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Larvicidal effects of bio-insecticide against filariasis vector, *Culex quinquefasciatus*

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Abstract

Background *Cx. quinquefasciatus* is a vector for lymphatic filariasis and West Nile fever, diseases for which no vaccine is available. Hence, controlling the population of this mosquito requires urgent attention. Synthetic insecticides are widely used for this purpose. However, the limitations associated with their usage, including the triggering of resistance development and non-target toxicities, necessitate the exploration of alternative, eco-friendly solutions. This study investigated the larvicidal effects of eight medicinal plants against *Cx. quinquefasciatus*. Notably, with the exception of *Ocimum basilicum* and *Tagetes erecta*, the insecticidal activity of the remaining six plants has not been previously studied, to our knowledge. Larvicidal efficacy was assessed following the WHO bioassay protocols.

Results The larvicidal bioassay results showed that stigmasterol isolated from the ethyl acetate fraction of *O. basilicum* leaves was highly effective as a larvicidal agent (LC₅₀ value of 34.97 ppm, i.e., < 50 ppm). To our knowledge, stigmasterol was isolated from *O. basilicum* for the first time in this study. Among the tested extracts of the eight selected plants, the ethyl acetate fraction of *O. basilicum* exhibited the next highest potency (LC₅₀ value of 97.72 ppm) with moderate larvicidal effectiveness ((LC₅₀ values > 50 ppm and < 100 ppm), whereas the chloroform fraction (LC₅₀ value of 115.32 ppm) and n-hexane fraction of *Pistacia integerrima* (LC₅₀ value of 298.02 ppm) were effective (LC₅₀ values > 100 ppm and < 300 ppm). In contrast, the crude methanolic extracts and their soluble fractions (n-hexane, chloroform, and ethyl acetate) from the remaining six plants, as well as the crude methanol extract and n-hexane fraction of *O. basilicum* and the crude methanol extract and chloroform and ethyl acetate fractions of *P. integerrima*, exhibited less effectiveness as larvicidal agents (LC₅₀ values > 300 ppm).

Conclusion These findings highlight stigmasterol as a highly effective natural larvicidal agent, offering a promising natural and sustainable alternative for integrated mosquito management.

Keywords Botanical insecticides, *Cx. quinquefasciatus*, Larvicidal effectiveness, Stigmasterol, Vector control

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Background

Mosquitoes, being blood-sucking insects, are perhaps the most dangerous vectors affecting human health worldwide. Due to their ability to transmit disease-causing pathogens, mosquitoes are likely to spread more diseases of pathogenic origin than other insects (de Omena et al., 2007). Mosquitoes, being vectors of many important diseases such as filariasis, haemorrhagic fever, yellow fever, chikungunya, malaria, and dengue (James, 1992), pose a significant global threat, contributing to millions of human deaths annually and remaining a major challenge. Annually, at least 500 million individuals are expected to be infected worldwide with mosquito-borne diseases (Madhumathy et al., 2007).

Among disease-transmitting mosquitoes, *Cx. quinquefasciatus*, the principal vector of life-threatening diseases like lymphatic filariasis (Fontes et al., 2012) and West Nile fever (WNV) (Molaei et al., 2007), is responsible for a significant number of mosquito-related deaths and has caused endemic and epidemic diseases in both in animals and humans for decades, predominantly in Asia, Africa, Europe, and the Middle East (Wilkins & Piero, 2004). For instance, lymphatic filariasis affects approximately 120 million people worldwide each year (Rahuman & Venkatesan, 2008), with the highest prevalence in Sub-Saharan Africa and India (Michael et al., 1996). Likewise, since its introduction in 1999, West Nile Virus has been the leading cause of arboviral encephalitis in the United States (Davis et al., 2007).

As *Cx. quinquefasciatus* is a vector for several fatal diseases, urgent control of its population is needed. Synthetic insecticides play a key role in *Cx. quinquefasciatus* control, but their usage has not been successful, as they not only negatively affect non-target organisms (Hoang & Rand, 2015) but also trigger resistance development in mosquitoes (Vaulin et al., 2019). In contrast, bio-insecticides of botanical origin are biodegradable, eco-friendly, non-toxic to beneficial insects (Khurshed et al., 2022). By exhibiting broad-spectrum activity against mosquitoes (Ghosh et al., 2012), they make it difficult for mosquitoes to acquire resistance, rendering them a promising alternative in integrated management of mosquitoes. Not only are they safer than synthetic insecticides, but they also play a key role in managing insecticide resistance in mosquitoes. Unlike synthetic insecticides, which most likely act as general toxicants, botanical insecticides not only act as toxicants but also function as chemosterilants, growth inhibitors, and repellents against mosquitoes (Prathibha et al., 2014). The use of botanical products as insecticides has proven to be not only safer for non-target organisms (Ravi et al., 2018; Souza et al., 2011) but also more effective than synthetic insecticides (Deletre et al., 2013). As a result, botanical insecticides from various plant families have focused on targeting larval

mosquitoes, offering a promising alternative for effective mosquito management (Nawarathne & Dharmarathne, 2024).

To address the global challenges posed by synthetic insecticides, including the triggering of insecticide resistance and non-target toxicity, this study evaluated the larvicidal efficacy of several medicinal plants, including *Asparagus officinalis*, *Leptopus cordifolius*, *Monotheca buxifolia*, *O. basilicum*, *Onosma hispidum*, *P. integerrima*, *Strobilanthes walichii*, and *T. erecta*, against *Cx. quinquefasciatus*. Although these plants have several medicinal properties, with the exception of *O. basilicum* and *T. erecta*, the insecticidal efficacy of the remaining six plants has not been previously studied, to our knowledge. Therefore, the present study aims to explore the larvicidal effectiveness of these plants against *Cx. quinquefasciatus*.

