

ANTIBACTERIAL AND LARVICIDE ACTIVITY OF THE ETHANOLIC EXTRACT OF *Azadirachta indica*. Juss (Meliaceae)

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ABSTRACT

Azadirachta indica, popularly known as "Indian neem" is a species widely used in folk medicine due to its antimicrobial properties. The present study aimed to evaluate the antimicrobial properties and analyze the cytotoxicity of the ethanolic extract of the *Azadirachta indica* plant (AiEE) against larvae of the *Aedes aegypti* mosquito. A preliminary phytochemical analysis by Thin-Layer Chromatography was used to determine the presence of secondary metabolites. The larvicidal activity was evaluated at 30 min, 1h, 2h, 4h, 8h, 16h and 24h, observing the larval mortality before different extract concentrations. The antibacterial activity of the extract against multiresistant strains of *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* in the presence or absence of antibiotics was analyzed using the broth microdilution method. The qualitative phytochemical analysis revealed the presence of coumarins, phenols, terpenes and triterpenes. AiEE displayed a concentration-dependent larvicidal activity although no clinically relevant antibacterial activity was observed, the extract potentiated the activity of conventional antibiotics. Our results suggest that AiEE has bioactive constituents with the potential to be explored in research aimed at combating dengue and antibiotic resistance.

Keyword: *Aedes aegypti*; *Azadirachta indica*; Bacterial resistance; Larvicidal activity

INTRODUCTION

Infectious diseases are among the leading causes of death worldwide. The viruses and bacteria are notable microorganisms with high pathogenic potential and demonstrated resistance to antimicrobial drugs (BARRETO; TEIXEIRA, 2008). Dengue is currently a major public health problem worldwide in terms of mortality and morbidity (DUARTE et al., 2013). This viral disease is transmitted by *Aedes aegypti*, an anthropophilic mosquito that also acts as a vector for other viral diseases, such as urban yellow fever, chikungunya and Zika (PICINATO et al., 2015). The impact of dengue fever on public health, the World Health Organization promoted a Global Strategy for Prevention and Control of Dengue 2012-2020, aiming to reduce global mortality and morbidity rates by 50% and 25%, respectively, by the year 2020 (WHO, 2012). Dengue control and prevention measures mainly involve the elimination of the vector through the use of chemical insecticides, such as temephos, malathion and fenitrothion. However, cases of mosquito resistance to these products have been reported in different parts of the world, indicating the need to develop new insecticides that meet safety, efficacy and selectivity parameters (FURTADO et al., 2005; MACORIS et al., 2003; MARANGONI; MOURA; GARCIA, 2012).

In the context of bacterial infections, drug resistance has become especially important and represents a significant threat to human health. The irrational use of antibiotics in different sectors of human activity has contributed to the selection of multidrug-resistant bacterial strains, which have resulted in increased death rates. Thus, the development of research aimed at the discovery of new antibiotics is of fundamental importance for the treatment of bacterial infections (OLIVEIRA-TINTINO et al., 2018).

Therefore, Brazil is remarkable in terms of its biodiversity, which provides promising potential for the discovery of new bioactive substances (LIMA et al., 2018; MORREIRA et al., 2007). Natural products represent therapeutic alternatives that offer easy access and are of great cultural importance. Despite this potential, therapeutic applications involving bioactive compounds require studies proving their effectiveness, safety and ecological viability (FIGUEREDO et al., 2013).

Azadirachta indica (Meliaceae) is a plant popularly known as neem. This species, native to India, has become naturalized in most tropical and subtropical countries, and is now widely distributed around the

world due to its known medicinal value (GIRISH; BHAT, 2008; MESQUITA et al., 2015; VINOTH; MANIVASAGAPERUMAL; RAJARAVINDRAN, 2012). The chemical and medicinal importance of this plant has led to its use in industrial activities and also constitutes a source of income for families living on small agricultural properties. Biologically active extracts obtained from the leaves, fruits, seeds and trunk of *A. indica* have been recognized for their insecticidal properties. Azadiractin, a secondary metabolite isolated from this plant, is a very potent active compound that acts as a growth regulator (MARQUES; MONTEIRO; PEREIRA, 2004).

