Borneo Journal of Resource Science and Technology (2025), 15(1): 97-104
DOI: https:doi.org/10.33736/bjrst.7706.2025

The In Vitro Ovicidal Activity of *Cassia alata* Methanolic Extracts on *Aedes aegypti* and *Aedes albopictus* Eggs

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Received: 26 August 2024 Accepted: 21 February 2025 Published: 30 June 2025

ABSTRACT

The widespread use of chemical insecticides for controlling *Aedes* mosquitoes has resulted in the development of insecticide resistance, prompting the need for alternative solutions. Botanical insecticides have gained attention as a promising approach in modern pest control. This study focuses on evaluating the ovicidal effects of methanol extracts from *Cassia alata* leaves on *Aedes* mosquitoes. The extracts were produced through the maceration-filtration technique, followed by the preparation of various concentrations (0.05-2.00 mg/ml) These concentrations were then tested to assess their impact on the fertility and egg viability of *Aedes aegypti* and *Aedes albopictus*. From the results obtained in the bioassay on egg fertility and viability, a significant difference in fertility was observed between *Ae*. *aegypti* and *Ae*. *albopictus* ($p<0.05$, $p = 0.044$). However, no significant difference was observed in overall egg viability between the two species ($p>0.05$, $p = 0.468$). The LC50 and LC90 values for *Ae*. *albopictus* were 0.323 mg/ml and 5.280 mg/ml, respectively, which were lower than those for *Ae*. *aegypti* 0.560 mg/ml and 11.480 mg/ml. This indicates that, *Ae*. *albopictus* is more susceptible to the methanolic *C. alata* extracts. These findings suggest that *C. alata* extracts could be a viable alternative to chemical insecticides in mosquito control programs, particularly for targeting *Ae*. *albopictus*.

Keywords: *Aedes*, *Cassia alata*, extracts, methanolic, ovicidal

INTRODUCTION

The increasing prevalence of mosquito-borne diseases, such as dengue, chikungunya, and Zika, has become a significant public health concern in Malaysia and other tropical regions of Southeast Asia (AbuBakar *et al*., 2022). These diseases are primarily transmitted by *Aedes aegypti* and *Aedes albopictus*, which have shown remarkable adaptability to urban environments and a high reproductive rate, contributing to their widespread distribution (Soni *et al*., 2023). To combat this, traditional chemical insecticides have been the mainstay in controlling mosquito populations. However, their prolonged use has led to the development of resistance among mosquito species, which reduces the effectiveness of insecticides and its becoming a critical global issue impacting public health and increasing the risk of diseases such as dengue and malaria (Richards *et al*., 2020). This resistance often arises from the frequent and repeated use of synthetic insecticides. To address this challenge, exploring alternative strategies, such as the use of bioinsecticides, is essential. Bioinsecticides offer a promising alternative to conventional synthetic insecticides like pyrethroids and organophosphates, posing challenges to their effectiveness. Additionally, the adverse effects of chemical insecticides on non-target organisms, including beneficial insects and aquatic life, as well as the potential for environmental contamination, have raised concerns about their continued use (Gan *et al*., 2021).

In response to these challenges, there is a growing interest in exploring alternative, environmentally friendly strategies for mosquito control. One promising approach is the use of natural plant extracts as insecticides. Plants have evolved a wide range of chemical defenses against herbivores and pathogens, many of which have been found to exhibit insecticidal properties (Şengül Demirak & Conpolat, 2022). Among these, *Cassia alata*, a medicinal plant native to tropical regions, has attracted attention for its potential as a natural ovicide against *Aedes* mosquitoes. In Southeast Asia, *C. alata* is commonly consumed as both food and spices.

However, they have also been traditionally utilized by local communities, particularly in countries such as Malaysia, Thailand, Indonesia, and Brunei, for medicinal purposes. One notable application among these communities is the management of skin diseases (Fatmawati *et al*., 2020). A comprehensive review of the traditional uses, phytochemical composition, and primary pharmacological activities of these herbs highlights their role in managing skin conditions, particularly atopic dermatitis (AD) (Chew *et al*., 2022).

