

Prospecting for special metabolites and larvicidal activity of ethanolic extracts from *Azadirachta indica* A. Juss. (Neem), collected in Tauá-CE against *Aedes aegypti* mosquito larvae

Prospecção de metabólitos especiais e atividade larvicida de extratos etanólicos de *Azadirachta indica* A. Juss. (Nim), coletada em Tauá-CE frente a larvas de mosquitos *Aedes aegypti*

Prospección de metabolitos especiales y actividad larvicida de extractos etanólicos de *Azadirachta indica* A. Juss. (Neem), recolectado en Tauá-CE contra larvas de mosquito *Aedes aegypti*

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Abstract

In the search for an alternative control against *Aedes aegypti*, many types of research are developed to discover substitutes for synthetic insecticides, including the use of oils, extracts, or active constituents from plants to find new insecticidal substances. The present work describes the phytochemical study results and evaluation of toxicity against *Aedes aegypti* larvae of ethanol extracts from *Azadirachta indica* A. Juss collected in Tauá-CE. Phytochemical tests were performed by different methods involving colorimetric, precipitation reactions, various metabolites such as alkaloids, anthocyanidins, anthocyanins, steroids, and flavonols, flavonones, triterpenoids was evident in the active extracts. For the toxicity tests, different concentrations of ethanolic extracts (250, 500, 1000 and 2000 µg / mL) were prepared with sterile distilled water and dimethylsulfoxide - DMSO (1%), and then 20 mL of each solution was added. And 25 3rd stage larvae in plastic cups. All bioassays were performed in quadruplicate. DMSO in an aqueous solution was used as a negative control. As a result, the tested extracts proved toxic to *Aedes aegypti* larvae, with an LC50 value higher than the control (10%) and ranging from 12% to 46%. The results obtained show that all extracts have an important pharmacological and toxicological potential. They deserve to be investigated in studies for the isolation and identification of bioactive compounds.

Keywords: *Azadirachta indica*; Phytochemical analysis; Toxicity; *Aedes aegypti*.

Resumo

Na busca por um controle alternativo contra *Aedes aegypti*, muitas pesquisas são desenvolvidas no intuito de descobrir substitutos para os inseticidas sintéticos contemplando inclusive a utilização de óleos, extratos, ou constituintes ativos provenientes de plantas visando encontrar novas substâncias inseticidas. O presente trabalho descreve os resultados do estudo fitoquímico e avaliação da toxicidade frente às larvas *Aedes aegypti* de extratos etanólicos de *Azadirachta indica* A. Juss coletada em Tauá-CE. Os ensaios fitoquímicos foram realizados por diferentes métodos envolvendo reações colorimétricas e de precipitação, evidenciou-se nos extratos ativos a presença de uma variedade de metabólitos como alcaloides, antocianidinas, antocianinas, esteroides, flavonóis, flavononas, triterpenoides. Para os ensaios de toxicidade, foram preparadas diferentes concentrações dos extratos etanólicos (250, 500, 1000 e 2000 µg/mL) com água destilada estéril e dimetilsulfóxido - DMSO (1%), e em seguida foram colocados 20 mL de cada uma das soluções e 25 larvas de 3º estágio em copos plásticos. Todos os bioensaios foram realizados em quadruplicata. Foi utilizado como controle negativo DMSO em solução aquosa. Como resultado, os extratos testados se mostraram tóxicos para as larvas *Aedes aegypti*, com valor de CL50 superior ao controle (10%) e variando entre 12% a 46%. Os resultados obtidos denotam que todos os extratos possuem um importante potencial farmacológico e toxicológico e que merecem ser investigados em estudos para o isolamento e identificação de compostos bioativos.

Palavras-chave: *Azadirachta indica*; Análise fitoquímica; Toxicidade; *Aedes aegypti*.