A. officinalis, known locally as Qanta in Pashto in Khyber Pakhtunkhwa province of Pakistan, is a perennial herb of the Asparagaceae family. It is known for diverse medicinal properties, including antioxidant (Fan et al., 2015), antimicrobial (Desoukey et al., 2020), and anticancer activities (Romani et al., 2021). It is also used to treat various conditions like kidney stone, hypertension, constipation, and infertility (Iqbal et al., 2017). The plant's biological activities stem from its secondary metabolites, including antioxidant and antimicrobial properties from saponins (e.g., sarsasapogenin, protodioscin, and shatavarin) and sterols (e.g., β -sitosterol), and anticancer properties from flavonoids (e.g., quercetin and rutin) (Guo et al., 2020). Other important compounds present in the plant include pyrazines and sulfur-containing volatiles, which contribute to the plant's characteristic aroma (Pegiou et al., 2019).

L. cordifolius, is a perennial shrub in the Phyllanthaceae family, known locally as Chaghji botey and topak lakhta in Pashto. It possesses antioxidant, antidiabetic, and anti-hyperlipidemic activities due to secondary metabolites like terpenoids (e.g., betulin), sterols (e.g., stigmasterol and β -sitosterol), and phenols (e.g., 2-Methoxy-4-vinylphenol) (Rahman et al., 2021).

M. buxifolia, locally called Gurgura in Pashto, is a perennial spiny tree from the Sapotaceae family, known for its anti-inflammatory, antinociceptive, antipyretic (Ullah et al., 2016), and cytotoxic, antimicrobial, and antioxidant activities (Ahmad et al., 2019). These biological effects are attributed to its secondary metabolites, including flavonoids like isoquercetin, kaempferol, and robinin, phenolics such as rutin and catechin, and terpenoids like lupeol, α -amyrin, oleanolic acid, and diosgenin, found in its various parts (Ali et al., 2020; Ullah et al., 2016, 2017).

O. basilicum, locally known as Kashmalay in Pashto, is a perennial herb from the Lamiaceae family, possessing anti-cancer (Purushothaman et al., 2019), antibacterial

(Nahak et al., 2011), antifungal (Elsherbiny et al., 2017), antioxidant (Gulcin et al., 2007), and insecticidal activities (Ileke & Adesina, 2019). These activities are attributed to its secondary metabolites: terpenoids (e.g., eugenol, linalool, camphor, and 1,8-cineole) contribute to its antibacterial, anticancer, antifungal, and antioxidant activities (Aburjai et al., 2020; Zhakipbekov et al., 2024); phenolics (e.g., apigenin-7-glucuronide) and terpenoids (e.g., ursolic acid) are linked to its antiviral activities (Kurnia et al., 2023); and phenolics (e.g., estragole), terpenoids (e.g., humulene, alpha farnesene, and 4-cerene), and flavonoids (e.g., rutin) are responsible for its insecticidal activities (Bincy et al., 2022; Prasannakumar et al., 2023; Darrag et al., 2024).

O. hispidum, locally known as Surkhai in Pashto, is a perennial herb in the Boraginaceae family, valued for its antibacterial (Nazir et al., 2022), anticancer (Asghar et al., 2018), antioxidant (Kumar et al., 2017), and wound healing effects (Ali et al., 2015), attributed to its secondary metabolites, including phenolics like ferulic acid and vanillic acid (antibacterial) (Naz et al., 2006) and flavonoids such as rutin (antioxidant) (Kumar et al., 2011).

P. integerrima, locally called Shnai in Pashto, is a dioecious tree from the Anacardiaceae family, known for its diverse medicinal properties, including antiasthmatic (Shirole et al., 2014), antioxidant (Eshwarappa et al., 2015), anti-microbial (Rahman et al., 2011), and leishmanicidal activities (Uddin et al., 2012). These activities are attributed to its secondary metabolites. Key compounds identified in the plant, such as the antioxidant quercetin (flavonoid) and pyrogallol (phenol) (Zahoor et al., 2018), the antiasthmatic and leishmanicidal naringenin and kaempferol (flavonoids) (Rauf et al., 2015, 2023), and the leishmanicidal pistagremic acid (terpene) (Uddin et al., 2012), justify these scientifically recognized activities.

S. walichii, commonly known as Kashmir acanthus, is a perennial herb from the Acanthaceae family. While other species from the genus are known for their anticancer, antidiabetic, antimicrobial, antioxidant, antiviral, and hepatoprotective activities due to the presence of compounds like stigmaterol (sterol), botulin, and lupeol (terpenoids) (Nair et al., 2016), *S. walichii* remains unstudied, to our knowledge. This lack of research means its bioactive compounds are currently unknown, presenting a knowledge gap for this plant.

T. erecta, locally known as Damberguley in Pashto, is an annual herb in the Asteraceae family. It is recognized for its diverse biological activities, including anticancer (Vedam et al., 2019), antioxidant (Burlec et al., 2021), antiparasmodial (Gupta & Vasuddeva, 2010), antimicrobial, hepatoprotective, and insecticidal properties (Sing et al., 2019). The plant's known compounds, which support these scientifically acknowledged activities, include

antioxidant flavonoids (e.g., quercetagitin) (Burlic et al., 2021), antiparasmodial phenolics (bithienyl) (Gupta & Vasuddeva, 2010), and anticancer, antimicrobial, and insecticidal terpenoids (lutein, and β -carotene) and phenolics (syringic acid) (Boruah et al., 2025).

Materials and methods

Plant material

Plant material, including shoots of *A. officinalis*, *L. cordifolius*, *O. hispidum*, and leaves of *M. buxifolia*, *O. basilicum*, *P. integerrima*, *S. walichii*, and *T. erecta*, were collected from the Ouch Khairabad area (34° 43' N, 72° 1' E), District Dir Lower, Khyber Pakhtunkhwa, Pakistan (Fig. 1S). The plants were identified by Dr. Gul Rahim, a renowned plant taxonomist and Lecturer at the Department of Botany, University of Malakand, Chakdara, Dir Lower, Khyber Pakhtunkhwa, Pakistan. Following identification, the plant materials (shade-dried and dust free) were ground into fine powder using an electric blender.