Recent studies have demonstrated the efficacy of *Cassia* sp. extracts in inhibiting the development of mosquito larv*ae*, suggesting its potential as a viable alternative to chemical insecticides (Raman Ibrahim *et al*., 2022). While significant research has focused on plant-derived larvicides and adult repellents, there has been relatively little investigation into the ovicidal activity of plant extracts for controlling mosquito eggs (Giovanni, 2015). Furthermore, the active compounds found in *C. alata*, such as anthraquinones and flavonoids (Fatmawati *et al*., 2020), have been reported to possess ovicidal properties that could disrupt the reproductive cycle of mosquitoes, thereby could reducing their population density (Lengai *et al*., 2020). This study aims to fill this gap by evaluating the potential of botanical insecticides as effective ovicides for mosquito vector control, potentially offering a new approach to managing mosquito populations and mitigating the spread of mosquito-borne diseases.

Ovicides are a class of insecticides specifically designed to target and eliminate mosquito eggs within their breeding habitats, thereby preventing the development and growth of mosquito populations. These substances work by either disrupting the oviposition process, thereby preventing adult mosquitoes from laying eggs, or by directly killing the eggs, preventing them from hatching into larv*ae*. Ovicides are particularly useful in breeding sites that are challenging to manage through source reduction or larvicide application, such as in wash basins (Andrew *et al*., 2016). Plant-derived compounds, including essential oils, have shown promise as effective ovicides for controlling mosquito populations (Ephantus *et al*., 2018). Hence, the use of *C. alata* as a mosquito ovicide is particularly relevant in Malaysia, where the plant is abundant and easily accessible. Despite the promising results from preliminary studies, there remains a gap in understanding the full potential of *C. alata* extracts in mosquito control, particularly concerning their ovicidal effects on *Aedes* species. This study aims to fill this gap by investigating the ovicidal activity of *C. alata* extracts against *Ae*. *aegypti* and *Ae*. *albopictus*, focusing on the efficacy, optimal concentrations, and potential mechanisms of action. By evaluating the effectiveness of *C. alata* as a natural ovicide, this research contributes to the ongoing efforts to develop sustainable mosquito control strategies that align with public health and environmental safety goals. Given the increasing resistance to chemical insecticides and the ecological hazards they pose, the exploration of plant-based insecticides like *C. alata* offers a promising alternative for mosquito control in Malaysia and other tropical regions. The findings of this study are expected to provide valuable insights into the potential of *C. alata* as part of an integrated vector management strategy, ultimately contributing to the reduction of mosquito-borne diseases in affected communities.

MATERIALS AND METHOD

Plant Preparation

Fresh, mature *C. alata* leaves were obtained from a local supplier and brought to the laboratory at the School of Biological Sciences, Universiti Sains Malaysia, Pulau Pinang. The leaves were thoroughly washed with tap water to remove any debris. After cleaning, they were spread out on newspapers on a laboratory table and left to air-dry at room temperature (27 ± 2 °C) for 5 - 7 days.

Plant Extractions

The extraction of *C. alata* leaves was performed using the maceration technique, which involves immersing coarsely powdered leaves in a solvent. Initially, the leaves were air-dried and then mechanically ground into a fine powder using an electric blender (Khind 1.2L BL1012). The powdered leaves were then subjected to maceration, where they were soaked in methanol at a ratio of 10 ml of solvent (methanol) per gram of leaf powder. A total of 160 g of powdered leaves were used, requiring 1600 ml of methanol for the extraction. To begin the extraction, 100 ml of methanol, serving as the menstruum, was

measured using a graduated cylinder and poured into a beaker containing the powdered leaves. The mixture was stirred thoroughly, covered tightly with aluminum foil, and left to macerate for at least three days in the laboratory. During this period, the beaker was occasionally shaken to enhance the extraction process. After the three-day maceration process, the mixture was filtered through filter paper to obtain the plant extract via the filtrate (Abdullahi & Mainul, 2020; Seng *et al*., 2023). The filtered extract was then placed in an oven set at 40 °C and left to evaporate for at least two days, allowing the solvent to fully evaporate and leaving a concentrated plant paste at the bottom of the beaker. This paste was carefully scraped out using a spatula, transferred to a Petri dish, and sealed with parafilm tape. The Petri dishes were then stored in a refrigerator to slow down the decay of the paste/extract, while sealing and moisture control were used to prevent microbial contamination.

