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ORIGINAL ARTICLE

Larvicidal Efficacy of *Andrographis paniculata* and *Tinospora cordifolia* against *Aedes aegypti*: A Dengue Vector

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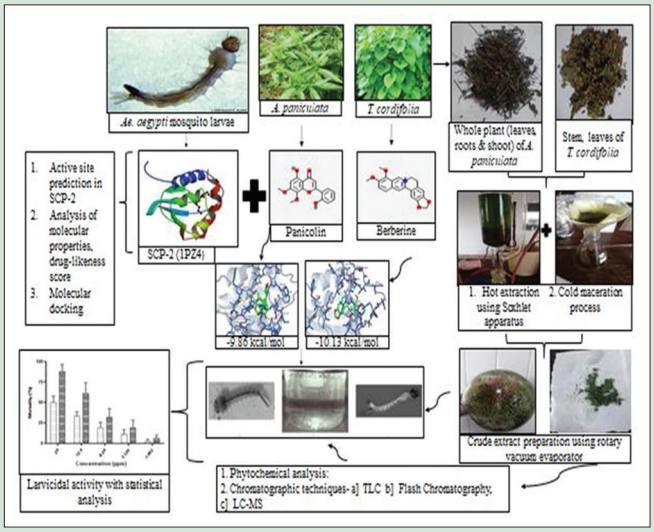
ABSTRACT

Background: *Aedes aegypti*, dengue fever mosquito, is predominantly accompanying the transmission of dengue in tropical and subtropical countries of the world, causing millions of deaths every year. **Materials and Methods:** The current research was carried out to evaluate the larvicidal efficiency of *Andrographis paniculata* and *Tinospora cordifolia* against third instar larvae of *A. aegypti* using *in silico* and experimental study. **Results:** The methanolic extract of *A. paniculata* and ethanolic extract of *T. cordifolia* showed the highest larvicidal activity with LC_{50} and LC_{90} value of 15.75 ppm; 349.60 and 100.64 ppm; 386.37 ppm, respectively after 48 h exposure, which was statistically significant ($P \leq 0.05$). However, the combined action of methanolic extract of *A. paniculata* + ethanolic (50:50) extract of *T. cordifolia* and the bioactive fraction of methanolic extract of *A. paniculata* showed the effective larvicidal activity with LC_{50} value of 113.20 ppm and 236.08 ppm respectively after 24 h treatment which was statistically significant ($P \leq 0.05$). The *m/z* values in liquid chromatography-mass spectrometry graph of bioactive fraction of methanolic extract of *A. paniculata* predicted the possible amount of bioactive compound class like flavone, phenols, phenylpropanoids, flavonoids, phenolic acid, alkaloids, isopalmitic acid, diterpenoids and quinic acid. The best energy binding affinity score against sterol carrying protein-2 of *A. aegypti,* panicolin (−9.86 kcal/mol), berberine (−10.13 kcal/mol) and their combination (50:50) showed effective larvicidal movement with LC_{50} value of 25.41 ppm, 23.15 ppm and 17.56 ppm after 24 h treatment. **Conclusion:** The crude extracts, bioactive fractions and synthetic bioactive compounds and their combination study of *A. paniculata* and *T. cordifolia* can be utilized as bio-control agents against *A. aegypti* mosquito.

Key words: *Aedes aegypti* mosquito larvae, *Andrographis paniculata,* chromatographic techniques, *in silico* study, larvicidal activity, phytochemical screening, *Tinospora cordifolia*

SUMMARY

- *Andrographis paniculata* and *Tinospora cordifolia* were used in traditional medicine for acute febrile illness treatment. The plants were collected from tribal areas of Purulia, West Bengal. The tribal peoples are using these plants for acute febrile illness treatment. But there are very less research on larvicidal activity against mosquito and larvae. Our research study expected that the crude extracts, bioactive fractions, and bioactive synthetic compounds, and some possible combinations (50:50) could be used as bio-control agents. Methanol, aqueous-methanol (50:50), ethanol, aqueous-ethanol (50:50) were used for crude extraction of *A. paniculata* and *T. cordifolia* using the (50:50) combination of Soxhlet hot extraction and cold maceration techniques. Chromatographic techniques like thin-layer chromatography, flash chromatography, and liquid chromatography-mass spectrometry were used to isolate bioactive fractions and prediction of bioactive compound class category. Hence for the fractionation process in chromatographic techniques, ethyl acetate and hexane solvent system was used in different ratio. Furthermore, we have done *in silico* study on 82 bioactive compounds present in these plants against *Aedes aegypti* larval receptor protein (sterol carrying protein-2: 1PZ4) to identify the best effective and possible compounds which could be used for the larvicidal activity.
- Therefore the methanolic extract of *A. paniculata* and ethanolic extract of *T. cordifolia,* the (50:50) combined treatment of methanolic extract of *A. paniculata* and ethanolic extract of *T. cordifolia* and the bioactive fraction of methanolic extract of *A. paniculata* showed the effective larvicidal activity showed the highest larvicidal activity after 24 h and 48 h exposure with LC_{50} and LC_{90} which was statistically significant ($P \leq 0.05$). The bioactive fraction of methanolic extract of *A. paniculata* predicted the possible amount of bioactive compound class like flavone, phenols, phenylpropanoids, flavonoids, phenolic acid, alkaloids, isopalmitic acid, diterpenoids and quinic acid. After *in silico* screening and docking of 82 bioactive compounds of *A. paniculata* and *T. cordifolia,* panicolin (−9.86 kcal/mol), berberine (−10.13 kcal/mol) and their combination (50:50) had been reported the larvicidal efficacy after 24 h treatment.



