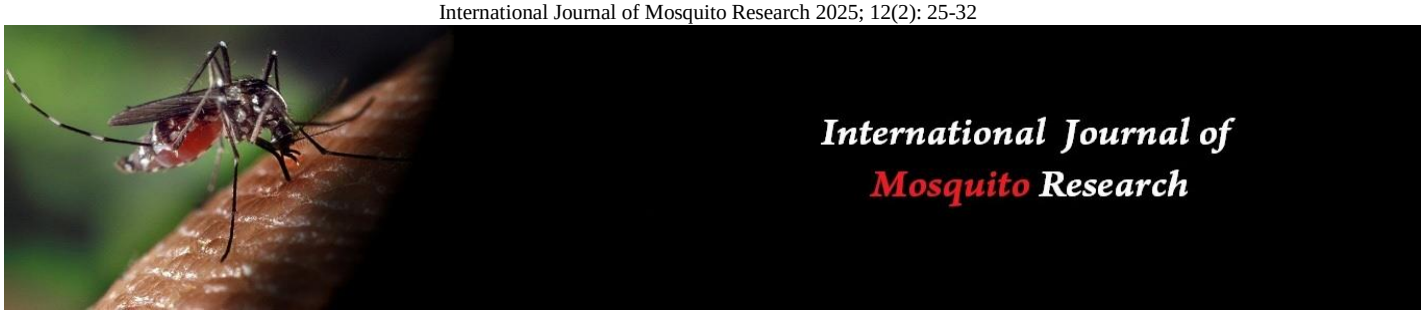

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Chemical composition and larvicidal activity of *Citrus hystrix* (kaffir lime) ethanolic leaf extract against *Aedes aegypti*

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Abstract
Mosquitoes are well-known carriers of dengue fever, underscoring their pivotal role in the global dissemination of infectious diseases with significant public health implications. This study employed an eco-conscious approach to combat these vector mosquitoes by assessing the phytochemical composition and the mosquito larvicidal potential of ethanolic leaf extract derived from *Citrus hystrix* against the dengue vector *Aedes aegypti*. A total of twenty-six (26) phytochemical constituents were detected from the ethanolic leaf extract of *C. hystrix* by GCMS. Citronellal (50.72%), 5-methyl-1-(4-methylpent-3-en-1-yl)-2-oxabicyclo[2.2.2]octan-4-yl]methanol (10.96%), caryophyllene (9.74%), and citronellol (3.87%) were identified as major chemical components followed by phytol (2.86%), citronellol acetate (2.75%), linalool (2.66%), copaene (1.59%), β-pinene (1.52%), and β-sabinene (1.46%). The mosquito larvicidal assay revealed promising effects of *C. hystrix* extract against *Aedes* mosquito larvae, varying efficacy based on the applied concentrations and duration of exposure. Higher extract concentration (2.0%) correlated with a greater mortality rate, while lower concentration (0.5%) exhibited reduced efficacy after 24 hours. Notably, a concentration of 2.0% demonstrated exceptional effectiveness, achieving a remarkable 95% mortality rate of mosquito larvae after 72 hours. Additionally, there is a notable increase in the number of dead mosquito larvae after 24 hours of treatment, further emphasizing the potency of the extract. This present study revealed that the ethanolic leaf extract of *C. hystrix* can be used as a new source of natural larvicidal agent and as an alternative for synthetic insecticides against *Ae. aegypti*.

Keywords: *Citrus hystrix*, larvicidal potency, mosquito, phytochemical screening

Introduction
Mosquitoes are tiny insects, yet they have had a major impact on humans and society. They spread dengue fever, dengue hemorrhagic fever, and other related diseases, especially in tropical and subtropical areas [1]. Dengue is prevalent in more than 120 countries, with 3.9 billion individuals at high risk of infection worldwide, and it causes morbidity and mortality [2]. Dengue virus carriers include *Aedes aegypti* and *Aedes albopictus* Skusse [3]. In the Philippines, dengue is a major public health concern. Between 2004 and 2010, the Philippines had the seventh-highest number of dengue cases globally [4]. Dengue cases were 220,705 in 2022, with 103 deaths in Central Visayas, 99 in Central Luzon, and 85 in Western Visayas [5]. The prevalence of dengue in the Philippines is influenced by various factors, including the inhabitants' knowledge, attitudes, and practices related to dengue prevention and control. These factors influence the presence of mosquito breeding sites and *Ae. aegypti* larval infestation rates. To manage these mosquitoes and the diseases they spread, preventive measures such as the use of traditional and synthetic insecticides are still required for situations in epidemic circumstances and in areas where adult mosquito populations are rapidly increasing [6, 7].
The most widely used vector control strategy is chemical control with bacterial larvicides, insect growth regulators, oils, and films. Nevertheless, the indiscriminate use of traditional and synthetic chemical control is contributing to a variety of issues, including widespread insecticide resistance, hazardous dangers to animals, adverse consequences on non-targeted species, and contamination of the surroundings [8-10].