Extractions and fractionations

The powdered plant materials (8 kg) from each plant were separately soaked in methanol for 15 days and then filtered using Whatman filter paper No. 42. The methanol solvent was evaporated from the filtrates under reduced pressure at 45 °C using a rotary evaporator to obtain crude methanol extracts. The methanol extracts were further fractionated into n-hexane, chloroform, and ethyl acetate soluble fractions in a separating funnel. Following bioassay-guided isolation, the crude methanol extracts and their n-hexane, chloroform, and ethyl acetate soluble fractions from each plant were tested for larvicidal efficacy against *Cx. quinquefasciatus* larvae. Notably, the ethyl acetate soluble fraction from *O. basilicum* leaves exhibited the highest larvicidal potential and was identified as the most active solvent fraction.

Bioassay guided isolation of larvicidal active compound

The most active ethyl acetate fraction (45 g) from *O. basilicum* was purified into stigmaterol through column chromatography using bioassay guided fractionation. Column chromatography fractions were obtained using a silica gel column (60, 230–400 mesh ASTM, (Merck, 0.040–0.063 mm) eluted with n-hexane: ethyl acetate in varying ratios (100:0, 95:5, 90:10, 85:15, 80:20, 70:30, 60:40, 50:50, 30:70, 10:90, and 0:100). Forty-two fractions were collected and analyzed using thin-layer chromatography (TLC) on silica gel plates (Merck, 60 F₂₅₄) using system of n-hexane: ethyl acetate (90:10, 80:20, and 70:30) as the mobile phase. Fractions with similar spots were pooled, resulting in 11 fractions, labelled as OF1 (4.09 g), OF2 (2.77 g), OF3 (2.14 g), OF4 (2.36 g), OF5 (3.12 g), OF6 (7.53 g) (eluted in n-hexane: ethyl acetate system in ratios of 90:10, 85:15, and 80:20, respectively),

OF7 (2.62 g), OF8 (3.5 g), OF9 (3.01 g), OF10 (2.74 g), and OF11 (3.12 g). These fractions were tested for larvicidal effectiveness against *Cx. quinquefasciatus*, and OF6 (7.43 g) was identified as the most active. OF6 (7 g) was further purified, yielding 19 subfractions, which were pooled into five subfractions, labelled as OF6-1 (1.4 g), OF6-2 (0.8 g), OF6-3 (0.7 g), OF6-4 (0.6 g), and OF6-5 (0.5 g) based on TLC analysis. These subfractions were tested for larvicidal efficacy against *Cx. quinquefasciatus* larvae, and OF6-3 exhibited the highest effectiveness. OF6-3 (0.7 g) was chromatographed using n-hexane-ethyl acetate as eluent, yielding a pure compound (MG-I) from fractions 78–100 of the 100 fractions collected, with each fraction consisting of 15 mL of eluting solvent.

The purity of the isolated compound was assessed by TLC and melting point determination. Its structure, as shown in Fig. 1, was elucidated using NMR (^1H , ^{13}C) and mass spectroscopy (Fig. 2S and 3S). Based on the melting point, which was found to be 167–169°C in the present study, and the spectral data from NMR (^1H , ^{13}C) and mass spectroscopy, the compound was identified as stigmasterol, consistent with previous reports (Falodun et al., 2008; Sianipar et al., 2021). The EI-MS spectra (Fig. 4S) were recorded on a JEOL 600 H-1 mass spectrometer. The ^1H NMR spectra were recorded on an Avance Neo 400 NMR spectrometer in CDCl_3 , and the ^{13}C NMR spectra were recorded on an Avance Neo 300 NMR spectrometer in CDCl_3 . EI-MS m/z (rel. int. %): $[\text{M}]^+$ 412.3 (34), 351.2 (15), 271 (35), 255.1 (70), 159 (75), 145 (82), 106.9 (76), 80.9 (92), 69 (64), 54.9 (100), 42.9 (78). ^1H NMR: 5.33 (1H, m, H-6), 5.16 (1H, dd, $J=15.3$, 8.0 Hz, H-22), 5.02 (1H, dd, $J=15.3$, 8.0 Hz, H-23), 3.54 (1H, m, H-3), 0.90 (3 H, d, $J=6.5$ Hz, CH_3 -21), 0.83 (3 H, d, $J=6.6$ Hz, CH_3 -26), 0.84 (3 H, t, $J=7.0$ Hz, CH_3 -29), 0.82 (3 H, d, $J=6.5$ Hz, CH_3 -27), 0.80 (3 H, s, CH_3 -19), δ 0.67 (3 H, s, CH_3 -18). ^{13}C -NMR: δ 140.8 (C-5, s), 138.3 (C-22, d), 129.2 (C-23, d), 121.7 (C-6, d), 71.9 (C-3, d), 56.8 (C-14, d), 56.0 (C-17, d), 51.2 (C-24, d), 50.1 (C-9, d), 45.9 (C-13, s), 42.3 (C-4, t), 40.4 (C-20, d), 39.8 (C-12), 37.2 (C-1, t), 36.6 (C-10, s), 35.6 (C-8, d), 33.6 (C-25, d), 31.9 (C-7, t), 31.7 (C-2, t), 29.3 (C-16, t), 28.9 (C-16, t), 28.1 (C-28, t), 24.2 (C-15, t), 23.0 (C-27, q), 21.0 (C-21, q), 19.8 (C-11, t),

19.3 (C-19, q), 18.6 (C-26, q), 17.8 (C-18, q), 12.0 (C-29, q).

Larvae collections

Larvae of *Cx. quinquefasciatus* were collected from a sewage water ditch located opposite the main gate of the University of Malakand (34° 38' 59.99 N, 72° 01' 60.00 E). Late 3rd instar larvae were used for the larvicidal bioassay. The larval breeding environment in this ditch is characterized by a subtropical climate with warm and humid conditions, featuring brief cold winters and prolonged hot summers.