Abbreviations Used: *A. aegypti*: *Aedes aegypti*, *A. paniculata*: *Andrographis paniculata, T. cordifolia: Tinospora cordifolia*, hrs: Hours, LC_{50}: 50% lethal concentration, LC_{90}: 90% lethal concentration, SCP: Sterol carrying protein, TLC: Thin layer chromatography, LC-MS: Liquid chromatography-mass spectrometry, WHO: World Health Organization, ppm: Parts per million, *A. aphylla: Anabasis aphylla*, DDT: Dichloro diphenyl trichloroethane, R and D: Research and Development, IC_{50}: 50% inhibition concentration, Castp: Computed Atlas of Surface Topography of Proteins, SMILES: Simplified Molecular Input Line Entry Specification, MoNA: MassBank of North America, LGA: Lamarckian Genetic Algorithm, kcal/mol: Kilocalorie per mole, CSIR-NISCAIR: Council of Scientific and Industrial Research-National Institute of Science Communication and Information Resources, g/l: Gram/litre, ESI: Electrospray ionization.

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INTRODUCTION

Dengue is a vital viral diseases spread by *Aedes aegypti* with acute febrile illness symptoms. Dengue vector is an ultimate concern because of its wide distribution in heavily polluted areas such as Asia, America, and some Pacific islands, cities of tropical countries.[1,2] Mosquito is commonly established due to deprived drainage system, particularly during rainy periods, fish pond, irrigation ditches, and rice fields. This provides a better breeding place for mosquitoes. *A. aegypti*, a vector of dengue and chikungunya is extensively spread in the tropical and subtropical sectors. About two-thirds of ecosphere's inhabitants live in areas diseased with dengue vectors, mainly *A. aegypti*.[3,4] To counter tropical disease, destroying the vectors or immediate hosts is unique of the strategies in the guidelines provided by WHO.[5]

For growth, egg production, and development, cholesterol is vital in mosquito, but they cannot synthesis. In the larval stage, mosquito converted phytosterol to cholesterol in their gut. To control mosquito population, targeting cholesterol metabolism is the main objective of vector-borne disease management. Sterol carrying protein (SCP)-2 is essential for the uptake, transport and storage mechanism of cholesterol in mosquito as well as insects in the life cycle.[6,7]

There is no vaccine and antiviral drugs available to prevent dengue disease and other mosquito borne viral diseases, so vector control is the most commonly chosen solution by killing or stopping mosquitoes from biting human beings to reduce disease transmission and morbidity. So, larviciding (control of larvae) and adulticiding (control of adults) are the main mosquito control methods. Though the larval stage of mosquito is the attractive target easy to control them in this habit easily for mosquitos' class in water. As in the third instar larvae stage or at the immature stage mosquito are immobile, thereby making efficient control in this stage.[8-10] The chemical method which targets the adult mosquito and third instar larvae through spraying chemical insecticides and synthetic larvicides in vector control in a traditional practice. As an example, temephos and permethrin are used for the control of third instar larvae and mosquito.[1] But the major problem of this method is that it has resulted in environmental hazards through injudicious and over use of the chemical insecticides in vector control. Hence, the effective bio-control agents can be utilized as sustainable solutions for suppressing the mosquito larval population.[8,11]

In tradition and modern medicines, plants are used to improve the healthcare and R and D sector. Natural products are less-destructive to the environment, non-target organisms and are easily biodegradable. Phytochemicals were used as a crucial mosquito vector management tactic since 1920's and they were gradually replaced by the synthetic chemicals after the induction of dichloro diphenyl trichloroethane.[12] In an earlier study, it was reported that the plant alkaloids resembling nicotine, anabasine, methyl anabasine and lupinine extracted from a weed *A. aphylla* are known as larvicidal agents which may act independently/combinations, have been assessed for novel and capable larvicides.[13] Many scientists are trying to discover a new insect repellent, which must be a plant source and do not have any dangerous effects on the environment and ecosystem.[14] Researchers have demonstrated the effect of bioactive compounds from plants, for example, saponins, steroids, flavonoids, alkaloids, tannins, and phenols, as mosquito larvicides.[15]

The present research study is to find out SCP-2 inhibitors from *Andrographis paniculata* and *Tinospora cordifolia* using computational screening and larvicidal activity of best binding affinity scored compounds could be a substitute technique to discover new and promising larvicides that would interpose the cholesterol uptake by mosquito third instar larvae. Moreover, our study discovered the larvicidal activity of *A. paniculata* and *T. cordifolia* crude extracts and their combination, active fractions, and bioactive synthetic compounds against *A. aegypti* third instar larvae.

