plasmodium in comparison with azadirachtin A, its most abundant constituent. Phytomedicine 23 (14), 1743–1752. https://doi.org/10.1016/j.phymed.2016.10.019.

Dembo, E.G., Abay, S.M., Dahiya, N., Ogboi, J.S., Christophides, G.K., Lupidi, G., Chianese, G., Lucantoni, L., Habluetzel, A., 2015. Impact of repeated NeemAzal®-treated blood meals on the fitness of anopheles stephensi mosquitoes. Parasit. Vect. 8 (1) https://doi.org/10.1186/s13071-015-0700-1.

Dhara, A., Hoon Eum, J., Robertson, A., Gulia-Nuss, M., Vogel, K.J., Clark, K.D., Graf, R., Brown, M.R., Strand, M.R., 2013. Ovary ecdysteroidogenic hormone functions independently of the Active Ingredient receptor in the yellow fever mosquito, aedes aegypti. Insect. Biochem. Mol. Biol. 43 (12), 1100–1108. https://doi.org/10.1016/J. IBMB.2013.09.004.

Dinan, L., Whiting, P., Girault, J.P., Lafont, R., Dhadialla, T.S., Cress, D.E., Mugat, B., Antoniewski, C., Lepesant, J.A., 1997. Cucurbitacins are insect steroid hormone antagonists acting at the ecdysteroid receptor. Biochem. J. 327 (3), 643–650. https://doi.org/10.1042/bj3270643.

Ekoka, E., Maharaj, S., Nardini, L., Dahan-Moss, Y., Koekemoer, L.L., 2021. 20-Hydroxyecdysone (20E) signaling as a promising target for the chemical control of malaria vectors. Parasit. Vect. https://doi.org/10.1186/s13071-020-04558-5. BioMed Central Ltd.

Ferdenache, M., Bezzar-Bendjazia, R., Marion-Poll, F., Kilani-Morakchi, S., 2019. Transgenerational Effects from single larval exposure to azadirachtin on life history and behavior traits of drosophila melanogaster. Sci. Rep. 9 (1), 1–12. https://doi.org/10.1038/s41598-019-53474-x.

Gabrieli, P., Kakani, E.G., Mitchell, S.N., Mameli, E., Want, E.J., Mariezcurrena Anton, A., Serrao, A., Baldini, F., Catteruccia, F., 2014. Sexual transfer of the steroid hormone 20E induces the postmating switch in anopheles gambiae. Proc. Natl. Acad. Sci. U.S.A. 111 (46), 16353–16358. https://doi.org/10.1073/PNAS.1410488111/SUPPL_FILE/PNAS.1410488111.SD02.XLSX.

Gáliková, M., Klepsatel, P., Senti, G., Flatt, T., 2011. Steroid hormone regulation of C. elegans and drosophila aging and life history. Exp. Gerontol. 46 (2–3), 141–147. https://doi.org/10.1016/J.EXGER.2010.08.021.

Gulia-Nuss, M., Robertson, A.E., Brown, M.R., Strand, M.R., 2011. Active Ingredient-like peptides and the target of rapamycin pathway coordinately regulate blood digestion and egg maturation in the mosquito aedes aegypti. PLoS One 6 (5), e20401. https://doi.org/10.1371/JOURNAL.PONE.0020401.

Habluetzel, A., Pinto, B., Tapanelli, S., Nkouangang, J., Saviozzi, M., Chianese, G., Lopatriello, A., et al., 2019. Effects of azadirachta indica seed kernel extracts on early erythrocytic schizogony of plasmodium berghei and pro-inflammatory response in inbred mice. Malar. J. 18 (1), 35. https://doi.org/10.1186/s12936-019-2671-8.

Hagedorn, H.H., O'Connor, J.D., Fuchs, M.S., Sage, B., Schlaeger, D.A., Bohm, M.K., 1975. The ovary as a source of alpha-ecdysone in an adult mosquito. Proc. Natl. Acad. Sci. U.S.A. 72 (8), 3255–3259. https://doi.org/10.1073/PNAS.72.8.3255.