Considering the pharmacological properties and the presence of bioactive substances derived from *A. indica*, this study aimed to evaluate the antimicrobial properties, as well as analyze cytotoxicity, of the ethanolic extract of this plant (AiEE) against the larvae of the *Aedes aegypti* mosquito.

METHODOLOGY

Plant material and extract preparation

The leaves of *Azadirachta indica* were collected in August 2018, in the municipality of Tarrafas (Ceará, Brazil) (Coordinates: 6° 68' 72.662" S 39° 75' 28.830" W). A voucher specimen was prepared and registered at the Agronomic Institute of Pernambuco (IPA, Recife, registry number 92,382).

For the preparation of the ethanolic extract, the leaves of *Azadirachta indica* were washed and dried for 48 h. The dried leaves were weighed, crushed and macerated in ethanol p.a. A total of 400 g of dry leaves were macerated in 4 L of ethanol over seven days. The extract was then filtered using a paper filter, evaporated in a rotary evaporator at 45 °C under reduced pressure. For solvent removal, the extract was then incubated in a water bath. The total yield of the extract was calculated and is expressed as a percentage of the dried leaf weight.

Phytochemical prospecting

The qualitative phytochemical analysis was performed by Thin-Layer Chromatography (TLC) on silica gel plates (MERCK-Germany, 15553) using 15 µL of extract and a solvent system.

Alkaloid presence was determined using

Dragendorff's reagent and scopolamine as a standard substance. To identify the presence of terpenes and triterpenes, a toluene:ethyl acetate mixture (93:7 v/v) was employed as a mobile phase additive in addition to the use of 1% sulfuric vanillin, followed by heating in an oven (100 °C). A change in the color of the solution to purple, green or blue was used as a diagnostic criterion. A ferric chloride solution was used to detect possible phenols. To detect coumarins, ether (1:1) was used as the mobile phase and, after drying, the KOH/EtOH developer was added and placed under UV light. To identify the presence of saponins, foam index was used after agitation using a solution of *Ziziphus joazeiro* extract as a control (HARBORNE, 1998; MATOS, 1988).

Evaluation of larvicidal activity

Colony establishment and maintenance

An *Aedes aegypti* colony was maintained at the Laboratory of Pharmacology and Experimental Cancerology (LAFACE) (Recife, Pernambuco). Mosquito eggs of the Rockefeller strain were deposited in plastic containers (15.0 cm x 5.0 cm) containing deionized water at a proportion of 1,000 eggs per 1 L of water with 1 g of larval diet and maintained at 27 °C under a relative humidity of 70-85%. After the fifth day, pupal phase larvae were collected and placed in plastic containers kept inside cages (40.0 cm x 15.0 cm). After emergence, mosquitos of both sexes were fed with a 9:1 sugar solution. Additionally, females were fed with rat (*Rattus norvegicus*) blood. Three days later, oviposition was stimulated under dark conditions in a recipient containing water and filter paper. The protocols used in this study were approved by the local institutional review board for animal experimentation (CEUA-LAFACE, protocol number 23076.049863/2015-91).

In vitro larvicidal assay

AiEE was dissolved in absolute ethanol, tween 80 and distilled water at five different concentrations (7.5; 5.0; 2.5; 1.0 and 0.5 mg/mL). A mixture of these solvents at identical proportions in the absence of extract was used as a control. After reaching the third and fourth instar, 30 larvae were placed in plastic recipients containing variable concentrations of AiEE. Mortality was monitored by eye observation within 24 hours after the start of treatment. All experiments were performed in triplicate (WHO, 1970).

