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Assessment of efficacy of *Diospyros kaki* fruit extracts against dengue vector *Aedes albopictus*

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Abstract
The best method for preventing mosquito-borne diseases is to reduce the mosquito population. The goal of the current study was to assess larvicidal efficacy of fruit extract of *Diospyros kaki* against *Aedes albopictus* larvae and to the non-target organisms. Bioassays were performed with crude and acetone solvent extracts on *Ae. Albopictus* larvae. Additionally, studies on the composition of phytochemicals were conducted. Through the use of Log-probit, regression, and ANOVA analyses, statistical explanations were derived. Both the crude and the acetone extract significantly reduced mosquito larvae. At 350 ppm concentration, 100% death of the 1st and 2ndinstar larvae was observed after 72 hours. Tannins, steroids, alkaloid, and flavonoids were present in fruits as secondary metabolites. Both the extract had no effect on a non-target organism. The fruits of *Diospyros kaki* have the capacity to target-specifically regulate *Ae. Albopictus* larvae.

Keywords: *Diospyros kaki*, *Aedes albopictus*, larvicide, non-target organism, phytochemical analysis, FT-IR analysis

1. Introduction
In many regions of the world, mosquitoes are responsible for spreading diseases like dengue, yellow fever, chikungunya, Zika, filaria, malaria and many more. One of the most significant species in the Culicidae family belongs to the genus *Aedes*. The Asian tiger mosquito, *Aedes albopictus*, is another name for this primarily forest-dwelling species. This species of mosquito has evolved throughout time to thrive in urban, suburban, and rural settings. At least 22 arboviral diseases, including dengue, chikungunya, yellow fever, and Zika, are spread by this species [1]. This mosquito is a vicious biter that can infect both domestic and wild animals in addition to humans. A method for controlling the diseases spread by mosquitoes is to disrupt transmission by either stopping adult mosquitoes from biting humans or by destroying the adults or larvae. Large-scale larval removal at breeding sites can be a tactic for controlling mosquito populations and diseases spread by mosquitoes. To eliminate mosquitoes, conventional synthetic insecticides are used. Long term use of these insecticides has negative effects on the environment and other life forms as well. However, some bio control agents, such as insects [2], air-breathing fish [3], and exotic fish [4], can control larvae. Some mosquito species may be reduced by the replacement host plant replacing [5]. In the programme to control mosquitoes, photochemical have the potential to take the place of synthetic insecticides. Nowadays, the use of photochemical as bacteriocides [6-7], anthelmintics [8-9], and mosquitocide [10-11] is favoured since they are readily available, efficient, biodegradable, and less likely to cause resistance [12-13].
Diospyros kaki is a member of the genus *Diospyros*, which belongs to the Ebenaceae family and has numerous species [14]. *D. kaki* is a medicinal plant that is indigenous to Myanmar, Pakistan, India, China, Pakistan, and Japan. Locals refer to *D. kaki* as "Parsimmon" and many regions cultivate it for its palatable fruits. *D. kaki* is traditionally used as an astringent and styptic. The fruit is typically laxative, astringent, stomachic, anti-tussive, and nutritious. The ripe fruit is used to treat haemorrhoids and constipation when consumed raw. It is also used to treat diarrhoea when cooked. The fruit can also be used to treat bronchial problems and dry cough when dried and powdered, respectively. Juice from unripe fruit can also be used to treat hypertension.

The fruits are thought to be demulcent, antivininous, and anti-febrile [15]. The fruit has ingredients that can neutralise the toxins produced by the bacteria *Staphylococcus alpha*, *Bordetella pertussis*, *Clostridium tetani*, and *Diptheria* [16]. The ripe fruit possesses detoxifying properties against the venoms of two snake species, *Trimeresums flavoviridis* and *Laticauda semifasciata* [15].

The primary objectives of our investigation were to identify the larvicidal effects of crude and acetone extracts of *D. kaki* fruits on *Ae. Albopictus* and to examine the presence of various photochemical in both crude and acetone extracts and various functional groups present in acetone extract using FT-IR analysis.

