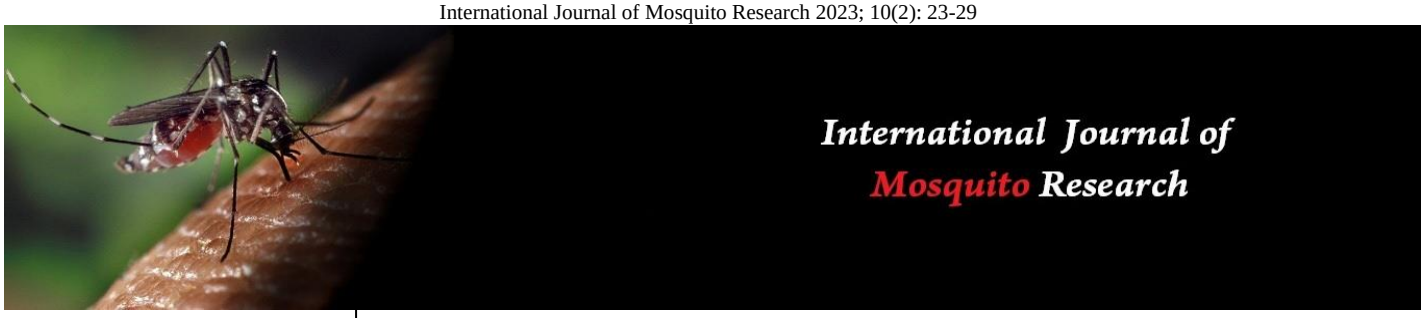

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Michelia champaca seed extract: A novel mosquitocide against the filarial vector *Culex quinquifasciatus*

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Abstract
Vector control is in risk as a result of the advent of synthetic pesticide resistance. Plant derived insecticides may be used as future bio control alternatives due to their target specificity and biodegradability. The aim of the present study was to determine the efficacy of Chloroform: Methanolic extract of *Michelia champaca* seeds against all the larval instars of *Culex quinquifasciatus*, the vector of *Bancroftian filariasis*. Chloroform: Methanolic extract showed remarkable larvicidal properties with LC_{50} and LC_{90} values as low as 15.33 ppm and 29.77 ppm recorded against the first instar larvae after 72 hours of treatment. There was a clear dose dependent increment in the death rate, which was positively correlated with the solvent concentration and had regression coefficient values close to 1. Because the investigated non-target organisms were unaffected, the plant-based mosquitocide that was thus discovered was proved to be environment friendly. FTIR analysis revealed the presence of various functional groups in the TLC fraction such as alcohol, alkane, methyl group, amine, aromatics, carboxylic acid and ether. So, *M. champaca* seeds extract can be proclaimed as an outstanding larvicidal agent against *Cx. quinquifasciatus*, and can serve as a source of potent mosquito larvicide in the near future.

Keywords: *Michelia champaca*, *Culex quinquifasciatus*, LC_{50} and LC_{90} values, larvicide, FTIR analysis

Introduction
There are more than 3500 species of mosquitoes in the globe, and thousands of them feed on the blood of different animals, with roughly 20% of them having the ability to transmit diseases to humans and other animals [1, 2]. Mosquito control is of grave concern as they transmit various diseases such as Malaria, Filariasis, Chikungunya, Dengue etc. that have the potential to cause a global lethality to more than a million in a year [3]. In many tropical nations, human filariasis continues to be a difficult socioeconomic issue and a serious threat to public health [4]. The Indian subcontinent has a higher prevalence of lymphatic filariasis, which is caused by the worm *Wuchereria bancrofti*, spread by the mosquito *Culex quinquefasciatus* [5]. Each year, this filarial vector infects around 100 million people around the world [6].
During previous years, eliminating and controlling the mosquito vector was mainly practiced through the application of synthetic organophosphate and organochlorine insecticides in larval habitat [7]. Synthetic pesticides are nor biodegradable, neither target specific and have negative impacts on beneficial organisms when applied in the vector control process. Therefore, the present need is to investigate a novel mosquitocide that would be biodegradable, target specific and non-detrimental to any other life forms [8] though some biocontrol agents are reported to reduce mosquito population [9-11]. Usually, some of the plant derived molecules are known to have such qualities that proclaim them to be most suitable to formulate mosquito larvicide [12, 13], pupicide [14], adulticide [15] and repellents [16] along with other attributes like antibacterials [17], anthelmintics [18, 19] etc.
Originating from South and Southeast Asia, *Michelia champaca* L. is a tall, evergreen tree that is mostly cultivated for its lumber. It is particularly renowned for its fragrant blossoms, which bloom during the monsoon season.