Hansen, I.A., Attardo, G.M., Rodriguez, S.D., Drake, L.L., 2014. Four-way regulation of mosquito yolk protein precursor genes by juvenile hormone-, ecdysone-, nutrient-, and Active Ingredient-like peptide signaling pathways. Front. Physiol. (March), 103. https:// doi.org/10.3389/FPHYS.2014.00103/BIBTEX, 5 MAR.

Hiwat, H., Bretas, G., 2011. Ecology of Anopheles darlingi Root with respect to vector importance: a review. Parasit. Vect. 4 (1) https://doi.org/10.1186/1756-3305-4-177.

Johnson, L.K., 2018. Regulation of Gene Expression Patterns during Reproduction in the Female, Aedes Aegypti. University of California Riverside. https://escholarship.org/content/qt7zf63036/qt7zf63036_noSplash_7f0d6f431f08ccaf0ee4c728257bf4aa.pdf?t=pmno1c.

Jones, I.W., Denholm, A.A., Ley, S.V., Lovell, H., Wood, A., Sinden, R.E., 1994. Sexual development of malaria parasites is inhibited in vitro by the neem extract azadirachtin, and its semi-synthetic analogues. FEMS Microbiol. Lett. 120 (3), 267–273. https://doi.org/10.1111/j.1574-6968.1994.tb07044.x.

Josling, G.A, Llinás, M., 2015. Sexual development in plasmodium parasites: knowing when it's time to commit. Nat. Rev. Microbiol. 13 (9), 573–587. https://doi.org/10.1038/nrmicro3519.

Kamaraj, C., Abdul Rahuman, A., Mahapatra, A., Bagavan, A., Elango, G., 2010. Insecticidal and larvicidal activities of medicinal plant extracts against mosquitoes. Parasitol. Res. 107 (6), 1337–1349. https://doi.org/10.1007/s00436-010-2006-8.

Kapitskaya, M.Z., Li, C., Miura, K., Segraves, W., Raikhel, A.S., 2000. Expression of the early-late gene encoding the nuclear receptor HR3 suggests its involvement in regulating the vitellogenic response to ecdysone in the adult mosquito. Mol. Cell. Endocrinol. 160 (1–2), 25–37. https://doi.org/10.1016/S0303-7207(99)00253-1.

Kaplan, E.L., Meier, P., 1958. Nonparametric estimation from incomplete observations. J. Am. Stat. Assoc. 53 (282), 457–481. https://doi.org/10.1080/01621459.1958.10501452.

Kilani-Morakchi, S., Morakchi-Goudjil, H., Sifi, K., 2021. Azadirachtin-based insecticide: overview, risk assessments, and future directions. Front. Agron. 3 (July), 32. https://doi.org/10.3389/FAGRO.2021.676208/BIBTEX.

Kruskal, W.H., Wallis, W.A., 1952. Use of ranks in one-criterion variance analysis. J. Am. Stat. Assoc. 47 (260), 583. https://doi.org/10.2307/2280779.

Kumar, M.B., Fujimoto, T., Potter, D.W., Deng, Q., Palli, S.R., 2002. A single point mutation in ecdysone receptor leads to increased ligand specificity: implications for gene switch applications. Proc. Natl. Acad. Sci. U.S.A. 99 (23), 14710–14715. https://doi.org/10.1073/PNAS.222278999/SUPPL_FILE/2789FIG7A.JPG.

Kumpula, E.P., Pires, I., Lasiwa, D., Piirainen, H., Bergmann, U., Vahokoski, J., Kursula, I., 2017. Apicomplexan actin polymerization depends on nucleation. Sci. Rep. 7 (1), 1–10. https://doi.org/10.1038/s41598-017-11330-w. *2017 7:1*.

Liu, L., Zhao, Y.-L., Cheng, G.-G., Chen, Y.-Y., Qin, X.-J., Song, C.-W., Yang, X.-W., Liu, Y.-P., Luo, X.-D., 2014. Limonoid and steroidal saponin from azadirachta indica. Nat. Prod. Bioprospect. 4 (6), 335–340. https://doi.org/10.1007/s13659-014-0042-2.