Rearing and Culturing *Aedes* Mosquitoes in the Laboratory

The laboratory strains of *Ae*. *aegypti* and *Ae*. *albopictus* mosquitoes were obtained from the Vector Control Research Unit (VCRU) at Universiti Sains Malaysia (USM). The mosquito eggs were placed in containers and reared with seasoned water under controlled room temperature (27 ± 2 °C). To maintain optimal conditions, the seasoned water was replaced twice a week, and the larv*ae* were fed fish food pellets once every two days to minimise bacterial growth, which could contaminate the water and harm the larv*ae*. Upon reaching the pupal stage, they were carefully transferred to small containers with seasoned water using a dropper. To prevent the emerging adult mosquitoes from escaping, the containers with pup*ae* were then placed inside a mosquito cage. Additionally, a small beaker with cotton soaked in a 10% sucrose solution was provided inside the cage to nourish the adult mosquitoes before they were subjected to blood feeding with rats.

Preparation of Stock Solution and Bioassay Concentrations

A stock solution with a concentration of 250 mg/ml was prepared by mixing 5000 mg of *C. alata* leaf extract paste with 20 ml of methanol in a beaker. The beaker was sealed with aluminum foil and stored in the fridge to prevent contamination. Ten (10) serial dilutions were performed from the stock solution by diluting it with methanol. The volumes of stock solution and methanol required for each concentration were calculated using the formula $M_1V_1=M_2V_2$ where V_1 is the volume of the stock solution needed. This was determined by dividing the desired concentrations and final volume by the concentration of the stock solution (M_1). The concentration of *C. alata* prepared for the ovicidal bioassay ranged from 0, 0.05, 0.10, 0.15, 0.20, 0.25, 0.50, 1.00, 1.50, and 2.00 mg/ml.

Ovicidal Bioassay of *Cassia alata* Extracts Against *Aedes* Eggs (Fertility)

The adult female mosquitoes were deprived of sucrose solution for 24 hours before being sent for the blood-feeding procedure. The blood-feeding procedure was conducted by the Animal Ethics unit of the School of Biological Sciences. Each session required at least 30 starved female mosquitoes and lasted between 30 to 60 minutes to ensure adequate feeding. Simultaneously, ten Petri dishes were prepared: one with 5 ml of seasoned water as a control and nine with 5 ml of *C. alata* methanol extracts at concentrations ranging from 0.05 to 2.00 mg/ml. Cone-shaped filter papers were placed in each Petri dish to serve as egg-laying substrates. After feeding, the mosquitoes were transferred to a cage containing the Petri dishes with the *C. alata* extracts where they were exposed to these extracts for 24 to 72 hours at 22.5 °C.

To maintain the moisture of the filter papers and ensure suitable breeding conditions, 2 mL of the respective test concentrations (0, 0.05, 0.10, 0.15, 0.20, 0.25, 0.50, 1.00, 1.50, and 2.00 mg/mL) was added daily to ensure consistency and comparability. Additionally, a sucrose solution was provided to prevent starvation. The filter papers with laid eggs were collected, dried (Figure 1), and stored in closed, labeled containers corresponding to their respective concentrations. The total number of eggs was counted under a stereo microscope (Model SZX16-CCD) and recorded. The bioassay was replicated five times.