2. Materials and Methods

2.1 Collection of mosquito larvae: *Ae. Albopictus* larvae were collected for the studies from the stock of the mosquito colony kept in the Mosquito, Microbiology, and Nanotechnology Research Units, Department of Zoology, The University of Burdwan, Burdwan. The colony provided protection from diseases, pesticides, repellents, and other dangerous agents. The colony was maintained at the temperature of 27 ± 2 °C and the relative humidity 80% - 85% under photoperiod of light and dark cycles in the ratio of 14:10 per day for the culture and experiments. Brewer yeast and dog biscuits were fed to the larvae in a 3:1 ratio [17].

2.2 Identification of plant: *D. Kaki* fruits were randomly collected from the campus of The University of Burdwan (23.16°N, 87.54°E). Plant taxonomist Professor Dr. Ambarish Mukherjee, Botany Department, The University of Burdwan recognised the plant sample. The voucher specimen (GCKKM/2017/S003) of the plant was submitted as herbarium in the Mosquito, Microbiology, and Nanotechnology Research Units, Parasitology Laboratory, Department of Zoology, The University of Burdwan.

2.3 Crude extract preparation: Mature fruits of *D. kaki* were harvested and thoroughly cleaned with tap water and then with the distilled water. To prepare the crude extract fresh fruits were first soaked in a tissue paper for a short while and then filtered through filter paper after being crushed in a grinder. For future tests, the undiluted extract was kept as a stock solution. To prepare the necessary concentrations, distilled water was added.

2.4 Solvent extract preparation: Fresh fruits were cut into small pieces and dried at room temperature. The thimble of the Soxhlet apparatus was filled with 200 gm. of dried fruit fragments. 2000 ml of acetone solvent were poured into the still pot of Soxhlet apparatus [18]. The extraction period was predetermined to be 72 hours, with a daily maximum of 8 hours. Extract was poured into a glass beaker. Extracts were maintained in a refrigerator at 4°C after evaporation with rotatory evaporator.

2.5 Larvicidal bioassay: The larvicidal bioassay was followed by the typical procedures suggested by the WHO [19] with a few alterations against all the larval instars (1st, 2nd, 3rd, 4th) of *Ae. Albopictus*. Five concentrations of crude extract of *D. kaki* fruits ranging from 0.2 ml to 0.6 ml was applied to each of 100 ml water containing beaker and 25 instar specific larvae of *Ae. Albopictus* were added to each one. The number of dead larvae was counted after 24, 48, and 72 hours of exposure. When the larvae remained still after being pricked, they were assumed to be dead. The studies took place in an environment with an average temperature of 28±2 °C, a relative humidity of 85±2%, and a photoperiod of 13.11 (Light: Dark). Similar sets of experiment were done with acetone extract of *D. kaki* fruits ranging from 150 ppm to 350 ppm. Additionally, a similar control trial without any extract was conducted. Three replicates were kept running simultaneously in the lab for each concentration of both crude and solvent extract.

2.6 Phytochemical analysis: The standard method of Sofowara, Trease and Evans, and Harborne [20-22] was used for the phytochemical examination of the acetone extract of the fruits. The presence or absence of secondary metabolites such as tannins, saponins, steroid, flavonoids, and terpenes in acetone extract was investigated.

2.7 FT-IR analysis: The functional group present in the acetone extract of *D.kaki* was examined using Fourier transformed infrared (FT-IR) spectroscopy. Using a mortar and pestle, the dried solvent extract and potassium bromide (KBR) were mixed at 1:10 ratio. A pallet was created with the help of hydraulic pressure. A blank pallet is also created to standardise the spectral analysis. The extract pallet was then subjected to FT-IR analysis using JASCO FT-IR Model-4700.

2.8 Test on non-target organisms: Species that reside in the same or an environment that is similar to that of the target mosquito larvae are considered non-target species. For dose-response bioassays against non-targets like *Chironomus circumdatus* larvae, the LC_{50} value of both crude and acetone extract of *D. kaki* fruits was applied, in which the target larvae died at the highest rate [23]. The average of three replicates for each was used to find mortality or any changes in behaviour.

