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Mosquito larvicidal bioassay with the fruit extracts of *Diospyros kaki* on filarial vector *Culex quinquefasciatus*

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Abstract

Due to the emergence of resistance of the commonly used synthetic pesticide, vector control is in grave danger. Application of biocontrol agents may be a suitable alternative approach in vector control. The purpose of this study was to evaluate the larvicidal effectiveness of crude and methanolic fruit extracts of *Diospyros kaki* against *Culex quinquefasciatus*, the carrier of human filarial worm. The methanol extract showed promising result in biocontrol assay than crude extract. The methanolic extract had the LC_{50} and LC_{90} values of 46 ppm and 186 ppm, respectively against 1st instar larvae after 72 hours of exposure, The rate of mortality was positively associated with the solvent extracts in increasing concentrations, having a regression coefficient value near to 1 in each case, showing a definite positive dose-dependent mortality. During chemical characterization of the solvent extract by GC-MS and FT-IR studies, some bioactive compounds like 5,8,11,14,17-Eicosapentaenoic Acid, Methyl 4,7,10,13,16,19-Docosahexaenoate, cyclotrisiloxane hexamethyl and Arsenous acid, tris(trimethylsilyl) ester were identified that showed the larvicidal activity against mosquito larvae either singly or jointly. Thus, *D. kaki* fruit extract showed excellent potential as a bio control agent for *Cx. quinquefasciatus* and the isolated bioactive phytochemical may be utilized as a source of a powerful insecticide.

Keywords: bioactive compounds; *Culex quinquefasciatus; Diospyros kaki*; FT-IR; GC-MS**;** LC_{50} and LC_{90} values

Introduction

One of the most important carriers of parasites and illnesses that continue to have a fatal effect on human population is the mosquito (Maheswaran *et al*., 2008). They not only transmit various diseases like malaria, filariasis, dengue, chikungunya etc. but also creates an intolerable biting nuisance (Youdeowei *et al*., 1983; De *et al*., 1994; Collins *et al*., 1995; Chatterjee *et al*., 2000). In addition to localized cutaneous reactions, allergic mosquito bites can also result in systemic reactions such as angioedema in people (Peng *et al*., 1999). Since, no vaccine or precautionary medication is widespread for the control of these diseases, preventive

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measures such as the use of larvicides, pupicides, and mosquito repellents are of utmost importance to reduce mosquito population density. Chemically synthesized pesticides and repellents bring out numerous ecological issues, including the emergence of insect strains that are resistant to them, biomagnification, etc. Natural products are typically favored due to their inherent biodegradability and the fact that they are target specific and less damaging to non-target organisms (Prabakar *et al.,* 2004). Plant products were also useful to control pathogenic bacteria and helminthes (Burman *et al*., 2018; Hossain *et al*., 2012).

A member of the Ebenaceae family, *Diospyros kaki*, or Oriental persimmon, is a native deciduous plant of China. With its broadly lanceolate, dark green leaves, the tree grows to a height of around 10 meters. In the spring, flowers bloom, and between September and October, fruits ripen. Fruits have a fibrous texture, are spherical, green when crisp and solid, and turn orange when mature. It has long been known that this plant is employed in traditional Chinese medicine. The fruits are used to treat hypertension, bronchial problems (Brown, 1995), and as an astringent and laxative (Duke *et al*., 1985).

The objective of the current study was to determine the mosquito larvicidal activities of *D. kaki* fruit extracts against *Cx. quinquefasciatus* and how they affect non target organism. Additionally, it involved isolating the bioactive chemicals that give fruit extract its larvicidal action by FTIR and GC-MS studies.

Materials and Methods

Rearing of mosquitoes

The current investigation was carried out in Burdwan, West Bengal, India (23°16′ N, 87°54′ E). Rafts of wild *Cx. quinquefasciatus* eggs laid in their native habitats like drains surrounding the University campus were randomly gathered. On hatching, larvae were given dog biscuits and dry yeast powder in a 3:1 powdered mixture (Rawani *et al*., 2009). The larvae were housed at a constant temperature of 25 to 30 °C and protected against diseases, pesticides, and repellents. Using a glass dropper and a glass beaker filled with tap water, the converted pupae were manually separated. The beaker was put into a mosquito cage to encourage the development of adult mosquitoes. The adult mosquitoes were fed with 10% glucose and periodically blood fed for their ovarian development.

