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RESEARCH ARTICLE

Phytochemical screening and ovicidal potential of neem (*Azadirachta indica*) leaf extracts using various solvents against *Aedes aegypti* L.

Syaliza Omar[1*], Norhayati Ngah[2] and Intan Haslina Ishak[3,4]

[1]Pharmaceutical Chemistry Department, Faculty of Pharmacy, University Sultan Zainal Abidin, Besut Campus, 22200 Jerteh, Terengganu, Malaysia
[2]Faculty of Bioresources and Food Industry, University Sultan Zainal Abidin, Besut Campus, 22200 Jerteh, Terengganu, Malaysia
[3]School of Biological Sciences, Universiti Sains Malaysia, 11800 Minden, Penang, Malaysia
[4]Vector Control Research Unit, School of Biological Sciences, Universiti Sains Malaysia, 11800 Minden, Penang, Malaysia

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☒ * **CORRESPONDING AUTHOR**
Dr. Syaliza Omar
Pharmaceutical Chemistry Department, Faculty of Pharmacy, University Sultan Zainal Abidin, Besut Campus, 22200 Jerteh, Terengganu, Malaysia.
Email: syalizaomar@unisza.edu.my

ABSTRACT

Dengue, caused by *Aedes aegypti*, continues to pose significant global public health challenges. Current vector control strategies include the extensive use of chemical insecticides, which have contributed to the development of insecticide resistance and potential harm to non-target organisms, including humans. Neem (*Azadirachta indica*) is renowned for its insecticidal activity against mosquitoes, exhibiting ovicidal, larvicidal, pupicidal and adulticidal properties. This study evaluated the phytochemical composition and ovicidal activity of neem leaf extracts against *Ae. aegypti* eggs. Neem leaves were extracted using ethanol, acetone, ethyl acetate and chloroform via the maceration method for 72 hours. Each extract was analysed through phytochemical analysis tests and ovicidal bioassay. Phytochemical screening tests revealed the presence of bioactive compounds such as alkaloids, saponins, tannins, steroids, glycosides, terpenoids, and flavonoids. Statistical analysis (ANOVA) revealed a significant difference ($p < 0.05$) in ovicidal activity across solvent extracts. The ethanolic neem leaf extract demonstrates ovicidal activity against *Ae. aegypti* with 79% egg mortality at 1000 mg/L. Probit analysis further showed that the ethanol extract had the lowest LC_{50} (289.47 mg/L) and LC_{90} (2083.29 mg/L) among other solvent extracts. These findings highlight the potential of ethanolic neem leaf extract as a natural ovicide and support its use as an alternative to synthetic insecticides. Its application could be especially valuable in small aquatic habitats or breeding sites near human dwellings.

1. INTRODUCTION

Dengue remains a major global health issue, with over 7.6 million cases reported to the World Health Organisation (WHO) by April 2024 (WHO, 2024). In Malaysia, dengue is endemic, and the cumulative number of dengue fever cases reported in 2022 has increased by 150% compared to cases in 2021. Until now, it remains a challenging socioeconomic problem in Malaysia. Dengue is a viral infection transmitted by the bite of an infected mosquito. The main vector is *Aedes aegypti*, which breeds in and around houses and buildings, including clogged drainage in the community gardens (Ali et al., 2020). In recent years, the trend of infection has been rapidly growing. Messina et al. (2019) predicted that dengue will expand to put more than six billion people, or 60% of the world's population, at risk of infection by 2080. The study identified climate change, population growth, unplanned or rapid urbanisation, ecological disruption, poor sanitation, increased trade, and international travel as contributory factors to the increase in dengue cases. This scenario will be worsened by the absence of a vaccine to prevent dengue. Therefore, the only way of reducing the incidence of dengue is by controlling the mosquito population.

In Malaysia, several strategies are employed to control Aedes populations, including insecticide application, release of Wolbachia-infected mosquitoes, and genetically modified Aedes (Ong, 2016; Wong et al., 2023). Among these, fogging with synthetic insecticides remains the most common short-term intervention in dengue outbreak areas (Ramlee et al., 2022). However, fogging targets only adult mosquitoes and does not affect eggs or larvae. Larviciding with insecticides such as temephos is also routinely carried out in water tanks and breeding sites. Nevertheless, long-term reliance on synthetic insecticides has led to widespread resistance in *Ae. aegypti* populations. Resistance to pyrethroids (e.g., deltamethrin, permethrin) and organophosphates (e.g., malathion) is now common in Southeast Asia (Amelia-Yap et al., 2018; Gan et al., 2021). Studies in dengue hotspot areas of Kuala Lumpur and Selangor confirmed resistance to permethrin, dichloro-diphenyl-trichloroethane (DDT), malathion, and propoxur (Ali

et al., 2020; Rasli et al., 2021). Beyond resistance, synthetic insecticides pose ecological and health risks (Oaya et al., 2019). They are non-selective, killing beneficial pollinators such as stingless bees and butterflies, and remain in the environment due to slow degradation. Temephos, for instance, has a long residual effect and is commonly applied to domestic water sources, raising concerns about potential neurotoxicity (Cilek et al., 1991; Martínez-Mercado et al., 2022). Pyrethroids used in fogging may also pollute the air and worsen respiratory conditions (Hamzah et al., 2022). These challenges highlight the urgent need for eco-friendly, biodegradable, and selective vector control strategies that effectively disrupt the mosquito life cycle.