$$PM = \frac{DL}{TL} \times 100$$

PM: Percentage of mortality

DL: Dead larvae

TL: Total larvae

Evaluation of antibacterial activity

Bacterial culture and antibiotics

Pseudomonas aeruginosa 24 (PA-024), *Staphylococcus aureus* 10 (SA-010) and *Escherichia coli* 06 (EC-06) multiresistant strains were kindly provided by Prof. S. Gibbons (University of London). These strains were initially kept in blood agar medium (Difco laboratories, Brazil) and then transferred to Heart Infusion Agar (HIA, Difco laboratories, Brazil) at 4° C. Samples were transferred from solid medium to test tubes containing sterile saline and turbidity was compared with the 0.5 value of the McFarland scale, corresponding to 10⁵ CFU. Gentamicin and Norfloxacin (SIGMA Chemical Co., St. Louis, USA) were dissolved in DMSO at 10 mg/mL and then diluted to 1,024 µg/mL testing purposes.

Minimum Inhibitory Concentration (MIC) determination

All bacterial strains were cultured as described above, peaked and transferred to Brain Heart Infusion broth (BHI, Difco laboratories, Brazil) and incubated at 37° C for 24 h. Next, a 100 µL solution containing 10% BHI: inoculum (10:1) was transferred to each well of a 96-well plate.

The extract and both antibiotics were prepared at initial concentrations of 1,024 µg/mL and serially diluted in test tubes. Next, 100 µL was transferred from each tube to wells containing the inocula in BHI. Wells containing only the inoculum in BHI were used as a growth control. Plates were incubated at 37 °C for 24 h (CLSI, 2013), 20 µL of 0.01% (w/v) sodium resazurin (Sigma) were added to each well, followed by an additional 1 h incubation period at room temperature. A change in the color of the solution (from blue to pink) due to resazurin reduction was used as an indicator of bacterial growth (PALOMINO et al., 2002). MIC values were defined as the lowest concentration capable of inhibiting bacterial growth. The potentiation of the antibiotic activity was analyzed by the values obtained in the MIC by the method of microdilution in broth in

wells containing AiEE in a subinhibitory concentration (MIC / 8) (COUTINHO et al., 2008).

Statistical Analysis

Data are expressed as arithmetic means ± standard deviations and were analyzed by analysis of variance (ANOVA), followed by the Bonferroni's post-test using GraphPad Prism software version 7.00. Statistical significance was considered when $p < 0.05$.

RESULTS

Phytochemical Analysis

The extraction of *Azadirachta indica* leaves in

ethanol presented a yield of 8.5%. Phytochemical analysis of the ethanolic extract by TLC identified the presence of different classes of secondary metabolites, including coumarins, phenols, terpenes and triterpenes. Alkaloids and saponins were not detected according to a table 1.

Larvicidal activity

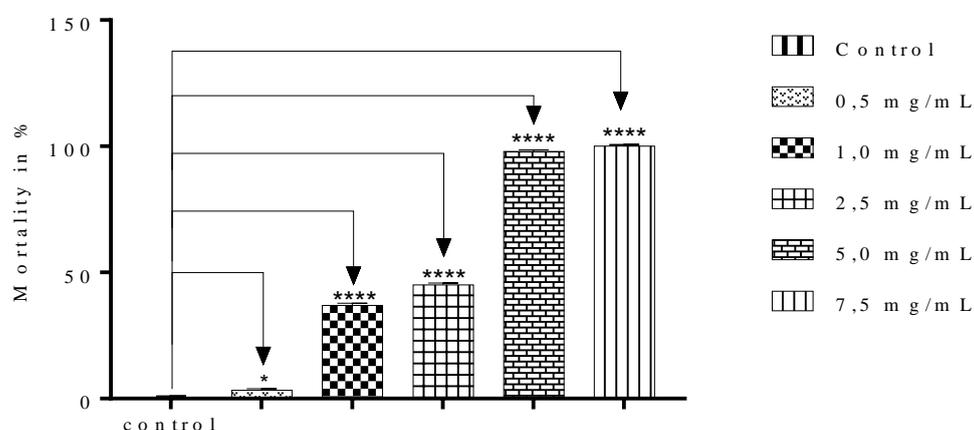
As can be seen figure 1, the concentrations of 7.5 mg / mL and 5 mg / mL were those that obtained the greatest larvicidal activity, reaching, respectively, the percentages of 100% and 97.77% when compared to the control. At concentrations of 2.5 mg / mL, 1.0 mg / mL and 0.5 mg / mL reached a mortality of 45%, 37% and 3.3%, respectively, after 24 hours of exposure to AiEE.

