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Phytochemical screening and larvicidal evaluation of leaf extract of *Tinospora cordifolia* against *Aedes aegypti* (Diptera: Culicidae)

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Abstract
Millions of individuals contract dengue each year from the *Aedes aegypti* mosquito. The most effective alternative methods under the environmentally sustainable control programs comprise investigating floral biodiversity and looking into the use of safer insecticides with botanical origins. Because synthetic chemical insecticides are so often used, the dengue vector has developed resistance to them. Examining the larvicidal activity, alternation in morphology and midgut histopathology by the extracts prepared from the leaf of *Tinospora cordifolia* on the dengue vector *Ae. aegypti*. The third instar larvae were used to assess the effects of aqueous, ethanolic, and petroleum ether extracts from *T. cordifolia* leaves. The results recorded after 24 h of treatment were shown significant ($P<0.05$) larvicidal activity. The aqueous, ethanolic, and petroleum ether extract with LC_{50} values of 380.18, 123.02, 213.79 ppm, and LC_{90} values of 707.94, 275.42, 457.08 ppm, respectively. Morphological and histopathological alternation in *Ae. aegypti* third instar larvae by leaf extracts included, short antenna, shrunken anal papillae, narrowed respiratory tube, partially darkened larvae and disorganized epithelial cells, damaged microvilli, necrotic nuclei, free cytoplasmic mass, diffused peritrophic membrane and the damaged basal lamina, respectively. The preliminary phytochemical analysis's findings demonstrated the existence of many phytoconstituents. The histopathological effects of *T. cordifolia* leaf extract on the midgut of *Ae. aegypti* larvae were first demonstrated in this work. Researchers hoped that the study's findings would aid them in better understanding the bioactivity of the plant's extract, which might be used to get rid of *Ae. aegypti*.

Keywords: *Aedes aegypti*, larvicidal activity, phytochemical screening, *Tinospora cordifolia*, histopathology

Introduction
Dengue is transmitted by *Aedes aegypti*. Rice fields, fish ponds, irrigation canals and artificial containers are common breeding grounds for mosquitoes, especially during rainy seasons. This gives mosquitoes a better place in tropical and subtropical nations, due to favorable environmental conditions (Ghosh *et al.*, 2012) [15]. There are 4 billion people who inhabit locations where dengue could occur. Every year, the dengue virus affects up to 400 million people. About 100 million individuals suffer from infection, and 40,000 people pass away from severe dengue (CDC, 2021) [8].

To eradicate tropical diseases, destroy the dengue vector by following the guidelines provided by WHO (2015) [47]. Dengue vectors have the ability to transmit the virus to multiple people during a single gonotrophic cycle. To prevent dengue fever, neither a vaccine nor antiviral medications are currently available. Therefore, vector control, which involves killing or preventing mosquitoes from biting people in order to decrease virus transmission, is the most frequently chosen remedy. The two main methods used to control mosquitoes are larvicidal and adulticidal sprays (Sharma et el., 2016) [39]. When controlling vectors in the past, the chemical method used spraying chemical insecticides and synthetic larvicides to target adult mosquitoes and instar larvae. For instance, temephos, permethrin, malathion, and deltamethrin are used as adulticides or larvicides (Vivekanandhan *et al.*, 2018) [44]. According to Jangir and Prasad *et al.*, (2022), the dengue vector, *Ae. aegypti* has become more resistant to cyfluthrin, lambda-cyhalothrin, DDT, and permethrin in the majority of districts of India [20].

However, the main issue with this approach is that it has caused environmental pollution by overusing chemical insecticides to control vectors. Chemical insecticides and larvicides are toxic to non-target organisms and are non-biodegradable. Alternative applications of plant-based bio-insecticides against *Ae. aegypti* may provide a more suitable and sustainable solution (Sharma *et al.*, 2016) [39]. Natural products are easier to biodegrade, less hazardous, and environmentally friendly. Due to insecticide resistance and the ineffectiveness of *Aedes* control methods (Benelli, 2015) [6]. It has become necessary to look for new insecticides, a safer alternative to chemical insecticides. Natural substances obtained from plants tend to have low mammalian toxicity and act quickly (compared to biological control) (Ghosh *et al.*, 2012) [15].

