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Larvicidal activity of ethanolic extract of *Azadirachta indica* against *Aedes aegypti* larvae

Actividad larvicida del extracto etanólico de *Azadirachta indica* contra larvas de *Aedes aegypti*

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ABSTRACT

Aedes aegypti is a mosquito that carries dengue virus, yellow fever and other diseases transmitted to humans. Organophosphorus larvicides are used to control the proliferation of this mosquito, which has generated a high degree of resistance; hence, new alternatives such as bio-larvicides formulated with plant extracts are of great interest. The aims of this study were to evaluate the ethanolic extract of *Azadirachta indica* leaves as a larvicide against *Aedes aegypti* and to determine the main compounds present in it by GC-MS. In the assay, three concentrations of ethanolic extract were used (10 mg L^{-1}, 20 mg L^{-1}, and 50 mg L^{-1}). This was performed thrice against a positive control (commercial larvicide: spores and endotoxic crystals of *Bacillus thuringiensis* var. israelensis Serotype H-14) and negative control (water). After 72 h of incubation, it was observed higher larval mortality (93%) in the ethanolic extract at a concentration of 50 mg L^{-1}; the extracts at 10 mg L^{-1} and 20 mg L^{-1} shown larval mortality of 47% and 70%, respectively. The majority compound determined by the GC-MS analysis was phytol (14.4% area). The results obtained in this study demonstrated the larvicidal potential of the ethanolic extract of *A. indica* against larvae of *A. aegypti*.

Keywords:
Bio-larvicide
Crude ethanolic extract
GC-MS
Mosquito
Neem
Phytol

RESUMEN

Aedes aegypti es un mosquito portador del virus del dengue, la fiebre amarilla y otras enfermedades transmitidas a los humanos. Los larvicidas organofosforados se utilizan para controlar la proliferación de este mosquito, el cual ha generado un alto grado de resistencia, por lo que las nuevas alternativas de biolarvicidas formulados con extractos de plantas son de gran interés. Los objetivos de este estudio fueron evaluar el extracto etanólico de hojas de *Azadirachta indica* como larvicida contra *Aedes aegypti* e identificar los principales compuestos químicos GC-MS. En el ensayo, se utilizaron tres concentraciones de extracto de etanol (10 mg L^{-1}, 20 mg L^{-1} y 50 mg L^{-1}), este se realizó por triplicado contra un control positivo (larvicida comercial: Esporas y cristales endotóxicos de *Bacillus thuringiensis* var. israelensis Serotipo H-14) y un control negativo (agua). Después de 72 horas de incubación, se observó una mayor mortalidad larval (93%) en el extracto etanólico a una concentración de 50 mg L^{-1}, y los extractos a 10 mg L^{-1} y 20 mg L^{-1} mostraron una mortalidad larval del 47% y 70% respectivamente. El compuesto con mayor abundancia determinado por el análisis CG-MS fue el fitol (área del 14,4%). Los resultados obtenidos en este estudio demostraron el potencial larvicida del extracto de etanol de *A. indica* contra las larvas de *A. aegypti*.

Palabras clave:
Biolarvicida
Extracto crudo de etanol
GC-MS
Mosquito
Neem
Fitol

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Aedes aegypti, known as mosquito, is widely distributed in America, except Canada and Chile. It is also the vector of several diseases transmitted to humans, including dengue, malaria, and, more recently, chikungunya and zika. Vector-borne diseases are the second group of pathologies with the highest morbidity in Ecuador, led by dengue and chikungunya (Ministerio de Salud Pública, 2015).

Dengue is caused by a virus of the Flaviviridae family, which presents different epidemiological patterns associated with four serotypes that cause this disease (DENV-1, DENV-2, DENV-3, and DENV-4) (WHO, 2020b). It has an alarming impact on human health worldwide due to its easy transportation from one place to another by infected travelers. In 2019, there were more than 3.1 million cases from which 28,000 were severe, and 1,534 ended up in death; currently, around 500 million people are at risk of contracting dengue in Latin America (PAHO, 2020). The WHO has reported several compounds to improve mosquito larvicides, among them are chemical and synthetic organic oils (WHO, 2009). Chemical and biological larvicides are insecticides widely used to kill insects as *A. aegypti* in the larval life stage and to control the pests. Synthetic organophosphate insecticides (temephos and malathion), and pyrethroids (deltamethrin, lambda-cyhalothrin, and cypermethrin) are used for the control of adults of *Aedes aegypti*, especially in the emerging stage; however, the pyrethroid and cypermethrin have become ineffective (Vargas-Miranda *et al*., 2019). Recently, it has been reported that *A. aegypti* has developed resistance to one of the four classes of insecticides most commonly used for its control (pyrethroids, organochlorines, carbamates, and organophosphates) (PAHO, 2020), and 26 countries have detected resistance to all four classes (WHO, 2020a).

