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Evaluation of the Toxicity of *Calotropis procera* Ait Leaf Extracts on *Aedes aegypti* (L) Larvae

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ABSTRACT

The study investigated the toxic effects of ethanol, aqueous, and hexane extracts of *Calotropis procera* Ait on *Aedes aegypti* (L) larvae. Leaf samples were collected in Riyadh, and then transported to the Department of Biology at the College of Science, Imam Mohammad Ibn Saud Islamic University, where they were manually cleaned and dried at room temperature in the shade. A phytochemical screening was performed to identify the chemical components of the plant according to the AOAC (1990) guidelines. Bioassay tests were conducted in a controlled environment with a temperature of 27 ± 2 °C, relative humidity of 75–85%, and a 12-hour light/dark cycle. The larvicidal activity of the plant extracts was evaluated using the methodology recommended by WHO (1996). Lethal concentrations (LC50 and LC95) were determined through regression analysis to assess larval mortality within 24 hours. The phytochemical analysis revealed the presence of tannins, cardiac glycosides, flavonoids, phenols, alkaloids, and terpenoids, while steroids and saponins were absent. Based on LC50 and LC95 values, the hexane extract showed the highest larvicidal potential (0.00250 ppm), followed by the ethanol extract (0.00251 ppm) and the aqueous extract (0.0028 ppm). These results indicate that *Calotropis procera* possesses strong larvicidal properties and can be used as an eco-friendly alternative for the control of *Aedes aegypti* larvae.

Keywords: *Aedes aegypti*, (*Colotropis procera* Ait), Lethal effect, Leaves extracts

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Introduction

Aedes aegypti (L) is a mosquito species that thrives in human environments and serves as a primary vector for various viral diseases globally, including dengue, yellow fever, chikungunya, and Zika [1]. In the 21st century, this mosquito was responsible for dengue outbreaks in Yemen and Saudi Arabia. The epidemiology of dengue and the likelihood of outbreaks are closely linked to climatic factors, particularly temperature and rainfall, as well as the abundance of the vector population. Effective control strategies require a comprehensive understanding of the vector's ecological dynamics and phylogenetic history. The evolutionary background of *Aedes aegypti* in the Arabian Peninsula, a transitional region between the Afrotropical, Palaearctic, and Oriental biogeographical zones, plays a crucial role in shaping its distribution. Strengthening knowledge about dengue transmission is essential for implementing effective prevention measures at both national and global levels [2]. In Saudi Arabia, mosquito-borne diseases pose a significant public health challenge, necessitating robust vector surveillance and management. A total of 51 mosquito species have been documented in the Kingdom; however, the presence of two remains uncertain, leaving 49 confirmed species, comprising eighteen anophelines and 31 culicines [3]. Since 1994, dengue fever (DF) cases have surged across Saudi Arabia, largely due to environmental and demographic conditions that favor its spread. Despite efforts to control Aedes mosquitoes, the primary carriers of the dengue virus (DENV), current strategies remain insufficient. In Makkah Al-Mokarramah, KSA, pinpointing the natural distribution of Aedes species is crucial for implementing targeted control measures [4, 5]. Research indicates that wild *Aedes aegypti* larvae in Makkah exhibit greater tolerance to insecticides than laboratory-reared larvae, suggesting an increasing resistance to widely used chemical treatments [6]. This growing resistance is not an isolated issue but part of a broader global challenge [7]. Dengue fever remains a major public health concern, primarily transmitted by Aedes mosquitoes. Existing vector control measures have proven ineffective in significantly reducing Aedes populations, highlighting the urgent need for alternative strategies to curb dengue transmission in different environments [8]. Biological control (biocontrol) relies on an organism's natural predators, parasites, or pathogens to regulate its population. Various biological control agents targeting mosquitoes have been identified and implemented worldwide [9, 10]. Focusing on eliminating mosquito larvae or restricting their access to breeding sites is a logical approach, as larvae are easier to eradicate while they remain confined to water, which is a manageable and contained habitat [11]. Targeting the larval stage remains the most effective approach for mosquito control. However, successfully managing larvae may not necessarily lead to a decrease in mosquito populations or biting frequency [12]. *Calotropis procera* Ait, commonly known as giant milkweed, thrives in open environments with minimal competition. Overgrazed rangelands and pastures often provide suitable conditions for its growth, along with disturbed urban areas, coastal dunes, and roadsides [13]. The plant's roots have been traditionally used to treat various ailments, including malaria, fever, leprosy, and snake bites. Despite its medicinal properties, the latex can cause blisters and skin irritation in sensitive individuals. Additionally, it serves as an important host plant for butterflies [14]. The active compounds identified belong to the class of cardio-glycosides. Cardiac glycosides are a significant group of natural substances known for their dual effects on the heart, both therapeutic and toxic. The use of plant-derived cardiac steroids dates back to 1500 B.C., serving various purposes such as heart medications, poisons, arrow toxins, emetics, and diuretics [15]. Natural compounds and biological agents offer promising alternatives for mosquito control and mitigating the health risks they pose. The secondary metabolic pathways in plants produce a diverse array of bioactive molecules, many of which have been utilized based on traditional medicinal knowledge. Often, there is a strong correlation between crude extracts historically used in traditional medicine and the purified compounds currently studied for their pharmacological potential. Plants remain a valuable source of novel bioactive substances, with many antifungal and antimicrobial agents still derived from botanical sources. Despite advancements in antimicrobial treatments, challenges persist with many conventional drugs [16]. There has been significant progress in developing plant-based products to target adult mosquitoes and other arthropods of medical and veterinary importance, such as ticks and lice. However, the development of botanical larvicides remains limited. This highlights the need for more research on natural alternatives to traditional chemical control methods [17]. Various natural substances have been proposed as viable alternatives [18]. Calotropis, known for its medicinal uses, includes around 175-180 genera and 2200 species across tropical and subtropical regions, many of which contain bioactive molecules. This genus consists of six species of small trees or shrubs found in Asia, North America, and tropical/subtropical Africa. In India, *C. gigantea* and *C. procera* are similar in both structure and function [19]. The primary aim of the study was to evaluate the lethal impact of aqueous, ethanol, and hexane extracts from *Calotropis procera* Ait on *Aedes aegypti* (L) larvae.