MATERIALS AND METHODS

In silico studies

From the protein databank (PDB) (www.rcsb.org/pdb/), the crystal structure (PDB ID: 1PZ4) of the SCP-2 of *A. aegypti* was acquired and SPDB viewer (www.expasy.org/spdbv/) was used for molecular visualization, energy minimization of SCP-2 and Castp online server was used for binding pocket active site detection of SCP-2. Based on IC_{50} value, toxicity, larvicidal activity and medicinal uses, structure of 82 bioactive compounds of *A. paniculata* and *T. cordifolia,* structure of were collected from Pubchem database, created from the Simplified Molecular Input Line Entry Specification notation by expending Open Babel Software. Geometry optimization and energy minimization were accepted out using the Chimera software after construct the structures. Molecular Docking calculations were carried out by Molecular Docking Server. Gasteiger partial charges, non-polar hydrogen atoms, rotatable bonds, essential hydrogen atoms, Kollman charges and salvation parameters were nominated and defined to implement for docking studies with 23 A^0 affinity grid point maps and 0.375 A^0 spacing using the auto grid program. Docking simulations were achieved using Lamarckian Genetic Algorithm and derived from 10 different runs, subsequently a maximum of 250000 energy estimations, translational step of 0.2 A^0.[16,17]

Experimental studies

Plant extraction, fractionation and characterization

Dried and powder samples of *A. paniculata* (stem, leaves, and roots) and *T. cordifolia* (stem, leaves) were extracted by Soxhlet hot extraction and cold maceration techniques with 250 ml of methanol, ethanol, aqueous-methanol (50:50) and aqueous-ethanol (50:50). The collected plant samples were authenticated in Council of Scientific and Industrial Research-National Institute of Science Communication and Information Resources, New Delhi. *A. paniculata*'s Ref. No. NISCAIR/RHMD/Consult/2018/3222-23-l and *T. cordifolia*'s Ref. No.-NISCAIR/RHMD/Consult/2018/3222-23-2. The resulting solution was filtered separately and the solvent was evaporated under reduced pressure, thereby the percentage of extraction was calculated by using following formula.[18]

$$\text{Extraction}(\%) = \frac{\text{Weight of the plant extract}(g)}{\text{Weight of the plant material}(g)} \times 100$$

The phytochemical screening was done following the standard procedure as described.[18] Evaluations of the major phytochemicals such as alkaloids, flavonoids, tannins, saponins, steroids, glycosides, phenols were conducted. After that, the dry methanolic extract of *A. paniculata* and ethanolic extract of *T. cordifolia* was subjected to flash chromatographic column over silica gel (200–400 mesh size), with a gradient of hexane-ethyl acetate and chloroform-methanol respectively, as the mobile phase, at increasing polarities. Thus in total, more than 200 fractions were obtained after flash chromatography and the fractions were analyzed using thin layer chromatography (TLC) plates and liquid chromatography-mass spectrometry (LC-MS), thus enabling molecular mass characterization of the active fractions by LC-MS analysis based on profiles and predictive analysis.[19] The MS analysis was performed using electrospray ionization (ESI) in the negative mode. The MS analysis was carried out using Mass Spectrometer. The MS parameters were: Retention time 0.268–0.451 min, 115 scans, and fragment = 60.0 Volt, *m/z* range = 0–400. The resolved compounds were then identified using online software, i.e., MassBank of North America, which is a

public repository natural product library for sharing mass spectral data. The identification of bioactive compound class was based on mass and intensity obtained via records.[20]

Collection and identification of mosquito third instar larvae

Mosquito third instar larvae were collected from open drains in Kolkata, West Bengal, (22.6686°N, 88.4089°E) India, which transported in plastic containers to the School of Tropical Medicine laboratory to identify the third instar larvae of dengue-carrying mosquito *A. aegypti* using stereo microscope. Therefore, *A. aegypti* mosquitoes' third instar larvae have a single hair, a three branch hair tufts which has two or more branches from the same socket on each side of the air tube which can be well-known from any other mosquitoes third instar larvae which have two or more hairs, branches and hair tufts on each side of the air tube. Besides the identified *A. aegypti* mosquitos' third instar larvae were sited in water-filled plastic dishes at 25°C–29°C and 80%–90% relative with larval food (yeast and biscuit in the ratio of 1:3).[21]

Larvicidal assay

By dissolving 100 mg of crude extracts of *A. paniculata* and *T. cordifolia*, 1000 ppm stock solution was prepared in 1 ml of methanol, ethanol, aqueous-methanol (50:50), aqueous-ethanol (50:50) to check larvicidal activity. 20 mosquitos' larvae were located in 250 ml glass beakers comprising 100 ml of 250, 125, 62.5, 31.25, 15.625 ppm concentration crude extracts with five replicates (n = 100 larvae) for each treatment and control of 0.2% at 20°C temperature for 24 h and 48 h. The number of surviving larvae and the percentage mortality was calculated.[22]

$$\text{Mortality}\,(\%)=\frac{\text{Observed mortality}\,(\%)-\text{Control mortality}\,(\%)}{100-\text{Control mortality}\,(\%)}\times 100$$