These mosquitocides are highly effective and work immediately however, due to improper administration, resistance develops in the vector population. Each year, more synthetic insecticides are used, increasing the dangers to humans and other organisms and causing progressive environmental harm [1]. To address these serious issues, a proper vector control strategy is required. One of the recommended environmentally friendly vector control approaches is to employ plant extracts that are biodegradable, readily available, and nontoxic, particularly to non-target organisms. Plant-based remedies were the only pest control available until the development of synthetic chemicals; nevertheless, only a handful of plant-based insecticides are commercially available. Moreover, most have lesser and short-lasting potency than synthetic compounds. On the other hand, plant-derived bio-products are currently gaining popularity as alternatives to synthetic pesticides that can be included in management programs to reduce adverse effects and toxic residue accumulation in the ecosystems. Furthermore, there have been no reported cases of insects becoming resistant to plant-based insecticides [11].

Natural products have attracted renewed interest as a potential and viable source of natural larvicides against several mosquito species. Because adulticide application may offer a short-term reduction in the adult mosquito population [12], larvicides provide a more efficient and desirable approach to reducing mosquitoes by targeting them in their early life stage [13, 14]. Plant extracts have been reported to be practical and potent in larval control management due to the high concentrations of bioactive components present in the extracts [15-17]. *Citrus* plants, especially *C. hystrix*, are well-known for their fruit used in cooking and traditional medicine [1]. *C. hystrix* is also known as kaffir lime, Thai lime, and makrut lime. In Malay and Indonesia, it is known as *Limaupurut* and *Jerukpurut*. This *citrus* plant belongs to the *Rutaceae* family [18]. The *C. hystrix* plant has a thorny bush and a height ranging from 2 to 11 meters (6 to 35 feet), with distinctive scented and oddly formed "double" leaves [19, 20]. These hourglass-shaped leaves are made up of a flat blade and a narrow, leaf-like stalk. The fruit is characterized by its small size, measuring roughly 4 cm in diameter, and its rough, warty, green skin that turns yellow upon ripening [20]. The leaves, peel, rind, and fruit juice of *C. hystrix* have long been used to lessen the severity of specific ailments. The fruit juice, in particular, is used not only as a shampoo to get rid of head lice in Cambodia [20] but also to clean garments, especially in Thailand [21], and infrequently in Cambodia. *C. hystrix* oil is a raw ingredient utilized in a variety of industries, including pharmaceuticals, agriculture, food, sanitation, cosmetics, and perfumes. It is also widely used in aromatherapy and is vital to many cosmetic and cosmetics products [22].

As carriers of the dengue virus, mosquitoes lay their eggs in clean and stagnant water containers or any receptacles that accumulate water, especially during rainfall. Controlling mosquitoes while they are still in the water is one of the best methods to prevent the spread of the dengue virus to humans because it is much easier than adult control or when they are already flying. There are some ways to control them when they are still in the water. One is the application of biological control using ethanolic leaf & fruit extracts. This study aims to investigate ethanolic leaf extracts of *C. hystrix* using GCMS to determine its chemical profile and the mosquito larvicidal activity of these extracts against *Ae. aegypti*.

Materials and Methods

Plant Collection and Preparation of *C. hystrix* ethanolic leaf extracts

C. hystrix mature leaves were collected from the researcher's residence in Liloan, Cebu. After the collection process, the leaves were washed and air-dried for three days. The leaves of the petiole and midrib were removed, leaving only the blade that was finely shredded and homogenized with an electric blender. In a beaker, 20 grams of finely powdered leaves were combined with 250 mL of 95% ethanol. The resulting mixture was manually shaken every thirty (30) minutes for six (6) hours (about one minute) and then kept at room temperature for forty-eight (48) hours. The jar was shaken again for about a minute before being filtered with a Whatman No. 1 qualitative filter paper (125 mm). After filtration, the collected filtrate was stored at 4 degrees centigrade and used as the stock solution. This stock solution was further used to prepare the different concentrations of the extracts (0.5%, 1%, 1.5%, and 2%).

