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EVALUATION OF THE TOXIC AND REPELLENT POTENTIAL OF THE CRUDE LEAF EXTRACT AND FIXED OIL OF *Azadirachta indica* A. JUSS

AVALIAÇÃO DO POTENCIAL TÓXICO E REPELENTE DO EXTRATO BRUTO DAS FOLHAS E DO ÓLEO FIXO DE Azadirachta indica A. JUSS

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ABSTRACT

The applicability of plant-based products in the health sector aligns with their traditional use, which, over time, has gained value and now serves as an efficient pharmacotherapeutic basis for numerous medications. Pre-clinical evaluation stages for new pharmaceutical product candidates include ensuring safety and efficacy. The species *Azadirachta indica* is known for its insecticidal properties and is an alternative for controlling arboviruses, currently managed with synthetic repellents that are developing resistance in target insects. The present study evaluated the toxic and repellent potential of the fixed oil and crude extract of *A. indica* leaves, with the aim of potential application in pharmaceutical formulations. The methods were tested with the *Artemia salina* and *Euschistus heros* models. The results classified both samples as non-toxic, with Mean Lethal Concentrations of 11622.4 and 1609.6 $\mu g\ mL^{-1}$ for *A. salina* exposed to the extract for 24 and 48 h, and 2009.3 and 2009.3 $\mu g\ mL^{-1}$ for the oil. The oil was classified as very repellent at 10.0%, and the extract as repellent at 2.5%. The samples represented an alternative for formulating repellents for human use, due to their non-toxicity and efficacy.

Keywords: Neem; Pharmacotoxicity; *Melia azadirachta* L; Repellency.

RESUMO

A aplicabilidade de produtos de origem vegetal no ramo da saúde decorre do uso tradicional de produtos naturais, que, ao longo do tempo, agregaram valor e hoje constituem a base farmacoterapêutica de inúmeros fármacos. As etapas pré-clínicas de avaliação de novos candidatos a produtos farmacêuticos incluem garantir a segurança e os efeitos. A espécie Azadirachta indica é conhecida por seu efeito inseticida e é uma alternativa para o controle de arboviroses, atualmente mediado por repelentes sintéticos, que vêm adquirindo resistência aos insetos-alvo. O presente trabalho avaliou o potencial tóxico e repelente do óleo fixo e do extrato bruto das folhas de A. indica, visando à possível aplicação em formulações farmacêuticas. Foram testados os métodos com os modelos Artemia salina e Euschistus heros. Os resultados classificaram ambas as amostras como atóxicas, com Concentração Letal Média de 11.622,4 e 1.609,6 µg mL^{-1}, para a exposição de 24 e 48 h de A. salina ao extrato e de 2.009,3 e 2.009,3 µg mL^{-1}, para o óleo. O óleo foi classificado como muito repelente a

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10,0% e o extrato como repelente a 2,5%. As amostras representaram uma alternativa para a formulação de repelentes de uso humano, devido à sua não toxicidade e à eficácia do efeito.

Palavras-chave: *Neem; Farmacotoxicidade; Melia azadirachta L; repelência.*

INTRODUCTION

Pharmacotherapy using natural sources is a traditional medicine system that originated in the early days and remains essential today (ANSEL JUNIOR, POPOVICH, & ANSEL, 2013). The wide range of phytocompounds present in botanical species exhibits relevant biopharmaceutical activities, adding value to pharmaceutical formulations. The evaluation of natural product candidates for new pharmaceutical formulations must be approved by regulatory agencies to ensure their efficacy and safety (SIMÕES *et al.* 2019).

Toxicological studies can be divided into *in vitro*, *in vivo*, and other models, such as aquatic animals and *Artemia*, whose percentage of agreement is comparable to that of mammalian models (OGA, CAMARGO, & BATISTUZZO, 2021). The alternative lethal dose assessment test in the *Artemia salina* model is recognized for its speed, ease, reproducibility, reliability, and low cost, and is applicable as a bioindicator of preclinical toxicity (NECO *et al.* 2022). *Artemia salina* is a species of marine microcrustacean in the family Artemiidae. It has low tolerance and responds readily to environmental changes (Santos *et al.* 2023). The larval stage of *A. salina* is known as nauplii, and is used in toxicity tests, where MEYER *et al.* (1982) establish a direct relationship between the degree of acute toxicity and the Mean Lethal Concentration (LC_{50}), considering non-toxic LC_{50} values above 1000.0 $\mu g\ mL^{-1}$.

The preliminary evaluation of repellent or attractive action can be conducted in the laboratory (MCDONALD, GUY, & SPEIRS, 1970). The knockdown effect can also be observed when the sample contains a substance that causes a state of intoxication and partial paralysis, which usually precedes death (WICKHAM, CHADWICK, & STEWART, 1974). The species *Euschistus heros* is among the most abundant in Brazil, and 3º-instar nymphs are effective for evaluating the chemical control of new products, as they help control pests without unbalancing the ecosystem (BETINELLI *et al.* 2023).