Materials and Methods

Plant collection

The leaves were collected from the Riyadh area and transported to the Biology Department's laboratory at Imam Mohammad Ibn Saud Islamic University. After manual cleaning, the leaves were dried in the shade at 27 °C. The dried leaves of *Calotropis procera* Ait were then blended for five minutes to create a powder, which was stored until required for use.

Phytochemical screening of the extract

A preliminary phytochemical analysis of *Calotropis procera* Ait leaves was conducted to determine the chemical components. The screening for tannins, flavonoids, alkaloids, cardiac glycosides, phenols, saponins, terpenoids, and steroids followed the method outlined by AOAC [20].

Extract preparation

Aqueous extract preparation

A portion of 20 g of powdered plant material was measured and mixed with one hundred milliliters of distilled water, then allowed to stand overnight. After 24 hours, the mixture was blended using a magnetic stirrer and filtered through Whatman No. 42 filter paper. The resulting filtrate was used to prepare the aqueous extract concentration and stored at 5 °C in a refrigerator.

Preparation of ethanol and hexane extracts

The leaf sections of *C. procera* were utilized for the preparation of extracts. A total of 20 g from each sample was combined with 100 milliliters of ethanol and hexane, respectively, and allowed to stand for 24 hours. After this period, the extract from each solvent was separated into the supernatant, filtered, and then evaporated. The resulting residue was collected as a dry powder, weighed, and reconstituted to the original concentration for subsequent use.

Breeding site

Mosquito larvae were gathered in plastic trays filled with tap water from various breeding sites in Riyadh. These larvae were then transported to the Biology Department at the College of Science, Imam Mohammad Ibn Saud Islamic University. Any other mosquito species or aquatic predators unintentionally collected alongside *Aedes aegypti* larvae were promptly removed from the rearing containers. The larvae were typically nourished with pre-prepared fish food, though occasionally, small amounts of yeast granules were used. Once the larvae developed into pupae, they were carefully moved to the adult cage using an appropriate dropper. The pupae were then placed into a similar rearing dish inside the adult cage.