Understanding the mosquito's life cycle, which progresses from egg to larva, pupa, and finally adult, is critical for effective population control. One approach is targeting the egg stage, which is frequently neglected in conventional vector control programs. This oversight is likely because mosquito eggs are not considered a direct problem, are immobile, and are less visible than other life stages. However, one should bear in mind that *Ae. aegypti* eggs can survive long latency periods without water and can resist desiccation for several months in the environment (Farnesi et al., 2015; Rezende et al., 2008). This means that even though the effects of fogging and larvicide diminish over time, the eggs remain and wait for the right conditions to hatch. In addition, eggs that survive insecticidal interventions aimed at larvae and adults remain viable and can subsequently hatch, contributing to reinfestation and sustaining mosquito populations. Nevertheless, not many studies have addressed egg-targeted control against mosquitoes. Therefore, this study specifically focuses on ovicidal strategies to target the egg stage, aiming to prevent hatching and break the mosquito life cycle at its earliest point. In this context, plant-based insecticides have been established to offer a safer and more environmentally friendly alternative to synthetic mosquitocides, as they are typically biodegradable, pose lower risks to human health, and contain diverse bioactive compounds with multiple modes of action (George et al., 2000; Hikal et al., 2017; Khursheed et al., 2022). Additionally, botanical insecticides are generally less likely to induce insect resistance due to their complex chemical profiles (Isman, 2006; Vollinger, 1987).

Neem (*Azadirachta indica*) is widely discovered in Malaysia and is globally recognized for its insecticidal properties in agriculture (Adusei and Azupio, 2022; Usharani et al., 2019). It is a tree in the family Meliaceae (Figure 1) locally known as 'semambu' among Malays in Malaysia. The neem-based pesticides are popular due to their repellent, antifeeding, ovicidal, fecundity-suppressing, deterrence of egg-laying, disruption of growth and reproduction, and inhibition of metamorphosis effects (Benelli et al., 2017). It is also safe towards non-target organisms (Raguraman and Kannan, 2014). The primary product of neem is seed oil, which contains over 100 active compounds, including azadirachtin, meliantriol, salannin, nimbin, nimbidin, and nimbolides (de Alba et al., 2023; Fernandes et al., 2019). Azadirachtin, a complex tetra-nor-triterpenoid limonoid, is widely distributed throughout the tree and serves as a key biomarker (Kilani-Morakchi et al., 2021). In addition to oil, various parts of the neem tree, such as leaves, bark, and stems, are rich in limonoids and other phytochemicals, including flavonoids, many of which exhibit insecticidal properties (Hikal et al., 2017; Mitchell et al., 1997; Roy and Saraf, 2006).

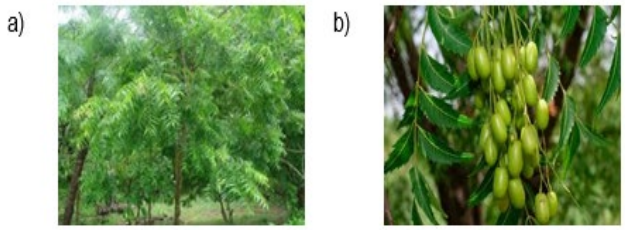

Figure 1: Neem tree (a) and seeds (b). Photos are adapted from Debnath et al. (2025).

A previous study reported that a neem product containing commercial azadirachtin at 1 ppm demonstrated almost 100% mortality in eggs against *Culex tarsalis* and *Culex quinquefasciatus* (Su and Mulla, 1998). Subsequently, Maheswaran and Ignacimuthu (2012) prepared PONNEEM, a novel herbal formulation using the oils of neem seed and *Pongamia glabra*, which exerted 100% egg mortality against *Ae. aegypti* and *Ae. albopictus* at 0.1 ppm as compared to temephos and azadirachtin as positive controls. Mirza and Zehra (2018) demonstrated that clove oil and neem oil (from seed) at 150 ppm reduced *Ae. aegypti* eggs hatch to 90%. Kala et al. (2019) demonstrated that their formulated tablet suspension of neem oil provided an 82% reduction in hatching of *Anopheles culicifacies* eggs as compared to the negative control (tablet without neem oil). Furthermore, the tablet suspension of neem oil was revealed to be non-toxic to the non-target fish, *Poecilia reticulate*. Ramkumar et al. (2019) reported that the egg hatching inhibition of *C. quinquefasciatus* was higher in eggs exposed to azadirachtin (75%) than in buprofezin (55%) at a 0.1mg/ mL concentration.