The use of *E. heros* is feasible when analyzing the mechanisms of action of compounds in insects. TIWARI & SOWDHAMINI (2023) report that the host-seeking behavior of mosquitoes targeting humans is mediated by odor detection via odor-activated ion channel complexes, which are expressed in most insects, including vector *A. aegypti*. The mosquito community is a serious public health problem because it serves as a vector for several diseases, and most known species are found in tropical and subtropical regions (SANTOS *et al.* 2021). Arboviruses are viral diseases transmitted mainly by arthropods and can cause a variety of potentially fatal symptoms. The main vectors are mosquitoes, in particular, the genera Aedes, Culex, and Anopheles (BRASIL, 2025).

The presence of these vectors poses an ongoing risk of arbovirus spread in affected regions. One of the vector control strategies is the use of products to ward off mosquitoes, the most common being the application of synthetic products, such as N,N-diethyl-meta-toluamide (DEET), but the continuous and indiscriminate use has led to the development of chemical resistance, environmental impacts, and unwanted effects on non-target organisms, including humans (SANTOS *et al.* 2021). The use of plant-derived natural products for controlling mosquito vectors offers attractive characteristics, such as biodegradability, low toxicity, and greater selectivity (DEHAIBES *et al.* 2023).

The *Azadirachta indica* plant A. Juss (neem) is a tree that predominates in regions of tropical and subtropical climates, easily and action different insects (FUNDAÇÃO JOAQUIM NABUCO, 2021). Neem is among the most studied biopesticides due to its toxic effects on pests and compounds that promote insecticidal and antifeedant activity, making it a potential alternative to synthetic compounds (DEHAIBES *et al.* 2023). However, evaluating different plant parts extracted by different methods across studies provides a more in-depth analysis of the botanical sample's potential, since the extraction method influences the yields of therapeutic and toxic compounds.

The present study aims to analyze the toxic potential and repellency of *A. indica* oil extracted from the seeds in a 1:1 hexane: methanol solvent (v:v) via bath-ultrasound and maceration (1 and 24 hours, respectively); and of the dried crude extract of the leaves obtained in ethanol: water 8:2 (v:v), via bath-ultrasound (2 hours), later resuspended in dichloromethane: methanol 1:1 (v:v) and dry.

METHODOLOGIES

EXTRACTION

The leaves and fruits of *Azadirachta indica* were collected in the municipality of Barra do Garças, state of Mato Grosso, Brazil, at geographic coordinates 15.899390° S - 52.2276279° W. The leaves were washed to remove impurities and dried in an oven at 40 °C ± 5 °C for 7 days, and the fruits were pulped to obtain the seeds, dried in an oven at 40 °C ± 5 °C for 48 hours; subsequently, they were manually hulled, and the seeds were dried at 40 °C ± 5 °C. Both samples were ground in an electric grinder and stored in airtight containers. The powdered leaves were extracted in a solvent mixture at a 1:3 ratio (sample: solvent), using 100 mL of P.A. ethanol and distilled water in an 8:2 ratio (v:v) as the extraction solvent. The mixture was homogenized and subjected to extraction in an ultrasonic bath (SolidSteel® model SSBu, 40 kHz, 35 °C) for two hours. Subsequently, it was filtered under vacuum using filter paper, and the filtrate was subjected to solvent evaporation in a rotary evaporator (Fisatom® model 802) at 60 °C ± 5 °C, at 7 rpm. The concentrated extract was dried in an oven at 45 °C ± 5 °C until the solvent evaporated, resuspended in a 1:1 (v:v) mixture of analytical-grade dichloromethane and methanol, and dried to constant weight. The pulverized seeds were

extracted using a solvent mixture in a 1:15 ratio (sample: solvent), with 300 mL of a 1:1 (v/v) mixture of hexane and analytical-grade methanol as the extraction solvent. The mixture was homogenized and subjected to an ultrasonic bath for 60 minutes. Afterward, it was allowed to stand for 24 hours, and vacuum filtration was performed. The solvent was removed in a rotary evaporator at 40 °C ± 5 °C at 7 rpm. The solvent residue was dried in an oven at 45 °C ± 5 °C until a constant weight was reached.

ANALYSIS OF TOXIC ACTIVITY

The evaluation of acute toxicity was performed by the aquatic model of *Artemia salina Leach*, as described by MEYER *et al.* (1982), with support from NAGANO & BATALINI (2021). The eggs were incubated for 24 h in an aqueous solution of 0.037 g mL^{-1} sea salt, with constant illumination in a 40 W lamp, and at room temperature (28 ± 2 °C) for the hatching of the nauplii. The ethanolic solution of *A. indica* oil stock was prepared at a concentration of 5,000.0 $\mu g\ mL^{-1}$ and from this, aliquots were transferred to test tubes in the volumes of 25.0 to 2000.0 µL. The tubes were stored in an oven at 40 ºC ± 5 °C for 24 h to allow solvent evaporation. Before the complete drying of the tubes with oil samples, the stock solution of the crude extract of the leaves of *A. indica* was prepared at a concentration of 5000.0 $\mu g\ mL^{-1}$, using the aqueous solution of sea salt (0.037 g mL^{-1}) as a solvent, and aliquots were also transferred to test tubes in the same volumes (25.0 to 2,000.0 µL). After 24 h of drying, 100 µL of dimethyl sulfoxide (DMSO) was added to each tube, and an additional 2.0 mL of aqueous sea salt solution was added to both samples, which were then homogenized in an ultrasound bath for 5 minutes. Subsequently, all tubes received 10 live nauplii of *A. salina* and the volume of the tubes was completed until reaching the final volume of 5.0 mL, obtaining dilutions at concentrations of 25.0, 50.0, 100.0, 300.0, 500.0, 700.0, 1000.0, 1300.0, 1600.0 and 2000.0 μg mL^{-1}. The control tubes used were with aqueous solution of sea salt and with 2% DMSO, diluted in the same saline solution. All test tubes were conditioned under constant lighting at room temperature. The assay was performed in triplicate, both in the tests and in the controls. Surviving nauplii were counted after 24 and 48 h. The Mean Lethal Concentration (LC_{50}) was calculated from the nonlinear regression curve of the percentage of deaths and the logarithm of the doses tested. The toxicity classification was based on NGUTA *et al.* (2011) and COSTA *et al.* (2022), being considered highly toxic for LC_{50} < 100.0 $\mu g\ mL^{-1}$, moderately toxic CL_{50} between 100.0 and 500.0 $\mu g\ mL^{-1}$ weakly toxic for LC_{50} between 500.0 $\mu g\ mL^{-1}$ and 1,000.0 $\mu g\ mL^{-1}$ and non-toxic for LC_{50} > 1,000.0 $\mu g\ mL^{-1}$. The death results in the control sample were corrected by Abbott's formula (1925), as per Equation (1).