The aril-coated seeds of this tree are very appealing to birds [20]. It is reported to be used as a febrifuge and to cure fever, leprosy, postpartum protection, and labour according to traditional literature. Additionally, it is acknowledged to possess antipyretic and anti-inflammatory qualities [21].
The present study specifically focused at evaluating the larvicidal potential of the Chloroform: methanol extract of *M. champaca* seeds against *Cx. quinquifasciatus* larvae.

Materials and Method

Rearing of test mosquito

The current study was carried out in Burdwan, West Bengal, India (23°16′ N, 87°54′ E). Randomly selected rafts of eggs from wild *Cx. quinquefasciatus* laid in their natural habitat, such as the nearby cement drains in and around the university campus, were gathered. Larvae were given dog biscuits and dried yeast powder in a 3:1 powdered mixture after hatching [22]. The larvae were reared at a constant temperature of 27 °C and protected from pathogens, insecticides, and repellents. Using a glass dropper and a glass beaker filled with tap water, the transformed pupae were carefully separated. The beaker was put into the enclosures to encourage the development of adult mosquitoes. Adult insects were given glucose meal on a cotton ball dipped in a 10% glucose solution. The laid eggs were raised in similar fashion. First generation laboratory-bred larvae were used during the present experiment.

Collection of Seeds

Fresh and matured fruits of *M. champaca* were collected from the plants growing at the outskirts of Chandan Nagar, Hooghly and West Bengal. The plant along with fruits was authentically identified by Dr. Ambarish Mukherjee and the specimen copy had been deposited at the departmental herbarium (BURD), Department of Botany, The University of Burdwan, having Specimen Id: GCMD/2018/S002. The collected fruits were rinsed well with distilled water, soaked in paper towels. They were slit opened to separate the seeds from the pericarp of the fruits. These seeds so obtained were further processed for experimentation.

Preparation of Solvent Extract

The separated seeds were air dried for about 15 days in a shed. About 200 grams of seeds were put in the thimble and 2L Chloroform:Methanol (1:1 v/v) solvent was poured in the still pot of the Soxhlet apparatus. Hot extraction method was performed with a total extraction span of 72 hours at a temperature of about 40 °C. The obtained extract was dried by using a rotary evaporator [12]. The sample was then lyophilized to produce a powdery version. The powdered extract was preserved at 4 ℃.

Larvicidal Bioassay

Larvicidal bioassay was carried out obeying the standard protocols of World Health Organization with some minor alterations [23]. Based on initial larvicidal screening with the prepared solvent extract, the base concentration was set at 15ppm and gradually increasing through 30, 45, 60 and 75 ppm. Dried extract was dissolved using Tween 20 and transferred to sterilized glass beakers, volume was made up to 100 ml using distilled water. Twenty-five *Cx. quinquefasciatus* first-, second-, third-, and fourth-instar larvae were separately added to various beakers having graded concentrations. They were given proper larval food and temperature was kept at 27 °C with relative humidity at 80–90%. Larval mortality was assessed at 24, 48, and 72 hours postexposure. After being pricked in the syphon or cervical area with a sharp needle, larvae that weren't responsive were assumed to be deceased. Three times each experiment was conducted in the same lab environment.

Mortality Curves

For all the larval instars of *Cx. quinquifasciatus*, their mortality percentages had been plotted along the Y axis against increasing doses of the solvent concentration in ppm as plotted along X axis. These curves were meant to give a clear pictorial demonstration of the larval mortality rates against the graded solvent extract concentrations (ppm).