Most research on neem has focused primarily on its seeds, while comparatively fewer studies have explored its leaves for mosquitocidal properties. Nevertheless, studies have demonstrated that the phytochemicals identified in neem seeds are also present in the leaves, albeit in varying quantities. Moslem and Kholie (2009) reported that both neem leaf and seed extracts were effective against the fungi *Alternaria solani*, *Fusarium oxysporum*, *Rhizoctonia solani*,

and *Sclerotinia sclerotiorum*. Furthermore, neem leaves have been revealed to possess insecticidal activity against various insect species (Endris & Mekonnen, 2023; Li et al., 2019; Tobing et al., 2023). Given these findings, neem leaves were selected for this study as they are available year-round, can be harvested without harming the tree, and are easier to process than seeds. They also represent a sustainable and cost-effective alternative, as neem trees require several years to produce seeds. Substituting leaves for seeds could reduce dependence on seed harvests, providing a more practical and scalable option for community-level mosquito control programs. To the best of the current knowledge, this is the first study to investigate both the phytochemical composition using solvents of varying polarities and the ovicidal activity of neem leaf extracts against *Ae. aegypti* eggs. Previous studies have not reported the use of different solvent polarities to extract phytochemicals from neem leaves for both phytochemical profiling and ovicidal determination against *Ae. aegypti*. Due to this limitation, this study was undertaken to provide valuable insights into the classes of phytochemicals present and the ovicidal efficacy of neem leaf extracts obtained using solvents of different polarities (ethanol, acetone, ethyl acetate, and chloroform).

2. MATERIALS AND METHODS

2.1 Collection and preparation of neem leaves

The method of neem leaves processing was adapted from Al-Hashemi and Hossain (2016) with slight modifications. Neem leaves were obtained from Kuala Besut, Terengganu, Malaysia (5.8261° N, 102.5498° E) in October 2023. To ensure cleanliness, the leaves were thoroughly washed to remove dirt and any foreign particles. Then, the leaves were carefully removed from the twigs and placed in a basket, which was then placed inside an incubator set to 45°C. They were left in the incubator (Memmert, Germany) for 5 days until thoroughly dried. The dried neem leaves were stored in a vacuum bag and placed at room temperature (25°C). For extraction, the dried leaves were ground into fine powder using an electric blender (Panasonic, Japan). The powder was kept in a ziplock bag at 4°C until further use.

2.2 Extraction of the plant

The extraction procedure was performed using the methods adapted from Tourabi et al. (2023) and Felhi et al. (2017) with slight modifications. For the extraction, a sample-to-solvent ratio of 1:20 was employed, comprising 50 g of powdered leaves and 1 litre of solvent. The powdered neem leaves were macerated with each of ethanol (HiMedia Laboratories, India), ethyl acetate (Merck, Germany), acetone (Merck, Germany), and chloroform (Merck, Germany) at 25°C for 72 hours. This provides enough time to break the cell wall to release soluble phytochemicals. The media was agitated manually every 12 hours to increase the extraction rate until the soluble matter was appropriately dissolved. The extract was then filtered using Whatman No. 1 filter paper and concentrated at 50°C using a rotary evaporator (Vivo RT2, Heidolf, Germany) to remove the solvent. The extract (in paste form) was removed from the round-bottom flask (Borosil, India) by dissolving in 10-20 mL of acetone, transferred to a glass petri dish (Pyrex, France), and left in a fume hood (ESCO, Singapore) for 24 hours. The final dried extract was stored in a freezer (Haier, China) at -20°C until further use.

2.3. Phytochemical screening assays

Phytochemical screening of neem leaf extracts was performed using standard qualitative assays to identify major classes of bioactive constituents. Seven assays were conducted targeting saponins, flavonoids, tannins, steroids, glycosides, alkaloids, and terpenoids using modified standard procedures (Shaikh et al., 2020). The presence of each phytochemical class was confirmed based on characteristic colourimetric reactions observed in the extracts. The stock solution (1000 mg/L) of each extract, prepared as in Section 2.4, was used for the screening tests.

2.3.1 Test for phenols (ferric chloride test)

One ml of extract was diluted with 2 ml of distilled water, followed by the addition of a few drops of 10% ferric chloride (Bendosen, Malaysia) solution. A dark green colouration confirms the presence of phenolic compounds.

2.3.2 Test for flavonoids

Two ml of extract were mixed with 2-3 drops of sodium hydroxide (Bendosen, Malaysia) solution, producing a yellow colouration. The addition of 0.01 M hydrochloric acid (Merck, Germany) caused the yellow colour to fade, confirming the presence of flavonoids.

2.3.3 Test for tannins

One ml of extract was combined with 2 ml of 5% ferric chloride solution. The development of a green or yellow colour indicates the presence of tannins.

2.3.4 Test for saponins

Two ml of extract were mixed with 20 ml of distilled water and shaken vigorously for about 15 minutes. Persistent foam formation of at least 1 cm indicates the presence of saponins.