$$\text{Mortality (\%)} = \frac{\text{test} - \text{control}}{100 - \text{control}} \cdot 100 \quad (1)$$

EVALUATION OF REPELLENT POTENTIAL

The analysis of the potential repellent effect of the dried crude leaf extract and the fixed oil of *A. indica* was carried out according to the tests described by MCDONALD, GUY, & SPEIRS (1970) and TALUKDER & HOWSE (1994), with adaptations. The repellency test was performed using insects of the species *Euschistus heros,* obtained from the MT Foundation in Rondonópolis, Mato Grosso (MT). Solutions were prepared at concentrations of 1000.0 to 10000.0 $\mu g\ mL^{-1}$, diluted in analytical grade solvents: DMSO for the crude dry extract of the leaves and acetone:DMSO: ethanol with surfactant Tween® 20 for the oil. The assay was performed in glass Petri dishes (9.5 cm in diameter), lined with aluminum foil cut to the same diameter. Filter papers of the same diameter were cut in half, half of which were treated with 0.5 mL of the appropriate dose, and the other half (negative control) received 0.5 mL of the solvent used. The filter papers were dried at room temperature for 15 min to completely evaporate the solvent, then placed in petri dishes. A total of 100 *E. heros* nymphs were used in each sample, with n = 20 for each concentration, distributed among four Petri dishes (n = 5). The total number of insects present in the treated halves and in the control of the filter papers was counted and recorded after 15 min, 30 min, 1, 2, 3, 4, and 5 h of exposure. The percentage of repellency (PR) was calculated by assigning different repellency classes using the scale in Table 1 and Equation (2), where PR is the percentage of repellency, N_t is the number of insects present in the treated area after exposure, and N_c is the number of insects present in the untreated area after exposure (LUKER, 2024; LOPEZ *et al.* 2018).

$$PR = \frac{N_c - N_t}{N_c + N_t} \cdot 100 \quad (2)$$

Table 1 - Repellency classification according to Repellency Percentage (PR).

Class 0	PR < 0.1%	Not repelente
Class I	PR = 0.1 - 20.0%	Very weak repelente
Class II	PR = 20.1 - 40.0%	Weak repelente
Class III	PR = 40.1 - 60.0%	Moderate repellent
Class IV	PR = 60.1 - 80.0%	Repellent
Class V	PR = 80.1 - 100.0%	Very repelente

Source: MCDONALD, GUY e SPEIRS (1970).