Crude extract preparation

During the experimental session, fresh, green *D. kaki* fruits were randomly collected from plants growing in the University campus. A voucher specimen (Specimen No. GCKKM/2017/S003) of the plant was submitted to the Department of Botany, The University of Burdwan for proper identification. Fruits that had been collected were rinsed in distilled water. These cleansed fruits were smashed in a mixer grinder to produce the crude extract, which was then filtered through No. 1 Whatman filter paper. By combining the crude extract with the appropriate quantity of sterilized distilled water, several graded concentrations of crude extracts were formulated.

Solvent extract preparation

Mature fruits weighing about 200 g were collected, cleaned, and shed dried. The extract was prepared by adding 2000 ml of methanol to the still-pot and the dried fruits in the thimble of the Soxhlet apparatus with an extracting span of 72 hours. A rotary evaporator operating at 40 °C was used to evaporate the eluted extract thus making it concentrated. The sample was then lyophilized to make a powdered form for larvicidal bioassay. The extract was dissolved in tween 20 to create progressive concentrations in ppm.

Larvicidal bioassay

Larvicidal bioassay was carried out in accordance with World Health Organization standard protocols (WHO, 1992), albeit with a few alterations. For experiments, graded concentrations of crude extract ranging from 0.2% to 0.6% were prepared and then placed in sterilized glass beakers with 100 ml of distilled water in each. 25 individuals of each larval instar of *Cx. quinquefasciatus* were placed into distinct beakers separately holding the graded crude extract concentrations. Larval mortality was measured after exposure for 24, 48, and 72 hours. When larvae were not responsive after being pricked with a sharp needle in the siphon or cervical region, it was determined that they were dead. Each experiment was repeated three times in same lab setting with 25-30 °C temperature and 80-85% relative humidity. Similarly, mortality was measured after 24, 48, and 72 hours of testing in larval bioassays using graded concentrations of methanol extract from 60 ppm to 300 ppm.

Phytochemical analyses

The phytochemical screening was carried out in accordance with the techniques employed by Harborne and Sofowara (Harborne, 1984; Sofowora, 1993), to confirm the presence of tannins, terpenoids, steroids, saponins, alkaloids, flavonoids, anthraquinones, and glycosides in the methanolic extract of *D. kaki* fruits.

Thin layer chromatography

Thin-layer chromatography was used for the separation of the bioactive components on glass plates covered with Silica-gel "G" (Merck, India) (0.5 mm thickness). For this separation, the mobile phase consisted of a 1:1 v/v mixture of chloroform and methanol. After the entire run, the plates were taken out of the TLC chamber, allowed to dry in the open air, and scrutinized under a UV light to identify the bioactive bands properly. The discovered spot's Rf value was determined.

Following that, bands with a similar Rf value were collected by scraping for further research, including IR and GC-MS investigations.

FT-IR analysis

The highly effective method for determining the chemical constitution, chemical bonds, or functional groups of the bioactive compounds present in plant extract is FTIR analysis. For this analysis, dried powdered methanol extract from *D. kaki* fruits was employed. Using a hydraulic press, 10 mg of dried sample and 100 mg of KBr pellet were combined to create small, discoidal tablets. For blank reference a set of pure KBr discs was used. The scan range used for the analysis was 400 to 4000 cm^{-1}.

GC-MS analysis

The chosen bands with desired bio-actives were removed from the glass plates together with the silica gel, and they were dissolved in absolute alcohol, which was then filtered using Whatman No. 1 filter papers and the supernatant was collected. The purified fraction was directly examined utilizing the Trace GC Ultra (THERMO SCIENTIFIC) GC model, Polaris Q MS model, Ion Trap Technology, and the XCALIBUR data storage software and NIST mass library. Helium served as the carrier gas, and a sample run duration of 60 minutes was combined with a flow rate of 1 ml/min.