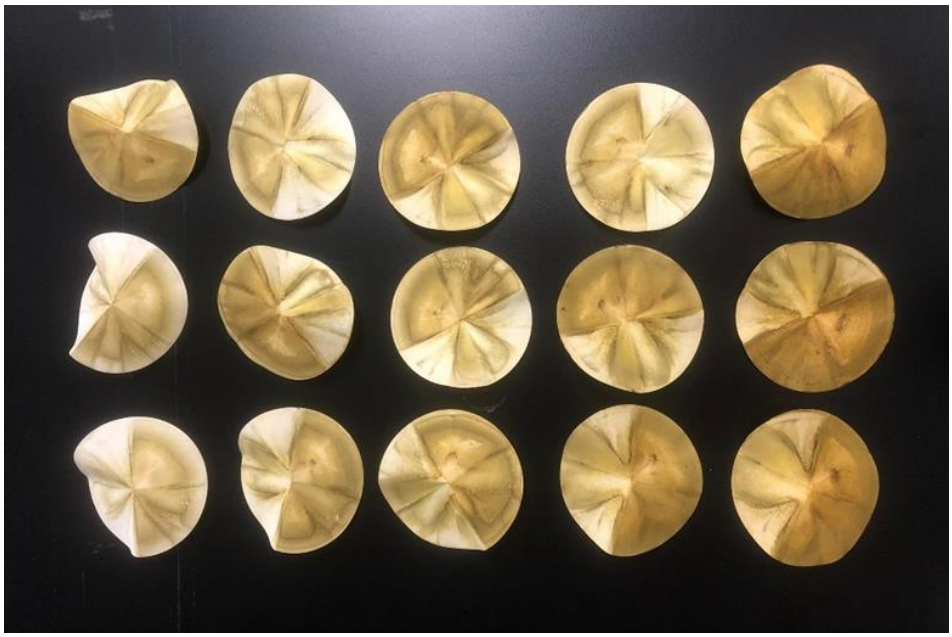

Figure 1. Cone-shaped filter paper used as a substrate for egg laying. The filter papers were removed from the cage for egg counting

Ovicidal Bioassay of *Cassia alata* Extracts against *Aedes* Eggs (Viability)

The viability assay was conducted to evaluate the effectiveness of *C. alata* extracts in controlling *Aedes* eggs over a seven-day period. The assay followed WHO guidelines (2005), with slight modifications. Unlike the standard procedure, which uses larv*ae* as the testing specimens, this assay utilised mosquito eggs. Targeting the egg stage allows to assess their fertility and viability after treatment, providing key insights into how treatments affect early development and helping to tailor mosquito control strategies to different life stages. Ten plastic cups were prepared with varying concentrations of the plant extracts, including a control. The control cup was filled with 200 ml of seasoned water, while the remaining nine cups contained 199 ml of seasoned water and 1 ml of plant extract at concentrations ranging from 0.05 to 2.00 mg/ml Four additional replicates were set up following the same procedure. Each cup was stocked with 125 mosquito eggs which were exposed to the test solutions for one week at approximately 22.5°C. During the assay, larv*ae* were provided with fish food pellets.

Data and Statistical Analysis

The number of eggs hatched was recorded by counting the larvae present in each plastic cup. Egg mortality was determined by using the following formula; Eq.(1):

$$Egg\ mortality\ (\%) = \frac{Total\ number\ of\ eggs - Total\ number\ of\ eggs\ hatched}{Total\ number\ of\ eggs} \times 100\% \quad \textbf{Eq.(1)}$$

The data were analysed using SPSS v27. A t-test was conducted to compare the fertility rates of *Ae*. *aegypti* and *Ae*. *albopictus*. The Mann-Whitney U Test was applied to assess significant differences in fertility rates and egg viability between the two species under different concentrations of *C. alata* extracts. The data were log-transformed prior to analysis. Additionally, lethal concentrations that kill 50% (LC50) and 90% (LC90) of the tested populations for egg viability of *C. alata* extracts were calculated using Probit analysis.

RESULTS AND DISCUSSION

Figure 2 shows the mean number of eggs laid by *Aedes* mosquitoes across the tested concentrations of methanolic *C. alata* extract. The results indicate that the fertility of both *Ae*. *aegypti* and *Ae*. *albopictus* decreases as the concentration of the extract increases. For *Ae*. *aegypti*, the highest mean number of eggs laid was 35.2 in the control group, while the lowest was 1.00 at 2.00 mg/ml. In contrast, *Ae*. *albopictus* laid the highest mean number of eggs (42.4) in the control group but none at concentrations of 0.50 mg/ml and higher.