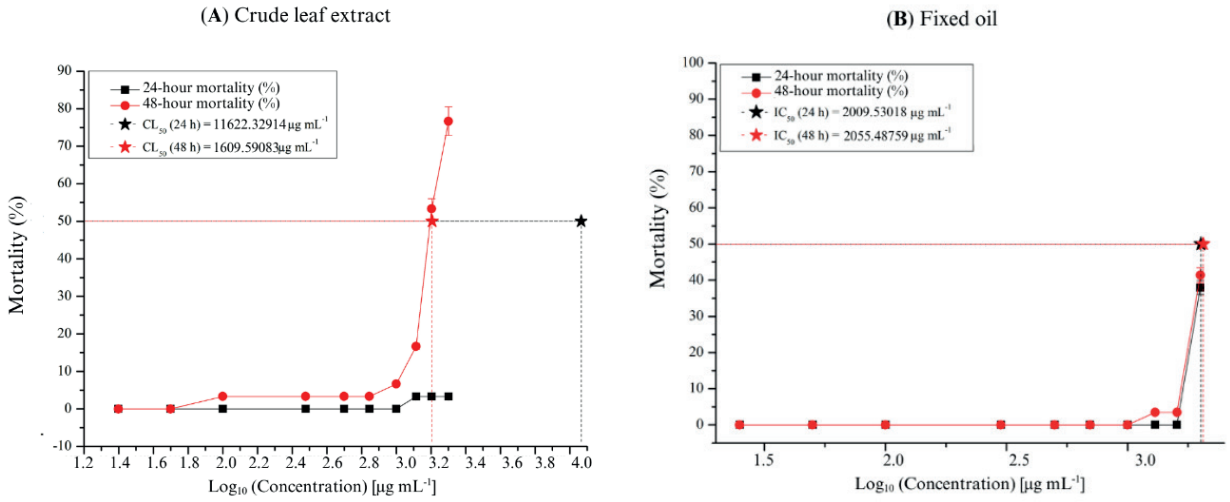
RESULTS AND DISCUSSION

ANALYSIS OF TOXIC ACTIVITY

The result of the percentage of mortality of *A. salina* exposed to the doses of crude extract of the leaves and to the fixed oil of *A. indica* is shown in Graph 1 and Table 2. In the 24-h period of exposure to the extract, the dose-response logistic regression model showed $R^2 = 0.80$, the LC_{50} was

estimated at 11,622.4 $\mu g\ mL^{-1}$ and the mean mortality rate was 1%. The data violated the assumption of normality (Shapiro-Wilk, $p < 0.0001$), and there were no significant differences between doses (Student's t, $p > 0.05$), as confirmed by a nonparametric test (W= 0.1489). In the 48-h period (extract), the adjustment was $R^2 = 0.99$, the LC_{50} was estimated at 1,609.6 $\mu g\ mL^{-1}$ and the mortality rate was 16.7%. The data did not meet the normality assumption (Shapiro-Wilk, $p < 0.0003$), and the doses did not differ significantly (Student's t, $p > 0.05$), but the non-parametric Wilcoxon test revealed a significant difference ($p = 0.0131$). The exposure of the extract sample, for the period of 24 and 48 h, was classified as non-toxic ($LC_{50} > 1000.0\ \mu g\ mL^{-1}$, according to NGUTA *et al.* (2011).

Graph 1 - Mortality curve of *A. salina* exposed to crude leaf extract (A) and fixed oil of *A. indica*.



Source: Prepared by the author.

Table 2 - Mortality of *A. salina* exposed to crude leaf extract and fixed oil of *A. indica* at 24 and 48 hours.

Concentration (µg mL⁻¹)	Mortality (%)			
	Crude leaf extract		Fixed oil	
	24 h	48 h	24 h	48 h
25.0	0.0	0.0	0.0	0.0
50.0	0.0	0.0	0.0	0.0
100.0	0.0	3.4	0.0	0.0
300.0	0.0	3.4	0.0	0.0
500.0	0.0	3.4	0.0	0.0
700.0	0.0	3.4	0.0	0.0
1000.0	0.0	6.7	0.0	0.0
1300.0	3.4	16.7	0.0	3.5
1600.0	3.4	53.4	0.0	3.5
2000.0	3.4	76.7	37.9	41.4

Source: Prepared by the author.

The analysis of variance of the percentage of mortality of the extract confirmed that there were no significant differences between the periods (ANOVA, $p = 0.944$) and the doses (ANOVA, $p = 0.5247$), but they violate the assumption of normality (Shapiro-Wilk, $p < 0.05$). However, the

significant difference between the Wilcoxon test (W > 0.8) and the result of the two-factor analysis of covariance (concentration and period), which does not violate the assumption of normality of the data (Shapiro-Wilk, p = 0.3134) shows that there is toxicity, but this dose-dependent effect is low (ANCOVA, p = 0.3830). The data suggest a dose-response relationship with time (ANCOVA, p = 0.0803), as evidenced by a decrease in the LC_{50} value and p-value, which reinforces the assumption that the observed toxic effect is cumulative with long periods of exposure.

The 24-h mortality rate in the oil dose assay did not converge efficiently in the dose-response non-linear regression model, likely due to the low percentage of deaths at most concentrations tested. Still, the mean mortality rate was 3.8% and the preliminary adjustment estimated a LC_{50} value of 2009.3 $\mu g\ mL^{-1}$, which should be interpreted with caution. The data violated the assumption of normality (Shapiro-Wilk, p < 0.0001), and the doses did not show significant differences (Student's t, p = 0.3434), as confirmed by the nonparametric test (W = 1.00). In the 48-h period, the dose-response model showed an adjustment of $R^2 = 0.99$, an estimated LC_{50} of 2,055.5 $\mu g\ mL^{-1}$, and the mean mortality rate remained low (4.9%). No significant differences were detected between doses (Student's t, p = 0.2678), but the data distribution indicated a violation of normality (Shapiro-Wilk, p < 0.0001), as confirmed by a non-parametric test (Wilcoxon, p = 0.1736). The oil sample was also classified as non-toxic, to NGUTA *et al.* (2011).

The analysis of variance of the percentage of toxicity of the oil detected significant differences among doses (Fisher) with homogeneous variances (ANOVA, p < 0.0001), but not among periods (ANOVA, p = 0.8549). The data violate the assumption of normality (Shapiro-Wilk, p ≈ 0.0001), but the nonparametric test suggests a greater difference between doses (Wilcoxon, 0.73608) than between periods (W = 0.41663). However, the existence of a low p-value, confirmed by the Wilcoxon test, and the slight increase in the mean mortality rate, with increasing time, suggests that the effect of mortality is notable only at high concentrations, but that the period of exposure does not influence so much - this is observed in the Tukey test, due to the significant difference between the concentration of 2000.0 $\mu g\ mL^{-1}$ and the others (p < 0.0001). The analysis of covariance confirms a stronger association between toxicity and dose (ANCOVA, p < 0.0001) and a smaller effect of time (ANCOVA, p = 0.811), indicating that the slight toxic effect is observed only at high doses, without a significant increase in toxicity with longer exposure periods.

