Neem (Azadirachta indica): larvicidal properties - a review

Neem (Azadirachta indica): propriedade larvicidas – uma revisão

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Abstract

Introduction: Disease vector mosquitoes are a public health problems worldwide. However, controlling these insects is a challenge, so natural insecticides are a promising strategy due to their potential and low toxicity. Neem (Azadirachta indica A. Juss, Meliaceae) is a tree that has several bioactive compounds and a wide spectrum of action, including repellency and larvicide. Objective: This review aims to show the use of Neem-based products used against disease vectors. Several articles were analyzed and showed that different derived compounds are being evaluated, such as extracts, oils, neem cake (extraction by-product), in addition to synthesized nanoparticles. Results: Based on the results, we provide a list of compounds evaluated that have shown to be variable in relation to the LC₅₀ values in relation to the three species of mosquitoes (Culex quinquefasciatus, Anopheles stephensi and Aedes aegypti). Conclusion: Prove the medical relevance of this tree in combating these disease vectors.

Keywords: Neem; Culicidae; Larvicidal; Insecticide; Resistance.
Resumo

Introdução: Os mosquitos vetores de doenças são um problema de saúde pública em todo o mundo. Entretanto, controlar esses insetos é um desafio, e, assim, os inseticidas naturais são uma estratégia promissora devido seu potencial e baixa toxicidade. O neem (Azadirachta indica A. Juss, Meliaceae) é uma árvore que possui vários compostos bioativos e um amplo espectro de ação, incluindo repelência e larvicida. Objetivos: Esta revisão tem como objetivo mostrar o uso de produtos à base de Neem usados contra vetores de doenças. Metodologia: Vários artigos foram analisados e mostraram que diferentes compostos derivados têm sido avaliados, como extratos, óleos, bolo de neem (subproduto da extração), além de nanopartículas sintetizadas. Resultados: Com base nos resultados, forneceu-se uma lista de compostos avaliados que mostraram ser variáveis em relação aos valores de CL50 em relação a três espécies de mosquitos (Culex quinquefasciatus, Anopheles stephensi e Aedes aegypti). Conclusão: Comprovou-se a relevância médica dessa árvore no combate a esses vetores de doenças.

Palavras-chave: Neem; Culicidae; Larvicida; Inseticida; Resistência.

Introduction

The Brazil is one of the largest consumers of pesticides in the world and the excessive dependence on chemical insecticides used against agricultural pests and public and animal health can still present health and environmental risks. However, even with various strategies for controlling insect vectors, the use of insecticides is still the main form of control of these vectors and since 2012 World Health Organization has been working on a Global Plan for Insecticide Resistance Management, especially for mosquitoes.

These insects belong to the Culicidae family and are primarily responsible for the transmission of most vector-associated diseases. Their larvae are aquatic and feed on particulate organic matter or detritus and biofilm. The abundance of these insects is believed to be related to the availability of the host, climate and habitat of the larvae as temperature and precipitation contribute to a faster development cycle.

Aedes aegypti and A. albopictus are the main transmitting species of dengue, Chikungunya, Zika and yellow fever viruses. Larvae of A. aegypti predominate in highly urbanized habitats, while A. albopictus predominates in rural areas. However, A. albopictus has been adapting to urban environments with larvae reproducing in artificial containers.

The genus Anopheles is the transmitter of the protozoan that causes malaria. This species has increased its geographical distribution but has not become as invasive as Aedes species. Larvae of this mosquito genus are adapting to new environments created by urbanization.

Another species, Culex quinquefasciatus, in turn, is the vector of Wuchereria bancrofti, among other phylarids and different arboviruses, being more prevalent in the tropics and subtropics, but predicted to spread to climate countries temperate as Canada. Their larvae require high nutrient content for development, and some species are able to sustain at high temperatures and extreme values pH.

Thus, glimpsing the combat of these insects and especially their larvae, without the use of chemical pesticides today, is still an attitude that human beings cannot overlook. However, the use of botanical insecticides has gained prominence, especially with the use of substances derived from the Neem tree (Azadirachta indica A. Juss, Meliaceae). From this tree, it is possible to generate products with a broad spectrum of activity against pests, and has been shown to be an
alternative means of combating various insects of medical and veterinary importance, including mosquitoes and their larvae.\textsuperscript{28}

In this context, a bibliographic review was made about Neem tree and the activity of different neem-based products employed against Culicidae mosquito larvae, vectors of important diseases.