Statistical analysis revealed a significant difference in fertility between the two species (Z = -2.012, p = 0.044), with *Ae*. *albopictus* showing lower fertility compared to *Ae*. *aegypti* at all concentrations except the control. These findings suggest that methanolic *C. alata* extracts are more effective in deterring oviposition *Ae*. *albopictus* than in *Ae*. *aegypti*, particularly at concentrations of 0.50 mg/ml and above.

Figure 3 presents a bar graph depicting the mean number of eggs hatched (egg viability) in *Ae*. *aegypti* and *Ae*. *albopictus* across various concentrations of methanolic *C. alata* extract (ranging from 0 to 2.00 mg/ml). The data show that the egg viability of *Ae*. *albopictus* decreases steadily with increasing extract concentration, with the highest mean number of eggs hatched being 22.0 in the control group and the lowest at 1.2 eggs at 2.00 mg/ml. For *Ae*. *aegypti*, while there is some fluctuation in egg viability, the overall trend also indicates a decrease as the concentration of the extract increases. The highest mean number of eggs hatched was 22.4 in the control group, with the lowest being 1.4 eggs at 2.00 mg/ml. Statistical analysis (t=0.741, P=0.468) reveals no significant difference in egg viability between *Ae*. *aegypti* and *Ae*. *albopictus*. However, *Ae*. *albopictus* generally exhibits lower egg viability compared to *Ae*. *aegypti*, showing similar trends in the fertility assay.

In this study, a significant reduction in the fertility and egg viability of *Ae*. *aegypti* and *Ae*. *albopictus* was observed with the application of methanolic *C. alata* extracts. This ovicidal activity is likely driven by the diverse phytochemical compounds in the leaves, including flavonoids and alkaloids, which are known for their insecticidal, repellent, and oviposition- inhibitory properties (Meryem & Emel, 2022). Solvent polarity significantly affects the extraction of bioactive compounds, with methanol being particularly effective due to its higher polarity (Anabela *et al*., 2020). Truong *et al*. (2019) found that methanol effectively extracts key phytochemicals, which may explain the strong ovicidal effects observed. Flavonoids like quercetin and k*ae*mpferol disrupt embryogenesis, while alkaloids such as berberine and quinine exhibit insecticidal properties that reduce egg viability (Simmonds, 2001). Similarly, Govindarajan (2011) reported that methanolic extracts of Cassia fistula reduced *Ae*. *aegypti* egg viability. Given the similarity to *C. alata*, its ovicidal activity may stem from the same bioactive compounds. Further analysis is needed to confirm their role in mosquito control. These findings highlight the potential of methanolic *C. alata* extracts as an effective natural ovicide for controlling *Aedes* mosquito populations.

To quantify this potential, Table 1 presents the LC50 and LC90 obtained from assays of egg mortality with varying concentrations of *C. alata* extracts. At a 95% confidence level, the LC50 and LC90 values for *Ae*. *albopictus* were lower than those for *Ae*. *aegypti*. Specifically, the LC50 for *Ae*. *albopictus* was 0.323 mg/ml, and the LC90 was 5.280 mg/ml while for *Ae*. *aegypti* the LC50 was 0.560 mg/ml and an LC90 of 11.480 mg/ml. These results indicate that a lower concentration of methanolic *C. alata* extracts is sufficient to achieve lethal effects on the egg populations of *Ae*. *albopictus* compared to *Ae*. *aegypti*. This research highlights that *Ae*. *albopictus* is more susceptible to these methanolic *C. alata* extracts.

The reduced susceptibility of *Ae*. *aegypti* might be due to genetic resistance developed over successive laboratory generations, with resistance genes potentially being passed down. Meryem & Emel (2022) reported that mosquitoes surviving insecticide exposure can transmit resistance traits to their offspring, resulting in increased resistance in subsequent generations. This resistance is often mediated by detoxifying enzymes like cytochrome P450 monooxygenases, which degrade insecticides and reduce their effectiveness. Continuous exposure can lead to increased enzyme activity, further contributing to resistance development. Dong-jiang *et al*. (2022) observed that plant secondary metabolites could induce cytochrome P450 gene expression, aiding in the detoxification of toxic compounds. Kamal *et al*. (2022) corroborated this by demonstrating that the LC50 values of eugenol (a component of basil and clove oils) for *Ae*. *aegypti* increased from the F10 to the F24 generation, paralleling a rise in cytochrome P450 activity.