The comparison of the percentage of toxicity between the samples did not show significant differences between the extract in the period of 24 h and the oil in 24 and 48 h (Student's t p > 0.5), whose data showed violation of the assumption of normality (Shapiro-Wilk, p < 0.0001) - although the non-parametric test suggested a difference (W > 0.3) the Tukey test, of the covariances confirm that there are no significant differences (p ≈ 0.9). No significant differences were detected between the extract, in the period of 48 h (extract), and the oil in 24 and 48 h (Student's t > 0.05), whose normality test suggests violation of the assumption (Shapiro-Wilk, p = 0.001) - the Wilcoxon test also suggests a difference (W > 0.7), but Tukey's p-value confirms that there are no significant differences (p > 0.1).

The analysis of the one-factor variance of the samples over the 24-h period did not show a significant difference (ANOVA, $p = 0.3171$), and the data violated the assumption of normality (Shapiro-Wilk, $p < 0.0001$); however, the non-parametric test suggested differences ($W = 0.54947$). In the 48-h period, the analysis of variance of the samples showed significant differences ($p = 0.286$), but the data suggest a violation of the assumption of normality (Shapiro-Wilk, $p = 0.0063$) - but the Wilcoxon test suggests a difference and normality ($W = 0.85419$). However, the multifactorial analysis of variance between doses across both periods revealed a significant difference in mortality rate as a function of concentration (ANOVA, $p = 0.0016$) and time (ANOVA, $p = 0.0315$), but the data violated the assumption of normality (Shapiro-Wilk, $p < 0.0001$).

Tukey's test did not indicate significant differences in the 24-h period, but the 48-h exposure resulted in significant differences between the samples at concentrations of 2000.0 and those from 25.0 to 700.0 $\mu g\ mL^{-1}$. The analysis of covariance confirms that the variation in concentration significantly affects the toxic potential of the samples (ANCOVA, $p < 0.0001$) and has a significant effect with the period set at 24 and 48 h (ANCOVA, $p = 0.390$) - but the covariation of the periods with the fixed doses suggests that the toxicity has a difference only between the doses (ANCOVA, $p = 0.0098$) and time had little influence (ANCOVA, $p = 0.628$), which demonstrates that assays to evaluate chronic toxicity can obtain similar results in these samples, evidencing their biological safety.

The literature corroborates the positive and superior effect of the extract and oil samples analyzed in this study. NAGANO & BATALINI (2021) reported LC_{50} of 596.7 $\mu g\ mL^{-1}$, for the crude hydroethanolic extract of *A. indica* leaves (extracted by maceration, with occasional shaking, 40 days), exposed for 24 h in *A. salina*, while in this study the LC_{50}, for the same period of exposure was 11622.4 $\mu g\ mL^{-1}$, for the leaf extract. The LC_{50} value for the 48-h exposure period also shows a lower Mean Lethal Concentration for the extract (1609.6 $\mu g\ mL^{-1}$, - which highlights the low toxic potential for the sample evaluated, even in the face of exposure for long periods), which may have been influenced by the choice of the extractive method.

In the study carried out by MORAES *et al.* (2019) the results show a dose-response pattern, where the increase in the mortality rate is proportional to the growth of concentrations, which showed LC_{50} of 14.3 $\mu g\ mL^{-1}$, for the assay with doses between 0.1 and 70.0 $\mu g\ mL^{-1}$, exposed for 24 hours - classifying high toxicity of the essential oil. While in this work the fixed oil presented LC_{50} of 2009.3 $\mu g\ mL^{-1}$, for the exposure period of 24 h and of 2055.5 $\mu g\ mL^{-1}$, for 48 h - where it presents a constant response pattern at the lower concentrations. The data suggest that the use of organic solvents in the extraction resulted in a sample with less toxic potential.

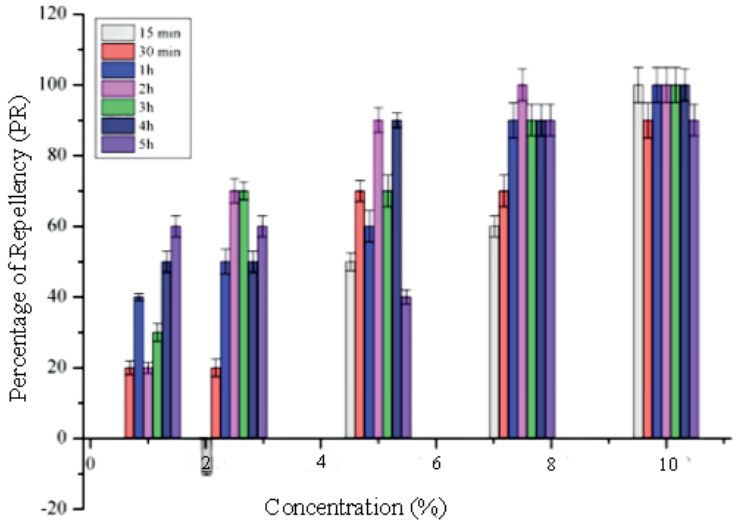
EVALUATION OF REPELLENT POTENTIAL

The Percentage of Repellency (PR) for *E. heros* exposed to *A. indica* oil is shown in Graph 2 and Table 3. Analysis of variance (ANOVA) indicated significant differences among concentrations