Resumen

En la búsqueda de un control alternativo contra *Aedes aegypti*, se desarrollan numerosas investigaciones para descubrir sustitutos de insecticidas sintéticos, incluyendo el uso de aceites, extractos o componentes activos de plantas para encontrar nuevas sustancias insecticidas. El presente trabajo describe los resultados del estudio fitoquímico y evaluación de la toxicidad frente a larvas de *Aedes aegypti* de extractos etanólicos de *Azadirachta indica* A. Juss recolectados en Tauá-CE. Las pruebas fitoquímicas se realizaron por diferentes métodos que involucran reacciones colorimétricas y de precipitación, la presencia de una variedad de metabolitos como alcaloides, antocianidinas, antocianinas, esteroides, flavonoles, flavononas, triterpenoides fue evidente en los extractos activos. Para las pruebas de toxicidad se prepararon diferentes concentraciones de extractos etanólicos (250, 500, 1000 y 2000 µg / mL) con agua destilada estéril y dimetilsulfóxido - DMSO (1%), y luego se agregaron 20 mL de cada solución. y 25 larvas de tercer estadio en vasos de plástico. Todos los bioensayos se realizaron por cuadruplicado. Se utilizó DMSO en solución acuosa como control negativo. Como resultado, los extractos probados demostraron ser tóxicos para las larvas de *Aedes aegypti*, con un valor de CL50 superior al del control (10%) y que oscila entre el 12% y el 46%. Los resultados obtenidos muestran que todos los extractos tienen un importante potencial farmacológico y toxicológico y que merecen ser investigados en estudios para el aislamiento e identificación de compuestos bioactivos.

Palabras clave: *Azadirachta indica*; Análisis fitoquímico; Toxicidad; *Aedes aegypti*.

1. Introduction

Dengue is a viral disease of 4 serotypes that can be transmitted mainly by female mosquitoes of the *Aedes aegypti*. This mosquito can also transmit the virus of chikungunya, yellow fever and Zika. Dengue is endemic in more than 100 countries in southeast asia, the americas, the western pacific, africa and the eastern mediterranean regions, and its incidence has increased 30-fold over the past 50 years (Who, 2020). About half of the world's population is at risk. Its expansion at a global level is related to demographic and social changes in this period, including unprecedented population growth, a growing

movement of people (and, consequently, viruses), uncontrolled urbanization, climate change, breach of public health infrastructure and programs of vector control, totaling approximately 100-400 million infections each year (Guzman & Harris, 2015; Who, 2020). In Fortaleza, between 2001 and 2012, there was an increase in the number of infected patients reaching 1561.1 per 100 thousand inhabitants in the last year (Oliveira et al., 2018).

The disease represents a major economic burden for governments and individuals, costing about the US \$ 2.1 billion per year on average in the Americas, excluding vector control, exceeding the costs of other viral diseases and leading individuals to death (Halasa et al., 2011). Dengue is no longer a sporadic disease and has become a public health problem due to its social and economic effects, increased geographic extent, number of cases and severity of the disease (Guzman & Harris, 2015). Prevention strategies and disease control depend on effective vector control measures, and continued community involvement can substantially improve the results achieved (WHO, 2020). Vector control is performed using insecticides, such as diflubenzuron and pyriproxyfen. Despite having a high efficacy for vectors, these present insecticides ecotoxicity in aquatic systems (Devillers, 2020; Montaña-Reyes et al., 2019).

Plants in this context become important to reverse the harm of pesticides, as they have unexplored supplies of bioactive compounds, low production cost, form safer compounds, fibers and phytochemicals for other species, are easy to grow, renewable and can be developed under broad conditions (Allison et al., 2013; Maurya et al., 2012). Besides, they offer chances to reduce environmental damage, slow deforestation, new opportunities for industries generating economic growth for the population. Research shows trends indicating that plant essential oils will continue to gain interest in developing potential botanical insecticides (Chellappandian et al., 2018).

Extracts of *Artemisia absinthium* and *Cichorium intybus* showed larvicidal activities against mosquito vectors of malaria, dengue and filariasis (Ali et al., 2018). Among other Meliaceae family species, *Azadirachta indica* stands out due to its therapeutic uses and pharmacological attributes of a wide spectrum. Some of its properties are antifungal, antibacterial, anti-inflammatory, sterilizing, antiscabic, anti-allergenic, analgesic, nematocidal, antipyretic and the properties are found in several plants at significant levels. The isolation of these secondary metabolites is essential to develop new, safer and more economical drugs (Saleem et al., 2018).