Qualitative phytochemical screening

The Choloroform: Methanol extract of *M. champaca* seeds was screened for the presence of tannins, terpenoids, steroids, saponins, alkaloids, flavonoids, anthraquinones, coumarins and glycosides in accordance with the methods established by Harbone and Sofowara [24, 25].

Thin Layer Chromatography (TLC)

About 30 glass plates were prepared with silica gel G using an Unoplan coating system. The powdered solvent extract was dissolved in Chloroform: Methanol and placed drop wise (by capillary tube) at the bottom of each of the preheated (100 °C for 30 min), silica coated glass plates. Each plate was put in its own glass chamber for thin-layer chromatographic (TLC) analysis after drying for 5 min. The mobile phase contained various solvent systems. After the solvent had travelled across the tops of the plates, each plate was taken out of its glass chamber and given a separate air-drying period. Both the distance between the spot location and the plate bottom, as well as the distance between the run of the developing solution and the plate bottom, were measured. The R_f values were deduced by the formula:

$$R_f = \frac{\text{distance of the spot centre from the start point}}{\text{distance of the solvent run from the start point}}$$

FT-IR Analysis

The primary spots that appeared on same R_f value were scrapped from the glass plates, dissolved in absolute alcohol, discarding the silica gel precipitate obtained at the bottom of the flask. The absolute alcohol was then evaporated using a water bath (~40 °C). Then the dried compound was dissolved distilled water and subjected to the bioassay experiment against the third-instar larval forms of *Cx. quinquefasciatus.* The spots with specific R_f value that have recorded considerable larval death, were subjected to infrared (IR) spectroscopic analysis using KBr plates (JASCO FT-IR Model-420) with 2 mm s^{-1} scanning speed. The analysis of the FT-IR graph was done by KnowItAll software. All of the reagents and solvents were analytical-grade and came from E. Merck in India.

Effect on Non-target organisms

In this study dipteran larvae of *Chironomus* sp. and hemipteran nymphs of *Diplonychus* sp. were chosen as non-target organisms, since they frequently coexist in the same habitats with mosquito larvae. For the bioassay, they were treated with the solvent extract at the LC_{50} concentration

required against 3rd instar *Cx. quinquifasciatus* larvae after 72 hours of exposure. Mortality or any physiological abnormalities were recorded for about 72 hours post exposure.

Statistical analyses

In order to remove the error in the percentage of mortalities (% M), Abbott's Formula was utilized [26]. Regression analysis was also performed using "MS Excel 2010," whereas log probit [27] and three-way ANOVA analysis were conducted using "Stat Plus 2009 Professional" and "Origin Pro 2021" respectively.

Results

Larvicidal bioassay

The highest larval mortality in the 72-h bioassay experiment using the Chloroform: Methanol extractive of mature seeds of *M. champaca* was obtained with 60 ppm concentration for all larval instars of *Cx. quinquefasciatus* (Table 1). There had been a gradual increment in larval mortality along with the increase in the dose of the solvent extractive and the exposure time. Log probit analysis demonstrated an inversely proportionate relation between exposure time and LC values. (95 percent confidence level). Lowest LC_{50} and LC_{90} values of 15.33 ppm and 29.77 ppm were recorded against the first instar larvae after 72 hours of treatment. In case of 3rd instar larvae, the LC_{50} and LC_{90} values accounted to be 18 and 32.86 ppm respectively. As per the results of regression analysis, the mortality rate (Y) is positively correlated with solvent extract concentrations, having regression coefficient (R^2) being close to 1 in each case (Table 2). According to the three-way ANOVA analysis, there was a significant difference in the rate of larval death (p0.05) when the Chloroform: Methanol extractive of *M. champaca* seed was studied with various concentrations, increasing time of exposure and different instars of *Cx. quinquifasciatus* (Table 3).

Mortality Curves

The mortality curves depicting the percent mortality of *Cx. quinquifasciatus* showed a gradual increase in percent mortality rates with increment in the concentration of Chloroform: Methanol extract after 24, 48 and 72 hours of exposure (Fig. 1). In case of 72 hours, a plateau phase was found at the right-hand side which was obtained after cent percent larval mortality.