($p < 0.0001$) but not over time ($p = 0.4920$). The data did not indicate a violation of the normality assumption over time ($p = 0.1227$). Tukey's test showed differences between the 1.0% and 5.0% doses, between the 2.5% and 7.5% and 10.0% doses, and between the 5.0% and 10.0% doses. Analysis of covariance confirmed the difference between doses ($p < 0.00001$) and demonstrated a relationship between effect and time ($p = 0.0086$).

The nonlinear regression analysis of the oil, of the $\log_{10}$ of the concentration under the different times, showed an adjustment of $R^2 = 0.90$ and Mean Effective Concentration (EC_{50}) of 5.6% and EC_{90} = 10.0%. The Effective Concentration of 90% increases to 12.5% in 30 minutes of exposure, to 13.5% in 1 hour of exposure, and after that period decreases to 4.4% (2 h), 8.1% (3 h), 8.6% (4 h). At 5 hours, the model does not converge, but raw data up to 4 hours of exposure suggest that the 10% dose is efficient, with a period of diminishing effect only in the initial 30 minutes of exposure. The formulation of a repellent for human use would indicate that protection begins after 30 minutes and that the product should be reapplied after 5 hours. The oil was considered highly repellent at a 10.0% dose, according to the classification of MCDONALD, GUY, & SPEIRS (1970).

Graph 2 - Bar graph of the Percentage of Repellency (PR) of *E. heros* exposed to the fixed oil of *A. indica*.



Source: Prepared by the author.

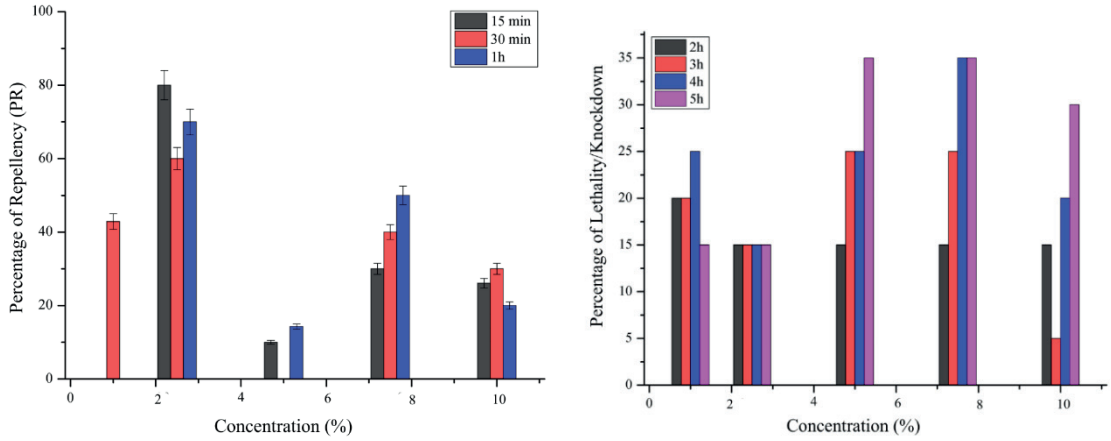
Table 3 - Percentage of Repellency of *E. heros* exposed to the fixed oil of *A. indica*.

Concentration (%)	Percentage of Repellency - PR (%)						
	15 min	30 min	1 h	2 h	3 h	4 h	5 h
1.0	0.0	20.0	40.0	20.0	30.0	50.0	60.0
2.5	-10.0	20.0	50.0	70.0	70.0	50.0	60.0
5.0	50.0	70.0	60.0	90.0	70.0	90.0	40.0
7.5	60.0	70.0	90.0	100.0	90.0	90.0	90.0
10.0	100.0	90.0	100.0	100.0	100.0	100.0	90.0

Source: Prepared by the author.

The Percentage of Repellency and Lethality/Knockdown of *E. heros* exposed to the crude extract of *A. indica* leaves is shown in Graph 3 and Table 4. Analysis of ANOVA indicated significant differences among doses ($p = 0.0024$) across the 15, 30, and 60 min exposure periods, but time was not a significant factor ($p > 0.05$). The data did not violate the assumption of normality (Shapiro-Wilk, $p > 0.2$). Tukey's test revealed a difference between the 2.5% concentration and the 1.0, 5.0, and 10.0% concentrations ($p < 0.05$). Analysis of ANCOVA confirmed the relationship between effect and dose ($p < 0.005$), but the model did not indicate a time-dependent effect ($p = 0.7971$). The nonlinear regression analysis in the dose-response model did not show an efficient fit, with $R^2 < 0.1$ across all repellency periods. The extract was considered repellent at a dose of 2.5%, according to the classification of MCDONALD, GUY, & SPEIRS (1970).

Graph 3 - Bar graph showing the percentage of Repellency and Lethality/Knockdown of *E. heros* exposed to the crude extract of *A. indica* leaves.



Source: Prepared by the author.