**Methodology**

Literature on the Neem tree and its insecticidal action was taken from indexed scientific journals and online databases: Science direct, Pubmed and Google Scholar. The terms used in the research were: “Neem (Azadirachta indica); larvicidal activity, mosquitoes”. The research result was revised to identify relevant articles related to the plant and its activity against Culex, Aedes and Anopheles mosquitoes. Works available in English and Portuguese were included. The works were selected by reading titles and abstracts, where the inclusion criteria were the works published in the last 20 years, including scientific articles related to the proposed theme and excluding articles outside the time limit and outside the central theme of the research. Information on characteristics, active ingredients, mode of action and insecticidal effect were collected in tabulated disease vectors.

**Results and discussion**

**Literature overview**

**Neem (Azadirachta indica)**

Neem is a tree, widely cultivated in the Indian subcontinent, but rapidly expanding worldwide, including in subtropical regions of America\textsuperscript{29}. Its use has, among other advantages, a broad spectrum of activity, including medicinal, antibacterial, antifungal and insecticidal properties, of medical relevance, low resistance induction and low mammalian toxicity\textsuperscript{29,30}.

Today, the United Nations recognizes the tree as the “21\textsuperscript{st} Century Tree” \textsuperscript{31,32}. Among its features is its fast and easy growth, reaching up to 40 meters in height. In addition, its bioactive potential and low toxicity make it a frequent target for medical and environmental studies, such as insertion in insect control programs\textsuperscript{33}. Plant cultivation is already described in different countries in Asia, Africa and America, but India is still the main producer\textsuperscript{31}.

All parts of neem have phytochemicals with different action potentials, but commercial use is limited to removable parts such as leaves, fruits and especially seeds, which are one of the main materials used in the production of bioactive compounds\textsuperscript{31}. More than 100 of these compounds have been described, being alkaloids, flavonoids, saponins, tannins, phenols, cardiac glycosides and terpenoids. Among terpenoids the main constituents are azadiractin, nimbine, nimbidine and nimbolides\textsuperscript{34-36}.

Azaractin is found in neem leaves and bark, but a greater amount of this biologically active substance is found in seeds and is considered to be the richest plant part of this molecule\textsuperscript{36}. Being the basis of many insecticides, it has hormonal regulatory properties, acting in various physiological processes in larvae and adults of disease vectors\textsuperscript{38}.

One product whose research has reported insecticidal action is nanoparticles based on Neem seed extract. This has gained prominence with studies on green nanoparticles with insecticidal properties, bioactivity synthesis, large scale production and the lowest environmental impact\textsuperscript{39}.

Recently, neem cake has also been researched, a reusable byproduct of the extraction of neem oil with bioactive property, having great prominence in the studies of this plant. India has an annual potential of 80,000 metric tons of plant oil and 330,000 metric tons of neem cake. The use of this abundant raw material in the production of ecological insecticides, makes A. Indica even more economically favorable\textsuperscript{11,36}.

All the different Neem products have the advantage of having multiple modes of action, which makes insect resistance difficult, which together with environmental safety characterize them as excellent
candidates for insect control methods, especially mosquitoes\textsuperscript{14}.

Neem Activities

One of the activities of Neem based products is repellency. The use of repellents by the population is one of the most common and ancient practices in the protection of disease-carrying insect vector bites, but their application to the skin must be careful for the side effects they may cause\textsuperscript{11}.

Abiy et al. (2015)\textsuperscript{40} reported that Neem was 100\% effective for 3 consecutive hours in repelling \textit{A. Arabiensis}, the main vector of malaria in Ethiopia. The repellent effect of Neem oil was also evaluated against \textit{Culex}, with 12 hours of protection at 20\% concentration.

The study also indicated that protective activity time was directly proportional to concentration \textsuperscript{41}. Benelli et al. (2014)\textsuperscript{11} also reported that several neem cake fractions were able to exert good mosquito repellency (above 70\%) at a concentration of 100 ppm.