Statistical analysis

GraphPad Prism (version 5.01) software is used to calculate LC_{50} and LC_{90} which was subjected to a dose-response bioassay to determine LC of the plant extracts at different concentrations ranging 15.625 ppm to 250 ppm on *A. aegypti* mosquito larvae subsequently 24 h and 48 h of activity. Regression equation, R^2 and P value were determined using GraphPad Prism (version 5.01) software.[23]

RESULTS

In silico studies

A binding site analysis was done using CASTp online server by which selected ligand molecules were binding with the cavity of SCP-2 of *A. aegypti*. Thus, the active site was analyzed on the modeled target and binding site of amino acids structure is alpha-helix structure. The active site residues as follows V8, I12, R15, L16, I19, D20, N23, R24, Q25, V26, F32, M46, L48, L64, M66, M71, I74, G75, A81, M90, I99, F100, L102, E103 [Figure 1]. The target site was showing much electrostatic interaction between ligands to target and area (SA) was 160.211. Molecular docking is one of the most widespread methods to explore the interactions between ligand and proteins. 82 plant-derived compounds of *A. paniculata* and *T. cordifolia* docked with SCP-2 and among them, best docking results of nine compounds were taken based on estimation free energy of binding (kcal/mol), estimation inhibition constant (K_i) with the active site pocket [Figure 2]. During the docking analysis, there are participating so much of inter-atomic interactions H-bond binding affinity, electrostatic energy, pi–pi interactions and van der wall forces. These binding affinities are strongly relied on the contribution from other factors such as entropy, de salvation and flexibility of receptor molecule to the ligand. The best energy binding affinity score was −0.33 kcal/mol to −9.86 kcal/mol and −2.45 kcal/mol to −10.13 kcal/mol of bioactive compounds of *A. paniculata* and *T. cordifolia* against SCP-2 protein of dengue mosquito third instar larvae [Tables 1 and 2].

Figure 1: Active site of SCP-2 (1PZ4)

Experimental studies

The yields of extracts percentage of *A. paniculata* and *T. cordifolia* ranged from 24.90% to 81.66%. It revealed that the methanolic extracts of *A. paniculata* (65.33%) and *T. cordifolia* (24.90%) showed enough amount of yield of percentage. The phytochemical analysis of methanol, ethanol, aqueous-methanol (50:50) and aqueous-ethanol (50:50) extracts of *A. paniculata* (whole plants) and *T. cordifolia* (stem and leaves) indicated the presence of bioactive compounds or phytochemicals that are existing in extracts such as tannins, alkaloids, saponins, flavonoids, steroids, phenols, glycosides [Table 3].

Furthermore, the different fractions were isolated by using flash chromatography and its identity was confirmed by TLC. The results of TLC are presented in Table 4 as given definite single spot with a range of R_f value of 0.55-0.85 have different color in two solvent systems, i.e., hexane: ethyl acetate after keeping inside the iodine chamber which identified the presence of the phytoconstituents such as phenols, flavonoids, and alkaloids from the collected 3 fractions in TLC plate depend on color under UV-visible chamber.

In this case, when checked for the larvicidal activity of methanolic, ethanolic, aqueous-methanolic (50:50), and aqueous-ethanolic (50:50) extracts of *A. paniculata* and *T. cordifolia* and active fractions from flash chromatography and TLC against *A. aegypti* mosquitoes third instar larvae. Firstly, the mortality of the dengue mosquitoes third instar larvae were calculated in the different concentrations (250, 125, 62.5, 31.25, 15.625) ppm of crude extracts and (25, 12.5, 6.25, 3.125, 1.563) ppm of bioactive fractions of plant extracts at 24 h and 48 h in the laboratory.

Therefore, the results of larvicidal activity showed that 48 h exposure of the dengue vector mosquito larvae with 250 ppm methanolic extract of *A. paniculata* and ethanolic extract of *T. cordifolia* could result in 100% mortality. Mosquito larvicidal effect of ethanolic, aqueous-methanolic (50:50), aqueous-ethanolic (50:50) extract of *A. paniculata* and methanolic, aqueous-methanolic (50:50), aqueous-ethanolic (50:50) extracts of *T. cordifolia* showed 6% to 100% mortality rate at different concentrations (ppm) [Figures 3, 4 and Tables 5, 6]. No mortality was detected among maintained control third instar larvae group for 24 h and 48 h.