Larvicidal bioassay

The larvicidal effectiveness of the tested plants against *Cx. quinquefasciatus* was evaluated following the WHO (1996) procedure under laboratory conditions (temperature: 28–31°C, relative humidity: 70–75%). To achieve complete solubility, 0.2 g of each tested sample was dissolved separately in 1 mL of DMSO and then diluted to 200 mL with distilled water to obtain a 1000 ppm stock solution. From this stock solution, test concentrations of 250 mL volume were prepared in 500 mL glass beakers using distilled water. Twenty-five larvae, collected from the field were released immediately after collection into each of these beakers without being supplied with food during the experiment. Four replicates were set up for each concentration. Mortality rates were recorded after a 24-hour exposure period. A parallel control experiment was conducted to account for potential DMSO effects by adding 1 mL of DMSO to 200 mL of water (matching the stock solution) and adding the same volume of this DMSO-water mixture to the control as was added for the test concentrations.

Data analysis

The percentage mortality of larvae was calculated using the formula:

$$\text{Percentage mortality} = \frac{\text{number of dead larvae}}{\text{number of treated larvae}} \times 100$$

The results were presented as mean $\pm$ standard deviation of four replicates. The larvicidal effectiveness of column chromatography fractions was compared using one-way ANOVA followed by Tukey test. The mortality data (average) were subjected to probit analysis to calculate LC_{50} and LC_{90} values, and their 95% confidence limits (lower and upper) using SPSS version 16.0. Results with $p\text{-value} \leq 0.05$ were considered statistically significant.

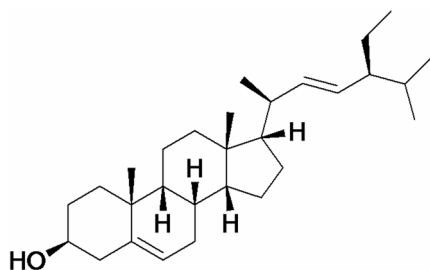


Fig. 1 Structure of Stigmasterol

Table 1 Larvicidal effectiveness of different solvent fractions obtained from the crude methanol extract of *O. basilicum* leaves against *Cx. quinquefasciatus*

Name of extract	Percentage of larval mortality $\pm$ SD			LC ₅₀ , LC ₉₀ and their 95% confidence limit	
	Concentration (ppm)			LC ₅₀	LC ₉₀
	20	40	80	(LCL-UCL)	(LCL-UCL)
n-Hexane	4 $\pm$ 4.61	9 $\pm$ 6.00	17 $\pm$ 2.00	422.17 (172.58– 23248.03.58.03)	3966.0 (687.46– 128100.0.46.0)
Chloroform	8 $\pm$ 3.27	22 $\pm$ 4.00	38 $\pm$ 6.92	115.32 (96.52– 148.14.52.14)	599.16 (389.84– 1129.08.84.08)
Ethyl acetate	10 $\pm$ 6.92	29 $\pm$ 8.24	42 $\pm$ 8.32	97.72 (71.83– 188.79.83.79)	539.97 (249.58– 3596.87.58.87)
Control	0.0 $\pm$ 0.0				

LC₅₀, concentration that causes 50% mortality; LC₉₀, concentration that causes 90% mortality; LCL, lower confidence limit; UCL, upper confidence limit

Results

Larvicidal effectiveness of crude methanol extracts and their solvent fractions from several medicinal plants against *Cx. quinquefasciatus*

The results showed that, among the crude methanol extracts and their solvent fractions from several medicinal plants, the ethyl acetate-soluble fraction from *O. basilicum* was the most effective larvicidal agent, exhibiting moderate larvicidal activity (LC₅₀ 97.72 ppm) against late 3rd instar larvae of *Cx. quinquefasciatus*. This was followed by the chloroform-soluble fraction from *O. basilicum* and the n-hexane-soluble fraction from *P. integerrima*, with effective larvicidal activity (LC₅₀ 115.32 ppm and 298.02 ppm, respectively) (Table 1). The n-hexane-soluble fractions from *A. officinalis*, *O. basilicum*, and *M. buxifolia* showed less effective against *Cx. quinquefasciatus* larvae (LC₅₀ values of 348.10 ppm, 422.18 ppm, and 513.16 ppm, respectively) (Tables 2 and 1). Additionally, the crude methanol extract from *S. wilchii*

Table 2 Larvicidal effectiveness of crude methanol extracts and different solvent fractions from several medicinal plants against *Cx. quinquefasciatus*