Larvicidal bioassay using bioactive compound

The selected band scrapings were warmed in a water bath for 5 minutes after being dissolved in 20 ml of pure alcohol (Temperature: 60-65 °C). The precipitate containing silica gel was discarded and the clear solution was transferred to a beaker. After the alcohol had evaporated, the solid mass at the beaker's bottom was gathered and weighed. By combining the obtained bioactive component with distilled water, graded concentrations of 10 ppm, 20 ppm, 30 ppm and 40 ppm were prepared. 25 *Cx. quinquefasciatus* larvae in their 3rd instar were

released to the abovementioned graded concentration of the active bio component for the experimental bioassay. After intervals of 24 hours, 48 hours, and 72 hours, larval mortality rate was reported. The bioassay tests were performed in triplicate on three separate days with a set of control each time (n=9).

Effect on non-target organisms

For this investigation, *Diplonychus annulatum* served as the non-target organism. 25 nymphs of *D. annulatum* were subjected to the LC_{50} concentration of the solvent extract in accordance with the procedure followed by Suwannee *et al*. (2006). After exposure for 24, 48, and 72 hours, nymphal mortality rates were recorded. The average mortality was calculated after the assay was triplicated using a set of controls each time.

Statistical analyses

During the bioassay, the measured percentage mortality was corrected using Abbott's methodology (Abbott, 1925). Additionally, MS EXCEL 2010 was used to conduct statistical analyses of the obtained data in order to determine the regression equations and the value of the regression coefficient. The Probit analysis to determine the LC50 and LC90 values as well as the three-way ANOVA analysis comparing various concentrations, various exposure times, and various instars of larvae were carried out using the "Stat Plus 2009 Professional" programme. Percent mortality determination, Log-Probit analysis and three-way ANOVA analysis were used as different statistical tools for determining the larval mortality rates and the effect of different randomized factors on larval mortality.

Results

Tables 1 and 2 reveal the percent mortality against *Cx. quinquefasciatus* larvae using the crude and solvent extracts of *D. kaki* fruits, respectively. The findings showed that larval mortality rates gradually increased with increasing extract concentration and exposure time. 100% mortality of 1st and 2nd instar larvae were recorded at 0.6% crude fruit extractive concentrative. For methanol extract from the fruits, 300 ppm concentration was sufficient to kill 100% of the 1st and 2nd instar larval population. Using the said crude and solvent extracts as a test substance for the *Cx. quinquefasciatus* mosquito, the results of probit and regression analyses have been summarized (Tables 3 and 4).

Table 1. Results of larvicidal bioassay using the crude extract of *Diospyros kaki* fruits against all the instars of *Culex quinquifasciatus*

Larval instars	Concentration (%)	Percent Mortality (Mean ± SE)		
		24 h	48 h	72 h
First	0.2	46.67 ± 0.33	56.00 ± 0.58	69.33 ± 0.68
	0.3	52.00 ± 0.58	62.67± 0.33	78.68 ± 0.68
	0.4	62.68 ± 0.33	72.00 ± 0.00	85.33 ± 0.33
	0.5	72.00 ± 0.58	86.68 ± 0.33	100.00 ± 0.00
	0.6	84.00 ± 0.58	94.68 ± 0.33	**100.00 ± 0.00**
Second	0.2	42.68 ± 0.33	58.68 ± 0.33	68.00 ± 0.00
	0.3	49.33 ± 0.33	62.68 ± 0.33	77.33 ± 0.33
	0.4	58.68 ± 0.33	72.00 ± 0.00	89.33 ± 0.33
	0.5	73.33 ± 0.68	92.00 ± 0.58	100.00 ± 0.00
	0.6	80.00 ± 0.00	94.68 ± 0.33	**100.00 ± 0.00**
Third	0.2	32.00 ± 0.00	44.00 ± 1.00	54.68 ± 0.68
	0.3	36.00 ± 0.58	50.68 ± 0.33	65.33 ± 0.68
	0.4	44.00 ± 0.00	64.00 ± 0.00	77.33 ± 0.33
	0.5	50.68 ± 0.33	69.33 ± 0.33	86.68 ± 0.33
	0.6	58.68 ± 0.33	76.00 ± 0.00	**96.00 ± 0.58**
Fourth	0.2	25.33 ± 0.33	41.33 ± 0.33	58.68 ± 0.33
	0.3	28.00 ± 0.00	50.68 ± 0.33	61.33 ± 0.33
	0.4	37.33 ± 0.33	56.00 ± 0.58	74.68 ± 0.33
	0.5	41.33 ± 0.33	60.00 ± 0.58	78.68 ± 0.33
	0.6	49.33 ± 0.68	68.00 ± 0.58	**85.33 ± 0.33**