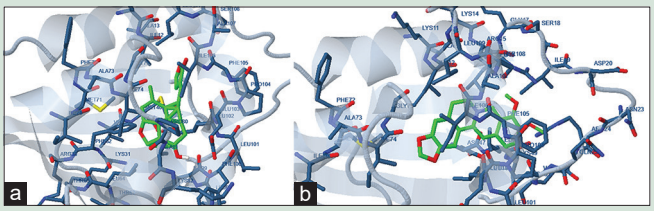

Figure 2: (a) Three-dimensional image of panicolin after molecular docking, (b) three-dimensional image of Berberine after molecular docking

Table 1: Docking of *Andrographis paniculata* compounds against sterol carrying protein-2

Compounds	Estimation free energy of binding (kcal/mol)	Estimation inhibition concentration, Ki
Paniculide-A	−8.68	433.77 nM
Panicolin	−9.86	151.50 nM
3-O-beta-D-glucosyl-14-deoxyandrographolide	−8.95	275.92 nM
6-acetylneoandrographolide	−9.13	203.19 nM
Andrographolactone	−9.37	136.28 nM
3-oxo-14-deoxyandrographolide	−8.83	334.67 nM

Table 2: Docking of *Tinospora cordifolia* compounds against sterol carrying protein-2

Compounds	Estimation free energy of binding (kcal/mol)	Estimation inhibition concentration, Ki
Beta-sitosterol	−9.18	37.39 nM
Berberine	−10.13	187.93 nM
Chasmanthin	−6.51	16.85 uM
Palmatine	−6.97	7.81 uM
Tetrahydropalmatine	−6.49	17.35 uM
Tinocordifolin	−6.35	22.26 uM

After studying individual plants larvicidal activity, we further investigated the larvicidal activity of the combination of maximum larval mortality rate of the two extracts and bioactive fraction of *A. paniculata* and *T. cordifolia,* which had not done in previous studies. The combination of methanolic extract of *A. paniculata* and ethanolic extract of *T. cordifolia* were established to be an effective larvicidal efficacy with LC_{50} = 15.75 ppm, LC_{90} = 349.08 ppm, Regression equation = Y = (0.12) x + 48.11, (R^2 = 0.60), P = 0.04 after 48 h treatment.

On the other hand, the combination of these two plants extracts also demonstrated valuable larvicidal mortality and activity in different concentrations after 24 h [Table 7], which was statistically significant ($P \leq 0.05$). After flash chromatography technique using hexane-ethyl acetate solvent, the collected bioactive fraction 1 of methanolic extract of *A. paniculata* was established to be an effective larvicidal activity with LC_{50} = 122.79 ppm, LC_{90} = 240.44 ppm, Regression equation = Y = 0.34x + 8.25, (R^2 = 0.95), P = 0.04 after 48 h treatment [Table 7] which was statistically significant ($P \leq 0.05$). The data were useful to identify and characterize the LC-ESI MS data obtained in a bioactive fraction of methanolic extract of *A. paniculata* at retention time 0.268–0.451 min had predicted molecular mass range with *m/z* values which will be the phytochemical class of flavone, phenols, phenylpropanoids, flavonoids, phenolic acid, alkaloids, isopalmitic acid, diterpenoids and quinic acid [Table 8]. After *in silico* docking study the best binding affinity scored compounds panicolin (*A. paniculata*), berberine (*T. cordifolia*) and their combination showed the larvicidal activity [Table 9].

DISCUSSION

Plant-derived larvicides are safe, availability and inexpensive to the elimination of mosquitoes either larvae or adult stage over chemical insecticides as observed by researchers in earlier studies.[10] Several medicinal plants have been investigated regarding their larvicidal activity against *A. aegypti.* In India, *A. paniculata* has shown insecticidal activity against mosquitoes, but there is no report on *T. cordifolia against* dengue vector. Phytochemical studies on *A. paniculata* and *T. cordifolia* have shown the presence of a variety of bioactive metabolites, including phenols, flavonoids, alkaloids, tannins, steroids, saponins etc., having modes of action, including contact toxicity, larvicides, repellent, insect growth regulators properties.

In silico studies have been done on mangrove-derived and other medicinal plants against SCP-2 protein of *A. aegypti*, but there is no study on *A. paniculata* and *T. cordifolia* plants against SCP-2 protein receptor of *A. aegypti*. Larson *et al.* reported that cholesterol is vital for growth, development, and egg production of mosquitoes.[24] Blitzer *et al.* stated that sterol carrier protein 2 (AeSCP-2) is responsible for transporting absorbed cholesterol from the gut.[25] As per previous *in silico* study, alpha-mangostin and panthenol had effective interactions against AeSCP-2 of *A. aegypti*.[6] Also Mangrove-derived compounds could be used as dengue mosquito larvicides against AeSCP-2.[7] The present *in silico* study shown that andrographolactone (−9.35 kcal/mol) from *A. paniculata* and beta-sitosterol (−10.13 kcal/mol) from *T. cordifolia* had best effective binding affinity energy interaction result against AeSCP-2. An important finding to emerge in this study was the bioactive compounds from *A. paniculata* and *T. cordifolia* can be used as bio-larvicides against dengue mosquito [Figure 2 and Tables 1 and 2].