2.3.5 Test for steroids (Salkowski's test)

One ml of extract was shaken with 1 ml of chloroform. Ten drops of acetic anhydride (Merck, Germany) were added,

followed by five drops of concentrated sulfuric acid (Merck, Germany) along the test tube wall. A colour change from violet to blue or green indicates the presence of steroids.

2.3.6 Test for alkaloids

Two ml of extract were treated with 1 ml of Dragendorff's reagent (Merck, Germany). The appearance of an orange-red precipitate indicates the presence of alkaloids.

2.3.7 Test for glycosides (Keller–Killiani test)

Two ml of extract were mixed with 0.5 ml of glacial acetic acid (HiMedia Laboratories, India). Carefully, 1 ml of concentrated sulfuric acid was added along the test tube wall. The formation of a dark blue colour at the interface indicates the presence of cardiac glycosides.

2.4. Ovicidal bioassay

The eggs of *Ae. aegypti* were obtained from Vector Control Research Unit (VCRU), Universiti Sains Malaysia (USM), Penang, Malaysia. They were lab strain and free from exposure to pathogens, insecticides and repellents. The use of these eggs was approved by the relevant ethics committee at VCRU. The eggs were kept dry in a ziplock bag to prevent dehydration at room temperature (25°C to 30°C) until further use. Eggs kept for more than one month following cultivation were discarded. The eggs on paper strips were observed under a stereomicroscope to evaluate viability before use. Egg hatchability was assessed using the method described by Govindarajan and Karuppannan (2011). A series of five concentrations (1000, 500, 250, 125, and 62.5 mg/L) was prepared through serial dilution using seasoned water. To prepare seasoned water, tap water was exposed to direct sunlight for a minimum of three days to eliminate residual chlorine. The stock solution (1000 mg/L) was prepared by first dissolving the extract in 1 mL of acetone due to its water insolubility, followed by dilution with 99 mL of seasoned water to obtain a final concentration containing 1% acetone (Zuharah et al., 2016). Subsequent serial dilutions with seasoned water were performed to achieve the desired concentrations. A negative control containing 1% acetone without neem extract was also prepared to ensure that any ovicidal activity observed was not due to the vehicle solvent (acetone). Approximately 30 to 50 *Ae. aegypti* eggs were introduced into each concentration in a 6-well microplate (LENGRUI, China), along with a pinch of fish pellets (AquaNice, Japan) as hatching stimulants. The microplate was then incubated under stationary conditions for 48 hours. Environmental conditions were controlled at 27°C to 30°C and 85% to 95% relative humidity. A negative control group containing only the vehicle solvent (1% acetone in seasoned water) was included and run concurrently for comparison. All treatments, including controls, were performed in four replicates. Egg hatching was assessed under a stereomicroscope (Leica EZ4E, Germany) after 48 hours of exposure and percentage of ovicidal activity was calculated using the following formula:

% Ovicidal activity = (Number of hatched eggs / Total number of eggs) × 100

2.5. Statistical analysis

The ovicidal activity was expressed as the mean standard deviation of four replicates. Probit analysis was employed to determine the LC_{50} and LC_{90} of the population exposed to the extracts, analysed with a 95% Confidence Interval (CI) level using Excel. One-way Analysis of Variance (ANOVA) was conducted to detect the significant differences in ovicidal activity between solvents, and the significance level was set at $p<0.05$. Prior to analysis, the data were tested for normality using Shapiro-Wilk test.

3. RESULT AND DISCUSSION

3.1 Influence of extraction solvent on the phytochemical screening of neem leaves

Phytochemicals are plant-derived compounds primarily responsible for the plant's pharmacological effects. Their solubility largely depends on the polarity of the extraction solvent, which can range from polar to nonpolar (Altemimi et al., 2017). The present study investigated the presence of phytochemicals in neem leaf extracts using four different solvents with different polarities. The results obtained are depicted in Table 1.

Table 1: Qualitative phytochemical screening of neem leaf extracts of various solvents.

Phytochemicals	Solvents			
	Ethanol	Acetone	Ethyl acetate	Chloroform
Saponins	+	+	+	+
Steroid	+	+	+	+
Alkaloids	+	+	+	+
Glycosides	+	+	+	+
Tannins	+	+	+	+
Flavonoids	-	+	-	-
Terpenoids	+	+	-	+

(+) detected, (-) non-detected

The tests revealed that saponins, tannins, steroids, glycosides, and alkaloids were consistently detected in all solvents, indicating these compounds are abundantly present in neem leaves and can be extracted regardless of solvent polarity. Interestingly, acetone was able to isolate all the phytochemicals tested. This can be attributed to acetone's intermediate polarity, which enables it to solubilize both polar and semi-polar compounds (Lee et al., 2024). However, this finding is in contrast to that of Sahrawat et al. (2018), who reported that the acetone extract of neem leaf prepared via

overnight maceration failed to detect flavonoids and alkaloids. The discrepancy may be attributed to the difference in extraction duration. In this study, the neem leaves were macerated for three days with frequent agitation to allow sufficient time for equilibrium to be reached between the plant cells and the solvent. As a result, sufficient amounts of flavonoids and alkaloids were extracted in the acetone extract, making them detectable in the screening tests.