A. indica or NIM can grow on stony and dry soils under different climatic conditions up to an altitude of 700 meters and survive for more than 200 years. It is abundant in places with precipitation between 450 to 1200 mm, with a temperature between 0 to 49 ° C and pH between 4 to 10. However, it can also be cultivated in regions with annual precipitation of 150–200 mm. In India, the plant's annual production of seeds and oil is 442,300 and 88,400 tons, respectively. Despite being a species with many advantages, attention should be paid to possible adverse effects on its constitution. Besides, only limited data are available on the pharmacokinetics and pharmacodynamics of NIM and its constituents (Sateesh, 1998; Jattan, Sushikumar & Pujar, 1995; Brahmachari, 2004; Hedge, 1995; Uchegbu et al., 2011; Gupta et al., 2017).

Based on the above, this study aimed to perform phytochemical bioprospecting and larvicidal activity of ethanolic extracts from the roots of *Azadirachta indica* A. Juss (Nim), collected in Tauá-CE against *A. Aegypti* mosquito larvae.

2. Methodology

Plant material

The collection and herbalization of plant material were carried out based on methodologies proposed by Cartaxo, Souza and Albuquerque (2010). The collection was carried out in the City of Tauá-Ce (040° 18 '05.4" W; 06° 01 '03.6" S). *Azadirachta indica* A. Juss was collected in plastic bags, barks and roots. (Neem). Subsequently, all plant material was transferred to the laboratory to prepare the desiccate and organic extracts. For herborization, parts of the plant that were in the

reproduction stage were collected in duplicate. An exsiccata from the plant was deposited and identified in the collection of Herbário Prisco Bezerra EAC-UFC, under number 56044.

Obtaining the extract

The preparation of organic extracts was carried out based on methodologies proposed by Pereira et al. (2009), with adaptations. All fresh plant material was dried in the open air. After drying, the weighing of each botanical material was carried out on an analytical balance. Then, all material was crushed, packed in glass containers and subjected to cold solvent extraction, using only commercial ethanol as an organic solvent, in varying amounts, for 96 hours. After this period, simple filtrations were performed, and the organic extracts were kept at room temperature ($30 \pm 2^\circ\text{C}$) for total evaporation of the solvent. All organic extracts obtained were weighed on an analytical balance and packed in ampoule bottles with the codes for each part of the plant used in organic extraction.

Preliminary phytochemical analysis

The extracts were subjected to preliminary chemical prospecting based on methodologies proposed by Matos (1997) and Da Silva et al. (2009), with adaptations. In these experiments, the characterization of the main classes of secondary metabolites was carried out through chemical reactions that resulted in the development of color and/or precipitate, characteristic for the following classes of metabolites: coumarins, phenols, tannins, flavonols, flavonones, steroids, triterpenoids, anthocyanins, anthocyanidins, flavonoids, xanthenes, catechins, leucoanthocyanidins, alkaloids. In these experiments, the extracts' solutions were prepared, dissolving 1.0 g of each extract with 100 mL of 70% alcohol. Subsequently, 3 mL aliquots of each extract solution were added to test tubes to characterize the groups. The constituents in the extracts were classified as strong (+++), medium (++), weak (+), suspicious (S) and absent (-).

***Aedes aegypti* mosquito larvae**

The bioassays were carried out with 3rd stage larvae of *A. aegypti*, as they are the most tolerant of the other stages (Silva et al., 2003). These were provided by the Health Department of the Municipality of Tauá-Ceará.