In the experimental study, we aimed to determine the larvicidal activity of *A. paniculata* and *T. cordifolia* against *A. aegypti* third instar larvae. Interestingly, we found that no study has been conducted on *T. cordifolia* and combination study of two plant extracts and synthetic bioactive compounds of *A. paniculata* and *T. cordifolia* against third instar larvae of *A. aegypti*. While only limited studies has been done on *A. paniculata*. In addition, we have checked for larvicidal activity of methanolic, ethanolic, aqueous-methanolic (50:50) and aqueous-ethanolic (50:50) extracts of *A. paniculata* and *T. cordifolia*, bioactive fractions and synthetic bioactive compounds against *A. aegypti* mosquitoes third instar larvae in the different concentrations (250, 125, 62.5, 31.25, 15.625) ppm of crude extracts and (25, 12.5, 6.25, 3.125, 1.563) ppm of bioactive fractions and synthetic bioactive compounds at 24 hrs and 48 hrs in the laboratory.

In a previous study, Sukhthankar *et al*. reported that using Soxhlet hot extraction technique, the methanolic extract of leaves of *C. odorata* showed the highest mortality against late instar larvae of disease vector *A. aegypti* with LC_{50} = 138 ppm; LC_{90} = 463 ppm respectively.[26] Moreover, Oliveira *et al.* revealed that *C. mollis*, *G. grazielae*, *M. aegyptia*, *R. doniana*, *S. verticillata*, and *T. americana* showed significant activity (>75% mortality) against the fourth instar larvae of dengue in Brazil.[27] Besides, Alagarmalai *et al.* showed the larvicidal activity of leaf extracts of *Breyenia vitis-idaea* in hexane, chloroform and ethyl acetate solvents against III instar larvae of mosquito with LC_{50} value of 126.18 ppm, 111.90 ppm and 98.21 ppm respectively.[18] Furthermore, Yadav *et al.* stated that acetone extract of *V. cinerea* and methanolic solution of *Prosopis juliflora* showed larvicidal activity 0.22 g/l and 0.44 g/l after 24 h treatment against dengue and chikungunya vector.[11]

Subsequently, Gautam *et al.* reported that the flavonoid extract of the whole aerial part of *A. paniculata* was established to be inactive against *A. aegypti* larvae at the concentration of 600 ppm and 70% mortality in *An. stephensi* at 200 ppm concentration.[28] Also, Thangavel *et al.* reported that the larvicidal activity of acetone extract of *A. paniculata* against the dengue vector *A. aegypti* (I, II III, IV instar third instar larvae) in 300 ppm after 24 h treatment and alkaloid, flavonoids, steroids, tannin, and phenolic compounds were present in acetone extract of *A. paniculata*.[29]

Table 3: Preliminary phytochemical analysis of *Andrographis paniculata* and *Tinospora cordifolia*

Plants	Solvents	Alkaloids	Flavonoids	Glycosides	Saponins	Phenols	Tannins	Steroids
Andrographis paniculata	Methanol	+	+	-	+	+	+	+
	Ethanol	+	+	+	+	+	+	+
	Aqueous-methanol	+	+	+	+	+	+	+
	Aqueous-ethanol	+	+	+	+	+	+	+
Tinospora cordifolia	Methanol	+	+	-	+	+	-	+
	Ethanol	+	+	+	+	+	+	-
	Aqueous-methanol	+	+	+	+	+	-	+
	Aqueous-ethanol	+	+	+	+	+	+	-

+: Presence of compound; -: Absence of compound

Table 4: Column elution and thin layer chromatography details of methanolic extract of *Andrographis paniculata*

Number of fractions	Percentage of solvent	Volume of solvent (ml)	TLC spot	R_f value	Color
1-30	95% Hex: 5% Etoac	500	Fraction 1	0.55	Bluish
31-70	85% Hex: 15% Etoac	400			
71-100	75% Hex: 25% Etoac	400			
101-120	65% Hex: 35% Etoac	400	Fraction 2	0.68	Blue green
121-140	55% Hex: 45% Etoac	600			
141-160	45% Hex: 55% Etoac	600			
161-180	35% Hex: 65% Etoac	400			
181-200	25% Hex: 75% Etoac	300	Fraction 3	0.85	Light orange
201-220	15% Hex: 85% Etoac	300			
221-240	5% Hex: 95% Etoac	200			