2.9 Statistical analysis: While observing the larvicidal potential of the fruit extracts, the percentage mortality was adjusted using Abbott's formula [24]. To determine the average larval mortality, Standard error (SE), regression equation, $LC_{50}$and LC_{90} values, and associated 95% confidence limits, probit analysis [25] was performed on the results. Additionally, a three-way ANOVA analysis was performed on the data. Results were considered statistically significant if $p<0.05$. The SPSS Statistical Software Package version 16.0 and MS Excel 2007 programmes were used to analyse the data.

3. Results

In laboratory tests, *D. kaki* fruit extract significantly triggered the mortality of *Ae. Albopictus* larvae in all instars. Within 48 hours, the 0.6 ml crude extract caused 100% mortality in 1stinstar larvae. After being exposed for 72 hours, 0.6 ml of the crude extract resulted in 100% death in the 2nd and 3rd instar larvae. 4th instar larvae showed more than 94% mortality at 0.6 ml of crude extract after 72 hrs. of exposure (Table 1). The mortality was also observed in acetone extract against all the instar (Table 2). The 350 ppm of acetone extract killed all 1st and 2nd instar larvae within 72 hours, resulting in 100% mortality. 350 ppm of the acetone extract resulted more than94% death in the 3rd and 4thinstar larvae after 72 hrs. of exposure.

Probit analysis of the crude extract revealed LC_{50} values of 0.13, 0.14, 0.20 and 0.23ml, respectively, against the 1st, 2nd, 3rd, and 4thinstar of larvae after 72 hours of exposure (Table 3). After being exposed for 72 hours, LC_{50} values of acetone extract of the fruits are 134.82, 126.32, 165.16, 193.75 ppm against the 1st, 2nd, 3rd, and 4th instar of larvae respectively (Table 4). According to the regression analysis, the percent mortality (Y) was positively correlated in both crude and acetone extract concentration (X).

The interaction among mortality (%), different instars, different concentrations of the crude and acetone extract, and different exposure periods was revealed by a three-way ANOVA. The results showed highly significant results (p<0.05) where differences in mortality (%) towards the variables were observed. Larval mortality and interactions among instar, concentration, and exposure time was strongly associated (table 5 and 6).

Photochemical analysis of both crude and acetone extract discovered the presence of different compounds which are presented in table 7. FT-IR analysis revealed the presence of different functional groups which are presented in table 8. FT-IR graph is depicted in Fig 1.

After exposure of 72 hours, treatment of non-target organisms with crude and solvent extracts showed no significant abnormality or mortality.

Table 1: Larvicidal bioassay using the crude extract of *Diospyros kaki* against all the instars of *Aedes albopictus*

Larval Instars	Concentration (%)	Percent Mortality (Mean ± SE)		
		24h	48h	72h
First	0.2	45.33±2.66	61.33±1.33	73.33±2.66
	0.3	54.66±1.33	80.00±2.30	89.33±3.52
	0.4	60.00±00.00	85.33±2.66	90.66±1.33
	0.5	81.33±3.52	92.00±00.00	98.66±1.33
	0.6	94.66±1.33	100±00.00	100±00.00
Second	0.2	37.33±2.66	50.66±1.33	70.66±1.33
	0.3	53.33±3.352	64.00±2.30	80±2.309
	0.4	57.33±2.66	73.33±2.66	88.00±00.00
	0.5	66.66±1.33	78.66±2.66	92.00±00.00
	0.6	76.00±00.00	97.33±1.33	100±00.00
Third	0.2	30.66±1.33	40.00±00.00	52.00±00.00
	0.3	42.66±1.33	57.33±1.33	70.66±1.33
	0.4	53.33±1.33	65.33±1.33	80.00±00.00
	0.5	68.00±00.00	72.00±00.00	85.33±00.00
	0.6	78.66±1.33	92.00±00.00	100±00.00
Fourth	0.2	30.66±1.33	33.33±1.33	48.00±00
	0.3	37.33±1.33	44.00±00.00	61.33±2.66
	0.4	50.66±1.33	52.00±00.00	68.00±00.00
	0.5	50.66±1.33	62.66±2.66	81.33±5.33
	0.6	60.00±00.00	81.33±2.66	94.66±1.33