Lucantoni, L., Giusti, F., Cristofaro, M., Pasqualini, L., Esposito, F., Lupetti, P., Habluetzel, A., 2006. Effects of a neem extract on blood feeding, oviposition and oocyte ultrastructure in anopheles stephensi liston (diptera: culicidae). Tissu. Cell 38 (6), 361–371. https://doi.org/10.1016/j.tice.2006.08.005.

Lucantoni, L., Yerbanga, R.S., Lupidi, G., Pasqualini, L., Esposito, F., Habluetzel, A., 2010. Transmission blocking activity of a standardized neem (azadirachta indica) seed extract on the rodent malaria parasite plasmodium berghei in its vector anopheles stephensi. Malar. J. 9 (1) https://doi.org/10.1186/1475-2875-9-66.

Mane-Padros, D., Cruz, J., Cheng, A., Raikhel, A.S., 2012. A critical role of the nuclear receptor hr3 in regulation of gonadotrophic cycles of the mosquito aedes aegypti. PLoS. One 7 (9), e45019. https://doi.org/10.1371/JOURNAL.PONE.0045019.

Mantilla, G., Oliveros, H., Barnston, A.G., 2009. The role of ENSO in understanding changes in Colombia's annual malaria burden by region, 1960-2006. Malar. J. 8 (1), 6. https://doi.org/10.1186/1475-2875-8-6.

Margam, V.M., Gelman, D.B., Palli, S.R., 2006. Ecdysteroid titers and developmental expression of ecdysteroid-regulated genes during metamorphosis of the yellow fever mosquito, aedes aegypti (diptera: culicidae). J. Insect Physiol. 52 (6), 558–568. https://doi.org/10.1016/J.JINSPHYS.2006.02.003.

McKinney, D.A., Strand, M.R., Brown, M.R., 2017. Ealuation of ecdysteroid antisera for a competitive enzyme immunoassay and of extraction procedvures for the measurement of mosquito ecdysteroids. Gen. Comp. Endocrinol. 253, 60. https://doi.org/10.1016/J.YGCEN.2017.08.028.

Mendonça Lopes, D., Provençano, A.F., Mello, C.B., Feder, D., Cunha, J.M.A.A., Sant'Anna, N.F., Lechuga, G.C., et al., 2022. Ecdysone modulates both ultrastructural arrangement of hindgut and attachment of trypanosoma cruzi DM 28c to the rectum cuticle of rhodnius prolixus fifth-instar nymph. Exp. Parasitol. 236–237, 108247 https://doi.org/10.1016/J.EXPPARA.2022.108247.

Mordue, A.J., Nisbet, A.J, 2000. Azadiractina Del Árbol De Neem Azadirachta Indica. Su Acción Contra Los Insectos. Departamento de Zoololgía de la Universidad de Aberdeen. http://www.agrogamacolombia.com.co/wp-content/uploads/2014/07/Azadiractina-del-árbol-de-Neem.pdf.

Morgan, E.D., 2009. Azadirachtin, a scientific gold mine. Bioorgan. Medic. Chem. 17 (12), 4096–4105. https://doi.org/10.1016/j.bmc.2008.11.081.

Nakanishi, K., 1971. The ecdysones. Pure Appl. Chem. 25 (1), 167–196. https://doi.org/10.1351/pac197125010167.

Orozco-Sánchez, F., 2009. Efecto De La Oferta De Oxígeno Sobre El Crecimiento y La Producción De Terpenoides Con Células De Azadirachta indica En Un Biorreactor. Tesis (Doctorado en Ciencias). Instituto Politécnico Nacional (México), Yautepec, Morelos, México. https://tesis.bnct.ipn.mx/bitstream/handle/123456789/5725/EFECTOFERTA.pdf?sequence=1&isAllowed=y.

Oulhaci, C.M., Denis, B., Kilani-Morakchi, S., Sandoz, J.C., Kaiser, L., Joly, D., Aribi, N., 2018. Azadirachtin effects on mating success, gametic abnormalities and progeny survival in drosophila melanogaster (diptera). Pest. Manag. Sci. 74 (1), 174–180. https://doi.org/10.1002/PS.4678.