The focus of this search shifts to plant-based herbal insecticides. Several studies have already isolated plant origins of larvicides including β—sitosterol, falcarinol, octacosane, geranial, pipernonaline, azadirachtin, and plumbagin (Kishore *et al.*, 2011) [24], and phytochemicals like saponins (Nya *et al.*, 2016) [29], steroids (Ghosh *et al.*, 2008) [16], flavonoids (Rajkumar and Jebanesan, 2008) [34], alkaloids (Saxena *et al.*, 1993) [38], and tannins (Silva *et al.*, 2004) [42] have the potential to be effective mosquito larvicides and alter the morphology and histology of larvae (Warikoo and Kumar, 2013; Sharma *et al.*, 2015; Yu *et al.*, 2015; Kim and Ahn, 2017) [45, 40, 49, 23].

Tinospora cordifolia, often known as Guduchi or Giloy and belonging to the Menispermaceae family. Renowned for its extensive use in the traditional Ayurvedic treatment of numerous diseases (Sinha *et al.*, 2004; Saha and Ghosh, 2012) [43, 37]. This plant's various parts have insecticidal or larvicidal properties that work against various insects (Sharma *et al.*, 2003; Abdullah *et al.*, 2012; Paul *et al.*, 2020) [41, 2, 30]. The current study's objective was to evaluate the larvicidal potential of extracts of *Tinospora cordifolia* leaves on *Ae. aegypti* third-instar larvae.

Material and methodology

Collection of plant material and extraction

Tinospora cordifolia's fully formed, fresh, and healthy leaves were gathered from a nursery in the Bulandshahr area, and the plant's identification was authenticated at the department of botany, Chaudhary Charan Singh University, Meerut. The material was delivered to the research laboratory, rinsed with distilled water, and left to air dry under shade for 21 days at 30°C. The dried leaf was mechanically ground into a fine powder using an electric grinder, put through a 2 mm sieve, and stored in sterile bottles.

Preparation of plant extracts

Separate glass Soxhlet extractors were used to extract leaf powder (500g) in ethanol and petroleum ether (Kasiramar, 2018) [22]. The extraction was conducted continuously for 4 days at a duration of six hours per day, with the Soxhlet extractor set in line with the boiling point of the solvent. The final step was to separate the extracted material and place it in a small beaker to allow the solvent to evaporate. The extracts were produced solvent-free in a water bath. The leaf residue was then weighed and redissolved in distilled water. For future usage, a stock solution of 2000 ppm/L was stored at 4ºC.

Preliminary phytochemical Screening and Physicochemical parameters

Following standard protocol, *Tinospora cordifolia* leaves were examined qualitatively for the identification of various phytoconstituents in different solvent extracts (Rajasudha and Manikandan, 2019) [33]. Evaluation of physicochemical parameters was done by following the methods outlined by WHO (2011) [48].

Larvae culture and identification

The larvae and eggs of Ae. aegypti were collected from mosquito ovitraps placed on the N.R.E.C. College campus and colonized in a research lab at a "temperature of 28°C, Relative Humidity (75%), and Light/Dark photoperiod (14:10)". On a plastic tray filled with tap water, the larvae were cultivated. The larvae were fed on yeast powder, powdered rice, and soybean powder in a ratio (3:1:1), and dechlorinated tap water was used to hatch the eggs (Kamaraj *et al*., 2009) [21]. The binocular microscope was used to identify the mosquito larvae morphologically by following the identification key (Christophers, 1960; Rueda, 2004) [11, 36]. "The third instar larvae have a single hair, a three-branch hair tuft on each side of the air tube. The hair tuft has two or more branches, that arise from the same socket" (Bruce, 2005) [7].

Larvicidal bioassay

The larvicidal activity of extracts of *Tinospora cordifolia* leaf was evaluated against *Aedes aegypti* third instar larvae by adhering to the established standards of WHO (2005) [46] with some changes. To test the larvicidal potential of leaves, a 2000 ppm stock solution was added with 10 ml of Tween-20 (emulsifying agent). During the initial testing, the plant extracts that achieved 100% larval mortality within 24 h were selected for larvicidal bioassay. Various dilute concentrations were prepared from this solution by adding distilled water 200, 400, 600, 800, and 1000 ppm (for aqueous extract) and 50, 100, 200, 400, 500, (for ethanolic extract) and 100, 200, 400, 600, 800 ppm (for petroleum ether extract). Five replicates of each treatment were used with batches of 20 larvae in a 250 ml beaker that comprised 150 ml of extracts. The control solution was 5% ethanol and tween-20 dissolved in distilled water.