TLC: Thin layer chromatography; Hex: Hexane; Etoac: Ethyl acetate

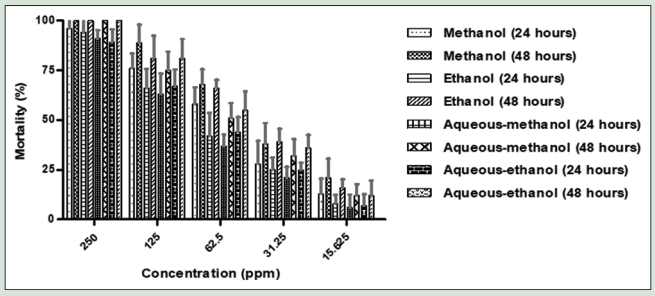

Figure 3: Larvicidal activity of different extracts of *Andrographis paniculata*

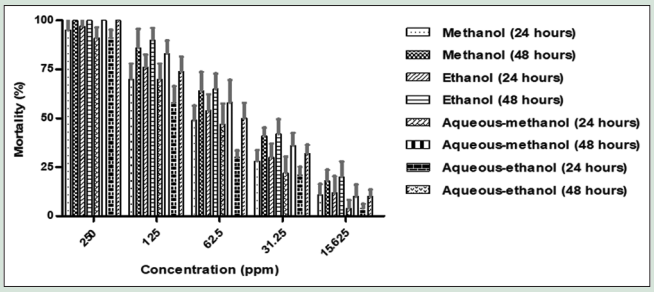

Figure 4: Larvicidal activity of different extracts of *Tinospora cordifolia*

Consequently, the methanolic, ethanolic, aqueous-methanolic (50:50), aqueous-ethanolic (50:50) extracts of *A. paniculata* showed 100% mortality at 250 ppm concentration after 48 h, but the methanolic extract presented the maximum value of LC_{50} = 101.21 ppm, LC_{90} = 386.92 ppm, Regression equation = Y = (0.14)x + 35.83, (R^2 = 0.75), P = 0.03. The least larval mortality of 12% was caused by the 48 h exposure to aqueous-ethanolic extract of *A. paniculata,* whereas methanolic extract of *A. paniculata* resulted in the highest observed mortality of 100% at 48 h treatment. Though, all the extracts from *A. paniculata,* which could result in statistically significant ($P \leq 0.05$) larval mortality from 250 ppm to 15.625 ppm were accepted for further investigations [Table 5 and Figure 3].

As a result, after 48 h at 250 ppm concentration 100% mortality was detected in methanol, ethanol, aqueous-methanol (50:50), aqueous-ethanol (50:50) extracts of *T. cordifolia* and the ethanolic extract explained the maximum value of LC_{50} = 100.06 ppm, LC_{90} = 386.37 ppm, Regression equation = Y = (0.14)x + 35.91, (R^2 = 0.75), P = 0.02. The least larval mortality of 10% was caused by the 48 h exposure to aqueous-methanolic extract of *T. cordifolia,* whereas ethanolic extract of *T. cordifolia* resulted in the highest observed mortality of 100% at 48 h treatment. Though, all the extracts from *T. cordifolia,* which could result in statistically significant ($P \leq 0.05$) larval mortality from 250 ppm to 15.625 ppm were accepted for further investigations [Table 6 and Figure 4].

Therefore, after studying individual plants larvicidal activity, we further investigated the larvicidal activity of the combination of maximum larval mortality rate of the two extracts and bioactive fraction of *A. paniculata* and *T. cordifolia* which had not done in previous studies. Though Subramaniam *et al.* reported that the combination study of *Aloe vera* + *Bacillus sphaericus* (1:2) showed the highest larvicidal activity with LC_{50} = 54.80 ppm; LC_{90} = 145.29 ppm against *A. aegypti.*[30] Present investigations clearly revealed that the combination of methanolic extract of *A. paniculata* and ethanolic extract of *T. cordifolia* were established to be an effective larvicidal efficacy with LC_{50} = 15.75 ppm, LC_{90} = 349.08 ppm, Regression equation = Y = (0.12)x + 48.11, (R^2 = 0.60), P = 0.04 after 48 h treatment, which was statistically significant ($P \leq 0.05$) [Table 7 and Figure 5].

In earlier research, Desiyamani *et al.* reported that the larvicidal activity showed in fractionated methanolic extract of *Rhizophora mucronata* and *Sesuvium portulacastrum* against dengue larvae with a minimum level of LC_{50} value 0.051 ± 0.2 ppm and 0.51 ± 0.29 ppm after 24 h treatment against 4th instar dengue larvae.[31] On the other hand, after chromatography technique using hexane-ethyl acetate solvent, the collected bioactive fraction of methanolic extract of

Table 5: Larvicidal activity of different extracts of *Andrographis paniculata* against *Aedes aegypti*

Concentration (ppm)		Mortality (%)		Regression equation		LC_{50} (ppm)		LC_{90} (ppm)		R^2		*P*	
		24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
Control	Methanol	0±0	0±0	y=0.16x+25.21	y=0.14x+35.83	154.94	101.21	404.94	386.92	0.83	0.75	0.02*	0.04*
250		96±4.18	100±0										
125		76±7.42	89±8.94										
62.5		58±8.37	68±7.58										
31.25		28±11.51	38±10.37										
15.625		13±7.58	21±9.62										
Control	Ethanol	0±0	0±0	y=0.17x+16.51	y=0.15x+32.64	197	115.73	432.29	382.4	0.92	0.78	0.006*	0.03*
250		94±6.52	100±0										
125		66±9.62	81±11.40										
62.5		42±11.50	66±4.18										
31.25		25±6.12	39±6.52										
15.625		8±4.47	16±4.18										
Control	Aqueous-methanol (50:50)	0±0	0±0	y=0.16x+17.70	y=0.18x+18.36	201.88	175.78	451.87	398	0.89	0.84	0.01*	0.02*
250		89±6.52	100±0										
125		67±8.37	81±9.62										
62.5		44±7.42	55±9.35										
31.25		25±3.54	36±6.52										
15.625		7±5.70	12±7.58										
Control	Aqueous-ethanol (50:50)	0±0	0±0	y=0.16x+12.95	y=0.16x+23.35	231.56	166.56	481.56	416.56	0.94	0.89	0.005*	0.01*
250		91±4.18	100±0										
125		63±10.4	75±9.35										
62.5		37±5.70	51±7.42										
31.25		21±5.48	32±8.38										
15.625		6±6.52	12±5.70										