Prinz, H.L, J.M Sattler, and F. Frischknecht. 2017. "Www.Bio-Protocol.Org/E2395" 7: 1–8. https://doi.org/10.21769/BioProtoc.2395.

Rattan, R., Summt, M., 2014. Plant secondary metabolites in the sustainable diamondback moth (Plutella Xylostella l .) management plant secondary metabolites in the sustainable diamondback moth (Plutella Xylostella l .) management. India. J. Fundam. Appl. Life Sci. 1, 295–309.

Redfern, C.P.F., 1982. 20-hydroxy-ecdysone and ovarian development in anopheles stephensi. J. Insect Physiol. 28 (2), 97–109. https://doi.org/10.1016/0022-1910(82)90117-2.

Ríos-Velásquez, C.M., Martins-Campos, K.M., Simões, R.C., Izzo, T., Santos, E.V.D., Pessoa, F.A., Lima, J.B., et al., 2013. Experimental plasmodium vivax infection of key anopheles species from the Brazilian Amazon. Malar. J. 12 (1) https://doi.org/10.1186/1475-2875-12-460.

Sappington, T.W., Kokoza, V.A., Cho, W.L., Raikhel, A.S., 1996. Molecular characterization of the mosquito vitellogenin receptor reveals unexpected high homology to the drosophila yolk protein receptor. Proc. Natl. Acad. Sci. U.S.A. 93 (17), 8934. https://doi.org/10.1073/PNAS.93.17.8934.

Sato, S.., 2021. Plasmodium—a brief introduction to the parasites causing human malaria and their basic biology. J. Physiol. Anthropol. https://doi.org/10.1186/s40101-020-00251-9. BioMed Central Ltd.

Rabinovich, 1978. Ecología De Poblaciones de Animales". Programa Regional De Desarrollo Científico y Tecnológico, 1ra Edition. Orlando Florida.

Schneider, L.C., Lauer, C., Vieira da, S., Conte, H., 2017. Toxic effect of commercial formulations of neem oil, *Azadirachta Indica* A. Juss., in pupae and adults of the sugarcane borer, *Diatraea Saccharalis* F. (lepidoptera: crambidae). Arquivos Do Instituto Biológico 84 (0), e0432014. https://doi.org/10.1590/1808-1657000432014.

Shaw, W.R., Catteruccia, F., 2018. Vector biology meets disease control: using basic research to fight vector-borne diseases. Nat. Microbiol. 4 (1), 20–34. https://doi.org/10.1038/s41564-018-0214-7. *2018 4:1*.

Shu, B., Yu, H., Li, Y., Zhong, H., Li, X., Cao, L., Lin, J., 2021. Identification of azadirachtin responsive genes in spodoptera frugiperda larvae based on RNA-Seq. Pestic. Biochem. Physiol. 172 (February), 104745 https://doi.org/10.1016/J.PESTBP.2020.104745.

Sun, G.Q., Zhu, J., Raikhel, A.S., 2004. The early gene E74B isoform is a transcriptional activator of the ecdysteroid regulatory hierarchy in mosquito vitellogenesis. Mol. Cell. Endocrinol. 218 (1–2), 95–105. https://doi.org/10.1016/J.MCE.2003.12.014.

Tapanelli, S., Chianese, G., Lucantoni, L., Serge Yerbanga, R., Habluetzel, A., Taglialatela-Scafati, O., 2016. Transmission blocking effects of neem (azadirachta indica) seed kernel limonoids on plasmodium berghei early sporogonic development. Fitoterapia 114, 122–126. https://doi.org/10.1016/j.fitote.2016.09.008.

Tricoire, H., Battisti, V., Trannoy, S., Lasbleiz, C., Pret, A.M., Monnier, V., 2009. The steroid hormone receptor ecr finely modulates drosophila lifespan during adulthood in a sex-specific manner. Mech. Ageing Dev. 130 (8), 547–552. https://doi.org/10.1016/J.MAD.2009.05.004.

Udeinya, I.J., Brown, N., Shu, E.N., Udeinya, F.I., Quakeyie, I., 2006. Fractions of an antimalarial neem-leaf extract have activities superior to chloroquine, and are gametocytocidal. Ann. Trop. Med. Parasitol. 100 (1), 17–22. https://doi.org/10.1179/136485906x78508.