Gas Chromatography-Mass Spectrometry (GC-MS) Profiling of *C. hystrix* ethanolic leaf extracts

The GCMS profile of the ethanolic leaf extract of *C. hystrix* was investigated on a Shimadzu GCMS-QP2020 NX gas chromatograph-mass spectrometer. This instrument is equipped with an automatic sample injector AOC-20i Plus. The column equipped in this GCMS instrument was SH-Rxi-5Sil MS, a low-polarity phase cross bond 1,4-bis (dimethylsiloxy) phenyl dimethylpolysiloxane (length, 30 m; i.d., 0.24 mm; and film thickness, 0.25 µm). Helium gas was utilized as a carrier gas (flow rate of one mL per minute). The initial temperature of the injector was set at 80 °C, while the ion-source temperature was 250 °C. The sample injection volume was set at one µL with a split ratio of 10:1. The oven temperature was kept at 70 °C for the first 2 minutes. The temperature was then increased to 260 °C at a rate of 6 °C per minute. The mass detector was operated at 70 eV ionizing energy, with a scan period of 0.5 seconds and fragment sizes ranging from 40 to 600 Da. The relative percentage of each constituent was calculated using the area normalization method. Furthermore, the phytochemical constituents were determined by comparing their mass spectral patterns to reference mass spectra deposited at the NIST library.

Larvicidal Potential of *C. hystrix* ethanolic leaf extracts collection of mosquito larvae

Mosquito larvae samples were collected in clean and stagnant water in Cebu City, Philippines. The collected larvae were brought to the laboratory and sorted for the succeeding test.

Evaluation of the *C. hystrix* ethanolic leaf extract by dose-response bioassay

The standard protocols recommended by WHO for larvicidal bioassay were used with slight modifications [23]. Four (4) dosages of the ethanolic extract (0.5%, 1%, 1.5%, and 2%) were prepared and tested against the larvae of the dengue vector. Four (4) batches of twenty (20) mosquito larvae were placed in a separate plastic cup containing 250 ml of aqueous solutions, yielding 100 larvae per concentration. Control setup was done using distilled water without any addition of the solution. The same method was applied in other concentrations. The number of dead mosquito larvae was monitored at different exposure periods (24, 48, and 72

hours). Larvae were considered dead if they did not move after being perforated with a needle in the cervical area. The plastic cups containing the larvae were kept in a controlled environment. The temperature was maintained at 25°C, the humidity was between 80% and 90%, and the light cycle was 12 hours of light followed by 12 hours of darkness [24, 25].

Statistical Analysis

Percent mortality with standard deviation for each concentration was determined at different exposure periods (24, 48, and 72 hours). Using probit regression analysis, lethal concentrations (LC_{50} and LC_{90}) were determined at a 95% confidence level. Statistical analyses of the experimental data were performed using different statistical software, IBM SPSS Statistics and Microsoft Excel, to determine the log probit at 95% confidence level, regression, and mean percentage mortality. The mean percentage mortality, standard error, and upper and lower bounds were calculated at 95% confidence.

Results and Discussion

Chemical Composition of *C. hystrix* ethanolic leaf extracts

Table 1 shows the phytochemicals of the ethanolic leaf extracts of *C. hystrix* analyzed by GC-MS, along with their corresponding retention time and peak area. In total, twenty-six (26) phytochemical constituents were detected by GCMS, as shown in Figure 1, belonging to different classes of bioactive compounds that exhibit several biological and pharmacological activities. Among these phytochemical constituents, citronellal (50.72%), 5-methyl-1-(4-methylpent-3-en-1-yl)-2-oxabicyclo[2.2.2]octan-4-yl]methanol (10.96%), caryophyllene (9.74%), and citronellol (3.87%) were identified as major chemical components illustrated in Figure 2, followed by phytol (2.86%), citronellol acetate (2.75%), linalool (2.66%), copaene (1.59%), β-pinene (1.52%), and β-sabinene (1.46%). Other phytochemicals are also present in trace amounts. Similarly, the GCMS profiling results in this present study coincide with the phytochemicals identified and reported previously in the earlier studies [26-28].