Table 1. Lethal concentrations (LC50 and LC90), of *Ae. aegypti* and *Ae. albopictus* eggs against *C. alata* extracts

Species	LC_{50} (mg/ml)	95% Confidence		LC_{90} (mg/ml)	95% Confidence	
		Lower	Upper		Lower	Upper
Ae. aegypti	0.560	0.465	0.690	11.480	6.973	22.502
Ae. albopictus	0.323	0.272	0.382	5.280	3.582	8.793

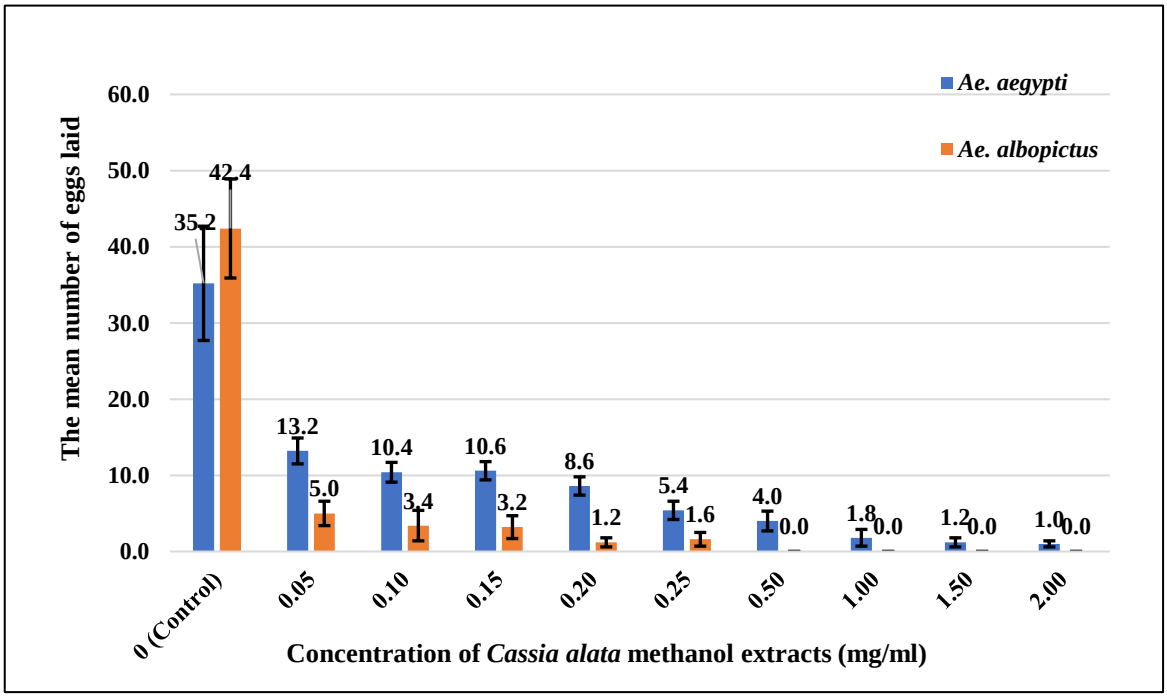

Figure 2. The mean number of eggs laid by *Aedes* mosquitoes across concentration (mg/ml)