Bioprospecting larvicidal activity

These tests were performed based on methodologies proposed by Silva et al. (2003) and Souza (2012), with adaptations. Initially, stock solutions of organic extracts (250, 500, 750 and 1000 ppm) were prepared with sterile distilled water and dimethyl sulfoxide, DMSO (1%). The bioassays were performed in cups with a capacity of 30 mL, properly insulated with nylon and elastic mesh. In these, 20 ml of each solution were placed and then 25 larvae of 3rd stage. All bioassays were performed in quadruplicate. To verify the lethality, the larvae mobility and reaction to external stimuli such as a light source (flashlight) and mechanical stimulus (touch with a stylus, touch with a glass stick on the outside of the container) were observed. Mortality was recorded every 24 h, dead larvae were removed from the cups, and 0.026 grams of cattle feed was added. DMSO in an aqueous solution was used as a negative control. The extracts were classified as: without larvicidal activity, SAL ($CL_{50} = 0\%$), with little larvicidal activity, PAL ($0\% < CL_{50} < 50\%$), with moderate larvicidal activity, MAL ($50\% \leq CL_{50} \leq 90\%$) and with high larvicidal activity, EAL ($LC_{50} > 90\%$), for each concentration tested.

Statistical analysis

The results were expressed in lethal concentration values to kill 50% (LC50) of the larvae, calculated using a straight-line regression equation. Pearson's correlation coefficient and standard errors were also calculated, both in the graphpadprism program (version 5.0) (Araújo, Cunha & Veneziani, 2010; Stefanello et al., 2006).

3. Results and Discussion

Currently, several types of research are being done focusing on the prospecting of special metabolites of vegetables through qualitative methods to detect the presence of phenols, tannins, anthocyanins, anthocyanidins, flavonoids, leucoanthocyanidins, catechins, flavones, flavonols, flavononols, xanthonones, saponins, coumarins and quinones (Lima et al., 2012; Rêgo Júnior et al., 2011; Araújo, Cunha & Veneziani, 2010; de Souza et al., 2008; Saraiva, 2007;). In the present work, the qualitative method was used to detect flavonols, tannins, phenols, flavonones, anthocyanins, anthocyanidins, flavonoids, and xanthonones triterpenoids, catechins, leucoanthocyanidins, steroids, alkaloids in ethanolic extracts of *Azadirachta i.* bark. Juss and they were classified according to their presence as strong, medium, weak and absent (Table 1).

Table 1. Preliminary phytochemical analysis of the leaves' ethanolic extracts, bark and branches of *Azadirachta indica* A. Juss collected in Tauá-Ce.

Special Metabolites	<i>Azadirachta indica</i> A. Juss	
	Bark	Branch
Alkaloids	-	-
Anthocyanidins	+++	-
Anthocyanins	+++	-
Catechins	-	++
Steroids	+++	-
Phenols	-	-
Flavonoids	-	+++
Flavonones	-	+++
Flavonols	-	-
Leucoanthocyanidins	-	++
Tannins	-	+++
Triterpenoids	-	++
Xanthonones	-	-

(+++) – Strong; (++) – Medium; (+) – Weak; (-) – absent. Fonte: Autores.

In the ethanolic extract of the branches of *Azadirachta indica* A. Juss, there was a strong presence of flavonols, flavonones and tannins, and a medium presence of triterpenoids, catechins and leucoanthocyanidins. The bark showed the lowest amount of special metabolites. However, all compounds found had a strong presence. The metabolites found were: Anthocyanidins, Anthocyanins and Steroids.

Research on metabolites' bioprospecting in the NIM also finds in ethanolic extracts the steroidal components, glycosides, flavonoids, triterpenoid, carbohydrates, alkaloids and antiquinone (Rapheal, 2012; Prashanth & Krishnaiah, 2014). In the evaluation of the phytochemical profile of the ethanolic extract of *Solanum lycocarpum* fruits (Lobeira or Fruta de Lobo), the presence of phenols, tannins, saponins, alkaloids and free steroids was detected (Araújo et al., 2010).

A phytochemical study used ethanolic extracts from leaves, bark, branches, and favelas (*Cndoscolus phyllacanthus*, Euphorbiaceae) with the same methodology and showed in all parts of the plant, the active extracts the presence of flavonols, flavonones and alkaloids (Lima et al., 2012).