Unfortunately, the ethanol and chloroform extracts did not detect flavonoids, while the ethyl acetate extract failed to detect both flavonoids and terpenoids. This is a significant contrast given that flavonoids and terpenoids are frequently reported as common phytochemicals in neem leaves and seeds and can be easily extracted by ethanol (Saleem et al., 2018; Siddiqui et al., 2004; Vergallo et al., 2019). For example, Nagano and Batalini (2021) used 80% aqueous ethanol to extract neem leaves over an extended maceration period (45 days). They reported the presence of phenols, tannins, flavonoids, and several other flavonoid subclasses. The difference between this study and Nagano and Batalini (2021) may be due to the shorter extraction duration (three days) or the absence of water in the ethanol solvent, as water can enhance the extraction of polar compounds like flavonoids (Chaves et al., 2020). Sutrisno et al. (2024) used methanol to extract neem leaves and identified flavonoid and phenolic contents, along with alkaloids, tannins, terpenoids, and saponins. Notably, the polarity of methanol is slightly higher than that of ethanol (Snyder, 1974), and both solvents are used interchangeably to extract polar compounds in many extraction conditions involving plants. Ethyl acetate's limited solubility for certain semi-polar compounds may explain its lower extraction efficiency, a result consistent with findings from Bolaji et al. (2024). In their study, neem leaf extraction using ethyl acetate also failed to detect flavonoids and terpenoids, although alkaloids, saponins, phenols, and cardiac glycosides were present.

Surprisingly, glycosides were detected in the chloroform extract in this study despite the general nonpolar nature of chloroform, which typically limits its ability to extract polar compounds. This could be attributed to the presence of less polar glycosides due to their large nonpolar aglycone moieties, which reduce overall polarity and enhance their solubility in nonpolar solvents (Chen et al., 2023; Han et al., 2024). Khanum et al. (2015) revealed that the chloroform extract of *Cajanus cajan* contained both polar and semi-polar compounds, including reducing sugars, phenols, saponins, flavonoids, and alkaloids. The detection of polar compounds in chloroform extracts in this study appears valid, as similar findings of detecting polar compounds in non-polar solvents have been reported. For instance, Nwanekezie et al. (2023) used Soxhlet extraction with n-hexane at 60°C and reported a wide range of compounds, including various flavonoids and glycosides in neem leaves. Since n-hexane is nonpolar in nature, it is unlikely to extract polar compounds such as flavonoids. However, the extensive list of polar compounds detected may be due to the temperature applied and the exhaustive nature of Soxhlet extraction. Similarly, Pineda-Cortel et al. (2019) demonstrated that hexane could extract predominantly polar compounds, such as saponins, glycosides, and tannins, from the leaves of *Artocarpus blancoi*.

Many factors influence the types and quantities of phytochemicals in plants, and these factors can contribute to the variations in phytochemical findings across studies. Pre-extraction factors, such as the plant's geographical origin, harvesting time, particle size of the leaf powder, moisture content, drying method, and storage conditions, can all affect the phytochemical content (Shaikh et al., 2020). In addition to these factors, the most crucial variables to consider are those related to the extraction process itself. These include the extraction method, solvent choice, solvent-to-sample ratio, temperature, and extraction duration, all of which can significantly influence the types and amounts of phytochemicals obtained (Krakowska-Sieprawska et al., 2022; Plaskova and Mlcek, 2023).

3.2 Influence of extracting solvent on the ovicidal activity of neem leaves

The ovicidal effect of neem leaf extract using solvents of different polarities was determined against *Ae. aegypti* eggs at concentrations ranging from 62.5 to 1000 mg/L. The percentage ovicidal activity after 48 hours of exposure and the LC_{50} and LC_{90} are presented in Tables 2 and 3, respectively. Notably, both ovicidal activity (percentage mortality) and lethal concentration values (LC_{50} and LC_{90}) provide complementary information when evaluating the biological efficacy of insecticides (De Franca et al., 2017). Percent mortality indicates the susceptibility of the extract to ovicidal effect at each concentration. Lethal concentration provides quantitative values of the potency and efficacy of each solvent extract in causing ovicidal activity across a range of concentrations, as determined by Probit analysis. The ovicidal activity of neem leaf extracts varied across different solvents and concentrations, as depicted in Table 2. One-way ANOVA showed a significant difference ($p < 0.05$) in ovicidal activity across solvent extracts. The mortality data reveal a concentration-dependent response for most extracts, with increased concentrations generally resulting in higher ovicidal effects. This concentration-dependence is consistent with previous studies that have reflected higher concentrations of plant-based extracts often lead to higher ovicidal rates in mosquitoes (Alves et al., 2020; Astreapuspita et al., 2025;

Pineda-Cortel et al., 2019). Among the solvents tested, the ethanol extract demonstrated the most effective ovicidal activity against *Ae. aegypti*, exhibiting the highest overall ovicidal activity with 79% egg mortality at 1000 mg/L. This ovicidal activity is further supported by Probit analysis for lethal concentrations, where ethanol extract recorded the lowest LC_{50} (289.47 mg/L) and LC_{90} (2083.29 mg/L) values (Table 3), indicating greater potency of ovicidal activity compared to the other solvent extracts.