Table 2: Larvicidal mortality of all the instars of *Aedes albopictus* against Acetone extractive of fruits of *Diospyros kaki*

Larval Instars	Concentration (ppm)	Percent Mortality (Mean ± SE)		
		24h	48h	72h
First	150	36.00±00.00	49.33±3.52	60.00±00.00
	200	42.66±1.33	61.33±1.33	77.33±1.33
	250	48.00±00.00	68.00±00.00	81.33±3.52
	300	60.00±00.00	77.33±1.33	90.66±3.82
	350	69.33±3.52	90.66±3.52	100±00.00
Second	150	40.00±00.00	52.00±00.00	68.00±00.00
	200	52.00±00.00	60.00±00.00	80.00±00.00
	250	60.00±00.00	70.66±1.33	88.00±00.00
	300	76.00±00.00	88.00±00.00	98.66±1.33
	350	82.66±00.00	96.00±00.00	100±00.00
Third	150	24.00±00.00	33.33±1.33	48.00±00.00
	200	32.00±00.00	38.66±1.33	60.00±00.00
	250	40.00±00.00	56.00±00.00	72.00±00.00
	300	56.00±00.00	72.00±00.00	85.33±00.00
	350	60.00±00.00	80.00±00.00	96.00±00.00
Fourth	150	20.00±00.00	28.00±00.00	36.00±00.00
	200	28.00±00.00	40.00±00.00	44.00±00.00
	250	41.33±00.00	54.66±2.66	69.33±1.33
	300	48.00±00.00	64.00±00.00	80.00±00.00
	350	60.00±00.00	76.00±00.00	94.66±00.00

Table 3: Log probit and Regression analyses on mortality of *Aedes albopictus* larvae treated with crude extracts of *Diospyros kaki*

Larval Instars	Period of Exposure	LC_{50} (%)	LC_{90} (%)	Regression	R^2- Value
1^{st}	24	0.2546	0.6951	Y=125.3x + 17.06	0.957
	48	0.17	0.4144	Y=89.34x +47.99	0.937
	72	0.1381	0.3254	Y=64.67x +64.32	0.857
2^{nd}	24	0.2942	1.284	Y=90.67x + 21.86	0.969
	48	0.2162	0.6241	Y= 108x +29.59	0.968

	72	0.1409	0.3963	Y= 70.68x+57.86	0.984
3rd	24	0.33861	1.0173	Y=121.3x + 6.126	0.997
	48	0.2613	0.7526	Y= 138.6x +7.864	0.938
	72	0.2039	0.4977	Y= 110.6x +33.33	0.963
4th	24	0.5035	2.167	Y= 92x +3.73	0.981
	48	0.3337	1.1156	Y=114.6x + 8.8	0.973
	72	0.2283	0.6584	Y=113.3x +25.33	0.989

Table 4: Log probit and Regression analyses on mortality of *Aedes albopictus* larvae treated with acetone extract of *Diospyros kaki*

Larval Instars	Period of Exposure	LC_{50} (ppm)	LC_{90} (ppm)	Regression	R^2- Value
1st	24	233.4016	844.6604	Y=0.168x+9.198	0.979
	48	160.8189	417.4241	Y=0.197x +20	0.988
	72	134.8291	275.7176	Y=0.186x +35.19	0.962
2nd	24	188.6898	476.595	Y=0.218x +7.472	0.987
	48	160.311	332.6539	Y=0.232x +15.33	0.983
	72	126.3228	233.4989	Y=0.165x +45.60	0.954
3rd	24	284.2156	825.6367	Y=0.192x-5.6	0.973
	48	216.6591	483.4813	Y=0.250x-6.404	0.980
	72	165.1604	334.5027	Y=0.242x +11.60	0.999
4th	24	300.0957	810.1327	Y=0.2x-10.53	0.991
	48	229.6447	540.7485	Y=0.24x-7.468	0.996
	72	193.7532	356.6906	Y=0.258x+4138	0.983

Table 5: ANOVA analysis using the crude extract of *Diospyros kaki* fruits against *Aedes albopictus*