Plant species	Name of extract	Percentage of larval mortality $\pm$ SD			LC ₅₀ , LC ₉₀ and their 95 % confidence limit	
		Concentration (ppm)			LC ₅₀	LC ₉₀
		56	168	504	(LCL-UCL)	(LCL-UCL)
<i>Asparagus officinalis</i>	Methanol	3 $\pm$ 3.82	17 $\pm$ 6	41 $\pm$ 8.87	670.82 (486.93-1141.72)	3899.0 (1963.39-13958.40)
	n-Hexane	5 $\pm$ 3.82	35 $\pm$ 5.03	58 $\pm$ 10.59	348.10 (274.06-479.60)	1796.0 (1081.50-4290.45)
	Chloroform	0 $\pm$ 0.00	0 $\pm$ 0.00	8 $\pm$ 3.27	2126.0 (1287.19-7415.99)	7599.0 (3151.41-70627.41)
	Ethyl acetate	0 $\pm$ 0.00	1 $\pm$ 2.00	10 $\pm$ 4.00	1890.0 (1073.56-11153.21)	7077.0 (2581.80-185179.60)
<i>Leptopus cordifolius</i>	Methanol	0 $\pm$ 0.00	5 $\pm$ 6	17 $\pm$ 3.82	1680.0 (906.50-10183.50)	8896.0 (2806.63-311155.18)
	n-Hexane	0 $\pm$ 0.00	9 $\pm$ 6.83	21 $\pm$ 2.00	1455.0 (831.89-5696.50)	8881.0 (2998.22-147895.93)
	Chloroform	0 $\pm$ 0.00	0 $\pm$ 0.00	0 $\pm$ 0.00	—	—
	Ethyl acetate	0 $\pm$ 0.00	0 $\pm$ 0.00	0 $\pm$ 0.00	—	—
<i>Monothea buxifolia</i>	Methanol	0 $\pm$ 0.00	0 $\pm$ 0.00	30 $\pm$ 8.32	674.94 (613.09-779.14)	1370.0 (1098.30-1964.52)
	n-Hexane	0 $\pm$ 0.00	0 $\pm$ 0.00	48 $\pm$ 13.06	513.16 (475.91-568.34)	815.81 (680.67-1419.61)
	Chloroform	0 $\pm$ 0.00	0 $\pm$ 0.00	0 $\pm$ 0.00	—	—
	Ethyl acetate	0 $\pm$ 0.00	0 $\pm$ 0.00	0 $\pm$ 0.00	—	—
<i>Onosma hispidum</i>	Methanol	11 $\pm$ 3.82	21 $\pm$ 2.00	37 $\pm$ 10.51	1154.0 (674.98-3319.19)	26380.0 (7052.84- 424428.98)
	n-Hexane	4 $\pm$ 4.61	11 $\pm$ 2.00	24 $\pm$ 5.66	2228.0 (1087.58-11451.33)	33080.0 (7592.09-1093760.81)
	Chloroform	4 $\pm$ 4.61	12 $\pm$ 5.66	25 $\pm$ 7.58	2001.0 (916.81-16898.89)	28340.0 (5660.92-2936594.76)
	Ethyl acetate	10 $\pm$ 8.32	21 $\pm$ 2	35 $\pm$ 6.83	1281.0 (661.17-6404.52)	30220.0 (6152.09-1960603.33)
<i>Pistacia integerrima</i>	Methanol	0 $\pm$ 0.00	11 $\pm$ 2.00	56 $\pm$ 4.61	444.23 (419.21-485.86)	1175.0 (1002.36-1434.72)
	n-Hexane	7 $\pm$ 5.03	24 $\pm$ 10.32	72 $\pm$ 3.26	298.02 (244.10-378.56)	1109.0 (768.59-1972.57)
	Chloroform	0 $\pm$ 0.00	3 $\pm$ 2.00	43 $\pm$ 3.82	564.00 (519.92-622.33)	1283.0 (1081.88-1613.08)
	Ethyl acetate	0 $\pm$ 0.00	2 $\pm$ 2.30	7 $\pm$ 5.03	4685.0 (1566.69-1022350.52)	33450.0 (5183.01-401300000)
<i>Strobilanthes wilchii</i>	Methanol	8 $\pm$ 9.23	18 $\pm$ 4.00	51 $\pm$ 3.82	525.09 (363.06-1016.38)	3453.0 (1545.09-19634.03)
	n-Hexane	1 $\pm$ 2.00	4 $\pm$ 4.61	22 $\pm$ 4.00	1168.0 (830.01-2175.59)	4882.0 (2509.06-17476.02)
	Chloroform	2 $\pm$ 4.00	16 $\pm$ 11.78	39 $\pm$ 6.83	700.51 (462.48-1680.48)	3807.0 (1613.12-31291.90)
	Ethyl acetate	0 $\pm$ 0.00	2 $\pm$ 2.30	15 $\pm$ 2.00	1465.0 (1076.82-2455.40)	5503.0 (3090.39-14881.86)
<i>Tagetes erecta</i>	Methanol	0 $\pm$ 0.00	5 $\pm$ 7.58	34 $\pm$ 6.92	712.31 (538.94-1251.02)	2132.0 (1224.009-7866.80)
	n-Hexane	0 $\pm$ 0.00	4 $\pm$ 5.66	32 $\pm$ 14.23	740.006 (546.89-148917)	2146.0 (1181.35-10870.10)
	Chloroform	0 $\pm$ 0.00	0 $\pm$ 0.00	0 $\pm$ 0.00	—	—
	Ethyl acetate	0 $\pm$ 0.00	0 $\pm$ 0.00	42 $\pm$ 9.52	542.68 (517.50-585.60)	868.14 (742.09-1219.37)

Values are mean $\pm$ SD of four replicates; SD, standard deviation; LC₅₀, concentration that causes 50% mortality; LC₉₀, concentration that causes 90% mortality; LCL, lower confidence limit; UCL, upper confidence limit; Mortality in the group of control was zero

Table 3 Probit analysis of larval mortality of column chromatography fractions from the ethyl acetate soluble fractions of *O. basilicum* leaves against *Cx. quinquefasciatus*

Fraction	LC ₅₀ (ppm)	95% confidence limit (ppm)		LC ₉₀ (ppm)	95% confidence limit (ppm)	
		LCL	UCL		LCL	UCL
OF1	155.90	109.98	294.77	720.70	358.76	2759.68
OF2	144.29	97.52	318.56	723.59	325.30	4093.47
OF3	108.17	73.52	247.04	496.23	225.40	3381.96
OF4	117.80	85.68	207.53	527.81	274.87	1868.39
OF5	113.00	87.20	168.58	532.56	309.33	1308.30
OF6	31.19	26.98	36.26	90.91	71.84	127.40
OF7	105.04	75.09	188.00	782.26	360.00	3423.23
OF8	147.56	101.13	299.63	785.46	363.40	3662.01
OF9	284.46	146.19	2036.23	1615.00	471.70	71291.17

Mortality in the group of control as well as in OF10 and OF11 was zero; LC₅₀, concentration that causes 50% mortality; LC₉₀, concentration that causes 90% mortality; LCL, lower confidence limit; UCL, upper confidence limit

Table 4 Larvicidal effectiveness of column chromatography fractions from the ethyl acetate soluble fraction of *O. basilicum* leaves against *Cx. quinquefasciatus*