Table 2. Larval mortality of all the instars of *Culex qunquifasciatus* treated with methanol extractive of fruits of *Diospyros kaki*

Larval instars	Concentration (ppm)	Percent Mortality (Mean ± SE)		
		24 h	48 h	72 h
First	60	25.33 ± 0.33	40.00 ± 0.58	54.68 ± 0.33
	120	36.00 ± 0.58	57.33± 0.33	72.00 ± 0.00
	180	49.33 ± 0.33	62.68 ± 0.33	78.68 ± 0.33
	240	66.68 ± 0.33	80.00 ± 0.58	92.00 ± 0.00
	300	80.00 ± 0.00	93.33 ± 0.33	**100.00 ± 0.00**
Second	60	26.68 ± 0.33	41.33 ± 0.33	57.33 ± 0.33
	120	38.68 ± 0.33	53.33 ± 0.33	72.00 ± 0.58
	180	53.33 ± 0.33	65.33 ± 0.33	82.68 ± 0.33
	240	72.00 ± 0.00	80.00 ± 0.00	93.33 ± 0.33
	300	82.68 ± 0.33	94.68 ± 0.33	**100.00 ± 0.00**
Third	60	18.68 ± 0.33	30.68 ± 0.33	41.33 ± 0.33
	120	28.00 ± 0.00	38.68 ± 0.33	49.33 ± 0.33
	180	42.68 ± 0.33	56.00 ± 0.00	73.33 ± 0.33
	240	60.00 ± 0.58	72.00 ± 0.00	82.68 ± 0.33
	300	74.68 ± 0.33	86.68 ± 0.33	**97.33 ± 0.33**
Fourth	60	16.68 ± 0.68	28.00 ± 0.00	44.00 ± 0.58
	120	25.33 ± 0.68	38.68 ± 0.33	56.00 ± 0.58
	180	36.00 ± 0.00	46.68 ± 0.68	60.00 ± 0.58
	240	44.00 ± 0.58	65.33 ± 0.33	74.68 ± 0.33
	300	56.00 ± 0.58	76.00 ± 0.00	**86.68 ± 0.33**

Table 3. Log probit and regression analyses of percent mortality of larva treated with crude fruit extract of *Diospyros kaki* against *Culex quinquifasciatus*

Larval instars	Period of Exposure	LC 50 (ml)	LC 90 (ml)	Regression	R^2 value
1st	24	00.26	0.81	y = 94.667x + 25.60	0.9861
	48	00.25	00.54	y = 101.33x + 33.87	0.9843
	72	00.23	00.37	y = 82.667x + 53.60	0.9468
2nd	24	00.31	00.86	y = 98.667x + 21.33	0.9828
	48	00.26	00.51	y = 101.33x + 35.47	0.9346
	72	00.24	00.37	y = 86.667x + 52.27	0.9414
3rd	24	00.47	02.19	y = 68x + 17.07	0.9897
	48	00.29	00.49	y = 166.67x + 2.93	0.9785
	72	00.25	00.52	y = 104x + 34.40	0.9974
4th	24	00.63	03.36	y = 61.333x + 11.73	0.976
	48	00.30	02.92	y = 62.667x + 30.13	0.9809
	72	00.22	00.76	y = 70.667x + 43.47	0.9594

Table 4. Log probit and regression analyses of percent mortality of larva treated with methanolic extract of *Diospyros kaki* fruits against *Culex quinquifasciatus*