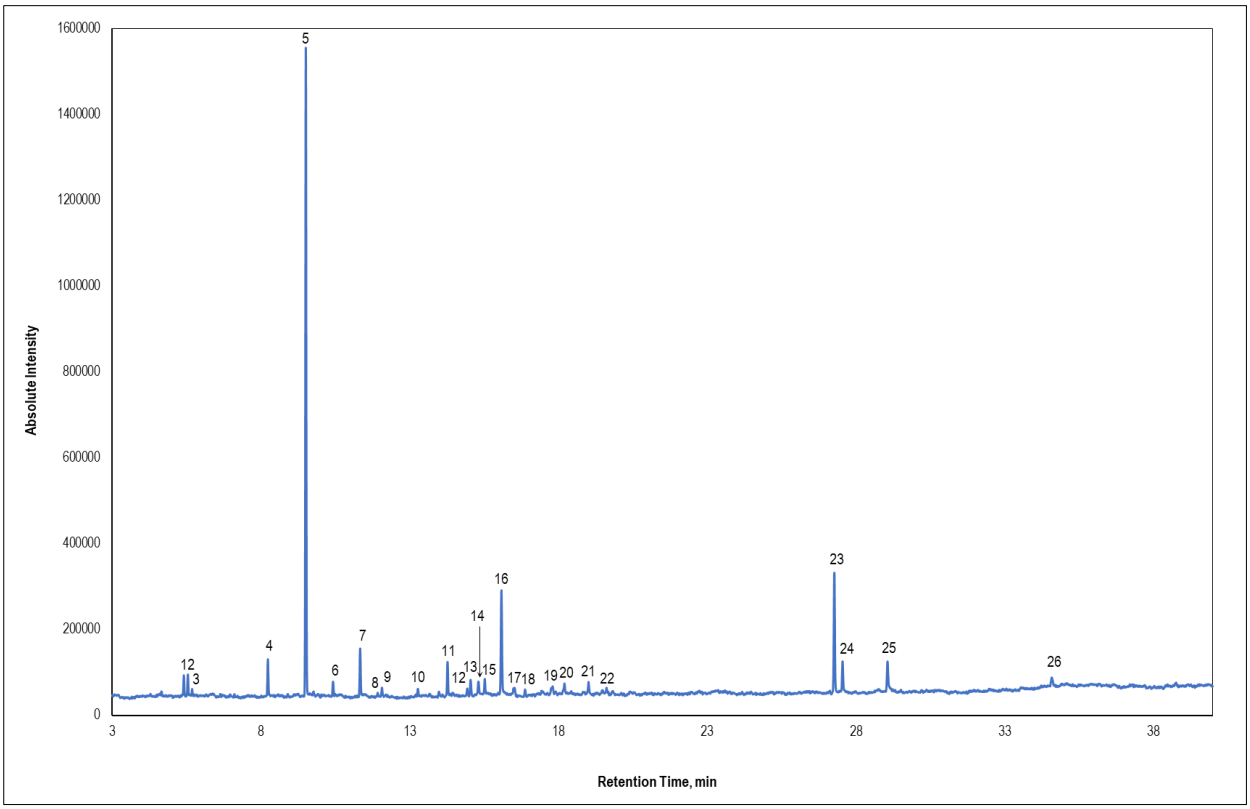

Fig 1: Gas chromatogram of *C. hystrix* ethanolic leaf extracts.

Table 1: Chemical constituents of *C. hystrix* ethanolic leaf extracts

Peak No.	Retention time (min)	Chemical composition[a,b]	Area (%)
1	5.414	β-Sabinene	1.46
2	5.550	βPinene	1.52
3	5.692	β-myrcene	0.40
4	8.239	Linalool	2.66
5	9.515	Citronellal	50.72
6	10.427	Napthalene	1.26
7	11.340	Citronellol	3.87
8	11.931	Nerol	0.26
9	12.073	α-Limonene diepoxide	0.66
10	13.278	Farnesyl acetate	0.60
11	14.277	Citronellol acetate	2.75
12	14.940	Nerol acetate	0.46
13	15.051	Copaene	1.59