Detection of morphological changes in larvae

The dead larvae were immediately examined for morphology under a light microscope; the degree of damage to the respiratory siphon, anal papillae, size, and colour, of the larvae, was documented.

Histopathological study

In order to compare *Ae. aegypti* third instar larvae treated with the different solvents of leaf with the untreated (control) larvae, a histopathology analysis was conducted. All treatment groups for the larvicidal test had their larvae collected after a 24-hour exposure period, and they were then fixed in 10% formalin for two days. For tissue processing, standard methods were used (Narciso *et al.*, 2014) [27], such as graded ethanol dehydration (30% to 100%), paraffin infiltration and embedding, 4 m thick sectioning using a microtome, and hematoxylin and eosin staining (Feldman and Wolfe, 2014) [13]. The midgut regions of larvae were descriptively inspected for histopathological changes under a light microscope.

Data analysis

The following statistical tools were applied. The mortality rate was corrected using Abbott's method (Abbott, 1925) [1]. Using Finney's table, the percentage mortality was converted to probit values and the LC_{50} and LC_{90} were calculated (Finney, 1971) by utilizing the program "Microsoft Excel 2021" including regression equations, and other statistics.

Results

The results of the phytochemical analysis were shown in Table 1. Several phytoconstituents were found in the aqueous, ethanolic, and petroleum ether extract of Tinospora cordifolia leaf after the qualitative phytochemical screening. Several phytoconstituents, including alkaloids, carbohydrates, glycosides, phenols, saponins, and tannins, were visible in the aqueous extract. A variety of phytoconstituents, including amino acids, anthraquinones, alkaloids, carbohydrates, glycosides, phenols, saponins, terpenoids, and steroids, were visible in ethanol extracts. Several phytoconstituents, including anthraquinones, alkaloids, glycosides, phenols, saponins, terpenoids, and steroids, were visible in petroleum ether extracts. The petroleum ether extract in ethanolic form had more phytoconstituents than the extract in watery form. Table 2 displays the results of the physicochemical parameters.

Table 1: Results of phytochemical analysis of *Tinospora cordifolia* leaf extracts

Phytoconstituents	Aqueous extract	Ethanolic extract	Petroleum ether extract
Amino acids	-	+	-
Anthraquinones	-	-	+
Phenols	+	+	+
Saponins	+	+	+
Alkaloids	+	+	+
Carbohydrates	+	+	-
Tannins	+	+	+
Terpenoids	-	+	-
Steroids	+	-	+
Flavonoids	-	+	-
Glycosides	+	+	+

Table 2: Physicochemical parameters of the leaf of Tinospora cordifolia

Parameters	Sub-parameters	Result (%)
Loss on drying		12.55±0.55
Ash value	Total ash value	8.76±0.41
	Water soluble ash	3.44±0.32
	Acid insoluble ash	1.09±0.08
	Sulphated ash value	3.88±0.18
Extractive value	Aqueous	26.45±0.83
	Ethanolic	19.40±0.57
	Petroleum ether	4.84±0.28

*Values presented in the table are the mean ± SD of three replicates

All the extracts of the leaf of *Tinospora cordifolia* which were extracted in different solvents were shown significant larvicidal potential during the *in vitro* larvicidal assay on *Aedes aegypti* third instar larvae (Table 3). The information in table 3 demonstrates that the ethanolic extract of leaf achieved 100% larval mortality at a higher concentration of 500 ppm with LC_{50} and LC_{90} values of 123.02 and 275.42 ppm, respectively, within 24 hours after the treatments were applied. Similar to this, a leaf extract in petroleum ether at 800 ppm concentration resulted in 100% mortality, with LC_{50} and LC_{90} values of 213.79 and 457.08 ppm, respectively (table 3). The aqueous extract of the leaf achieved 100% larval mortality at a higher concentration of 1000 ppm with LC_{50} and LC_{90} values of 380.18 and 707.94 ppm, respectively (Table 3).

Table 3: Larvicidal efficacy of aqueous, petroleum ether, and ethanolic extracts against *Ae. aegypti* in 24 hours.