Table 2: Ovicidal activity (% mortality) of neem extracts at different concentrations.

	Concentrations (mg/L)				
Solvent	62.5	125	250	500	1000
Ethanol	18±14.8	27±25.2	44±24.8	66±8.9	79±9.3
Ethyl acetate	31±11.6	32±1.9	34±7.2	42±12.3	79±9.0
Acetone	11±8.1	23±6.1	42±16.3	62±2.6	64±22.4
Chloroform	1±2.1	23±5.7	35±6.5	39±21.4	51±4.3

The strong ovicidal activity of the ethanol extract may be attributed to ethanol's high polarity and ability to extract a broad range of bioactive phytochemicals, including alkaloids, saponins, tannins, terpenoids, glycosides, and steroids from the neem leaves sample. Similarly, the ethyl acetate extract achieved 79% ovicidal activity at 1000 mg/L. However, the activity at intermediate concentrations (62.5-500 mg/L) was moderate, suggesting that even though ethyl acetate can extract some active compounds, it is less efficient than ethanol. This is possibly due to its semi-polar nature, which may limit its ability to solvate and extract polar bioactive compounds. The LC_{50} and LC_{90} values were 362.96 mg/L and 7737.16 mg/L, respectively, which were slightly higher than those of the ethanol extract. The acetone extract also demonstrated an increasing ovicidal pattern with concentration. Nevertheless, the ovicidal activity pattern was slightly lower than that of ethanol and ethyl acetate extracts at the same concentration. Egg mortality in the acetone extract was 64% at 1000 mg/L. The LC_{50} and LC_{90} values were 409.98 mg/L and 3315.64 mg/L, respectively, suggesting that the acetone extract is less potent than the ethanol and ethyl acetate extracts. Chloroform extract exhibited the lowest mortality rates across the concentrations tested, despite being positively detected for six phytochemicals tested. Mortality ranged from 1% at 62.5 mg/L to 51% at 1000 mg/L, indicating that chloroform was the least efficient in inducing egg mortality across all concentrations tested. The highest LC_{50} (675.66 mg/L) and LC_{90} (3724.28 mg/L) values for chloroform further support this observation, suggesting that it requires substantially higher concentrations to achieve 50% mortality compared to the other solvent extracts. This limited efficacy is possibly due to the inefficient extraction of bioactive compounds that contribute to the ovicidal activity.

Although acetone was the only solvent that successfully extracted all seven phytochemicals screened in this study, its lower ovicidal activity and higher lethal concentration values compared to ethanol and ethyl acetate extracts may be due to an insufficient concentration of active compounds with ovicidal activity in the extract.

Table 3: Lethal concentrations of neem extracts against *Ae. aegypti* eggs.

Extract	LC_{50} (mg/L) (95% LCL-UCL)	LC_{90} (mg/L) (95% LCL-UCL)	X^2	Slope ± SE
Ethanol	289.47 (246.39 – 340.08)	2083.29 (2083.18 – 2084.44)	0.0035	1.49 ± 0.76
Ethyl acetate	362.96 (99.31 – 1326.57)	7737.16 (7732.93-7742.22)	0.0756	0.96 ± 0.37
Acetone	409.98 (408.56-411.90)	3315.64 (3314.11-3317.15)	0.0171	1.41 ± 0.18
$CHCl_3$	675.66 (672.12-678.39)	3724.28 (3720-3727)	0.2098	1.72 ± 0.53

LC_{50}: lethal concentration for 50% mortality; LC_{90}: lethal concentration for 90% mortality; LCL: lower confidence limits; UCL: upper confidence limits; $\chi 2$: Pearson chi-square. $CHCl_3$: Chloroform

Additionally, possible antagonistic interactions between certain compounds could reduce ovicidal activity. Antagonistic interactions occur when active constituents are masked by other compounds in a complex mixture, thereby diminishing the overall potency of the extract (Chaachouay, 2025). This phenomenon is common in plant extracts (Caesar and Cech, 2019) and can also occur in mixtures of synthetic insecticides (Levchenko et al., 2019). A similar reason applies to the chloroform extract, which tested positive for six phytochemical groups (except flavonoids), yet exhibited the least ovicidal efficacy among the solvents tested. It is important to note that the hatchability in the negative control containing 1% acetone in water as a vehicle solvent was 85 ± 10.4%, indicating that the vehicle solvent had minimal effect on egg viability and development. This confirms that the observed ovicidal activity in the treatment groups can be attributed to the neem leaf extracts rather than to the vehicle solvent itself. These results highlight the importance of solvent choice in extracting active compounds from neem leaves, which are responsible for egg mortality.