14	15.317	Copaen-4-α-ol	1.01
15	15.529	Citronellal diethyl acetal	1.14
16	16.090	Caryophyllene	9.74
17	16.529	1,2-Epoxydodecane	1.04
18	16.881	Humulene	0.66
19	17.809	α-farnesene	1.24
20	18.207	1-isopropyl-4,7-dimethyl-1,2,3,5,6,8a-hexahydronapthalene	0.72
21	19.016	β-Nerolidol	0.90
22	19.622	Caryophyllene oxide	0.34
23	27.278	[5-methyl-1-(4-methylpent-3-en-1-yl)-2-oxabicyclo [2.2.2] octan-4-yl] methanol	10.96
24	29.065	Phytol	2.86
25	34.589	Oxypeucedanin	0.90
26	34.625	(R)-4-(3-(4-((3-fluorophenyl) (hydroxy)	
Total			100.00
Legend: [a]Compounds listed in order of elution from SH-Rxi-5Sil MS; [b]Compounds identified based on computer matching of the mass spectra with the NIST Library			

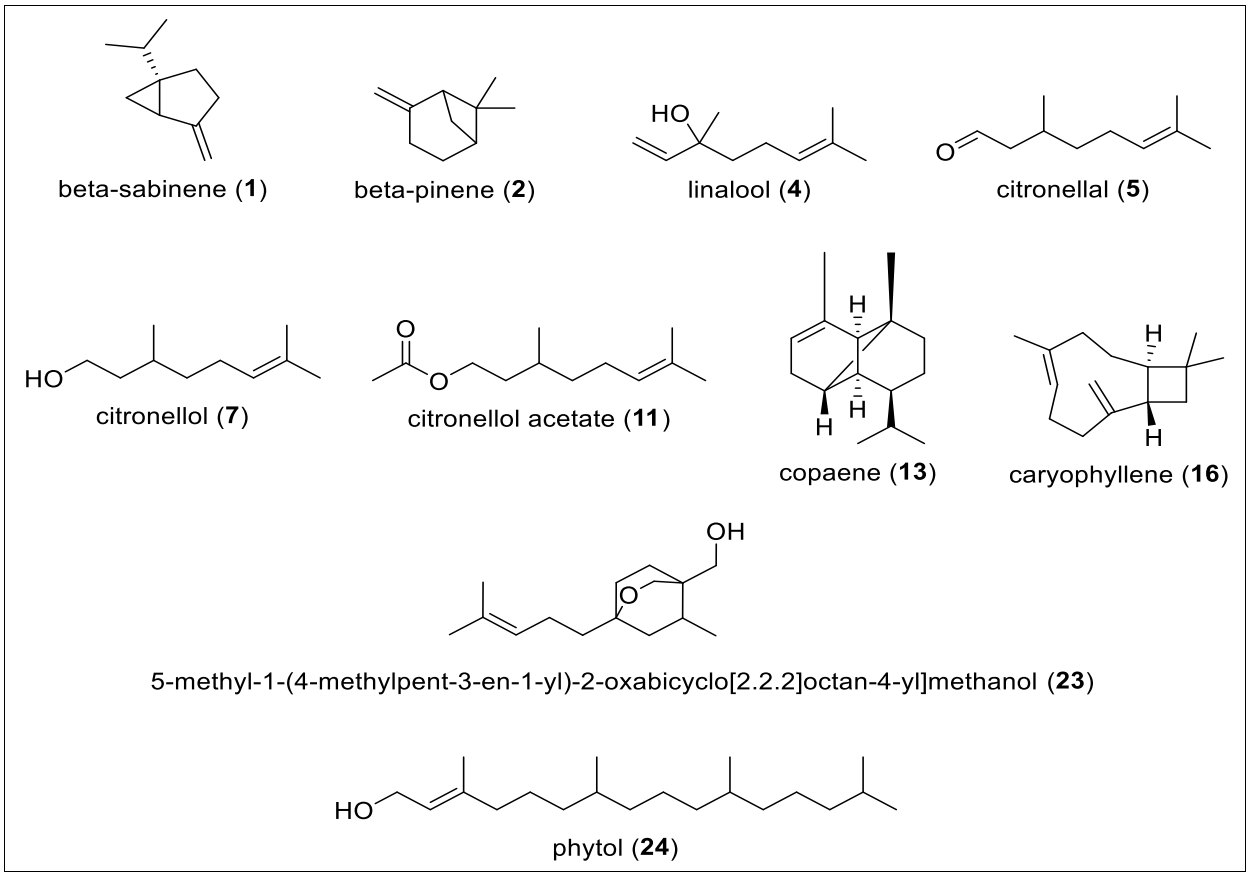

Fig 2: Major phytochemical components of *C. hystrix* ethanolic leaf extracts.