Insecticidal activity however is still the main search for Neem based products. In this sense, several researchers have evaluated the potential, larvicide of \textit{A. indica} in different formulations against mosquitoes of medical importance \textsuperscript{31,42}. Given this, it can be seen in \textbf{TABLE 1} that there are different formulations and LC\textsubscript{50} for mosquito species.

\begin{table}[h]
\centering
\caption{Larvicidal activities (LC\textsubscript{50}) off different nem products}
\begin{tabular}{|l|l|l|l|}
\hline
\textbf{Extracts} & \textbf{Larvae} & \textbf{LC\textsubscript{50}} (ppm) & \textbf{Reference} \\
\hline
Ethanol extract of endocarp fruits \textit{A. indica} & \textit{A. aegypti} Fed & 0.044 (%g) 440 ppm & Wandscheer et al., 2004\textsuperscript{43} \\
& No-fed & 0.056 (%g) 560 ppm & \\
Isolated extract of \textit{A. indica} seeds & \textit{A. stephensi} & 290.3 ppm & Murugan et al., 2016\textsuperscript{44} \\
Methanolic extract \textit{A. indica} & \textit{C. quinquefasciatus} & 74.04 ppm & Batabyal et al., 2009\textsuperscript{41} \\
\textit{A. indica} extract with carbon tetrachloride & \textit{C. quinquefasciatus} & 86.00 ppm & Batabyal et al., 2009\textsuperscript{41} \\
\textit{A. indica} extract with light petroleum & \textit{C. quinquefasciatus} & 79.17 ppm & Batabyal et al., 2009\textsuperscript{41} \\
\hline
\end{tabular}
\end{table}
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| Extract of *A. indica* | *C. pipiens* | 81.21 ppm | Shoukat et al., 2016<sup>45</sup> |
|-------------------------|--------------|-----------|----------------------------------|

### Oil

| Larvae | LC<sub>50</sub> | Reference |
|--------|----------------|-----------|
| Neem oil (leaves) | 0.05% (500 ppm) | Mukhtar et al., 2015<sup>46</sup> |

| Larvae | LC<sub>50</sub> | Reference |
|--------|----------------|-----------|
| *C. quinquefasciatus* | 1.8 ppm | Dua et al., 2009<sup>47</sup> |
| *A. aegypti* | 1.7 ppm | |
| *A. stephensi* | 1.6 ppm | |

### Formulação de óleo de Neem (32%) *An. gambiae* (8 dias)

| Larvae | LC<sub>50</sub> | Reference |
|--------|----------------|-----------|
| 10.7 ppm | Okumu et al., 2007<sup>48</sup> |

### Óleos de semente de Neem industriais

| Larvae | LC<sub>50</sub> | Reference |
|--------|----------------|-----------|
| *A. albopictus* | 171.735 ppm | Benelli et al., 2015<sup>33</sup> |

### Commercial products

| Larvae | LC<sub>50</sub> | Reference |
|--------|----------------|-----------|
| *A. stephensi* | 0.35 ppm | Vatandoost and Vaziri, 2004<sup>49</sup> |
| *C. quinquefasciatus* | 0.69 ppm | |
| *A. stephensi* | 1.923 ppm | Gunasekaran et al., 2009<sup>50</sup> |
| *A. aegypti* | 8.416 ppm | |
| *C. quinquefasciatus* | 15.866 ppm | |

### Cake of Neem

| Larvae | LC<sub>50</sub> | Reference |
|--------|----------------|-----------|
| Neem nanoparticles | Larvae | LC$_{50}$ | Reference |
|--------------------|--------|----------|-----------|
| Silver nanoparticles of aqueous extract of Neem leaves | A. aegypti | 0.006 ppm | Poopathi et al., 2015$^{35}$ |
| | C. quinquefasciatus | 0.047 ppm |

| Silver Nanoparticles (AgNPs) - Aqueous extract of leaves and bark | A. stephensi | 2 ppm | Soni et al., 2014$^{51}$ |
| | C. quinquefasciatus | 10 ppm |