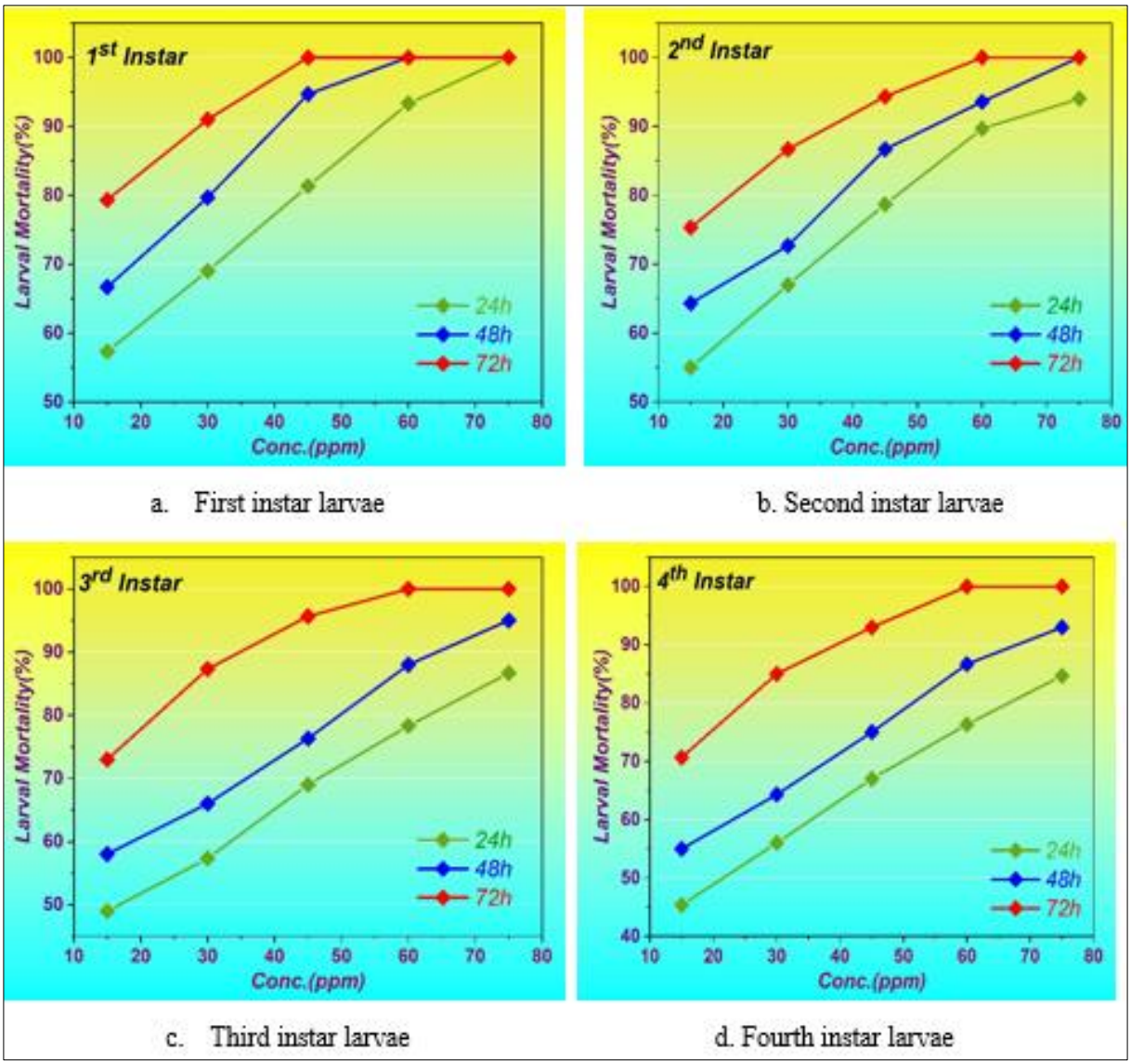

Fig 1: Mortality curves depicting percent mortality of all larval instars of *Culex quinquifasciatus* against graded concentrations of Chloform:methanol extract of *Michelia champaca* seeds

Phytochemical analyses
Chloroform: Methanol extractive of *M. champaca* seed upon different chemical treatment as per the protocol of Harbone and Sofowara revealed the presence of tannin, terpenoids, flavonoids, alkaloids and coumarins (Table 4).