Valzania, L., Mattee, M.T., Strand, M.R., Brown, M.R., 2019. Blood feeding activates the vitellogenic stage of oogenesis in the mosquito aedes aegypti through inhibition of glycogen synthase kinase 3 by the Active Ingredient and TOR pathways. Dev. Biol. 454 (1), 85–95. https://doi.org/10.1016/J.YDBIO.2019.05.011.

Vasil, I.K., Constabel, F., Jozef, S.(J.S.)S., Bogorad, L., 1984. Cell Culture and Somatic Cell Genetics of Plants, 1st Edition. Academic Press, Orlando, Florida. http://www.sciencedirect.com:5070/book/9780127150055/phytochemicals-in-plant-cell-cultures?via=ihub=.

Veitch, G.E., Boyer, A., Ley, S.V., 2008. The azadirachtin story. Angewand. Chem. Int. Edit. 47 (49), 9402–9429. https://doi.org/10.1002/anie.200802675.

Viñuela, E., Budia, F., Del Estal, P., 1991. Los insecticidas reguladores del crecimiento y la cutícula. Boletrín de Sanidad Vegetal y Plagas 17, 391–400. ISSN 0213-6910.

Vivekanandhan, P., Venkatesan, R., Ramkumar, G., Karthi, S., Senthil-Nathan, S., Shivakumar, M.S., 2018. Comparative analysis of major mosquito vectors response to seed-derived essential oil and seed pod-derived extract from Acacia Nilotica. Int. J. Environ. Res. Public Health 15 (2). https://doi.org/10.3390/ijerph15020388.

Werling, K., Shaw, W.R., Itoe, M.A., Westervelt, K.A., Marcenac, P., Paton, D.G., Peng, D., et al., 2019. Steroid hormone function controls non-competitive plasmodium development in anopheles. Cell 177 (2), 315–325. https://doi.org/10.1016/J.CELL.2019.02.036 e14.

WHO. 2017. "Global Vector Control Response 2017-2030″. https://www.who.int/publications/i/item/9789241512978.

WHO. 2022. *World Malaria Report 2022*. https://www.who.int/teams/global-malaria-programme/reports/world-malaria-report-2022.

Yao, T.P., Segraves, W.A., Oro, A.E., McKeown, M., Evans, R.M., 1992. Drosophila ultraspiracle modulates ecdysone receptor function via heterodimer formation. Cell 71 (1), 63–72. https://doi.org/10.1016/0092-8674(92)90266-F.

Yerbanga, R.S., Lucantoni, L., Ouédraogo, R.K., Da, D.F., Yao, F.A., Yaméogo, K.B., Churcher, T.S., et al., 2014. Transmission blocking activity of azadirachta indica and guiera senegalensis extracts on the sporogonic development of plasmodium falciparum field isolates in anopheles coluzzii mosquitoes. Parasit. Vect. 7 (1) https://doi.org/10.1186/1756-3305-7-185.

Zhang, J., Sun, T., Sun, Z., Li, H., Qi, X., Zhong, G., Yi, X., 2018. Azadirachtin acting as a hazardous compound to induce multiple detrimental effects in drosophila melanogaster. J. Hazard. Mater. 359 (October), 338–347. https://doi.org/10.1016/J.JHAZMAT.2018.07.057.

Zhu, J., Chen, L., Raikhel, A.S., 2007. Distinct roles of broad isoforms in regulation of the 20-hydroxyecdysone effector gene, vitellogenin, in the mosquito aedes aegypti. Mol. Cell. Endocrinol. 267 (1–2), 97–105. https://doi.org/10.1016/J.MCE.2007.01.006.

Zou, C., Liu, G., Liu, S., Liu, S., Song, Q., Wang, J., Feng, Q., Su, Y., Li, S., 2018. Cucurbitacin B acts a potential insect growth regulator by antagonizing 20-hydroxyecdysone activity. Pest Manag. Sci. 74 (6), 1394–1403. https://doi.org/10.1002/ps.4817.