| Neem oil cake | C. quinquefasciatus | 5600 ppm (0.56%) | Shanmugasundaram et al., 2008$^{52}$ |
| | A. aegypti | 2900 ppm (0.29) |
| | A. stephensi | 4500 ppm (0.45) |

| Neem Cake Extract Methanolic | An. culicifacies | 1.321 ppm | Benelli et al., 2017$^{53}$ |
| Ethyl acetate | | 1.818 ppm |

| Neem Cake Extract Fractions: NTMeOH, NTAcOEt, NRAcOEt, NRBuOH, NRH$_2$O | An. culicifacies | 1.321ppm | Chandramohan et al., 2016$^{50}$ |
| | | 1.504 ppm |
| | | 1.818 ppm |
| | | 1.950 ppm |
| | | 2.545 ppm |
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As mentioned, several studies are conducted against the different formulations of neem but extracts are the main ones in the tests of insecticidal activity. And as a way to improve the quality and protection of the active ingredients for a longer useful life, in one study the plant extracts were encapsulated by different materials, including alginate, which had a larval mortality of 98%. A. aegypti after 84 hours and 100% after 96 hours. There was no mortality in the control containers. One of the advantages of the emulsifier is biocompatibility, better dispersion in water and, consequently, causing the larvae to be exposed to higher concentrations of this substance. Despite the delayed effect, the authors observed that the alginate granule formulation showed potential against dengue mosquito larvae, and this fact was attributed to the alginate granule formulation which may have preserved the stability of the active ingredient.

As a way to improve the quality and protection of the active ingredients for a longer shelf life, in one study plant extracts were encapsulated by different materials, including alginate, which showed a larval mortality rate of 98% A. aegypti after 84 hours and 100% after 96 hours. There was no mortality in the control containers. One of the advantages of the emulsifier is biocompatibility, better dispersion in water and, consequently, causing the larvae to be exposed to higher concentrations of this substance. Despite the delayed effect, the authors observed that the alginate granule formulation showed potential against dengue mosquito larvae and this fact was attributed to the alginate granule formulation which may have preserved the stability of the active ingredient.

In turn, Anjali et al. (2012) carried out a study with neem oil nanoemulsion, and observed that the size of

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| Neem-Urea Nanoemulsion (NUNE) | A. aegypti | 99261 ppm | Mishra et al., 2018 |
| Silver nanoparticles with Neem extract | A. aegypti | 5.425 ppm | Chandramohan, 2016 |
| Nanoemulsion Neem Oil | C. quinquefasciatus | 11.75 ppm | Anjali et al, 2010 |
| | | 25.99 ppm | |
| | | 62.89 ppm | |

**LC50:** Median lethal concentration; ppm: part per million; nm: nanometers
the drop influences the effectiveness. The reduced size of nanoemulsion increased larvicidal efficacy, with lower LC50 value due to the greater dispersion of thin particles. Nanoemulsions have the advantage of being economically viable, less toxic when compared to synthetic insecticides and therefore an alternative to insect vector control55. Another strategic alternative to conventional pesticides is nanoparticle biosynthesis. The synthesis of nanoparticles is based on oxidation/reduction reactions and the appropriate choice of solvent. Plants have a wide variety of metabolites that assist in the reduction process. Neem is an example of this, in which terpenoids are reducing phytochemicals57.

Extending studies of the larvicidal effect of neem, Imbahale and Mukabana (2015) studied population control of vector larvae in six different Anopheine and Culicini mosquito habitat types. In this research, pure splinters from neem stem were used as raw material. Regarding the larvae, 7782 Anopheine (93% late instars) and 11,590 Culicini (71% first instars) were sampled. With the study, it was observed that in habitats treated with Neem, larvae did not develop, suggesting that the evaluated compound discourages the development of resistance in the vectors since more evolved larval stages and pupae of both groups were outnumbered. In addition, it is also suggested that pregnant female mosquitoes still oviposited in the treated habitats42.