Table 4 - Percentage of Repellency and Lethality/Knockdown of *E. heros* exposed to the crude extract of *A. indica* leaves.

Concentration (%)	Percentage of Repellency (%)			Percentage of Lethality/Knockdown (%)			
	15 min	30 min	1 h	2 h	3 h	4 h	5 h
1.0	0.0	42.9	-10.0	20.0	20.0	25.0	15.0
2.5	80.0	60.0	70.0	15.0	15.0	15.0	15.0
5.0	10.0	0.0	14.3	15.0	25.0	25.0	35.0
7.5	30.0	40.0	50.0	15.0	25.0	35.0	35.0
10.0	26.1	30.0	20.0	15.0	5.0	20.0	30.0

Source: Prepared by the author.

The data from 2 hours of exposure to the extract showed immobility and the immobile insects were accounted for, but the term "knockdown" was used in parallel with lethality to define these results, because at the end of the count and joining the insects for disposal, it was observed that when they were removed from the test environment with the extract, returned to moving normally,

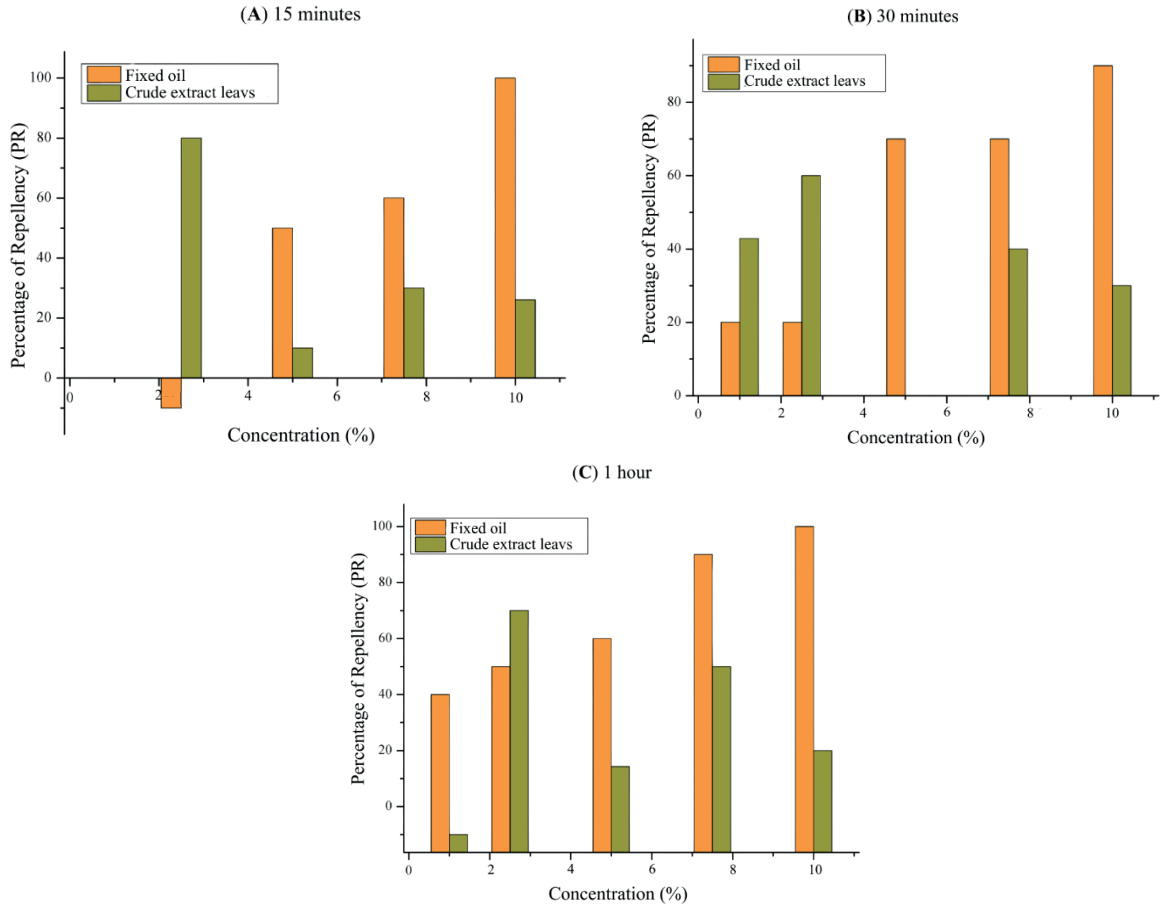
suggesting a temporary paralysis effect, but some units of *E. heros* die - this information is important data for future investigations on the potential knockdown effect of the crude extract sample of *A. indica* leaves.