Source of variation	Sum of squares (SS)	Degree of freedom (DF)	Mean of squares (MS)	F value	P-Level
Instars (I)	15318.044	3	5106.015	467.014	0.00
Hours (H)	20432.533	2	10216.267	934.415	0.00
Conc. (C)	37041.244	4	9260.311	846.980	0.00
I × H	511.822	6	85.304	7.802	0.00
I × C	734.400	12	61.200	5.598	0.00
H × C	573.689	8	71.711	6.559	0.00
I × H × C	985.067	24	41.044	3.754	0.00
Within groups				---	---
Total	75,595.978	59		----	---

Table 6: ANOVA analysis using the acetone extract of *Diospyros kaki* fruits against *Aedes albopictus*

Source of variation	Sum of squares (SS)	Degree of freedom (DF)	Mean of squares (MS)	F value	P-Level
Instars (I)	11836.711	3	3945.570	462.372	0.00
Hours (H)	24659.733	2	12329.867	1444.906	0.00
Conc. (C)	41093.689	4	10273.422	1203.917	0.00
H x I	295.289	6	49.215	5.767	0.00
C x I	717.511	12	59.793	7.007	0.00
H × C	363.378	8	45.422	5.323	0.00
H x C x I	402.489	24	16.770	1.965	0.009
Within groups				---	---
Total	79,367.356	59		----	---

Table 7: Results of Photochemical analysis of both crude and acetone extract of *Diospyros kaki* fruits

S. No	Secondary metabolites	Crude extract	Acetone extract
1.	Alkaloids	Present	Present
2.	Flavonoids	Present	Present
3.	Steroid	Present	Present
4.	Saponins	Present	Absent
5.	Glycosides	Present	Absent
6.	Tannins	Present	Present
7.	Triterpenoids	Absent	Absent
8.	Phenols	Present	Present
9.	Coumarins	Present	Present
10.	Protein	Present	Present

Table 8: FT-IR analysis of acetone extract of *Diospyros kaki* fruits showing presence of different functional groups

Absorption spectra peaks	Probable functional groups
3366.14	OH, OH stretching of alcohol NH stretching of amine
2921.63	CH stretching of alkanes OH stretching of carboxylic acid
2361.41	NH stretching of amino salt
1715.37	C=O stretching of amides C=O stretching of carboxylic acid
1373.07	C-N stretching of alcohol NH stretching of amide
1140	CO stretching of alcohol NH stretching of amide
1036.55	C-O stretching of alcohol C-C stretching of alkenes C-O-C stretching of ether