Bioassay tests

The experiments were conducted under a 12-hour light/dark cycle, with a relative humidity of 75–85% and an average temperature of 27 ± 2 °C. The larvicidal effects of *C. procera* leaf extracts were assessed following the WHO (1996) method [21]. In each test, 20 third and early fourth-instar larvae of *Aedes aegypti* were transferred into test cups containing 250 ml of tap water. Different concentrations (1, 1.5, 2, 2.5, 3, and 5 ml) of aqueous, ethanol, and hexane extracts were applied to the water. Each concentration was repeated three times for each extract. A control group was maintained separately and kept alive. After 24 hours, the mortality rate of the larvae was recorded.

Statistical analysis

The mean larval mortality (Y variable) after 24 hours was graphed against the corresponding concentrations (X variable) using regression analysis in Microsoft Excel 2016. Regression lines were generated to calculate the lethal concentrations for 50% and 95% mortality (LC50 and LC95) in *Aedes aegypti* larvae.

Results and Discussion

Phytochemical constituents of C. procera leaves

Table 1 presents the results of the phytochemical analysis, revealing the presence of tannins, cardiac glycosides, flavonoids, phenols, alkaloids, and terpenoids in *C. procera* leaves, while steroids and saponins were not detected.

Table 1. *C. procera* leaf phytochemical constituents

Compound	Leaves
Tannins	+
Saponins	-
Flavonoids	+
Steroids	-
Glycosides	++
Alkaloids	+

Phenols	+
Terpenoids	+

+: present, −: absent

The effect of Aqueous extract preparation of C. procera leaves on Aedes aegypti larvae

The *Aedes aegypti* larvae were exposed to aqueous extract doses of 0.0028, 0.0042, 0.0060, 0.0069, and 0.0080 ppm for 24 hours. Mortality rates at these doses were recorded as 55%, 65%, 75%, 80%, and 85%, respectively **(Table 2)**. The calculated LD50 and LD95 values were 0.0028 ppm and 0.011 ppm, respectively. At the lowest concentration (0.0028 ppm), mortality was observed at 55%, while the highest concentration (0.0080 ppm) resulted in 85% mortality. The regression analysis yielded an R-square value of 0.79, with a standard error for the log dose (SE(Y)) of 1.47 and SE(X) of 0.65. The aqueous extract was found to be toxic to *Aedes aegypti* larvae.

Table 2. The effect of aqueous extract preparation on *Aedes aegypti* larvae

DOSE		MORTALITY %		Probit	
ppm	**Log+3**	**Tested**	**Corrected**	**Tabulated**	**Calculated**
0.0028	0.45	55.0	55.0	5.13	5.00
0.0042	0.63	65. 0	65.0	5.39	5.46
0.0060	0.78	75.0	75.0	5.67	5.85
0.0069	0.84	80.0	80.0	5. 84	6.00
0.0080	0.91	85.0	85.0	6.04	6.18

Control mortality was 0.0% in all cases; regression equation: Y = 11.56 + 2.75X, SE(Y) = 1.47, SE(X) = 0. 65, R-square = 0.79, LD_{50} = 0.0028 ppm, LD $_{95}$ = 0.011 ppm

The effect of ethanol extract preparation of C. procera leaves on Aedes aegypti larvae

The ethanolic extract was tested for its effects on *Aedes aegypti* larvae at concentrations of 0.0011, 0.0017, 0.0022, 0.0028, 0.0032, 0.0038, 0.0048, and 0.0063 ppm. The mortality rates observed at these doses were 20%, 25%, 40%, 45%, 70%, 75%, 80%, and 85%, respectively **(Table 3)**. The calculated LD50 was 0.00251 ml/L, while the LD95 was 0.0096 ppm. At the lowest dose (0.0011 ml/L), 20% mortality was recorded, while the highest concentration (0.0063 ppm) resulted in approximately 85% mortality. The regression analysis provided an R-square value of 0.92.