Nevertheless, it is important to highlight that the results of phytochemical screening do not necessarily correspond directly with the observed biological efficacy. While the phytochemical screening results (Table 1) confirmed the presence of specific groups of compounds, the tests used in this study were qualitative in nature. The tests are relying solely on observable colour changes following reactions between compound functional groups and chemical reagents (Maheshwaran et al., 2024). Such colour changes indicate only the presence of phytochemical classes, without

providing information on concentration, relative abundance, or differentiation among structural variants within the same class. For instance, tannins represent a broad class of compounds that include gallotannins, ellagitannins, complex tannins, and condensed tannins (Khanbabaee and Van Ree, 2001). These tannin subclasses can differ markedly in their biological activities, with some exhibiting ovicidal effects while others may not. Furthermore, tannins can be extracted using a variety of solvents such as water, methanol, ethanol, and acetone (Das et al., 2020), meaning that different solvent systems may yield different tannin subclasses. Therefore, it is possible that the phytochemicals extracted in each solvent in this study are not necessarily the same compounds, despite falling under the same phytochemical class. This justifies that, despite acetone extract containing several phytochemical groups, their ovicidal efficacy and potency were lower than those of ethanol and ethyl acetate extracts. The identity of the individual compounds present in the extract can only be confirmed through quantitative analytical techniques such as high-performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS) or gas chromatography-mass spectrometry (GC-MS). These methods are essential for characterizing and comparing the constituents across different extracts.

The pronounced ovicidal effect of the ethanol extract in this study supports earlier studies emphasizing the efficiency of ethanol in extracting insecticidal constituents from neem. Wandscheer et al. (2004) demonstrated that ethanol extracts of ripe fruits from *Melia azedarach* and neem were lethal to third and fourth-instar larvae of *Ae. aegypti*. Similarly, Manzano et al. (2020) reported high larval mortality (93%) at 50 mg/L from ethanolic neem leaf extracts, with GC-MS analysis identifying phytol as the major constituent. More recently, Imakwu et al. (2024) confirmed the larvicidal activity of ethanolic neem leaf extracts against *Ae. aegypti*, revealing a strong positive correlation between larval mortality and extract concentration. Several studies have also utilized ethanol as an extraction solvent for different plant species. Torawane et al. (2021) employed ethanol to extract phytochemicals from *Cyathocline purpurea*, *Blumea lacera*, *Neanotis lancifolia*, and *Neanotis montholonii* for ovicidal and larvicidal assays against *Ae. aegypti*. LC-MS analysis confirmed the presence of alkaloids, tannins, saponins, and flavonoids in the ethanol extracts, all of which exhibited significant ovicidal activity. Similarly, Nurul Izzatin et al. (2025) revealed that a 96% ethanol extract of *Crescentia cujete* L. displayed an ovicidal effect (LC_{50} = 6.51 ppm) against *Ae. aegypti*, with LC-MS revealing a high content of flavonoids. Egunjobi and Okoye (2020) extracted *Duranta erecta*, *Tridax procumbens*, and *Pennisetum purpureum* using 95% ethanol, where *D. erecta* demonstrated the strongest ovicidal effect with an LC_{50} of 10.037 ppm. More recently, Suryadi et al. (2025) demonstrated the larvicidal potential of ethanol extract from *Averrhoa bilimbi* L. leaves against *Ae. aegypti*.

The relatively higher LC_{50} (289.47 mg/L) and LC_{90} (2083.29 mg/L) values observed in the ethanolic neem leaf extract may be attributed to the use of crude extract, which contains a mixture of both active and inactive constituents. The presence of non-bioactive or potentially antagonistic compounds may reduce the overall potency, as explained previously. Other reasons may be explained by differences in extraction techniques and other parameters such as solvent-to-plant ratio, maceration duration and temperature, all of which influence the concentration of bioactive constituents in the crude extract (Harborne, 1998). Moreover, variability in phytochemical content due to geographical origin, leaf maturity, and seasonal collection may have contributed to the lower concentration of active compounds with ovicidal activity in the samples. Variations in bioassay conditions, including egg age, water quality, and incubation period, may also have influenced the hatchability outcomes (Arévalo-Cortés et al., 2022; Fischer et al., 2025; Gopalsamy et al., 2021; Islami et al., 2025). Several studies have demonstrated that purification and fractionation of crude plant extracts can enhance biological activity by selectively enriching the active constituents and removing inactive or antagonistic compounds (Eukubay et al., 2023; Selim et al., 2022). Supporting this, Maduraiveeran et al., (2025) reported that the chloroform crude extract of the aerial parts of *Ocimum americanum* exhibited the highest activity, with LC_{50} and LC_{90} values of 337.94 and 918.79 ppm after 48 hours, respectively. Bioassay-guided fractionation of the extract led to the isolation of the major bioactive compounds, oleanolic acid and ursolic acid, which exhibited markedly lower LC_{50}/LC_{90} values (oleanolic acid LC_{50}/LC_{90}=42.41/78.39 ppm and ursolic acid LC_{50}/LC_{90}=26.09/62.06 ppm, respectively), confirming the benefits of purification in improving ovicidal efficacy.