Analysis of ANOVA for lethality or knockdown in the extract sample did not reveal significant differences with respect to concentration ($p = 0.1633$) or exposure periods of 2 to 5 hours ($p = 0.1656$); the data did not violate the assumption of normality (Shapiro > 0.1). ANCOVA analysis of covariance confirmed no difference in effect between doses ($p = 0.0633$) but indicated a time-dependent relationship ($p = 0.0108$). The Shapiro-Wilk test did not indicate a violation of normality ($p = 0.975$). Although the data do not indicate a significant difference between the concentrations of the extract, in terms of lethality/knockdown, the raw data demonstrate that the concentration of 2.5% presented consistent and low results over time, as well as the highest PR, even if the effect decreases over time, being a relevant concentration to be better evaluated in pharmaceutical matrices. The nonlinear regression model of lethality or knockdown dose-response showed an adjusted R^2 of 0.71 and a 20% Lethal Concentration (LC_{20}) of 0.6%, indicating that extremely low doses (close to 0%) are required for a 50% effect. Over 3 to 4 hours, the model adjustment was inefficient, with $R^2 < 0.1$ and PLK (%) at extremely low levels, close to 0%. After over 5 hours of exposure, the model fit was $R2 = 0.70$, with an estimated LC_{50} value of 32.6% and an LC_{80} of 49%.

The data suggest that the crude leaf extract evaluated is toxic at long exposure durations, even at low doses, making it ideal for use as an insecticide and potentially effective at knockdown. For the human pharmaceutical application of this plant material, as a repellent, the concentration of 2.5% becomes interesting to be better evaluated, due to the low and constant lethality of insects, and for having presented the best percentage of repellency, since the toxicity test in the *A. salina* model guarantees safety for human use, where the atoxicity of the sample was evidenced.

The results of the different portions extracted from *A. indica* were compared during exposure periods up to 1 h according to Graph 4. Time covariation indicated a significant relationship between the concentrations evaluated in the different samples (ANCOVA, $p < 0.005$). The Shapiro-Wilk test indicated no violation of the normality assumptions ($p = 0.4769$). Tukey's test detected a significant difference between the doses of 1.0% of oil and 2.5% of extract ($p = 0.0269$); between 1.0% of the extract and those of 5.0 ($p = 0.0311$), 7.5 ($p = 0.0036$) and 10.0% ($p < 0.0001$) of the oil; between 2.5% of the oil and 2.5% of the extract ($p = 0.0269$); between 5.0 % of the extract and 5.0 ($p = 0.0198$), 7.5 ($p = 0.0022$) and 10.0 % ($p < 0.0001$) of the oil; between 7.5% of the oil and 10.0% of the extract ($p = 0.0371$); and between 10.0 % of the oil and 7.5 ($p = 0.0092$) and 10.0 % of the extract ($p = 0.0008$). The non-significant difference between the 2.5% dose of the extract and the 10.0% dose of the oil indicates that they may be equivalent in repellency effect, and pharmaceutical formulations with only half the oil dose (5.0%) could have the effect compensated with half the extract dose (1.25%) - if the samples are compatible with the excipients of the chosen dosage form.

Gaph 4 - Bar graph of the Percentage of Repellency (PR) of *E. heros* exposed to fixed oil and crude leaf extract of *A. indica*, over periods of 15 min, 30 min, and 1 hour.



Source: Prepared by the author.

Studies on the repellency of *A. indica* oil, extracted by Soxhlet, in hexane solvent, carried out by AVINNDE, MORAKINYO, & SRIDHAR (2020) show efficacy with a PR of 100% at a dose of 10.0% at 0, but the effect decreased to 96.19% in 1 h of exposure, to 92.85% in 4 h. These data demonstrate the superior results obtained by this study, with oil obtained by the ultrasonic bath method and maceration using a hexane: ethanol solvent, which showed 100% PR during the first analysis (15 min) and between 1 and 4 hours of exposure. When compared to the extract sample, despite not reaching the mean repellency of 50% at the 10% dose, the concentration of 2.5% presented a profile similar to that of the authors' study, with a decrease in PR over time, with a repellency of 80% in the initial time, which decreased to 70% in 1 h of exposure.

Finally, very low concentrations of *Azadirachta indica* extracts appear to act as insect attractants, given their low repellency. This result is not associated with a limitation of the method, but may be linked to the various secondary metabolites present in the extract; at low concentrations, these extracts or oils would not possess repellent bioactive compounds strong enough to overcome the attractants present, which also justifies the "knockdown" concept, since the insects, despite reaching lethality when exposed to the leaf extract, underwent a period of temporary paralysis that could be

mistaken for lethality. This effect suggests that there are insecticidal and repellent bioactive compounds that act over time, but the synergy with other phytochemicals may modify their immediate lethal action.

CONCLUSION

The toxicity analysis using a nauplius model of *A. salina* found that both the crude leaf extract and the fixed oil of *A. indica* were non-toxic, indicating these samples are safe for use in pharmaceutical formulations. The test with the *E. heros* model characterized the tested oil sample as very repellent at a dose of 10% (v/v), with medium to moderate repellency at lower concentrations (5.0 to 10.0%), but very low concentrations can lead to ineffective repellency and even insect attraction (2.5% in 15 min). The leaf extract showed variable effects: the effective dose was 2.5%, but it had lower repellency than the oil sample. Based on the results of the two trials, it is suggested that repellents for human use, formulated from these samples under the same extraction conditions and at 10.0% (oil) and 2.5% (leaf extract), could be a safe alternative to synthetic repellents. The resulting products would be non-toxic to humans and low-toxic to insects, ensuring an effect without causing environmental imbalance across the entire ecosystem. Although *Artemia salina* assays serve as a useful preliminary bioindicator of toxicity, future work requires dermatological testing (skin irritation) to reinforce the safety of topical use in humans, as well as an assessment of absorption using printed biomembranes.

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