Moreover, phytochemicals including citronellal, citronellol and citronellol acetate were reported as major components of *C. hystrix* leaf and fruit peel extracts [29-31] and display diverse biological activities both *In vitro* and *in vivo* systems including antimicrobial [32-35], anti-inflammatory [36], antioxidant [35, 37], hepatoprotective [38], anticancer [39-41], cholinesterase inhibition [37, 42-44], insecticidal and larvicidal activities [45-47]. In addition, Figure 1 and Table 1 show that citronellal constitutes the highest peak area among the phytochemicals present in *C. hystrix* ethanolic leaf extracts.

Citronellal is an oxygenated monoterpene (a terpenoid) present in the essential oil of several aromatic plants, including *Citrus* spp., *Cymbopogon* spp., *Eucalyptus citriodora*, and *Melissa officinalis* [48]. Like many natural products, citronellal and its corresponding oxidation product citronellol have been reported to exhibit insect-repellent properties against mosquitoes, mites, ticks, fleas, and other varieties of insects such as moths, aphids, and lace bugs [49]. These monoterpenes can be used in various applications, including their use as a natural and nontoxic insecticide, an innovative and safer substitute over chemical and synthetic insecticides. Other biologically active and biocidal compounds, such as those listed in Table 1, may also contribute largely to the insecticidal and larvicidal activities of *C. hystrix* ethanolic leaf extracts against mosquitos.

Mosquito larvicidal activities of *C. hystrix* ethanolic extracts

The application of *C. hystrix* extract exhibited significant efficacy against *Ae. aegypti* mosquito larvae, as illustrated in Figure 3. Within 24 hours of application, 15%, 26.65%, 25%, and 63.3% mortality rates were observed at concentrations of

0.5, 1.0, 1.5, and 2.0%, respectively. Subsequently, after 48 hours, mortality rates increased compared to the initial 24-hour exposure, reaching 18.3%, 60%, 66.3%, and 81.65% at the same concentrations. Remarkably, by the 72-hour mark post-application, mortality rates surged to 55%, 70%, 66.65%, and 95% respectively for the concentrations mentioned.