Table 1. Thin-Layer Chromatography Profile of *Azadirachta indica* Ethanolic Extract

Class of Compound	Presence
Alkaloids	-
Coumarins	+
Phenols	+
Terpenes	+
Triterpenes	+
Saponins	-

Legend: Present (+), Absent (-).

Figure 1. Toxicity of AiEE against *Aedes aegypti* larvae. A total of 30 larvae per group were treated with increasing concentrations of AiEE for 24 h. Results are expressed as percentage of mortality. **** $p < 0,0001$

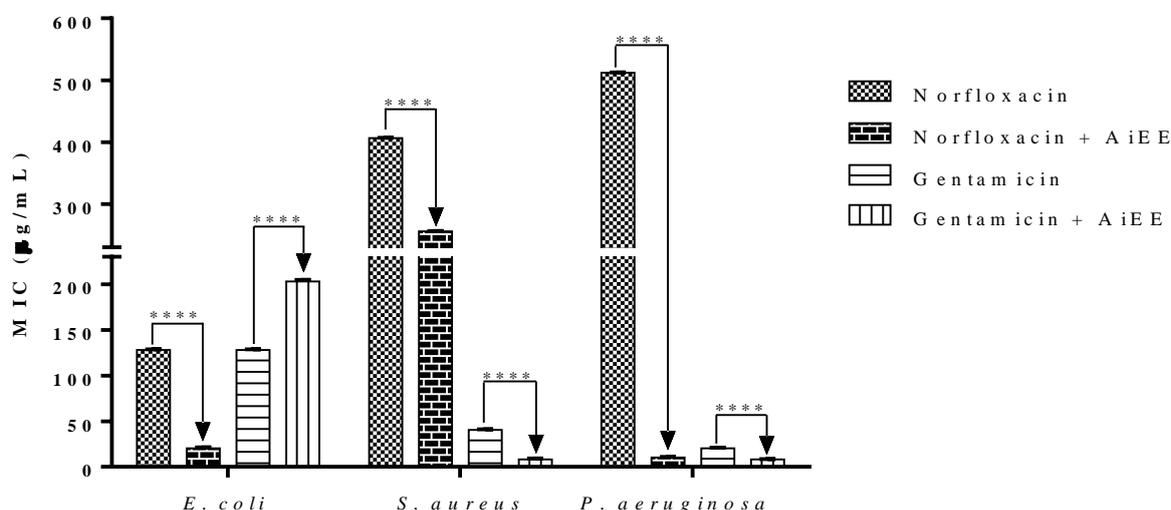


Antibacterial and Antibiotic-modulating activity

The antibacterial activity analysis revealed that AiEE presented a MIC value higher than 1,024 µg/mL, indicating that this extract did not present clinically relevant antibacterial activity (Coutinho et al., 2008). Nevertheless, a subinhibitory concentration (MIC/8) of the extract potentiated the effect of the Gentamicin and

Norfloxacin against resistant strains of *S. aureus* and *P. aeruginosa*. Tests with *E. coli*, demonstrate that although the extract potentiated the effect of Norfloxacin, it antagonized the effect of Gentamicin as seen in the figure 2. These data suggest that *A. indica* is species with potential to be used in research aiming development of new drugs against bacterial resistance.