Table 3. The effect of ethanol extract preparation of *C. procera* leaves on *Aedes aegypti* larvae

DOSE		MORTALITY%		PROBIT	
Ppm	**Log+3**	**Tested**	**Corrected**	**Tabulated**	**Calculated**
0.0011	0.04	20.0	20.0	4.16	3.98
0.0017	0.24	25.0	25.0	4.33	4.54
0.0022	0.35	40.0	40.0	4.75	4.85
0.0028	0.45	45.0	45.0	4.87	5.13
0.0032	0.55	70.0	70.0	5.52	5.30
0.0038	0.57	75.0	75.0	5.67	5.47
0.0048	0.68	80.0	80.0	5.84	5.78
0.0063	0.8	85.0	85.0	6.04	6.12

Control mortality was 0.0% in all cases; regression equation: Y = 12.33 + 2.82X, SE(Y) = 0.81, SE(X) = 0.31, R-square = 0. 92, LD_{50} = 0.0251 ppm, LD $_{95}$ = 0.0096 ppm.

The effect of hexane extract preparation of C. procera leaves on Aedes aegypti larvae

The toxicity of hexane extract on *Aedes aegypti* larvae was assessed at concentrations of 0.0011, 0.0017, 0.0022, 0.0028, 0.0032, 0.0038, 0.0048, and 0.0063 parts per million. The mortality rates at these concentrations were 25%, 35%, 40%, 50%, 55%, 65%, 75%, and 85%, respectively **(Table 4)**. The analysis revealed an LD50 of 0.00250 parts per million and an LD95 of 0.0296 parts per million. The lowest concentration (0.0011 ppm) resulted in 25% mortality, while the highest dose (0.0063 parts per million) caused an 85% mortality rate. The regression analysis showed an R-square value of 0.96. The hexane extract was found to be lethal to *Aedes aegypti* larvae.

Table 4. The effect of hexane extract preparation of *C. procera* leaves on *Aedes aegypti* larvae

Dose		Mortality (%)		Probit	
ppm	Log +3	Tested	Corrected	Tabulated	Calculated
0.0011	0.04	25.0	25.0	4.33	4.17
0.0017	0.24	35.0	35.0	4.61	4.62
0.0022	0.35	40.0	40.0	4.75	4.87
0.0028	0.45	50.0	50.0	5.00	5.10
0.0032	0.55	55.0	55.0	5.13	5.24
0.0038	0.57	65.0	65.0	5.39	5.38
0.0048	0.68	75.0	75.0	5.67	5.63
0.0063	0.8	85.0	85	6.04	5.90

Control mortality was 0.0% in all cases; regression equation: Y = 10.93 + 2.28X, SE(Y) = 0.47, SE(X) = 0.18, R-square = 0. 96, LD_{50} = 0.0251 ppm, LD_{95} = 0.0296 ppm.

Effective relatives of Calotropis procera preparation according to LD50

The analysis of LC50 and LC95 values indicated that the hexane extract demonstrated the strongest larvicidal effect against *Aedes aegypti* larvae, with a value of 0.00250 ppm, while the ethanol extract (0.00251 parts per million) and aqueous extract (0.0028 parts per million) followed in effectiveness, as illustrated in **Table 5** and **Figure 1**.

Table 5. Effective relatives of *Calotropis procera* preparation according to LD_{50}

Preparation	*Aedes aegypti* larvae	
	LD_{50} /ppm	LD_{95} /ppm
Aqueous extract	0.0028	0.011
Ethanol extract	0.00251	0.0096
Hexane extract	0.00250	0.0296