Larvicidal bioassay with TLC fraction
Percent mortality of the test mosquito larvae in their third instar have been depicted in Table 5. When they were treated with various concentration of the TLC fraction (RF = 0.57), 100% mortality was recorded at 30 ppm concentration after 72 hours of exposure.

FT-IR Analysis
Upon Infrared Spectroscopic analysis of the TLC fraction obtained from the Chloroform: Methanol extract of *M. champaca* seeds, revealed the presence of various functional groups such as alcohol (3335.28 cm^{-1}), alkane (2922.59 cm^{-1}), methyl group (2852.20 cm^{-1}), amine (2361.41 cm^{-1} and 2342.12 cm^{-1}), aromatics (1621.84 cm^{-1}), carboxylic acid (1218.79 cm^{-1}) and ether (1028.84 cm^{-1}), (Fig:2).

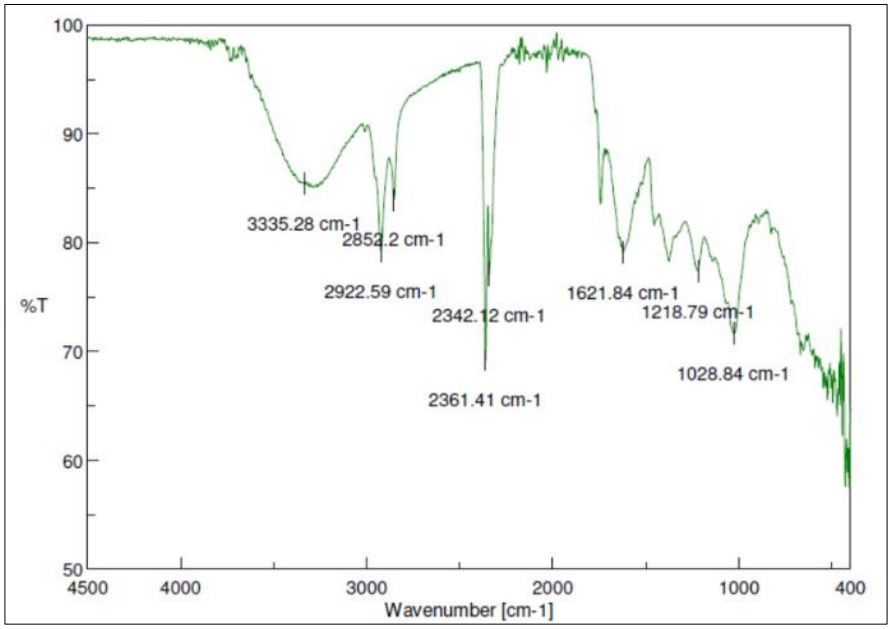

Fig 2: IR analysis of the bioactive TLC- fraction obtained from Chloform:methanol extract of *Michelia champaca* seeds

Effect on Non-Target organisms
Upon treatment with the LC_{50} concentration (for the 3rd instar mosquito larvae at 72 hour) of the solvent extract of *M. champaca* seeds, no difference in the survival rate and swimming behavior of the nontarget organisms such as Chironomid larvae and nymphs of *Diplonychus* sp. was seen after 72 hours of exposure (Table 6).