Figure 2. Antibiotic-modulating activity of *A. indica* ethanolic extract in association with Norfloxacin and Gentamicin against *E. coli* 06, *S. aureus* 10 and *P. aeruginosa* 24. **** p <0,0001



Discussion

The present study analyzed the larvicidal and antibacterial properties of an ethanolic extract obtained from the leaves of *A. indica*. The extraction process resulted in a yield 8,5% comparable to those of studies using the same plant material and solvent (SILVA et al., 2016). A TLC analysis of this extract revealed the presence of secondary metabolites such as coumarins, phenols, terpenes and triterpenes, corroborating previous studies which identified a similar composition in *A. indica* ethanolic extracts (BISWAS; BENERJEE; BANDYOPADHYAY, 2002), especially triterpenes (MACIEL et al., 2010). According to the literature, triterpenoids represent the main active components of this species, among which azadiractin, melianthriol, limonene and odoratone are worthy of mention (DELEITO; BORJA, 2008; PEREIRA et al., 2008; PAES et al., 2015). Flavonoids, phenols, carotenoids, steroids and ketones have also been identified in *Nem* extracts, including azadiractin, which has been

recognized as a highly active mixture of isomeric compounds (MOSSINI; KEMMELMEIER, 2005). The presence of saponins and alkaloids in *A. indica* extract was previously demonstrated (ALVARADO; ROSALES; MACHADO, 2016). It has been recognized that the composition of plant-derived extracts may vary significantly under the influence of several factors, such as: genetic variability, time and place of collection, part of the plant collected, environmental conditions, as well as methodology of extraction and analysis. Accordingly, the pharmacological properties of a given species might be affected by changes in its chemical composition (TRINDADE et al., 2000; CHAGAS; VIEIRA, 2007)

According to figure 1, the concentration of 7.5 mg/mL obtained a larval mortality of 100%, thus being more effective than the other concentrations. Through the results observed it is possible to verify that this larvicidal action of the extract depends on the concentration.

Behavioral changes were seen when the extract

was associated with the liquid medium. According to the exposure of the larvae as a function of the 24-hour time, the movements of the larvae were gradually impaired, a situation not observed in the control group, whose movements remained normal.

This behavior of decreased response and mortality was also seen by Beserra et al., (2014), in which the larvae of *Ae. aegypti* submitted to the raw ethanolic extract of *Jatropha curcas*, had their movements decreased after one hour of exposure, being completely immobilized after 24 hours from the beginning of the experiment. The authors related this behavioral variation with the mechanisms of action of vegetables in the larvae penetrating by ingestion or by direct contact.

The highest concentration of the tested extract showed a mortality of 100%, an effect that is correlated with the work of (WANDSCHEER, 2004), who also carried out tests with ethanolic extract of neem leaves against *Ae. aegypti* larvae, in concentrations ranging from 3mg / mL to 10mg / mL. Pereira et al., (2009) when checking larval activity of neem ethanol extract in a period of 48 hours, obtained mortalities that varied from 70 to 100%. Dua et al., (2009) working with neem seed oil over a period of 48 hours, noticed a larvicidal activity of up to 99.7% of *Ae. aegypti*.

Hashmat and collaborators (2012), when performing tests with neem ethanolic extract with concentrations ranging from 1 to 5 mg/mL, it obtained mortality rates ranging from 70 - 99%. The study by Indrayani and Sudarmaja (2018) showed different results of this work regarding the analysis of the concentration of 2.5mg / mL, which according to the author the concentration showed 100% larvicidal activity.

The deaths of the larvae of *Ae. Aegypti* after exposure to AiEE may have resulted from the interruption of its development, as mentioned (Pereira et al., 2009). In works already published with extract of *A. indica*, they showed that the larvae can develop normally until the next seedling, however, they cannot complete them (TUNAZ; UYGUN, 2004) This impediment may be the result of several morphogenetic defects that end up causing the death of the larvae.