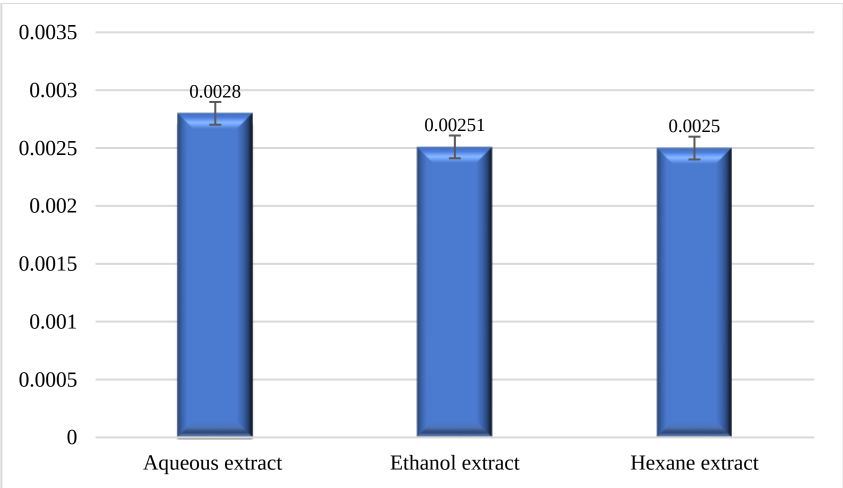

Figure 1. Histograms of the obtained LC_{50} values of all extracts on *Aedes aegypti* larvae

Various control methods, such as insect growth regulators, pesticides, and microbial agents, are frequently used to target mosquito larvae. In addition to these, insecticide-treated bed nets and indoor residual spraying are employed. However, the use of such chemicals can have detrimental effects on the environment and human health and often lead to resistance in mosquito populations [22]. As a result, finding alternative, eco-friendly insecticides is critical. For this study, the plant *Calotropis procera* (Aiton) Dry, native to Saudi Arabia, was chosen for investigation. LC-MS/MS analysis was employed to examine the metabolic content of different extracts of *C. procera*. The cardenolides calactin, uscharidin, 15-hydroxy-calactin, 16-hydroxy-calactin, and 12-hydroxy-calactin were identified as the primary compounds in the leaves of *C. procera* [23]. Secondary metabolites like those produced by *C. procera* have various biological and physiological effects, including deterrent and antifeeding actions [24]. Earlier research, such as the work by Doshi *et al*. [25], also explored the phytochemical and biological properties of *C. procera*, with results comparable to those of the current study. The aqueous leaf extract of *C. procera* was found to be highly toxic to the larvae of mosquitoes *Anopheles arabiensis* and *Culex quinquefasciatus*. The LC50 values for the second, third, and fourth instar larvae were found to be 273.53, 366.44, and 454.99 parts per million for *A. arabiensis* and 187.93, 218.27, and 264.85 parts per million for *C. quinquefasciatus*, respectively [26]. The current study confirms these findings, showing that the aqueous extract of *C. procera* has significant larvicidal potential against *Aedes aegypti* larvae, with an LC50 of 0.0028 ppm. These results suggest that the use of this plant extract could help replace harmful chemical pesticides, offering a natural, environmentally friendly alternative [27]. The fresh leaf extract of *C. procera* has demonstrated larvicidal effects, particularly against mosquito larvae in the Diptera order. However, methanolic extracts from the same plant proved to be more potent as a larvicide [28]. This finding is in line with the present study, which confirms the toxic effects of *C. procera* against mosquito larvae. At a concentration of 0.6 mg/mL, the extract achieved a complete mortality rate of 100% for the L1, L2, and L3 stages of *C. quinquefasciatus*. The LC50 values for these larvae stages were 0.194, 0.251, 0.258, and 0.284 mg/mL for L1, L2, L3, and L4 larvae, respectively, underscoring its potent larvicidal capabilities [29]. These bioassays highlight the potential of *C. procera* as a source of natural alternatives for mosquito control and entomological monitoring [30]. In contrast, hexane extracts were less effective than chloroform extracts from *A. indica* and *D. metal*, which achieved 62% and 87% mortality at a concentration of 1000 ppm, respectively, against late-third instar larvae. These plant extracts could offer alternatives to synthetic insecticides for controlling mosquito populations [31]. Further tests with *C. procera* hexane leaf extract against dengue vectors revealed LC50 and LC90 values of 78.39 parts per million and 100.60 parts per million, respectively. The extract's toxicity increased with longer exposure times, reducing the LC50 value by 2.3%. Additionally, larvae exposed to *C. procera* exhibited faster wriggling and erratic vertical movements [32].