According to Gomes et al. (2011), they were aiming to detect phytochemical analysis in extracts of jurubeba (*Solanum paniculatum* Linnaeus), capim santo (*Cymbopogon citratus* Stapf), purging potato (*Operculina hamiltonii*) and São Caetano melon (*Momordica charantia* Linnaeus). To obtain the extract, powder from each plant's parts was used and ethanol PA was used as a solvent. Four tests (phenols and tannins; anthocyanins, anthocyanids and flavonoids; leucoanthocyanidins, catechins and flavonones; and for alkaloids.) Were carried out to prospect for constituents of the hydroalcoholic extract and concluded that the ethanol extracts of the four plants presented compounds such as tannins and catechins and absence of phenols, anthocyanins and anthocyanidins. Tests with São Caetano melon revealed the compounds Flavones, flavonols, xanthonas, Chalcones, Auronas, Flavanonols and Leucoanthocyanidins. Jurubeba and capim santo showed the presence of Flavones, flavonols, xanthonas Flavonones and alkaloids. The purge potato also had Flavonones.

The pharmacognostic screening of ethanol extracts from leaves of *Vitex megapotamica* (Trumã), Brum et al. (2011) carried out a phytochemical analysis and were able, as a result, to verify the presence of extracts of anthocyanin heterosides, phenols and tannins, catechins, flavonols, flavanones, flavanonols and xanthonas, steroids and triterpenoids (free steroids), cardioactive heterosides, phenols with ortho and position meta free, phenols with the free position, coumarins, organic acids and phenols.

According to Franca et al. (2013), to carry out a phytochemical study through colorimetric reactions and precipitation used ethanolic extracts of leaves and branches of catingueira (*Caesalpinia pyramidalis*). There was the average presence of coumarins, tannins, flavonols and flavonones, and a low presence of steroids on the leaves. In the branches, a strong presence of flavonols and flavonones was detected, a medium presence of tannins and alkaloids and a weak presence of coumarins and steroids.

Each plant species or part of the same plant has different concentrations of metabolites, thus influencing the effect that the compounds will cause. Among the desired effects of research with organic plant extracts is the focus on bioassay of lethality against dengue mosquito larvae, in order to assess acute toxicity and biological activity, is currently accepted by the scientific community (Simas et al., 2004; Pimenta et al., 2006; Kanis et al., 2009; Silva et al., 2003; Souza, 2012). Next, in Table 2, we can see the lethality bioassay's effectiveness against larvae of *Aedes aegypti* mosquitoes against the ethanolic extracts from the barks and branches of *Azadirachta indica* A. Juss (NIM) collected in Tauá, CE.

Table 2. Average mortality of *Aedes aegypti* CL50 mosquito larvae after 48 hours treated with extracts of *Azadirachta indica* A. Juss. Collected in Tauá-Ce.

Concentration	<i>Azadirachta indica</i> A. Juss	
	Bark	Branch
250ppm	20 ± 5.7	12 ± 0.0
	LAL	LAL
500ppm	30 ± 2.8	28 ± 2.8
	LAL	LAL
1000ppm	28 ± 0.0	26 ± 5.7
	LAL	LAL
2000ppm	46 ± 2.8	32 ± 5.7
	LAL	LAL

SAL -No larvicidal activity; PAL - Little larvicidal activity; MAL - Moderate larvicidal activity; EAL- High larvicidal activity. Fontes: Autores.

As a result, the tested extracts proved toxic to *A. aegypti* larvae, with LC CL50 values varying from 12 to 46% higher than the control LC50 (10%). The ethanolic extract of neem peels, collected in Tauá-Ce, showed more significant toxicity, with an LC50 of 46% of larval death treated in the 48 hours in which the experiment was carried out a concentration of 2000 ppm.

The extract does not instantly kill the larvae but prevents them from continuing to feed, interfering in their development, demonstrating to be a regulator of insect growth (Pereira et al., 2009). However, it was possible to observe the effectiveness of the liquid extract of Neem in treatments performed in different doses and concentrations, with mortality of LC50 in the period of 24 to 48 hours, higher than the control (10%), which contained 1% DMSO in sterile water and feed for the larvae.