The aqueous extract of neem fruit, bark and leaf was evaluated for the percentage of mortality of C. quinquefasciatus larvae after 24 hours of exposure, as well as larval susceptibility to raw neem leaf dust. In the study by Kudon et al (2011)58, bark, fruit and leaf extract (0.1 g/mL) showed an average 24-hour larval mortality of 72.7%, 68.7% and 60% respectively. Leaf powder caused 100% mortality at a concentration of 1 g/mL. However, at a concentration of 0.1 g/mL, mortality was less than 20%. It is noteworthy that neem powder floats in water; thus, in larger doses, it may have acted as a barrier and not a pesticide that prevented the larva from breathing and resulting in death by suffocation; but also with an efficient effect on vector control.

Several neem oils, commercial or not, are also evaluated for larval insect susceptibility. Silapanuntakul et al, 201656 showed that Thai Neem Oil caused 97% mortality of A. aegypti larvae, while Thai Neem Alginate bead formulation resulted in a 62% mortality rate. Demonstrating the best oil efficiency, which is justified by easier and more uniform dispersion in water.

Neem oil toxicity against A. aegypti was also evaluated by Gomes et al. (2016)59, who observed a mortality rate of 82% after 7 days and resulted in a 50% reduction in survival rates over 2 days, with a concentration of 1%. Compared to the 0.001% neem concentration, 77% of the larvae survived after the seventh day of evaluation, similar to the control (86%). However, survival rates were significantly lower when applied the combination of neem and the fungus Metarhizium anisopliae against vector of dengue, proposing a reduction in the chance of developing resistance.59.

The synergism of natural products in turn is also a strategic alternative to further enhance Neem’s pesticidal activity. Assifuah-Hasford (2018)60 showed that neem oil and fresh extracts of sweet orange (Citrus sinensis), pennyroyal leaves (Mentha pulegium) and garlic (Allium sativum) have insecticidal properties similar to chemical pesticides. The 100% efficacy in larvicidal activity was also observed in the tests performed against A. stephensi and C. quinquefasciatus using PONNEEM at 0.1 ppm. PONNEEM is a biopesticide prepared from oils of A. indica and Pongamia glabra. The synergism of phytoconstituents was a crucial factor as it caused restlessness, slowness and convulsions that triggered insect death61.

A large number of published studies on green nanoparticle synthesis show their potential for controlling disease vectors. More than 5,600 publications in the SCOPUS database were retrieved in a survey by Mirsha et al. (2018)62. In 2017, using as keywords “mosquito nanoparticles” more than 200 published articles were surveyed, reporting the effectiveness of plant-synthesized nanoparticles as pesticides.
Regarding the studies of Neem's silver-based nanoparticles (AgNPs), Poopathi et al. (2015)\textsuperscript{35} synthesized AgNPs from Neem leaf extract, showing activity against LC\textsubscript{50} and LC\textsubscript{90} against A. aegypti and C. quinquefasciatus (TABLE 1). In contrast, silver nanoparticles (AgNO\textsubscript{3} - 1Mm) used as control showed no larvicidal activity. Particle size may be directly associated with the mechanism of action. Nanoparticles with a size between 41 and 60 nm allow the passage through the insect cuticle and individual cells, where they interfere with molting as well as other physiological processes.

In another study, silver nanoparticles (AgNP) were synthesized using neem cake and larvicidal activity evaluated against A. aegypti. The study showed efficacy at low doses for vector control. In this paper, the authors report a higher toxicity of AgNPs compared to Neem bolus. The biosynthesized particles presented LC\textsubscript{50} about 27 times lower than the extract alone\textsuperscript{36}.