Mosquito species	Plant part used	Solvent	Conc. (PPM)*	Mortality (%)	LC_{50} (PPM)	LC_{90} (PPM)	95% confidence interval		Regression equation	R^2	P-value (P<0.05)
				24 h	24 h	24 h	Lower bound	Upper bound	24 h	24 h	24 h
Ae. aegypti	leaf	Aqueous extract	1000	100±0.0	380.18	707.94	0.0834	9.8408	Y=4.8787x - 7.5967	0.76	0.05
			Control	00±00							
		Ethanol extract	500	100±0.0	123.02	275.42	0.8845	6.3877	Y=3.6362x-2.6065	0.85	0.02
			Control	00±00							
		Petroleum ether extract	800	100±0.0	213.79	457.08	0.6230	7.1010	Y=3.862x-4.0142	0.82	0.03
			Control	00±00							

*PPM- Part per million.

Figure 1-3 demonstrates that as the concentration of extracts rises, the death rate also rises. A positive correlation exists between the dependent variable (Y=mortality) and the independent variable (X=concentration) according to the results of regression analysis. The results of Log probit analysis demonstrated that the ethanolic extract of leaf was shown more larvicidal potential than the other solvents extract (Table 3).

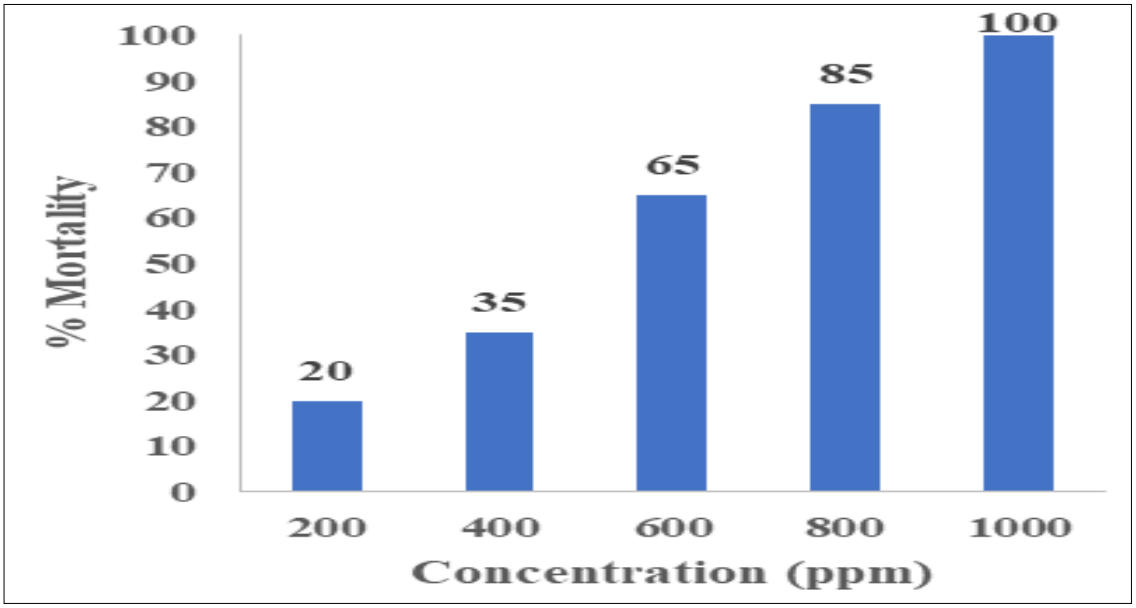

Fig 1: Larvicidal action of aqueous extract of leaf.