The most effective repellent activity was observed from the leaves of *C. procera*, with a weight loss of 0.034%, indicating its strong anti-termite properties [33]. In our study, however, hexane extracts demonstrated the highest larvicidal efficacy against *Aedes aegypti* larvae, with the most effective concentration being 0.00250 parts per million. This result is consistent with earlier research. Furthermore, the hexane extract from the leaves showed LC30, LC50, and LC90 values of 67, 83, and 140 parts per million, respectively, while the extract from the stem produced LC30, LC50, and LC90 values of 55, 68, and 115 parts per million. The larvae exposed to these extracts showed noticeable damage, such as shrinkage, distortion, and vacuolization of the gut tissues and peritrophic membranes at different lethal concentrations [34]. Additionally, hexanoic and ethanolic extracts from 27 plant species native to Brazil's Cerrado biome were tested for their larvicidal effects on third-stage *Aedes aegypti* larvae at a concentration of 500 micrograms per milliliter. Fourteen of the extracts, sourced from seven species, demonstrated activity against the larvae, causing mortality rates greater than 65%. Some of the active species included *Dugeutia furfuracea*, *Piptocarpha rotundifolia*, *Casearia sylvestris* var. *lingua*, *Serjania lethalis*, and *Xylopia aromatica*, with LC50 values ranging from 56.6 to 384.37 micrograms per milliliter. Other species, like *Annona crassiflora* and *Cybistax antisyphilitica*, had effective LC50 values of 23.06 and 27.61 micrograms per milliliter, respectively. These findings support the potential for isolating active compounds from these species to explore further larvicidal activities [35]. All plant extracts showed larvicidal effects, although there was an important difference between ethanolic and aqueous extracts. Due to their safety, environmental compatibility, and widespread availability, plant-derived insecticides are considered a promising alternative to synthetic chemical insecticides in the future [36]. The use of plant-based insecticides with proven efficacy may reduce the reliance on traditional chemical pesticides [37]. The present study demonstrated that the ethanol extract (0.00251 ppm) exhibited toxicity with larvicidal activity against *Aedes aegypti* larvae, which aligns with findings from previous studies, although variations in LC50 values were observed due to environmental differences. Since ancient times, plant-derived toxic agents have been used as an alternative strategy for mosquito control. These agents have proven effective against various vector mosquitoes due to their inexpensive, non-toxic, biodegradable, and broad-spectrum properties. Numerous studies have focused on phytoconstituent sources and quinone activity, their mechanisms of action on target populations, instar specificity, variations in larvicidal activity across mosquito species, extraction-solvent polarity, the nature of active ingredients, and significant advances in biological mosquito control through plant-derived secondary metabolites [38]. The rise of resistance to synthetic insecticides presents a major challenge to vector control strategies. Plants, rich in bioactive compounds, produce several secondary metabolites that act as defensive chemicals to control insect pests. Compared to synthetic pesticides, plant-based solutions offer several advantages, increasing their preference over chemical insecticides [39]. However, certain components and metabolites have low bioavailability and poor host solubility [40]. Tailoring and directing the most effective material for specific challenges is essential [41]. Insects from the same species in different environments may maintain a broad genetic base, potentially leading to speciation [42]. As a result, mosquitoes have developed resistance to many pesticides, making the discovery of new plant-derived pesticide materials crucial. While synthetic pesticides are an essential tool for pest control, they have detrimental effects on the environment and are incompatible with organic farming practices [43]. Biological data from breeding and field studies are compared and discussed concerning established life-cycle data, which is a useful method for assessing the sensitivity of plant extracts as insecticides [44, 45].

Conclusion

The results based on the LC50 and LC95 values revealed that the hexane extract of *C. procera* exhibited superior larvicidal activity against *Aedes aegypti* larvae (0.00250 parts per million), followed by the ethanol extract (0.00251 parts per million) and aqueous extract (0.0028 parts per million). Due to its high toxicity to *Aedes aegypti* larvae and its eco-friendly nature, *C. procera* has significant potential for large-scale use in mosquito control.

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Conflict of Interest: None

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