Larval instars	Period of Exposure	LC_{50} (ppm)	LC_{90} (ppm)	Regression	R^2 value
1st	24	167.6624	451.0956	y = 0.2333x + 9.4667	0.994
	48	115.8397	320.6707	y = 0.2156x + 27.867	0.9822
	72	89.1448	207.4874	y = 0.1844x + 46.267	0.9797
2nd	24	155.5556	404.1757	y = 0.2422x + 11.067	0.9934
	48	123.0615	296.4398	y = 0.2222x + 26.933	0.9972
	72	88.3556	196.7035	y = 0.1778x + 49.067	0.9834
3rd	24	193.1158	496.9222	y = 0.24x + 1.6	0.9908
	48	152.4344	367.6766	y = 0.2422x + 13.2	0.9891
	72	124.8574	259.8057	y = 0.2422x + 25.2	0.9777
4th	24	267.0626	1156.6422	y = 0.1556x + 8	0.9919
	48	168.1258	548.5405	y = 0.2044x + 14.133	0.9833
	72	115.522	418.0908	y = 0.1733x + 33.067	0.9769

The three-way factorial ANOVA analysis is a definite statistical test through which the effect of all the factors such as fruit extract concentrations, different instars, and exposure times is ascertained on larval mortality. According to three-way ANOVA analyses (Table 5), all the three factors significantly influenced the mortality rates of the wrigglers. It clearly showed the statistically significant variations in larval mortality at p 0.05). It was also discovered that complex interactions between the three entirely randomised independent factors mentioned above had a controlling effect on larval mortalities.

Table 5. Three-way ANOVA analysis of larva treated with methanol extract of *Diospyros kaki* fruits

Source of variation	Sum of squares (SS)	Degree of freedom (df)	Mean of squares (MS)	F value	p-level
Instars (I)	549.9778	3	183.3259	434.1930	0.00
Hours (H)	1326.9000	2	663.4500	1571.3289	0.00
Conc. (C)	3609.7444	4	902.4361	2137.3487	0.00
I × H	6.7889	6	1.1315	2.6798	0.02
I × C	60.7444	12	5.0620	11.9890	0.00
H × C	20.8222	8	2.6028	6.1645	0.00
I × H × C	34.1556	24	1.4321	3.3706	0.00
Within groups	50.6667	120	0.4222	---	---
Total	5659.8000	179	31.6190	----	---

The existence of alkaloids, flavonoids, steroids, glycosides, and saponins in the methanolic extract was confirmed by phytochemical tests. Both the fruit's crude extract and its methanol extract had very little effect on non-target organisms.

FT-IR spectroscopic studies, highlighted that the methanolic extract was composed of the following functional groups such as alcohol (3246.57 cm^{-1}), amine (2337.3 cm^{-1} and 2360.44 cm^{-1}), azides (2166.63 cm^{-1}), aromatics (1578.45 cm^{-1}) and alkanes (1035.59 cm^{-1}) (Figure 1).