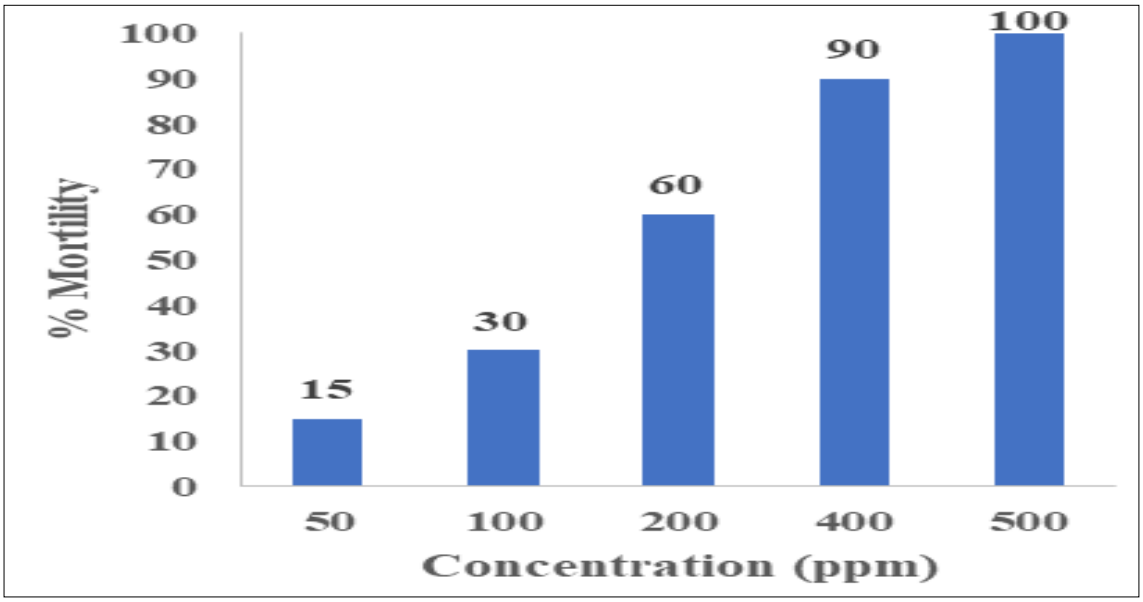

Fig 2: Larvicidal action of ethanolic extract of leaf.

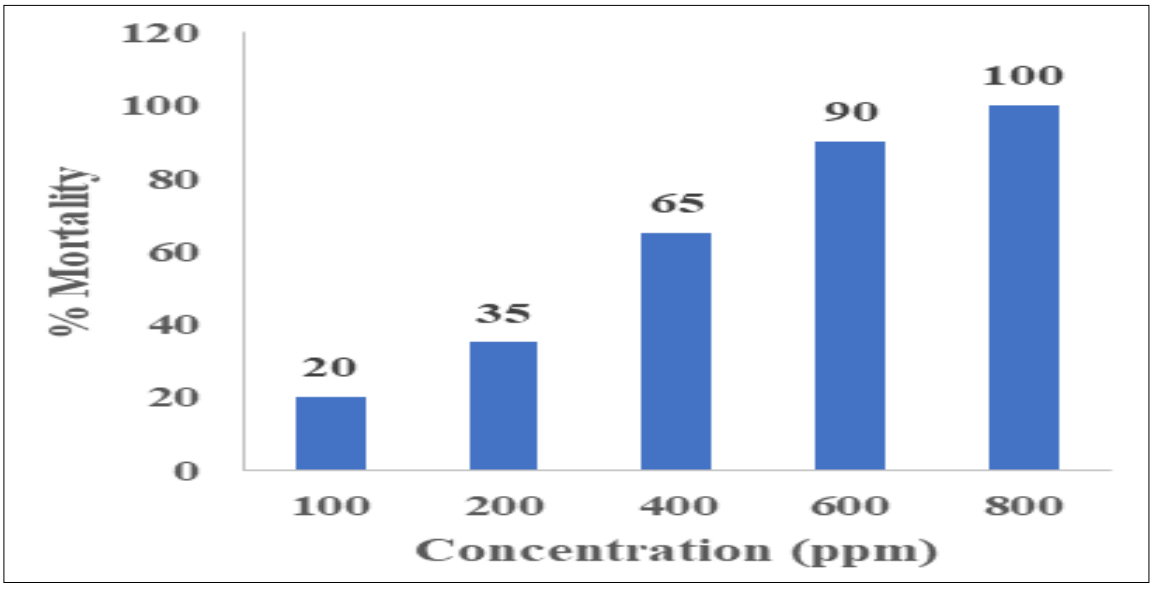

Fig 3: Larvicidal action of petroleum ether extract of leaf.