Concerns about environmental pollution and the development of insect resistance to chemical larvicides have stimulated the search for natural insecticides derived from plants (Howard *et al*., 2010). Larvicides from botanical origin have increased the general interest, like the ones derived from *Azadirachta indica* (Meliaceae family, commonly known as neem tree), whose insecticidal, pesticidal and larvicidal extracts have been widely studied (Vietmeyer, 1992).

The neem tree is considered one of the most versatile trees in the world, thanks to its ability to grow in areas that reach high temperatures. Its extract composition is considered one of the richest and most complete because of the presence of a great variety of alkaloids, flavonoids, phenolic compounds, steroids and ketones (Imam *et al*., 2012). These compounds can be extracted from the entire tree, seeds, stem, flowers and fruits, varying in concentration depending on the region and the time of year the tree is collected (Fernandes *et al*., 2019). Extracts of *Azadirachta indica* mixed with other plant extracts and synthetic products have been evaluated against different mosquitoes, such as *Cx. quinquefasciatus*, *Cx. pipiens*, *Ae. aegypti, Ae. togoi, and An. stephensi;* showing synergistic, additive and antagonistic effects (Shaalan *et al*., 2005).

Schneider *et al*. (2017) reported the high potential of neem oil to control pupae and adults of *D. saccharalis* present in sugarcane. The study conducted by Lin *et al*. (2016) provided insight into the gene expression of *Monochamus alternatus* (vector of the destructive forest pest pinewood nematode) at the transcriptional level when subjected to azadirachtin, an active compound of neem, confirming its potential against the pest. This enhances the value of azadirachtin as a potential insecticide of natural origin. Besides, the neem extract is considered a growth regulator insecticide for the control of the lesser mealworm beetle *Alphitobius diaperinus* (Coleoptera: Tenebrionidae) (Zorzetti *et al*., 2015). In other applications, Forim *et al.* (2013) developed a method to prepare nanoparticles loaded with neem (*A. indica*) extracts, which presented a promissory larvicidal activity against *Plutella xylostella* with 100% larval mortality.

Azadirachtin's terpenoids and limonoids are the chemicals responsible for the insecticide, larvicide and antibacterial activity, as was reported by Su and Mulla (2003), Ndione *et al*. (2007) and Liu *et al*. (2014). The insecticide activity by the limonoids in azadirachtin is related to the direct inhibition of chitin synthesis and changes of pupation and metamorphosis related to the hormone ecdysone (Wandscheer *et al*., 2004).

Considering the above mentioned, the present study aimed to evaluate the ethanolic extracts of *Azadirachta indica* leaves, which grows in the Ecuadorian coastal zone, against *Aedes aegypti* larvae and to identify the compounds present in the extract responsible for the larvicidal activity.

MATERIALS AND METHODS

Plant material

Leaves of *A. indica* from Guayaquil – Ecuador (2°07'32" S, 79°50'48" W) were collected in February 2015, during the tree flowering stage. A sample of the plant material was taken for botanical identification, being herborized in the National Herbarium of Ecuador (QCNE), keeping an herbal control (Code: CIBE019) in the Bioproducts laboratory of CIBE-ESPOL, Guayaquil, Ecuador.

Plant extract

The plant extract was obtained according to the procedure described by Maragathavalli *et al.* (2012). Fresh leaves were dried in an oven with air recirculation for 24 h at 55 °C for subsequent manual milling and sieving, the selected fraction was the one remaining on the 2-mm mesh sieve. Three successive macerations were performed with ethanol at 96% (100 g dry sample per 500 mL of solvent) under stirring at room temperature for 48 h and then filtered. The solvent of the combined maceration was removed by roto-evaporation at 40 °C, and the extracted material was kept at 4 °C until use (Heidolph, 40001). Three samples (100, 200, 300 mg) were dissolved on 100 mL of distilled water, giving an initial concentration of 1, 2, and 5 mg mL^{-1}.

GC-MS analysis

The plant extract was analyzed by GC-MS according to the method of Umar *et al.* (2014) with some modifications, using Agilent Technologies gas chromatography and mass spectrometry equipment (7890A GC and 5975C XL MSD inert with triple-axis detector). The samples dissolved in distilled water were filtered or centrifuged to remove any insoluble matter. The injection of 2.0 µL of a sample (10 mg mL^{-1}) was performed at 250 °C with no division mode; the detector temperature was 280 °C, and the oven was equipped with an HP-5 capillary column (30 m×0.25 mm ID×0.25 µm film thickness). The GC-MS was programmed with a ramp of 70 °C for 2 min, at a speed of 5 °C min^{-1} until reaching 285 °C. The carrier flow of helium was adjusted at a speed of 1.2 mL min^{-1} to have a run of 45 min. Electronic ionization was set at 70 eV (135 and 230 °C), and the data were collected in full scan mode (40-1000 amu). Lastly, the compounds were identified by comparing their retention index and the Wiley 9th mass spectrum data with the NIST 2011 MS Library.