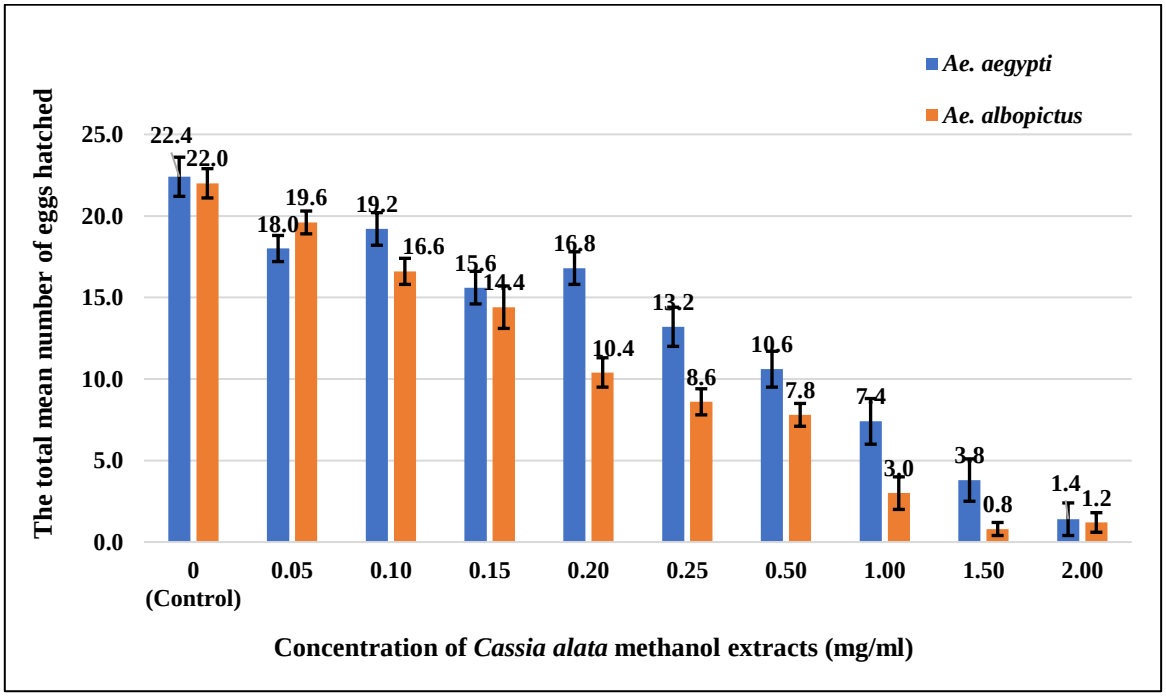

Figure 3. The mean number of eggs hatched by *Aedes* mosquitoes across concentrations (mg/ml)

As reported, *C. alata* contains flavonoid compounds, that likely interact with cytochrome P450 enzymes. Petr *et al*. (2002) found that flavonoids can either induce or inhibit cytochrome P450 expression affecting its metabolic activity. This interaction suggests that *Ae*. *aegypti* may have developed genetic adaptations to enhance survival against *C. alata* extracts potentially through cytochrome P450 mechanisms. Senthil-Nathan (2020) identified cytochrome P450 as a key indicator of metabolic resistance, which can lead to cross-resistance, a significant challenge in managing insecticide resistance. In this study, *C. alata* extracts were less effective against *Ae*. *aegypti* than *Ae*. *albopictus*. This difference in susceptibility warrants further investigation, as it may be influenced by species-specific physiology, detoxification mechanisms, or prior exposure to similar compounds, including potential cross-resistance to synthetic insecticides (Lin *et al*., 2024).

CONCLUSION

In conclusion, methanolic *C. alata* extracts are promising botanical insecticides with the potential to replace chemical insecticides in *Aedes* control. These extracts have shown high effectiveness, particularly against *Ae*. *albopictus*, achieving 100% repellency with no eggs laid at concentrations ranging from 0.50 to 2.00 mg/ml. The LC50 and LC90 values for *Ae*. *albopictus* were significantly lower than those for *Ae*. *aegypti*, indicating greater susceptibility. These eco-friendly, biodegradable extracts are less persistent and less toxic to non-target organisms, making them suitable for integrated pest management (IPM) programs (Şengül Demirak *et al*., 2022). Future research should focus on improving the extraction methods and solvents used for *C. alata* to maximize its effectiveness. It is also important to investigate its potential as a larvicide or adulticide against *Aedes* mosquitoes under different environmental conditions. These efforts could help establish *C. alata* as a reliable and sustainable option for mosquito control.

ACKNOWLEDGEMENT

We extend our gratitude to the School of Biological Sciences, USM Pulau Pinang, for their facilitation and support during this study. We also wish to acknowledge that this research did not receive funding from the university or any other agency.

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