In order to identify the injured target body part following treatment with *Tinospora cordifolia* leaf extract and compare the treatment groups to a control group of larvae, morphological changes were seen in *Ae. aegypti* third instar larvae. Figure 5 gives a summary of the morphological alterations. In control groups, no damage was observed in larval body parts (Figure 4, A-C). Those larvae were treated with different solvents extracted from leaves, were shown different morphological alternations (Figure 4, D-L). In the treatment of petroleum ether extract, exhibits shortening of antennae, constricted or folded midgut region, narrowed respiratory tube and shrinking anal papillae (Figure 4, D-F). The treatment of ethanolic extract exhibits shrunken anal papillae and narrowed respiratory tube (Figure 4, G-I). In addition to the constricted lower midgut area, no harm to the head, thorax, or abdomen was seen after treatment with an aqueous extract (Figure 4, J-L).

Fig 4: Shows the morphological changes in the head, thorax, and abdominal segments of *Ae. aegypti* third instar larvae. (A to C- Control larvae, D to F- In petroleum ether extract of leaf, G to I – In ethanolic extract of leaf, J to L-In aqueous extract of leaf).

In order to better understand the targets of action of *Tinospora cordifolia* leaf extract in *Ae. aegypti* third instar larvae, a histopathology study was carried out. Figure 5 (A–E) shows the variations in the histopathology between the control larvae and the treatment groups. Third-instar control *Ae. aegypti* larvae contain typical gastrointestinal with apparent peritrophic membranes in the midgut region, epithelial cells with compactly pigmented cytoplasm, clearly defined chromatin, and spherical nuclei. Furthermore, most microvilli where epithelial cells were still connected to the basement lamina were normal looking.