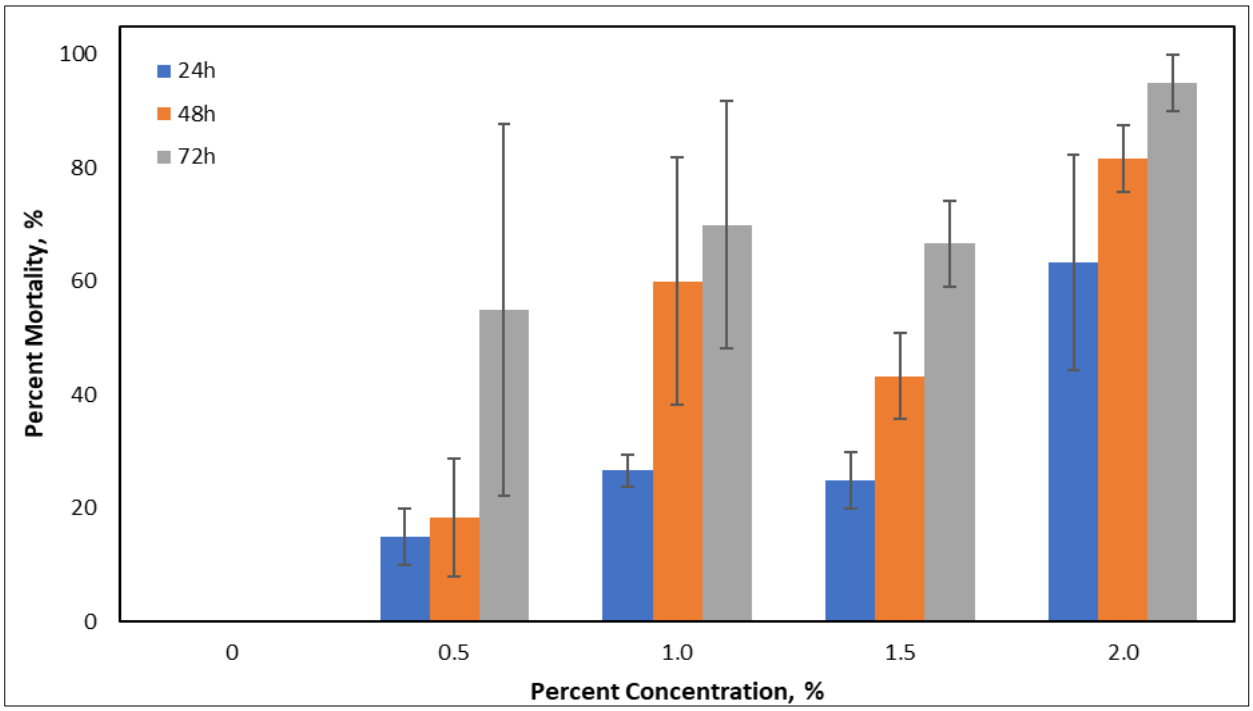

Fig 3: Percent mortality of mosquito larvae at different exposure periods to the *C. hystrix* ethanolic leaf extract.

Table 2: Larvicidal properties of *C. hystrix* ethanolic leaf extract against the larvae of *Ae. aegypti* at different exposure periods

Time	Concentration (%v/v)	Mortality% (Mean ± SD)[a]	LC_{50} (LCL-UCL)[b]	LC_{90} (LCL-UCL)[b]	$\chi 2$ (DF=10)[c]
After 24 h	0.5	15.0 ± 5.0	1.981 (1.579-3.030)	8.762 (4.813-35.407)	17.978
	1.0	26.7 ± 2.9			
	1.5	25.0 ± 5.0			
	2.0	63.3 ± 18.9			
After 48 h	0.5	18.3 ± 10.4	1.095 (0.718-1.597)	3.779 (2.231-30.798)	28.555
	1.0	60.0 ± 21.8			
	1.5	43.3 ± 7.6			
	2.0	81.7 ± 5.8			
After 72 h	0.5	55.0 ± 32.8	0.470 (0.000-0.000)[d]	2.829 (0.000-0.000)[d]	39.809
	1.0	70.0 ± 21.8			
	1.5	66.7 ± 7.6			
	2.0	95.0 ± 5.0			

Legend: LCL-lower confidence level, UCL-upper confidence level; [a]Mean replicates of three measurements; [b]95% Confidence interval; [c]Degrees of freedom; $\chi 2$ chi-square value; [d]No confidence limit estimated.

Table 2 illustrates that an LC_{50} of 1.98 after 24 hours of extract application to the mosquito larvae suggests the requirement of 1.98% to eradicate 50% of the mosquito larvae. Similarly, an LC_{90} of 8.76 indicates that only 8.76% of extract is necessary to eliminate 90% of the mosquito larvae within the same time frame. Conversely, after 48 hours of extract application, the table reveals an LC_{50} of 1.09 and an LC_{90} of 3.78, implying the necessity of 1.09 and 3.78% of the extract, respectively, to eliminate 50% and 90% of the mosquito larvae. Furthermore, the table indicates that after 72 hours of application, an LC_{50} of 0.47 and an LC_{90} of 2.82 are required to eradicate 50% and 90% of mosquito larvae, respectively.

Furthermore, the results highlight the significant larvicidal effects of *C. hystrix* extract against *Ae. aegypti* mosquito larvae, with mortality rates increasing over time and with higher extract concentrations. The increasing mortality rates over 24, 48, and 72 hours show the importance of exposure duration in achieving optimal results. The significant improvement in efficacy with time suggests that the extract's active components continue to affect the larvae beyond the initial exposure period higher concentrations of *C. hystrix* extract yield higher mortality rates, consistent with the principles of dose-response relationships. However, the dramatic increase in mortality at lower concentrations over time indicates that even minimal amounts of the extract can be highly effective given sufficient exposure. Over time, the decreasing LC_{50} and LC_{90} values reflect the extract's increasing potency with prolonged exposure. These metrics are crucial for determining the optimal concentration and exposure time for effective mosquito control.

Conclusion

GCMS profiling identified twenty-six (26) phytochemicals in the ethanolic leaf extract of *C. hystrix*, with citronellal, 5-methyl-1-(4-methylpent-3-en-1-yl)-2-oxabicyclo[2.2.2]octan-4-yl]methanol, caryophyllene and citronellol as major components. The extract also demonstrates significant potential as an effective natural larvicide against *Ae. aegypti* mosquito larvae. Its increasing efficacy over time and at varying concentrations provides a strong foundation for developing environmentally friendly mosquito control strategies.

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