Larvicidal assay

The Chemistry Faculty of Universidad de Guayaquil provided the *A. aegypti* larvae. The assay was developed according to the methodology of Wandscheer *et al.* (2004) and Cruz-Estrada *et al.* (2013). Three concentrations of neem extract were prepared by diluting 1 mL of the initial extracts (1, 2, and 5 mg mL^{-1}) in 100 mL of water, obtaining final doses of 10 mg L^{-1}, 20 mg L^{-1}, and 50 mg L^{-1}; dose range was selected based on Wandscheer *et al.* (2004). Ten larvae (III and IV instar stage) were placed in each polyethylene plastic containers with test solutions (100 mL) at a temperature from 25 to 30 °C and 12 h photoperiod. The tests were performed thrice. Negative control (NC) was water, and positive control (PC) was Bactivec (Labiofam), bio-larvicide which active ingredient (0.6%) is *Bacillus thuringiensis* var. israelensis; for the assay, it was diluted to the recommended application (1% v/v). After 24, 48, and 72 h, the percentage of mortality was registered.

Statistical analysis

Assumptions of normality and homogeneity of variances were corroborated, then the data was submitted to 2 way ANOVA. Tukey's test at a 5% probability was used for the comparison of means. The statistical software use was MINITAB 16.

RESULTS AND DISCUSSION

In the comparative study of the percentage of mortality (%) of each extract vs. the exposure time, the mortality was proportional to the exposure time ($P<0.05$). Among the different concentrations, significant differences were found ($P<0.05$), by comparing each independent extract, differences between extracts 10 mg L^{-1} and 20 mg L^{-1}, 10 mg L^{-1} and 50 mg L^{-1}, 20 mg L^{-1} and 50 mg L^{-1} were demonstrated ($P<0.05$), indicating that a higher concentration greater the mortality over time, observing a dose-dependent behavior. The extract 50 mg L^{-1} exhibited the highest mortality with values close to the positive control. The mortality percentages of positive control were 50%, 80%, and 100 % at 24, 48, and 72 h, respectively. The negative control had no larvicidal effect, ensuring the results of this study (Figure 1).

The results of this research demonstrate the larvicidal action of neem extract on larvae of *A. aegypti*. The best larvicidal activity (mortality 93%) was obtained at 50 mg L^{-1} of the extract at 72 h. Nour *et al.* (2012) reported the larvicidal

activity against *Aedes aegypti* mosquitoes' larvae of extracts from different parts of *A. indica* (leaves, stem, root, and seed), being the leaves the ones that produced the greatest activity. In their study, they reported a larvicidal activity between 60-70% in 48 h using a concentration of 50 mg L^{-1} of ethanolic *A. indica* leaf extract, similar to those reported in this study. However, here, the result was superior compared to the ethanolic extract obtained by reflux (50% mortality).

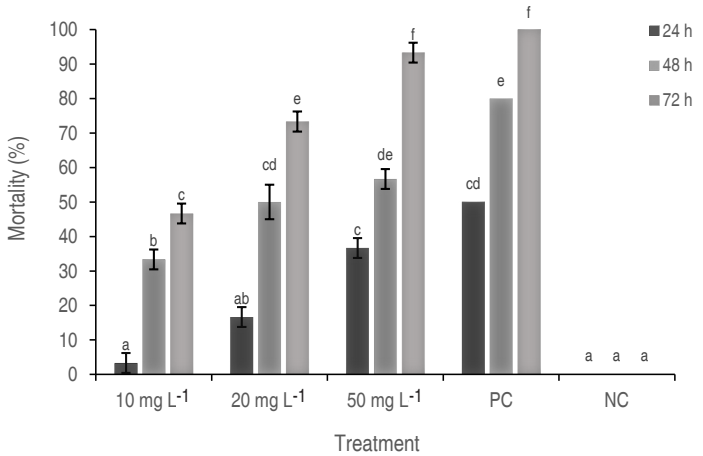

Figure 1. Larval mortality (%) of *A. aegypti* treated with the ethanolic extract of *A. indica* at different concentrations 10 mg L^{-1}, 20 mg L^{-1}, and 50 mg L^{-1}, and Positve (PC, Bactivec) and Negative (NC, water) Control, observed at 24, 48 and 72 h of exposure. Different lowercase letters indicate significant differences among the concentrations by the Tukey test (P<0.05).

Previous research of larvicidal action of ethanolic extract of *A. indica* seeds against *Aedes aegypti* was reported by Wandscheer *et al.* (2004), obtained an LC_{50} value of 440 mg L^{-1}. On the other hand, Shaima *et al.* (2006) reported a larvicidal activity of the methanolic *A. indica* leaf extract against *Anopheles stephensi* with LC_{50} values of 18.2 and 13.1 ppm, after 24 and 48 h, respectively. This demonstrates the larvicidal potential of *A. indica* extract for different species of mosquitoes.