It is worth mentioning that the phytochemicals identified in the extracts, regardless of the solvent employed, are well documented for their insecticidal and mosquitocidal activities across mosquito life stages (Tufan-Cetin et al., 2023; War et al., 2011). These bioactive constituents, such as saponins, alkaloids, flavonoids, tannins, glycosides, terpenoids, and steroids, play crucial roles in insect control. Saponins, which are water-soluble, interact with the larval cuticle and alter cell membrane microstructure, ultimately leading to membrane disruption (Egunjobi and Okoye, 2020). Alkaloids primarily act on the insect gamma-aminobutyric acid (GABA) receptor, disturbing the nervous system (Coquerel et al., 2021), whereas flavonoids exert their effect through interference with the octopaminergic system in larvae (Perumalsamy et al., 2015). Tannins readily form complexes

with proteins and macromolecules (Petchidurai et al., 2023), thereby impairing larval growth and development and preventing successful adult emergence (Farahat et al., 2021; Khanna and Kannabiran, 2007). Glycosides have also been reported to possess insecticidal activity (Zeng et al., 2014). Terpenoids are well recognized for their ovicidal effects (Kaur et al., 2023; Sarma et al., 2019), primarily through blocking the micropylar region of the egg's chorion, which results in oxygen depletion or disrupts the embryos' ability to detoxify volatile components (Nattudurai et al., 2017). Plant-derived steroids such as brassinosteroids have been demonstrated to interfere with insect molting and reproduction (Davison et al., 2003).

The ovicidal effect observed in the ethanol extract may be attributed to the ability of the compounds to block the micropyle region of the egg, hindering gas exchange and ultimately leading to embryo death (Egunjobi and Okoye, 2020). Nonetheless, confirmation of this mechanism would require ultrastructural analysis using a transmission electron microscope (TEM) or scanning electron microscope (SEM). Similar mechanisms have been reported in previous studies. For instance, Kala et al. (2019) demonstrated that a neem oil tablet suspension reduced hatching of *A. culicifacies* eggs by 82% compared to the control. The ovicidal action was attributed to neem oil clogging aeropyles or pores, as well as damaging multiple chorionic layers (Suman et al., 2013). Such clogging prevents oxygen supply to the embryo, while the accumulation of carbon dioxide causes further damage, ultimately leading to egg mortality (Mehlhorn et al., 2011). Alves et al. (2020) reported that lectins from *Moringa oleifera* and *Myracrodruon urundeuva* exhibited ovicidal activity against *Ae. aegypti* by altering the chorionic surface and penetrating the embryos. In another study, Moungthipmalai et al. (2023) reported that essential oils, specifically trans-cinnamaldehyde from *Cinnamomum verum* and geranial from *Cymbopogon citratus*, acted synergistically as ovicides against *Ae. aegypti* eggs. Scanning electron microscopy of unhatched eggs revealed damage to the exochorionic layer, with oil layers covering cell borders, papillae, and aeropyles. Such blockage of aeropyles disrupts embryonic respiration, while geranial exerts an additional neurotoxic effect by inhibiting acetylcholinesterase (AChE) activity (Soonwera and Sittichok, 2020) and penetrating the serosal cuticle to interfere with embryogenesis (Castillo-Morales et al., 2021).

4. CONCLUSION

The present study demonstrates that neem leaf extracts of various solvents possess rich phytochemical compounds with promising ovicidal activity against *Ae. aegypti* eggs. Phytochemical screening tests revealed the presence of bioactive compounds such as alkaloids, saponins, tannins, steroids, glycosides, terpenoids, and flavonoids. Among the solvents tested, ethanol emerged as the most effective, extracting a wide range of phytochemicals and exhibiting the highest ovicidal activity, with 79% egg mortality at 1000 mg/L and the lowest LC_{50} (289.47 mg/L) and LC_{90} (2083.29 mg/L) values. Thus, the ethanol extract of neem leaf warrants further investigation for the development of eco-friendly ovicidal formulations suitable for integrated vector management programs. The extract might be used directly as an ovicidal agent in small-volume aquatic habitats or breeding sites of limited size around human dwellings. Furthermore, studies are in progress to purify the active compounds of crude ethanolic neem leaf extract, identify the active constituents, and optimise the process using alternative extraction techniques in the laboratory. These efforts aim to enhance the potency and standardize the bioactive composition of the extract.

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