Fraction	% Mortality				Control
	Concentration (ppm)				
	10	20	40	80	
OF1	0.0 ± 0.0 ^b	4.0 ± 4.61 ^{bc}	18.0 ± 5.16 ^{bc}	25.0 ± 3.82 ^{cd}	0.0 ± 0.0
OF2	0.0 ± 0.0 ^b	6.0 ± 5.16 ^{bc}	22.0 ± 8.32 ^{bc}	27.0 ± 6.83 ^{bcd}	0.0 ± 0.0
OF3	4.0 ± 4.61 ^{ab}	6.0 ± 6.92 ^{bc}	17.0 ± 11.49 ^{bc}	43.0 ± 15.09 ^{bc}	0.0 ± 0.0
OF4	3.0 ± 2.0 ^{ab}	5.0 ± 7.57 ^{bc}	16.0 ± 7.30 ^{bc}	39.0 ± 8.24 ^{bc}	0.0 ± 0.0
OF5	4.0 ± 3.27 ^{ab}	6.0 ± 2.30 ^{bc}	16.0 ± 3.27 ^{bc}	42.0 ± 7.66 ^{bc}	0.0 ± 0.0
OF6	12.0 ± 5.66 ^a	25.0 ± 5.03 ^a	59.0 ± 5.03 ^a	90.0 ± 11.54 ^a	0.0 ± 0.0
OF7	8.0 ± 9.23 ^{ab}	14.0 ± 2.30 ^{ab}	23.0 ± 2.00 ^b	46.0 ± 4.00 ^b	0.0 ± 0.0
OF8	2.0 ± 4.00 ^b	7.0 ± 5.03 ^{bc}	14.0 ± 4.00 ^{bc}	33.0 ± 8.24 ^{bcd}	0.0 ± 0.0
OF9	0.0 ± 0.0 ^b	3.0 ± 6.00 ^{bc}	9.0 ± 2.00 ^{cd}	16.0 ± 7.30 ^{de}	0.0 ± 0.0
OF10	0.0 ± 0.0 ^b	0.0 ± 0.00 ^c	0.0 ± 0.00 ^d	0.0 ± 0.00 ^e	0.0 ± 0.0
OF11	0.0 ± 0.0 ^b	0.0 ± 0.00 ^c	0.0 ± 0.00 ^d	0.0 ± 0.00 ^e	0.0 ± 0.0

Values are mean ± SD of four replicates. Means sharing same letters in a column are not significantly different at $p < 0.05$ (one – way ANOVA, Tukey test)

(LC₅₀ 525.09 ppm) and the ethyl acetate-soluble fraction from *T. erecta* (LC₅₀ value of 542.68 ppm) (Table 2) were also less effective larvicidal agents against *Cx. quinquefasciatus*. The classification of the larvicidal activity as “highly effective” was based on a published study (Pavela, 2015), which reported phytochemicals with LC₅₀ values < 50 ppm to be highly effective, LC₅₀ values > 50 ppm and < 100 ppm to be moderately effective, LC₅₀ values > 100 ppm and < 300 ppm to be effective, and > 300 ppm to be less effective against mosquito larvae.

Larvicidal effectiveness of column chromatography fractions against *Cx. quinquefasciatus*

The larvicidal bioassay results of column chromatography fractions from the most active ethyl acetate-soluble fraction of *O. basilicum* leaves revealed that, among the 11 isolated fractions, OF6 was the most effective larvicidal agent against *Cx. quinquefasciatus* larvae, with LC₅₀ value of 31.19 ppm (Table 3). Notably, OF6 exhibited a concentration-dependent larval mortality of 12%, 25%, 59%, and 90% at tested concentrations of 10 ppm, 20 ppm, 40 ppm, and 80 ppm, respectively (Table 4). No mortality was observed in the control group.

Larvicidal effectiveness of column chromatography sub-fractions against *Cx. quinquefasciatus*

The larvicidal bioassay results of column chromatography sub-fractions from the most potent column chromatography fraction (OF6) revealed that, among the five isolated sub-fractions, OF6-3 was the most effective larvicidal agent, with LC₅₀ value of 25.67 ppm. It exhibited mortality rates of 12%, 29%, and 77% at tested concentrations of 10 ppm, 20 ppm, and 40 ppm, respectively (Table 3). The control group exhibited zero mortality.

Larvicidal effectiveness of purified stigmasterol isolated from Ethyl acetate-soluble fraction of *O. basilicum* against *Cx. quinquefasciatus*

Stigmasterol showed high larvicidal effectiveness against *Cx. quinquefasciatus* larvae, exhibiting 12% and 30% mortality at tested concentrations of 10 ppm and 20 ppm, respectively (Table 5), with LC₅₀ value of 34.97 ppm (Table 5). Mortality in the control group was zero.

Spectroscopic characterization

Spectroscopic analysis conclusively confirmed the successful isolation and purity of stigmasterol, with close agreement between the calculated and experimental values.

Table 5 Larvicidaleffectiveness of OF6 chromatographed sub-fractions and purified stigmasterol against *Cx. quinquefasciatus*

Fraction	Percentage of larval mortality $\pm$ SD			LC ₅₀ , LC ₉₀ and their 95 % confidence limit	
	Concentration (ppm)			LC ₅₀	LC ₉₀
	10	20	40	(LCL-UCL)	(LCL-UCL)
OF6-1	6 $\pm$ 4.00 ^{abc}	12 $\pm$ 3.27 ^b	26 $\pm$ 8.32 ^b	106.82 (65.11-352.63)	718.56 (251.38-10058.59)
OF6-2	9 $\pm$ 2.00 ^{ab}	14 $\pm$ 4.00 ^b	30 $\pm$ 8.32 ^b	100.18 (63.99-262.67)	818.21 (297.68-7861.64)
OF6-3	12.0 $\pm$ 3.27 ^a	29.0 $\pm$ 3.82 ^a	77.0 $\pm$ 3.82 ^a	25.67 (23.22-28.69)	63.36 (52.32-82.93)
OF6-4	4 $\pm$ 3.27 ^{bc}	10 $\pm$ 2.30 ^{bc}	23 $\pm$ 6.83 ^b	109.79 (70.45-282.60)	621.47 (251.94-4548.14)
OF6-5	0.00 $\pm$ 0.00 ^c	3 $\pm$ 3.82 ^c	9 $\pm$ 2.00 ^c	144.76 (78.24-1575)	508.74 (173.83-36794)
Stigmas-terol	12 $\pm$ 3.26	30 $\pm$ 5.16	—	34.97 (28.04-52.40)	136.98 (80.21-379.89)

Values are mean $\pm$ SD of four replicates. Means sharing same letters in a column are not significantly different at $p < 0.05$ (one – way ANOVA, Tukey test).