Differences between larvicidal activities could be attributed to the different methods of drying employed. The oven with recirculating air used in this research could cause thermal degradation of the active compounds present in the leaves, reducing the larvicidal activity of extract compared to those reported by Maragathavalli *et al.* (2012) for the same species in another geographical ecological environment.

GC-MS analysis

In Figure 2, the gas analytical chromatogram of the compounds identified by GC-MS of the ethanolic extract is shown. The significant component detected in the extract was phytol (14.24%). The presence of terpenes and fatty acids was also reported (Table 1).

The presence of the terpene phytol, as the main component of the ethanolic extract of *A. indica* is consistent with that reported by Cruz-Estrada *et al.* (2013). Furthermore, previous reports suggest that phytol extracted from plants have larvicidal activity against mosquitoes (Renjana and Thoppil, 2013). Phytol presented in the leaf extract of *Lantara chamber*, *Azadirachta indica* and *Ocimum gratissimum* possess larvicidal properties against *Aedes aegypti* and *Culex quinquefasciatus* (Maneemegalai and Sathish, 2008; Maragathavalli *et al.*, 2012; Pratheeba *et al.*, 2015), and *Premna latifolia* extract against *Aedes albopictus* as observed in the studies conducted by Krishnaveni and Ramamurthy (2014). The combination of neophytadiene and phytol has been shown to have higher insecticidal activity than its components alone (Cáceres *et al.*, 2015). Additionally, potent toxicity of 2-Furancarboxaldehyde has been shown against *Drosophila melanogaster* larvae (Miyazawa *et al.*, 2003). On the other hand, linoleic acid has larvicidal activity against *Aedes aegypti* with LC_{50} and LC_{90} values of 35.39 and 96.33 ppm, respectively (Rahuman *et al.*, 2008), so this compound in the present study could be contributing to larvicidal activity.

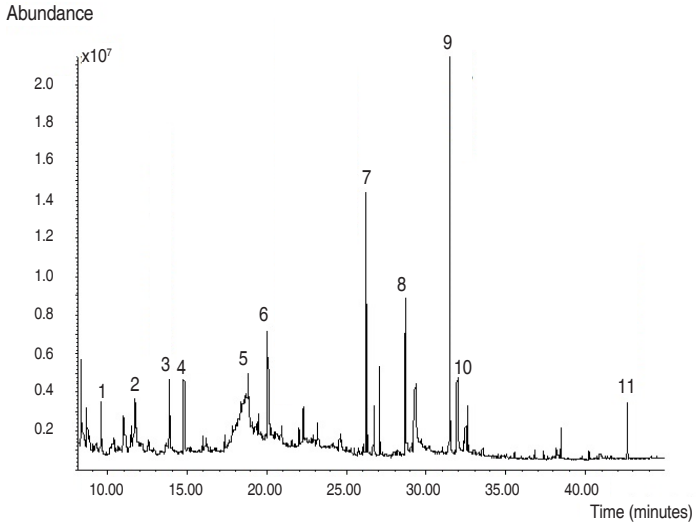

Figure 2. Chromatogram of the ethanolic extract of the leaves of *Azadirachta indica*.

Table 1. Chemical Compounds identified by GC-MS in the ethanolic extract of *Azadirachta indica* leaves.

Peak	Retention Time (min)	Relative abundance (%)	Name of compound
1	9.543	2.53	2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one
2	11.675	6.29	2-Furancarboxaldehyde
3	13.866	4.58	2-Methoxy-4-vinylphenol
4	14.787	3.22	Syringol (Ether)
5	18.814	4.87	Phenol, 2,4-bis(1,1-dimethylethyl)
6	20.054	2.93	2,6-Dimethyl-3-(methoxymethyl)-p-benzoquinone
7	26.205	8.10	Neophytadiene
8	28.701	7.23	Palmitic acid
9	31.465	14.24	Phytol
10	31.955	4.07	Linolenic acid
11	42.630	2.02	Squalene oil

According to the results mentioned above may be noted that the neem tree leaves have a high amount of terpenes, which is corroborated by this study where phytol was the most abundant metabolite, a component that confers the insecticide property and possibly the responsible of the reported larvicidal action.

CONCLUSIONS

In this research, it was observed higher larval mortality (93%) after 72 h incubation in the ethanolic extract at a concentration of 50 mg L^{-1}, compared with the extracts at 10 mg L^{-1} and 20 mg L^{-1} shown larval mortality of 47% and 70%, respectively. In the analysis by GC-MS, the presence of phytol was evident as a majority component in the ethanolic extract (14.24%).

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