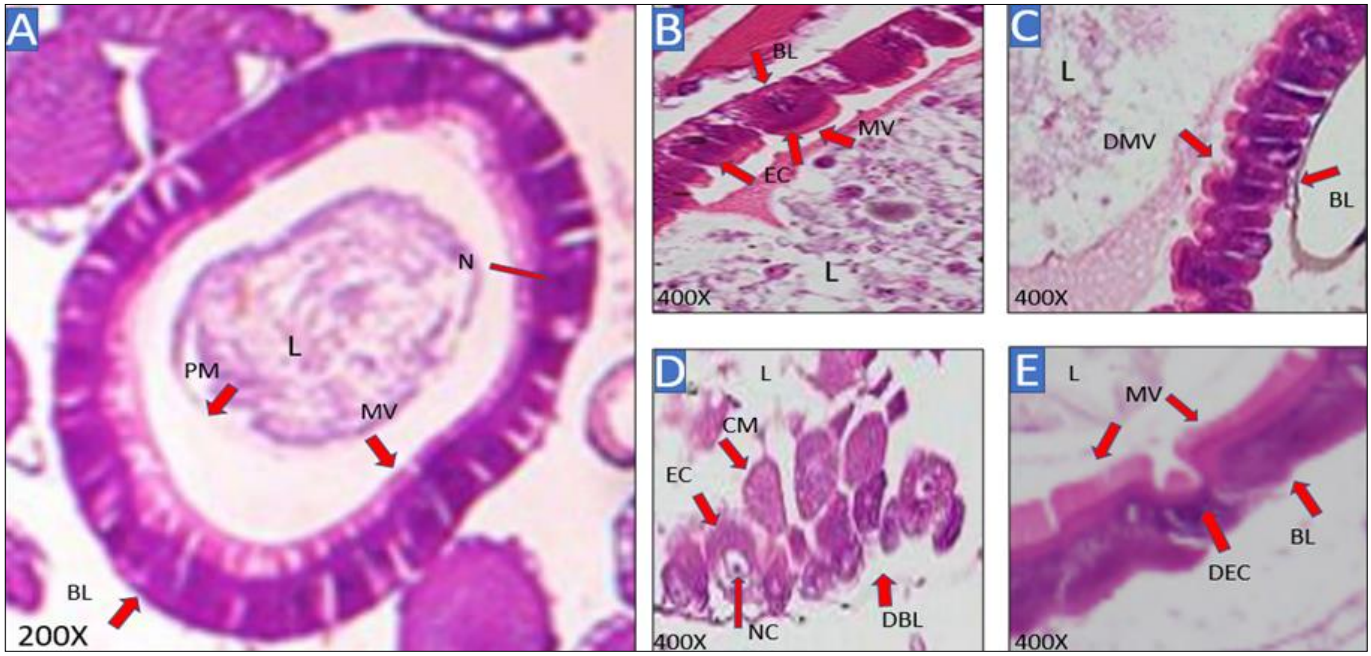

Fig 5: Shows the histopathological changes in the midgut region of *Ae. aegypti* third instar larvae. Midgut region of control group larvae (A), Treatment groups (B-E), Aqueous extract treatment (B), Petroleum ether extract treatment (CD), and Aqueous extract treatment (E). (L; Lumen, MV- Microvilli, BL; Basement lamina, N; Nucleus, PM; Peritrophic membrane, EC; Epithelium cell, NC; Necrotic nuclei, DMV; Degenerating microvilli, DBL; Damaged Basement lamina, DEC; Damaged epithelial cell, CM; Cytoplasmic mass).

No histological changes were observed in the treatment of the aqueous extract of the leaf (Figure 5, B). In petroleum ether extract treatment (Figure 5, CD), the midgut region epithelium cells appeared disorganized, with the damaged basal lamina, necrotic nuclei, cytoplasmic mass, damaged microvilli, and peritrophic membrane appearing diffuse. In the treatment of ethanolic extract (Figure 5, E), exhibit diffuse epithelial cells in the basement lamina. Epithelial cells were intact with basement lamina where microvilli were looking normal.

Discussion

Plants produce phytochemicals that operate as growth inhibitors, growth regulators, repellents, larvicidal, ovicidal deterrents, and chemosterilants for insects. These chemicals also act as a wider pool for biologically active molecules. This may be the effect of a complex mix of phytochemicals contained in plants that may be acting together to cause such a response. Plant-derived insecticides rarely allow insects to develop resistance to them (Howard *et al.*, 2007; Maurya *et al.*, 2012) [18, 26].

Regarding their larvicidal activity against *Ae. aegypti*, several medicinal plants have been studied**.** In the present study, different phytoconstituents, including phenols, glycosides, flavonoids, steroids, tannins, saponins, and alkaloids, were detected in the leaf extracts of *Tinospora cordifolia* after being extracted with aqueous, ethanol, and petroleum ether (Table1). The actions of these metabolites vary, including contact toxicity, larvicides, repellents, and insect growth regulators (Maurya *et al.*, 2012) [26]. There is evidence that the compounds found in plants like flavonoids, alkaloids, tannins, and saponins are what make them toxic and insecticidal (Akinyemi *et al.*, 2005; Azmathullah *et al.*, 2011) [3, 5].

To determine the larvicidal effectiveness of *Tinospora cordifolia* against *Ae. aegypti* third instar larvae, we carried out an experimental investigation. Aqueous, ethanolic, and petroleum ether solvent extracts all demonstrated significant larvicidal activity at concentrations of 1000, 500, and 800 ppm, respectively. The aqueous, ethanol and petroleum ether extract with LC_{50} values of 380.18, 123.02, 213.79 ppm and LC_{90} values of 707.94,275.42, 547.08 ppm, respectively. Our finding supported by Paul *et al.*, (2020) [30] reported that this plant's metabolites have larvicidal activity, which lends weight to our conclusion. Additionally, 90 and 93% of the *Ae. aegypti* larvae died after 24 and 48 hours, respectively, when exposed to methanolic extracts of the stem and leaf of *Tinospora rumphii*, with LC50 and LC90 values of 10 mg/mL and 46 mg/mL (Gutierrez *et al.*, 2014) [17].

Earlier studies on various plants have discovered that phytochemicals extracted using various solvents have larvicidal efficacy against several medically significant insects. Nobsathian *et al.*, (2018) [28] reported that *Maerua siamensis* contains a compound capparilosides A and B that exhibits toxic action with LC_{50} values of 71.14 and 99.79 ppm against *Ae. aegypti* larvae. The methanol extract of the flower from *Clitoria ternatea* has been shown to have larvicidal potential on *Ae. aegypti* (Ravindran *et al.*, 2020) [35]. After 24 hours of exposure, *Asparagus setaceus* crude and ethanolic extracts showed effective larvicidal effects against *Ae. aegypti* first and second instar larvae (Chakraborty *et al.*, 2021) [10]. Moreover, it has been demonstrated that a Methanolic extract made from *Vitex ovata* has larvicidal action and has been proven to produce 76% and 84% larval mortality against *Ae. aegypti* at doses of 5000 ppm and 10000 ppm, respectively, within 24 hours (Aziz *et al.*, 2021) [4]. *Coleus aromaticus* and *Aegle marmelos* extract in methanol were tested against the *Ae. aegypti* larva (II, III, IV instars) and pupa, considerable larvicidal action was found in 24 h by Dass *et al.*, (2022) [12].