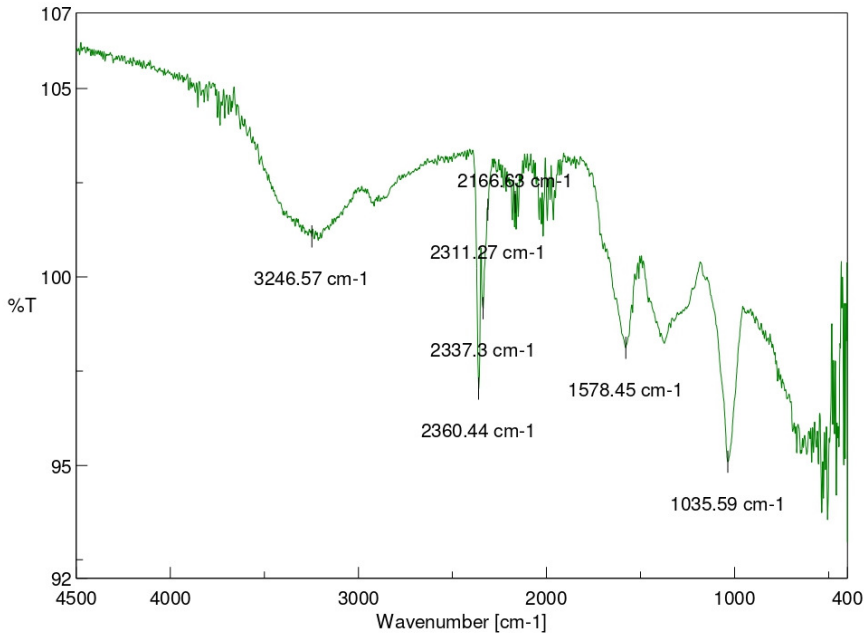

Figure 1. FT-IR analysis of the methanolic extract of *D. kaki* fruits

The major bioactive ingredients as identified from GC-MS analysis are 5,8,11,14,17-Eicosapentaenoic Acid, Methyl 4,7,10,13,16,19-Docosahexaenoate, cyclotrisiloxane hexamethyl and Arsenous acid, tris(trimethylsilyl) ester which are hypothesized to have the mosquito larvicidal activity (Figure 2).

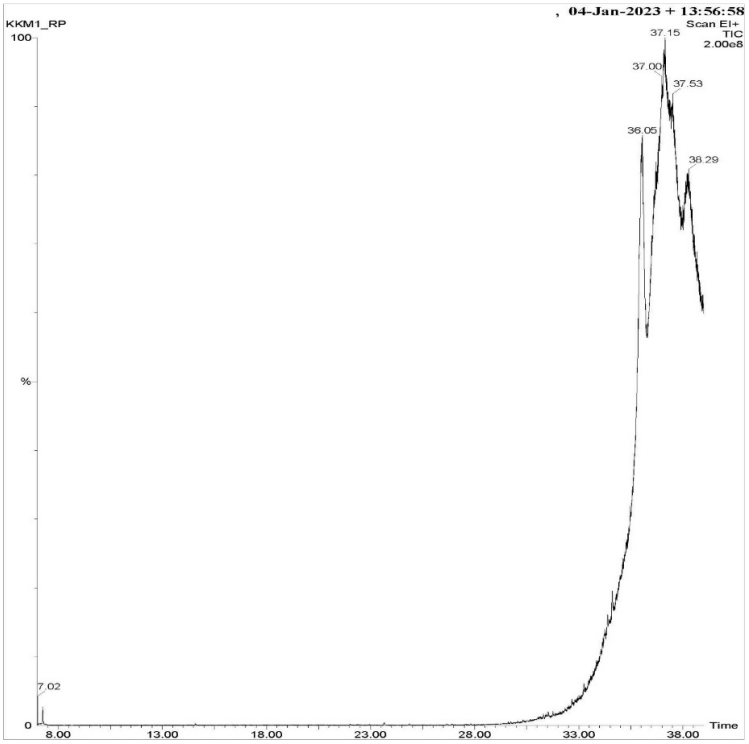

Figure 2. GC-MS analysis of the TLC scrapings obtained from the methanolic extract of *D. kaki* leaves

Table 6 shows the larvicidal activity of the bioactive compounds isolated from *D. kaki* fruits. On treatment against 3rd instar larvae, it caused 100% mortality after 72 hours at 40 ppm concentration, thus claiming it to be one of the most effective mosquito control agents.

Table 6. Percent mortality of third instar larvae of *Culex quinquefasciatus* treated with graded concentrations of bioactive ingredients isolated from fruits of *D. kaki*

Concentration (ppm)	Mortality percent (Mean ± SE)		
	24 h	48 h	72 h
10	49.00 ± 0.00	58.57 ± 0.00	70.57 ± 0.33
20	59.57 ± 0.00	72.33 ± 0.33	84.24 ± 0.57
30	70.91 ± 0.33	83.24 ± 0.33	94.00 ± 0.33
40	85.00 ± 0.57	98.00 ± 0.33	100.00 ± 0.00

Discussion

As an alternative method for controlling arthropods crucial to public health, the natural insecticides of plant origin have recently attracted attention since they are environment friendly and biodegradable. Household pests have traditionally been managed with plant-based treatments. Insecticidal agents that are natural and favorable to the environment are still being sought after and researched. Depending on the polarity of the phytochemicals, different types of solvents such as water, methanol, chloroform, and hexane can be used to extract the plant products from either the entire plant or a specific section. Thus, a botanical larvicide is ought to be safe for humans and other non-target creatures and using this in place of synthetic ones would not only be economical but also would create fewer negative impacts on the environment (Campbell *et al.,* 1993).