Similarly, studies of the efficacy of AgNPs produced with Neem bark and leaf extracts were carried out for the different developmental stages of C. quinquefasciatus and A. stephensi, noting the close relationship between mortality and life stages. Aqueous extracts of leaves and bark of A. indica were used in the synthesis, forming spherical AgNPs of varying size. Soni et al. (2014)\textsuperscript{32}, testaram diferentes concentrações e observaram um melhor efeito sobre o estágio larval de 1\textsuperscript{st} e 4\textsuperscript{th} instares de C. quinquefasciatus e 1 e 2\textsuperscript{nd} instar de A. stephensi com extrato de folhas. Larvae had a 100% mortality rate after the first hours of exposure (15 min and 1h: 30 min / 1h respectively). On the other hand, the effectiveness of the nanoparticles against the 2\textsuperscript{nd} and 3\textsuperscript{rd} instars of C. quinquefasciatus were verified after this exposure period. The 2\textsuperscript{nd} instar larvae presented LC\textsubscript{50} 6, LC\textsubscript{90} 12 and LC\textsubscript{99} 14 ppm; while the third instar larvae LC\textsubscript{50} 10, LC\textsubscript{90} 18, and LC\textsubscript{99} equal to 20 ppm. For A. stephensi, the LC\textsubscript{50} for 3\textsuperscript{rd} and 4\textsuperscript{th} instar larvae was determined after 12h and 17h respectively, showing a lower susceptibility of the larvae. In relation to nanoparticles synthesized with neem bark extract, again the larval stage was more susceptible. The first three stages presented 100% mortality for C. quinquefasciatus and the fourth presented an LC\textsubscript{50} of 2 ppm, half of that observed for pupae. For A. stephensi, 1\textsuperscript{st} and 2\textsuperscript{nd} instar larvae were 100% killed after 1h of exposure to nanoparticles\textsuperscript{32}.

As shown, larvae are the main insect elimination strategy as they cannot dissipate from their breeding grounds and control them more effectively. However, studies on other evolutionary stages of insects are performed. Authors describe the mosquito activity for Aedes, most effectively in later hours. After 24 hours of exposure to extracts of A. indica, they had a mortality ranging from 26.6 to 48.9%, much lower than the mortality observed after 48h, which reached 100%, with a lower LC\textsubscript{50}, passing from 1.17 mL to 0.09 mL\textsuperscript{63}.

The effect of azadirachtin was studied against C. piri, showing remarkable mosquito control. Again, time and dose were determining factors. At concentrations of 24 and 48 mg/ L the maximum value in mortality rate was evidenced, oviposition dropped from 91 in the control to 53 and 39 at concentrations of 3 and 6 mg/L respectively. In addition, a reduction in male fertility and increased male sterility has been observed\textsuperscript{64}.

Compared to A. sthephensi, in a study by Nathan et al (2005)\textsuperscript{68}, after 24h of azadirachtin (neem limonoid) exposure, the mortality rate was 69.3% (0.025 ppm), 80.4%. (0.05 ppm) and 95.4% (0.1 ppm).

It is noteworthy that, in addition to its activity against biological pest control, studies have shown that azadiractin has cellular action, with relevant cytotoxicity, as well as apoptotic induction in Si9 cells (Leptoptera cells), as demonstrated by the study by Huang et al. (2013)\textsuperscript{66}.

It should be noted that, in addition to its activity against biological pest control, studies have shown that azadiractin has cellular action, with relevant cytotoxicity, as well as apoptotic induction in Si9 cells (Leptoptera cells), as demonstrated by the study by Huang et al. (2013).

The study aimed to further investigate the mechanism of azadirachtin induced apoptosis in Lepidopteran. It was showed that the release of cytochrome c into the cytosol is an important event during
lepidopteran apoptosis, followed by the cytochrome c, the activation of initiator and effector caspases (caspase-9 and -3) also was detected. However, most importantly, in apoptosis induced by azadiractin, the released cytochrome c is accompanied by the generation of ROS (intracellular oxygen)\(^6\). In this study, after 12h and 48h of exposure, it was observed the formation of apoptotic bodies, as well as a significant increase in the reactive species of intracellular oxygen (ROS) after exposure to the active principle of Neem.

**Conclusion**

From this review, we can conclude that different by-products derived from the Neem tree have been studied and shown potential and effective effects in combating insect vectors of diseases. In addition, its importance is still high, since phytochemicals are promising because of their relative safety and low toxicity to the environment.

The vast activity of this plant makes it a great control tool. Its multiple modes of action, coupled with its different metabolites, means that a substantial number of studies are and are being conducted each year. Their goal is unique, it is common, to seek more and more information to complement the findings and enhance the effects on reducing mosquito vectors and diseases caused or not, with less damage to human health, animals and the environment.

**Conflicts of interest**

The authors declare no conflicts of interest.

**Acknowledgments**

We would like to thank Universidade Federal de São João del-Rei, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) by financial support.

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