The AiEE extract demonstrated a rapid insecticidal effect, presenting a high mortality potential (in the first 4 hours of the experimente). Natural insecticides are fast-acting, killing insects in short intervals or preventing them from feeding. This rapid action is associated with other advantages, such as, for example, rapid degradation under environmental conditions, reduced impacts on beneficial and non-

target organisms, and a high possibility of not presenting toxicity to mammals (MACHADO; SILVA; OLIVEIRA, 2007; RONDELLI et al., 2010).

Another major challenge for the medical profession has been the resistance of microorganisms such as bacteria especially opportunistic ones in the hospital environment such as *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus*. The resistance process occurs mainly due to the indiscriminate use of antibiotics, where strains by means of efflux pumps, enzymes and changes in the binding site acquire resistance to a certain medication.

Multiresistant strains of *E. coli*, *S. aureus* and *P. aeruginosa* were used to evaluate the antibacterial activity of AiEE alone or in combination with antibiotics. The extract had a MIC value greater than 1,024 µg / mL, indicating that it has no clinically relevant antibacterial effect. However, the combination of a subinhibitory concentration (MIC/8) with Norfloxacin caused a significant reduction in the MIC of this antibiotic against all strains, indicating the extract has a synergistic effect when associated with this antibiotic against resistant bacterial strains. Curiously, when associated with Gentamicin, AiEE presented synergistic effects against *S. aureus* and *P. aeruginosa*, but antagonized the effect of the antibiotic against *E. Coli*, an enterobacterium with remarkable resistance to multiple drugs (OLIVEIRA et al., 2016). These data suggest that the modulating effect of the extract varies according to the type of strain and antibiotic associated.

Staphylococcus aureus is a species found in the normal flora of the skin and mucous membranes, such as nasal passages, hands, throat and intestines. Nevertheless, under opportunistic conditions, this bacterium cause severe infections such as pneumonia, fatal septicemia, endocarditis and boils. In addition, *S. aureus* has been associated with cross infection, which occurs mainly in immunodepressed subjects in contact with highly pathogenic microorganisms (ALEMÁN, 2013; MIRANDA et al 2015; TAVARES et al., 2015).

According to Kong et al (2015), *P. aeruginosa* is responsible for a significant number of hospital infections, in addition to be the leading cause of death in patients with cystic fibrosis. *P. aeruginosa* can form biofilm, a sessile community of bacteria surrounded by an extracellular matrix of extracellular DNA, proteins and exopolysaccharide. Biofilms can originate in a variety of surfaces, impairing the action of antibiotics, as well as the action of the immune system. Thus, they act as a site for bacterial resistance and therefore are directly related to persistence of chronic infections (FERREIRA & LALA, 2010; SOTO, 2013).

A study by Cristo et al., (2016) using an extract prepared from the *A. indica* bark obtained results that corroborate our findings with regard to modulation Gentamicin activity against *P. aeruginosa* and *S. aureus*. However, their results in the tests with *E. coli* differed significantly from ours, suggesting occurrence of pharmacological and possibly chemical variation among different parts of the plant. Our group have demonstrated the antibiotic-potentiating effect of several species and different families, such as: *Turner ulmifolia*, *Momordica charantia*, *Mentha arvensis* and *Cordia verbenaceae*. In fact, association between natural products and synthetic drugs represent has demonstrated to be an excellent strategy against bacterial resistance, since bioactive compounds found in plant extracts can interfere with several bacterial resistance mechanisms (COUTINHO et al., 2009;

COUTINHO et al., 2010; FERREIRA et al., 2009).

Conclusion

In conclusion, *A. indica* ethanolic extract presented a larvicidal activity against *Aedes aegypti* larvae and potentiated the activity of conventional antibiotics against resistant bacteria. The results of the present study encourage the development of further research aimed at investigating the use of *A. indica* extracts as larvicidal agents, as well as the potential of its secondary metabolites, particularly triterpenes, on development of new drugs against bacterial resistance.

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