Mass spectroscopy

The mass spectral data of the isolated compound showed that the molecular ion peak appeared at m/z 412.3, corresponding to the molecular formula $C_{29}H_{48}O$. Distinct ion peaks appeared at m/z 336.1, 319.1, 306.0, 279.2, 256.2, 185.0, 167.0, 149.0, 125.0, 113.0, 97.0, 83.0, 71.0, 56.9, and 42.9. The spectroscopic data matched the data available in the literature (Falodun et al., 2008). Thus, the compound was determined to be stigmasterol.

NMR spectroscopy

In the 1H -NMR spectra, all protons correspond to the stigmasterol structure. The 1H -NMR spectrum showed a multiplet appearance for two tertiary methyl groups at δ 5.33 (1H) and 5.16 (2 H), for three olefinic protons (showing trans-olefinic coupling to C-22 and C-23) at δ_H 5.33 (1H), 5.16 (1H), and 5.02 (1H), and another multiplet at δ 3.54 for the carbinylic proton (Falodun et al., 2008, Sianipar et al., 2021). The data thus established the structure as stigmasterol.

The ^{13}C -NMR spectrum displayed 29 carbon signals corresponding to the stigmasterol structure, comprising six methyl carbon signals at δ_C 17.8 (C-18), 12.0 (C-29), 23.0 (C-27), 18.6 (C-26), 21.0 (C-21), and 19.3 (C-19), nine methylene carbon sp^2 signals at δ_C 19.8 (C-11), 28.1 (C-28), 24.2 (C-15), 28.9 (C-16), 31.7 (C-2), 31.9 (C-7), 37.2 (C-1), 39.98 (C-12), and 42.3 (C-4), seven metine carbons sp^3 at δ_C 33.6 (C-25), 35.6 (C-8), 40.4 (C-20), 51.2 (C-24), 50.1 (C-9), 56.0 (C-17), and 56.8 (C-14), one oxygenated metine carbon at δ_C 71.9 (C-3), three metine carbons sp^2 at δ_C 121.7 (C-6), 138.3 (C-22), and 129.2 (C-23), two quaternary carbon sp^3 at δ_C 36.6 (C-10) and 45.9 (C-13), and one quaternary carbon sp^2 at δ_C 140.8 (C-5) (Falodun et al., 2008, Sianipar et al., 2021).

Discussion

Managing *Cx. quinquefasciatus*, the primary vector of West Nile Virus encephalitis, requires effective alternatives to synthetic insecticides, which trigger resistance development and non-target toxicity. Botanical bio-insecticides offer a promising alternative, as they manage insecticide resistance, exhibit broad action against mosquitoes, and are eco-friendly, natural, and sustainable. This study explores the larvicidal effectiveness of several medicinal plants, including *A. officinalis*, *L. cordifolius*, *M. buxifolia*, *O. basilicum*, *O. hispidum*, *P. integerrima*, *S. walichii*, and *T. erecta* against *Cx. quinquefasciatus*. Notably, with the exception of *O. basilicum* and *T. erecta*, the insecticidal activity of the remaining six plants has not been previously studied, to our knowledge. Previous studies have investigated the mosquitocidal effectiveness of various plants belonging to the genus *Ocimum* (Kamaraj & Rahuman, 2010; Shahi et al., 2010), and they have reported *O. basilicum* to be more effective (Rajamma et al., 2011). The findings of the present study showed that among crude methanol extracts and their n-hexane, chloroform and ethyl acetate soluble fractions evaluated from eight medicinal plants, the ethyl acetate soluble fractions from *O. basilicum* was the most effective mosquito larvicide, with LC₅₀ value of 97.72 ppm against late 3rd instar larvae of *Cx. quinquefasciatus*. The results obtained in the present study are consistent with those reported in a previous study on the larvicidal effectiveness of crude methanol extract and its solvent fractions by Bagavan et al. (2009). They reported that the ethyl acetate-soluble fraction from *Ocimum canum* and *Ocimum sanctum* leaves to showed good activity against *Anopheles subpictus* larvae (LC₅₀ = 88.15 ppm and 21.67 ppm, respectively). However, our findings contradict those of Nzelibe and Chintem (2015), who reported the n-hexane-soluble fraction (LC₅₀ 2.5 ppm) as the most effective among the tested crude ethanol extract and its n-hexane, ethyl acetate, and aqueous fractions from *Ocimum gratissimum* leaves against late 3rd instar larvae of *Cx. quinquefasciatus*. Additionally, Opiyo (2020) in his study reported the n-hexane fraction as the most effective in his study, which assessed the methanol extract and its n-hexane and ethyl acetate-soluble fractions, as well as essential oil extract from *Ocimum suave* leaves for their repellent, growth inhibitory, and insecticidal activity against *Sitophilus zeamais* (commonly called maize weevil).

In the present study, among the crude methanol extracts and their n-hexane, chloroform and ethyl acetate-soluble fractions evaluated from the remaining seven plants, the most effective were in the order of: n-hexane-soluble fraction from *P. integerrima* (LC₅₀ = 298.02 ppm) > n-hexane-soluble fraction from *A. officinalis* (LC₅₀ = 348.10 ppm) > n-hexane-soluble fraction from *M. buxifolia* (LC₅₀ = 513.16 ppm) > crude methanol extract

from *S. wilchii* (LC_{50} = 525.09 ppm) > ethyl acetate soluble-fraction from *T. erecta* (LC_{50} = 542.68 ppm) > crude methanol extract from *O. hispidum* (LC_{50} = 1154.0 ppm), and n-hexane-soluble fraction from *L. cordifolius* (LC_{50} = 1455.0 ppm). Our study on *T. erecta* yields distinct findings compared to previous research on the larvicidal activity of crude methanol extract and its solvent fractions from this plant against *Cx. quinquefasciatus*, highlighting the variability in plant extract efficacy. Notably, Jayaraman et al. (2015) reported that the leaf methanol extract from *T. erecta* was the most active against *Cx. quinquefasciatus* larvae (LC_{50} = 273.78 ppm), followed by chloroform-soluble fraction (LC_{50} = 370.81 ppm), ethyl acetate-soluble fraction (LC_{50} = 397.08 ppm), and n-hexane-soluble fraction (LC_{50} = 273.78 ppm). Similarly, Govindarajan and Sivakumar (2014) reported that *Asparagus racemosus* root methanol extract (LC_{50} = 115.13 ppm) exhibited significantly higher activity against *Cx. quinquefasciatus* larvae than its n-hexane, chloroform, and ethyl acetate-soluble fractions. These discrepancies highlight the complexity of plant extract bioactivity, emphasizing the need for further research.