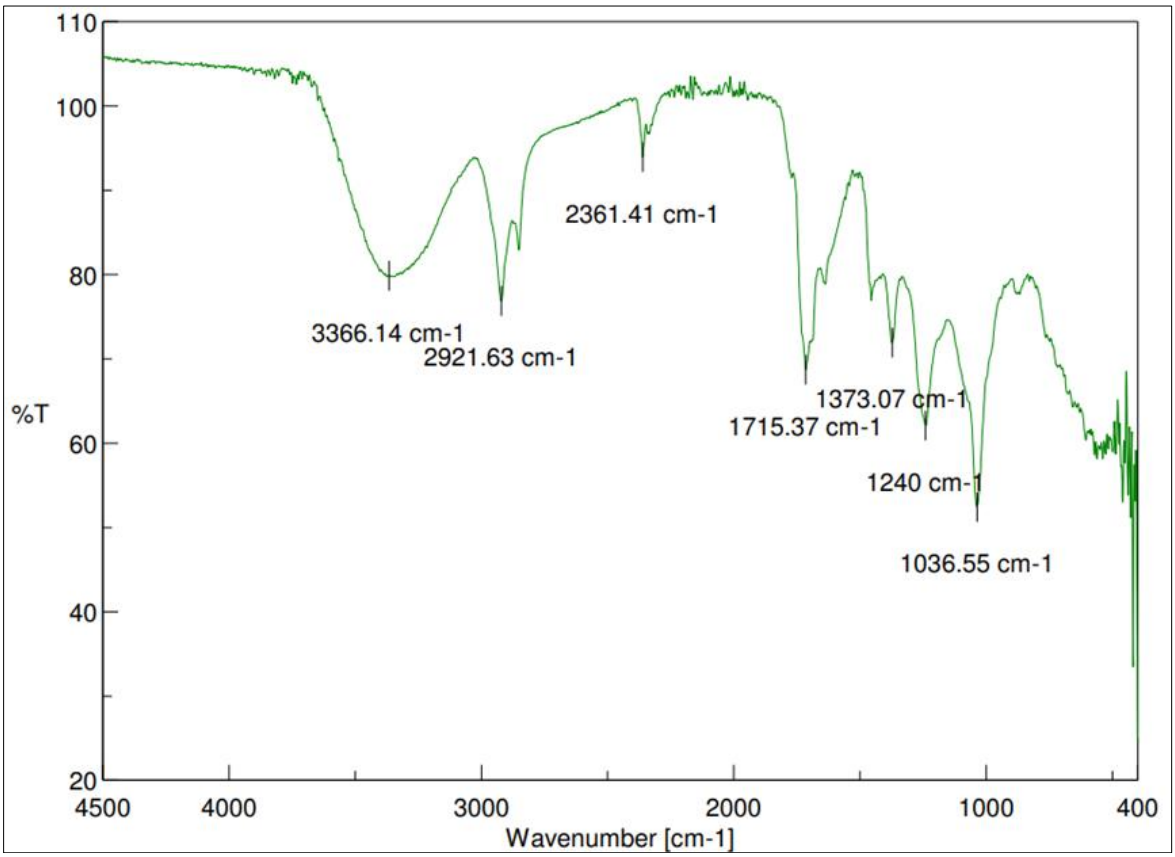

Fig 1: FT-IR analysis of acetone extract of *Diospyros kaki* fruits showing presence of different peaks of different peaks

4. Discussion

One of the most significant public health issues in developing nations is the spread of mosquito-borne diseases. It can be controlled by insecticides that kill mosquitoes and eventually reduces the risk of mosquito –borne diseases. The development of newer insecticides or the emergence of insecticide resistance in vector mosquitoes poses a serious threat to vector control. However, better alternatives to common synthetic insecticides are sought. In the future, photochemical may be a good substitute for synthetic insecticides because they are generally safe, affordable, biodegradable and widely accessible. There is a lot of research that highlights the value of plant extracts as effective insecticides [26]. Several experiments have reported a variety of plants with larvicidal, pupicidal, adulticidal, phago-deterrence, oviposition deterrence, repellent, and smoke toxic effects against mosquitoes [27-29].

Previously, mosquitocidal activity was found from ethyl acetate extract of *Ocimum sanctum* leaves against *Ae. Aegypti* and *Cx. quinquefasciatus* with LC_{50} values of 425.94 ppm and 592.60 ppm respectively [30]. Seaweed, *Bryopsis pennata* aqueous extracts exhibited larvicidal activity against *Ae. Aegypti* and *Ae. Albopictus* with LC_{50} values 591.77 and 692.45ppm respectively [31].

Crude and acetone extracts of *D. Kaki* fruits demonstrated potent insecticidal activity against *Ae. Albopictus* larvae and resulted in 100% mortality after 72 hours of exposure with both crude and acetone extract in both 1st and 2nd instar larvae. LC_{50} and LC_{90} values in crude and acetone extract are significantly low in comparison to other previous studies in 1st and 2nd instar larvae after 72 hrs. of exposure. Non target organism is non-responsive against both the extract. Presence

of different compound like alkaloids, tannins, flavonoids, phenol may be responsible for its mosquitocide property.

5. Conclusion

This leads to the conclusion that the larval stage of the medically significant mosquito *Ae. Albopictus* can be controlled by using the crude and acetone extracts of fruits of *D. kaki*. The *D. kaki* extracts may be a more effective natural insecticide and an affordable, secure substitute for synthetic insecticides. More research is needed to determine the identity and chemical composition of the active molecules and their mechanisms of action in the larvae. A proper field assessment is required before using them in the mosquito control programme.

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7. Conflict of interest: We have no conflict of interest.

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