An evaluation of the effect of the crude extract of Neem against the mosquito *Aedes aegypti* was carried out for the forms of egg and larva using the alcoholic extract of the leaves of the Neem. It was verified the delay in developing the mosquito eggs about the control, after an exposure period of 48 hours. He concluded that the crude extract *Azadirachta indica* did have an ovicidal and larvicidal action *in vivo* on the mosquito strains (Pereira et al., 2009).

Alcoholic extracts from vegetable fruits of *Capsicum frutescens* L. (Chili Pepper), leaves and seeds of *Momordica charantia* L. (Melon-de-São-Caetano) and leaves and fruits of *Azadirachta indica* A. Juss (Nim) were tested as alternative ways to control *A. aegypti* larvae at concentrations of 105 ppm and 2x105 ppm. At concentrations of 2x105 ppm, there was considerable mortality for the chilli pepper extract, which caused the death of 43% of the larvae, followed by the extracts of leaves of Melon-de-São-Caetano with 37%, of seeds of melon-de-São -Caetano with 30%, Neem leaves with 23% and Neem seeds with 20% mortality.

Studies of the Nim plant demonstrate the species as a natural insecticide alternative because they are biodegradable, so they do not leave toxic residues or contaminate the environment and are safe for non-target organisms (Chutulo and Chalannavar, 2018; Drabu et al., 2012; Debashri & Tamal, 2012). In experiments, it was seen the actions of alcoholic extracts of the leaves of Neem (*Azadirachta indica* A. Juss), being proven results of repellent and natural insecticide in African Bee (*Apis mellifera* L.) and Housefly (*Musca domestica* L.). The insects exposed to the extract of *Azadirachta indica* A. Juss, for approximately 30 min irritability, were observed in the presence of the extract, some avoided landing on the table, and others landed. Still, when they came into contact with the extract, they started to behave agitated (Silva et al., 2012).

Gomes (2012) investigated the toxicity of *Azadirachta indica* plant extracts in *Aedes aegypti* mosquito larvae, evaluating the toxicity of Neem oil in different concentrations. Larvae exposed to concentrations of 10-3%, 10-2%, 5x10-2%, 10-1, and 1% survived 74.4%, 18.12%, 22.43%, 16.7% and 5.6%, respectively, the performance of vegetable oil from Neem, which has insecticidal activity in combination with *M. anisoplae*, was also verified. Larvae exposed to vegetable oil from Neem (10-4%) combined with the fungus have a mortality rate of 70%, demonstrating a synergistic action between the fungus and Neem oil.

The use of vegetable oils as an insecticide alternative is possible. These substances are rich in bioactive metabolites, low cost and effective in reduced dosages than the currently commercialized synthetic products. The extracts of hexane, methanol and ethyl acetate from the neem cake are very promising as larvicides and prevent oviposition against a number of important vector mosquitoes, including *A. albopictus*, currently the most invasive mosquito worldwide. Besides, it proved to be effective as a larvicidal and oviposition deterrent also in field conditions (Benelli et al., 2015).

Based on the previous reports, as well as on the fact that there are no reports of preliminary chemical prospecting and lethality against larvae of the *Aedes aegypti* mosquito from organic extracts from the barks and branches of *Azadirachta indica* collected in the Yam region, we can infer that our the results presented in this work are considered important in directing the isolation and identification of bioactive compounds.

4. Conclusion

Because of the results obtained, we can observe the absence of phenols in all parts of the plant and the presence of the other metabolites tested, whether in a strong, medium or weak presence. The branches presented the highest number of metabolites in a total of six metabolites. Both extracts proved toxic to *A. aegypti* larvae, with an LC50 value higher than the control (10%), varying from 12% to 46% in the concentrations studied against *Aedes aegypti* larvae. It is believed that this species has an important pharmacological and toxicological potential. However, further studies should verify the true effects of Neem extracts on the dengue mosquito larvae and the isolation and identification of bioactive compounds.

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