Discussion
The natural insecticides of plant origin have recently gained attention as a substitute strategy for controlling arthropods that are related to public health as transmitter of diseases since they are environmentally safe and biodegradable. Plant-based remedies have been used to control household pests from ancient times. Natural and environment friendly insecticides are continuously being sought after and investigated. Different types of solvents, including water, methanol, chloroform, acetone, hexane, petroleum ether can be used to extract specific phytochemicals from the whole plant or any particular part. The larvicidal effect of plant extracts result from alkaloids, flavonoids, polyphenols, steroids, terpenoids, and other compounds singly or in combination that are synthesized in varying degrees from various plant parts [28]. Therefore, utilizing a botanical larvicide in place of a synthetic one would not only be more cost-effective but also will be safe for people and other non-target animals [29].
The current study evaluated the toxicity of chloroform: Methanolic extract of *M. champaca* seeds against *Cx. quinquefasciatus* larvae. The toxicity of *M. champaca* extracts against mosquito larvae has never been documented before. Chloroform: Methanol extract demonstrated 100% mortality in the third instar larvae of *Cx. quinquefasciatus* at 60 ppm concentration, with the LC_{50} and LC_{90} values of 18 and 32.86 ppm respectively after 72 hours of exposure (Table 1 and Table 2).
Previously, numerous studies have already examined how phytosteroid affects mosquito larvae. When used against *Aedes aegypti* and *Cx. quinquefasciatus*, the *Ocimum sanctum* leaf extract produced significant mortality with LC_{50} values of 425.94 and 592.60 ppm, respectively [30]. The leaves of *Aegle marmelos* (L) were highly larvicidal against *Anopheles subpictus* and *Culex tritaeniorhynchus* with LC_{50} values of 167.00 and 99.03 ppm [31]. Banerjee, *et al.* looked into the efficiency of chloroform: Methanol (1:1 v/v) extracts of mature *Limonia acidissima* leaves against the larval form of *Cx. quinquefasciatus* where the LC_{50} values for the bioactive substances in the mature plant's leaves after 72 hours of exposure were 1.73, 5.01, 17.37, and 29.19 ppm for each instar of *Cx. quinquefasciatus* [32].
Compared to the above studies, it can be claimed that the chloroform: Methanol extract from *M. champaca* seeds is much more potent to diminish *Cx. quinquifasciatus* larvae since it has exhibited very low LC_{50} and LC_{90} values. Moreover, the non-target organisms remained unaffected by the said extract, which makes it more suitable as a novel larvicide and confirms its target specificity.

Table 1: Percent mortality of all the four instars of *Culex quinquefasciatus* upon exposure to chloroform: Methanol (1:1) extractive of *Michelia champaca*

Larval Instars	Concentration (ppm)	Percent Mortality (Mean±SE)		
		24h	48h	72h
First	15	57.33 ± 0.33	66.67 ± 0.33	79.33 ± 0.67
	30	69.00 ± 0.00	79.67 ± 0.33	91.00 ± 0.33
	45	81.33 ± 0.67	94.67 ± 0.33	100.00 ± 0.00
	60	93.33 ± 0.57	100.00 ± 0.00	100.00 ± 0.00
	75	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
Second	15	55.00 ± 0.57	64.33 ± 0.33	75.33 ± 0.58
	30	67.00 ± 0.33	72.67 ± 0.33	86.67 ± 0.33
	45	78.67 ± 0.33	86.67 ± 0.00	94.33 ± 0.33
	60	89.67 ± 0.57	93.57 ± 0.67	100.00 ± 0.00
	75	94.00 ± 0.33	100.00 ± 0.00	100.00 ± 0.00
Third	15	49.00 ± 0.57	58.00 ± 0.00	73.00 ± 0.58
	30	57.33 ± 0.67	66.00 ± 0.58	87.33 ± 0.00
	45	69.00 ± 1.20	76.33 ± 0.33	95.67 ± 0.33
	60	78.33 ± 0.33	88.00 ± 0.00	100.00 ± 0.00
	75	86.67 ± 0.00	95.00 ± 0.57	100.00 ± 0.00
Fourth	15	45.33 ± 0.57	55.00 ± 0.58	70.67 ± 0.00
	30	56.00 ± 0.67	64.33 ± 0.33	85.00 ± 0.33
	45	67.00 ± 0.00	75.00 ± 0.00	93.00 ± 1.20
	60	76.33 ± 0.67	86.67 ± 0.33	100.00 ± 0.00
	75	84.67 ± 0.00	93.00 ± 0.67	100.00 ± 0.00