In the current study, the microscopic investigation showed the shortening of antennae, shrinking of anal papillae, narrowed respiratory tube, and constricted or folded midgut region of *Ae. aegypti* third instar larvae after being exposed

to ethanol and petroleum ether extract of leaf of *Tinospora cordifolia*. These outcomes are consistent with earlier research. *Ae. aegypti* larvae were treated with high amounts of *Argemone mexicana*, which caused damage to the anal papillae according to Warikoo and Kumar (2013) [45]. Furthermore, *Sharma et al.,* (2015) [40] found that the anal papillae of *Ae. aegypti* larvae in the early fourth instar displayed structural damage after exposure to extracts of the stems and leaves of *Achyranthes aspera*.

Furthermore, the ethanol extract of *Kaempferia galanga* caused cuticle shrinkage and anal papillae injury in *Culex quinquefasciatus* larvae, according to Insun *et al.,* (1999) [19]. Chaithong *et al.,* (2006) [9] speculate that osmotic and ionic dysregulation may be the cause of the structural malformation of the anal papillae. So, it is probable that osmotic and ionic dysregulation contributed to the death of larvae.

The color of the extract-exposed larvae changed from being transparent like the control larvae to becoming paler and even darker, indicating the presence of dead or dying tissues. (Kim and Ahn, 2017) [23]. An essential component of the larval respiratory system is the siphon. The hydrophobic surface of the larvae is lost when the siphon is damaged, which prevents water from entering the larval body and deprives the larvae of oxygen. As a result, the siphon and anal papillae that were damaged had an impact on the larvae's capability to survive (Yu *et al.,* 2015) [49].

Alkaloids may also have the ability to poison the stomach, which would upset the digestive process and kill *Ae. aegypti* larvae (Kim and Ahn, 2017) [23]. The findings of this experiment corroborate Liu *et al.,* (2012) [25] observation that larvae that have consumed food from the water's surface may be in danger from alkaloids. Alkaloids also block the hormone (ecdysone) that controls the growth of third-instar larvae. If these hormones do not function properly, the larvae will eventually stop developing into pupae and will eventually die or extend the larval duration.

Saponin is widely known for having insecticidal properties because it reduces the action of digestive enzymes and food absorption. During the digestive process in insects, saponin bind to free sterols and decrease the concentration of free sterols. The ecdysone hormone is derived from free sterols. Low free sterol concentrations stop the process of moulting. Moreover, saponin interacts with the cuticle membrane, limits larval growth, and has antifeedant effects. Later, they injure the larvae and eventually die. (Podolak *et al.,* 2010) [31].

The midgut of the larva plays a significant role in secreting digestive enzymes and absorbing nutrition (Liu *et al.,* 2012) [25]. In this study, the midgut injury in the larvae consisted of disorganized epithelial cells, damaged microvilli, necrotic nuclei, free cytoplasmic mass, diffused or damaged peritrophic membrane and the damaged basal lamina. The injury affected the way food was digested in the midgut of the larvae because some metabolites of *Tinospora cordifolia* act as a poison. According to the following mechanism, "after many hours of exposure, the toxins in the crude extract cross the cell membrane and attach to certain receptors on the epithelial cells to produce tiny pores. They facilitate the entry of ions and water into the cells, disrupting the osmotic balance, causing the cells to expand and subsequently lyse". The basal membrane was torn apart, releasing the lysed cells into the lumen of the midgut (Procopio *et al.,* 2015) [32].

Conclusion

In conclusion, *Tinospora cordifolia* showed significant larvicidal activity against the third instar larvae of *Aedes aegypti*. Preliminary phytochemical analysis reveals that the selected plant is a rich source of different phytoconstituents, and alters the larval morphology and histoarchitecture of the midgut of larvae, which results in the death of larvae. These plant metabolites might be used in vector control programs.

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Conflicts of interest

No conflict of interest

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