Moreover, in the present study, the most effective ethyl acetate-soluble fraction from *O. basilicum* was subjected to column chromatography, yielded the compound stigmasterol. Among the 11 isolated fractions from the *O. basilicum* ethyl acetate-soluble fraction, fraction OF6 was found to be more effective against late 3rd instar larvae of *Cx. quinquefasciatus*, with LC_{50} value of 31.19 ppm, and was therefore subjected to further column chromatography fractionations, separating into five sub-fractions. When tested against *Cx. quinquefasciatus* larvae, sub-fraction 3, labelled as OF6-3, was found to be the most effective (LC_{50} 25.67 ppm) against *Cx. quinquefasciatus* larvae. Subsequent fractionation of OF6-3 through a silica gel column using n-hexane-ethyl acetate as the eluent yielded pure stigmasterol from fractions 78–100 of the 100 fractions collected, with each fraction consisting of 15 mL of eluting solvent. The structure of stigmasterol was elucidated by mass spectrometry and NMR spectroscopy, with spectra comparable to those reported earlier (Falodun et al., 2008; Sianipar et al., 2021). Interestingly, previous studies have also explored the insecticidal potential of column chromatography fractions and isolated compounds from the most potent solvent fractions of the genus *Ocimum*. For instance, Nzelibe and Chintem (2015) reported that column chromatography fraction three (F3) from the most active n-hexane soluble-fraction of *O. gratissimum* exhibited the highest larvicidal effectiveness (LC_{50} 1.49 ppm) against late 3rd instar larvae of *Cx. quinquefasciatus*. Opiyo (2020) purified chromatography fractions II and III from the most potent n-hexane fraction of *O. suave* leaves into stigmasterol and evaluated its efficacy against *S. zeamais*. Notably, our study

revealed that stigmasterol exhibits high larvicidal activity against *Cx. quinquefasciatus* late 3rd instar larvae, with LC_{50} value of 34.97 ppm (Table 5). This finding is consistent with earlier research, where stigmasterol isolated from *Vitex schiliebenii* leaves acetone extract showed similar larvicidal potential (Nyamoita, 2013). Our results demonstrate that chromatography fractions containing stigmasterol exhibit high larvicidal effectiveness, highlighting the potential of plant derived stigmasterol in controlling mosquito populations. These findings highlight the significance of further exploring plant-based compounds for mosquito control.

The mosquito larvicidal potential of stigmasterol against *Cx. quinquefasciatus* can be attributed to its neurotoxic effects, specifically to its ability to inhibit acetylcholinesterase activity in mosquitoes (Gade et al., 2017). This inhibition of acetylcholinesterase leads to hyper-excitability in the mosquito's olfactory system, resulting abnormal neural function. This distinct mode of action of stigmasterol offers an alternative solution to overcome insecticide resistance. Unlike synthetic insecticides, which often target sodium channels and are vulnerable to resistance mechanisms like the kdr mutation (Uddin et al., 2025), stigmasterol neurotoxicity via acetylcholinesterase inhibition provides a novel pathway for *Cx. quinquefasciatus* control (Muthu et al., 2025). Given its natural origin and unique mechanism, stigmasterol emerges as promising agent for integrated mosquito management programs, potentially revolutionizing the fight against *Cx. quinquefasciatus*.

Conclusion

Cx. quinquefasciatus, the vector for West Nile virus encephalitis, poses a significant threat due to the lack of a vaccine for this disease; making this mosquito control as the only viable option. However, worldwide dependence on synthetic insecticides has rendered them ineffective by triggering the development of resistance in mosquitoes and causing non-target toxicity. This study highlights the potential of stigmasterol isolated from *O. basilicum* as a natural and effective larvicidal agent against *Cx. quinquefasciatus*. Considering the issues of insecticide resistance, non-target toxicity, and environmental safety, further research on plant-derived bio-insecticides, especially from understudied plants, is important for the development of sustainable mosquito management strategies.

Abbreviations

ANOVA	Analysis of variance
<i>A. officinalis</i>	<i>Asparagus officinalis</i>
<i>L. cordifolius</i>	<i>Leptopus cordifolius</i>
<i>M. buxifolia</i>	<i>Monothea buxifolia</i>
<i>O. basilicum</i>	<i>Ocimum basilicum</i>
<i>O. hispidum</i>	<i>Onosma hispidum</i>
<i>P. integerrima</i>	<i>Pistacia integerrima</i>
<i>S. wilchii</i>	<i>Strobilanthes wilchii</i>

<i>T. erecta</i>	<i>Tagetes erecta</i>
Cx	<i>Culex</i>
g	Gram
kg	Kilogram
mg	Milligram
mL	Milliliter
LC ₅₀	Lethal concentration causing 50 % of mortality
LC ₉₀	Lethal concentration causing 90 % of mortality
OF	Ocimum fraction
ppm	Parts per million
NMR	Nuclear magnetic resonance
¹³ CNMR	Carbon-13 nuclear magnetic resonance
¹ H NMR	Proton nuclear magnetic resonance
TLC	Thin layer chromatography
WHO	World Health Organization

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s41936-025-00531-9>.

Supplementary Material 1.

Author contributions

All authors contributed to the study conception and design. M. Zahoor, MN. Umar, M. Zahid and M. Gul conceived and designed the study. M. Gul collected the materials and conducted the experiments. M. Gul and SWA. Shah wrote the main manuscript. M. Zahid supervised the research work. M. Zahoor statistically analyse the data, while the others edited the manuscript writing. All authors read the manuscript and approved its publication.

Funding

This research work has received no funding.

Data availability

The data associated with this study are included in the manuscript.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Received: 28 February 2025 / Accepted: 27 November 2025

Published online: 11 December 2025

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