Table 2: Regression and log-probit analyses using the chloroform: Methanol (1:1) extractive of *Michelia champaca* seeds against *Culex quinquifasciatus*

Larval Instars	Period of Exposure	LC_{50} (ppm)	LC_{90} (ppm)	Regression	R^2- Value
1st	24	23.75	50.56	Y= 0.7311x + 47.297	0.99
	48	21.66	36.27	Y= 0.5799x + 62.105	0.88
	72	15.33	29.77	Y= 0.3356x + 78.964	0.76
2nd	24	21.51	61.83	Y= 0.6711x + 46.667	0.98
	48	21.50	46.33	Y= 0.6149x + 55.776	0.98
	72	17.15	33.75	Y= 0.4178x + 72.465	0.90
3rd	24	25.97	95.21	Y= 0.6423x + 39.164	0.99
	48	22.79	63.70	Y= 0.64x + 47.866	0.99
	72	18.00	32.86	Y= 0.4445x + 71.199	0.85
4th	24	26.48	106.05	Y= 0.6601x + 36.163	0.99
	48	23.16	68.53	Y= 0.6556x + 45.298	0.99
	72	18.27	35.20	Y= 0.4911x + 67.636	0.89

Table 3: Three-way ANOVA analysis of mortality of all the four larval instars, different hours of exposure and different concentrations of Chloroform: Methanolic (1:1) extract as variables

Source of variation	Sum of squares (SS)	Degree of freedom (DF)	Mean of squares (MS)	F value	P-Level
Instars (I)	256.4167	3	85.47222	236.6923	0
Hours (H)	591.8778	2	295.9389	819.5231	0
Conc. (C)	1802.967	4	450.7417	1248.208	0
I × H	39.36667	6	6.561111	18.16923	0
I × C	14.05556	12	1.171296	3.24359	0.000456
H × C	65.23333	8	8.154167	22.58077	0
I × H × C	25.07778	24	1.044907	2.89359	0
Model	2794.994	59	47.37279	131.1862	0
Error	43.3333	120	0.361111	0	0
Corrected	2838.328	179	0	0	0

Table 4: Results of Phytochemical analyses of Chloroform: Methanol (1:1) extract of *Michelia Champaca* seeds

Phytochemicals	Chloroform: Methanol (1:1) extract
Tannin	+
Terpenoids	+
Sterols	_
Saponin	_
Flavonoids	+
Alkaloids	+
Coumarins	_
Glycosides	_
Cardiac glycosides	_

Table 5: Percent mortality of third instar larvae of *Culex quinquifasciatus* against Chloroform: Methanol (1:1) extract TLC fraction with R_f value 0.57 isolated from the seeds of *Michelia Champaca*

Concentration (ppm)	Percent Mortality (Mean±SE)		
	24h	48h	72h
10	48.57 ± 0.00	62.33 ± 0.57	70.67 ± 0.33
20	64.00 ± 0.57	75.33 ± 1.20	86.33 ± 0.57
30	85.24 ± 0.33	91.33 ± 0.33	100.00 ± 0.00

Table 6: Effect of Chloroform: Methanol (1:1) extract of *Michelia Champaca* seeds on non-target organisms at laboratory conditions

Non-Target Organism (Nymphs)	Percent Mortality (Mean±SE)		
	24h	48h	72h
Diplonychus sp.	0.00 ± 0.00	1.00 ± 0.00	1.67 ± 0.00
Control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Chironomus sp.	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Conclusion
Overall, it can be said that *M. champaca* seeds showed a strong larvicidal capacity against *Cx. quinquifasciatus* larvae. Moreover, it is environmentally safe to use it as larvicide since it is are not hazardous to other life forms. Therefore, both the solvent extract and the TLC fraction are capable of acting as potent larvicidal agents, in accordance with the aforementioned findings.

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Declarations
Competing interest
We have no conflict of interest.

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Authors contribution
All the experiments were done and manuscript was written by MD, while the work plan and final checking was done by GC.

Data availability statement
The databases used and analyzed during the current study is available from the corresponding author on reasonable request.

Ethical approval
Not applicable for the study.

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