In the current study, 100% larval death was observed when first instar larvae were exposed to 0.6% crude extract concentration and 300 ppm of methanolic extract of *D. kaki* fruits within 72 hours of exposure. Both crude and Methanol extract regression studies revealed a positive connection between mortality and extract concentration, with a regression coefficient close to 1.

According to log-probit analyses (Finney, 1971), with increasing concentration and exposure time, the LC50 and LC90 values gradually declined. For the crude fruit extract, LC50 and LC90 values against 1st instar larvae after 72 hours of exposure were 0.23 ml and 0.37 ml respectively (Table 3). The least LC50 and LC90 values for the methanol extract were determined to be 89.14 ppm and 207.48 ppm respectively, against the same instar and exposure span as above (Table 4).

According to many researchers, phytochemicals have been successfully used as larvicidal agents to reduce mosquito populations (Chowdhury *et al*., 2007; Singha *et al*., 2011). The impact of phytosteroid on mosquito larvae has already been covered by a number of studies (Ghosh *et al*., 2012). Previously, with LC50 values of 425.94 and 592.60 ppm, respectively, the *Ocimum sanctum* leaf extract produced considerable mortality against *Aedes aegypti* and *Cx. quinquefasciatus* (Anees, 2008). According to Elango *et al*. (2009) the leaves of *Aegle marmelos* (L) had high larvicidal capabilities against *Anopheles subpictus* and *Culex tritaeniorhynchus*, with LC50 values of 167.00 and 99.03 ppm (Elango *et al*., 2009). The effectiveness of chloroform: methanol (1:1 v/v) extracts of mature *Limonia acidissima* leaves against the larval form of *Cx. quinquefasciatus* was investigated by Banerjee and his co-workers (Banerjee *et al*., 2011). After 72 hours of exposure, the LC50 values for the bioactive chemicals in the mature plant's leaves were 1.73, 5.01, 17.37, and 29.19 ppm for 1st, 2nd, 3rd and 4th instar larvae of *Cx. quinquefasciatus*, respectively.

The current study, however, focused on the selective mortality of larval instars of the *Cx. quinquefasciatus* upon crude and methanolic extracts treatment of *D. kaki* fruits. After 72 hours of exposure,

the obtained LC50 and LC90 values for the 1st instar larvae are discovered to be significantly lower than those previously reported. The larvicidal property is specifically brought about by the bioactive substances discovered through FTIR and GC-MS investigations are 5,8,11,14,17-Eicosapentaenoic Acid, Methyl 4,7,10,13,16,19- Docosahexaenoate, cyclotrisiloxane hexamethyl and Arsenous acid, tris(trimethylsilyl) ester which are active either singly or combinedly. After 72 hours of exposure, the bioactive chemicals identified from the analyses demonstrated 100% larval mortality at 40 ppm concentration. Additionally, the experiment also revealed the safety of non-target organism, demonstrating the target specificity of the extract.

Conclusions

In summary, it can be concluded that *D. kaki* fruit extracts has a high larvicidal capacity against the *Cx. quinquefasciatus* and is not harmful to other life forms, indicating that it is environment friendly. According to the aforementioned findings, the active components are therefore capable of acting as powerful larvicidal agents.

Authors' Contributions

Conceptualization and supervision: GC. Investigation and Methodology: KKM, PM. KKM performed all the larvicidal bioassays. Chromatographic techniques performed by PM. Statistical analyses: PM. Writing - original draft and Writing - review and editing: MD. Final checking of the manuscript: GC. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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