Significant at $P<0.05$ level. Value represents mean±SD of five replications. Mortality of the larvae observed after 24 and 48 h. LC_{50}: Lethal concentration brings out 50% mortality; LC_{90}: Lethal Concentration brings out 90% mortality; SD: Standard deviation

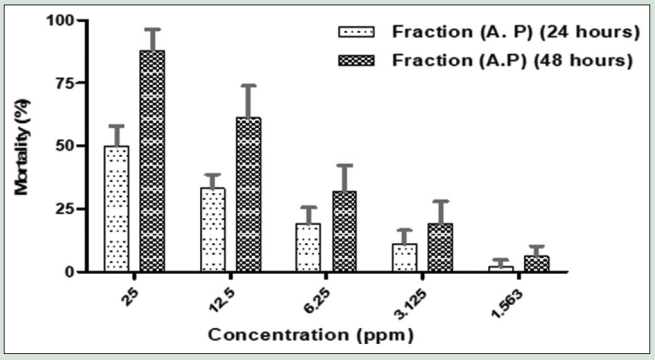

Figure 5: Larvicidal activity of bioactive fraction of methanolic extract of *Andrographis paniculata*

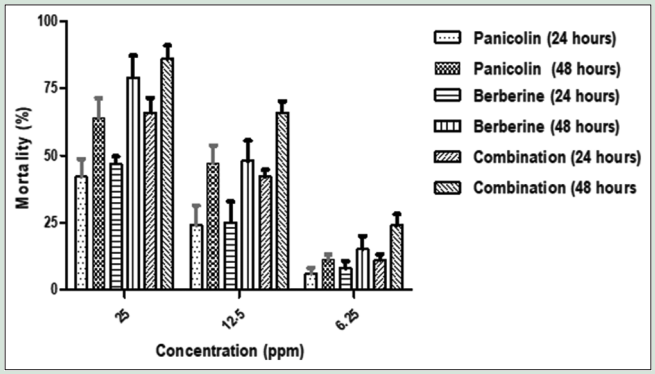

Figure 6: Larvicidal activity of docked compounds and combination study

A. paniculata was established to be an effective larvicidal activity with LC_{50} = 122.79 ppm, LC_{90} = 240.44 ppm, Regression equation = Y = 0.34x + 8.25, (R^2 = 0.95), P = 0.04 after 48 h treatment, which was statistically significant ($P \leq 0.05$) [Table 9 and Figure 6].

Recently, Nobsathian *et al.* revealed that cappariloside A and cappariloside B from *Maerua siamensis* were showing larvicidal activity against *A. aegypti* larvae with LC_{50} = 71.14 ppm and 99.79 ppm at 24 h and 48 h, respectively.[32] The present study panicolin (*A. paniculata*) and berberine (*T. cordifolia*) and their combination (50:50) showed the effective larvicidal efficacy against *A. aegypti* with LC_{50} = 25.41 ppm, 23.15 ppm and 17.56 ppm at 24 h treatment [Table 9 and Figure 6].

CONCLUSION

The study findings suggest that the crude extracts, combination extracts, and bioactive fractions of *A. paniculata* and *T. cordifolia* plant have the potential to be used as larvicides against dengue third instar larvae. However, the isolated and identified bioactive fractions need for further structure characterization and larvicidal activity against dengue and other mosquito species. In addition, the bioactive compounds panicolin (*A. paniculata*), berberine (*T. cordifolia*) and their combination can be prospective candidates against third instar larvae of *A. aegypti.* Supplementary investigation is required for enriching the larvicidal

Table 6: Larvicidal activity of different extracts of *Tinospora cordifolia* against *Aedes aegypti*

Concentration (ppm)		Mortality (%)		Regression equation		LC_{50} (ppm)		LC_{90} (ppm)		R^2		*P*	
		24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
Control	Methanol	0±0	0±0	y=0.16x+20.94	y=0.15x+33.97	181.63	106.87	431.63	373.53	0.88	0.77	0.01*	0.03*
250		95±6.12	100±0										
125		70±7.91	86±9.62										
62.5		49±7.42	64±9.62										
31.25		28±5.70	41±4.18										
15.625		11±5.48	18±5.70										
Control	Ethanol	0±0	0±0	y=0.16x+23.94	y=0.14x+35.91	162.88	100.64	412.88	386.37	0.85	0.75	0.02*	0.04*
250		97±4.47	100±0										
125		76±6.52	90±6.12										
62.5		54±8.22	65±7.91										
31.25		30±7.07	42±7.58										
15.625		12±8.37	20±7.91										
Control	Aqueous-methanol (50:50)	0±0	0±0	y=0.16x+16.13	y=0.16x+26.86	211.69	150.88	461.68	394.63	0.87	0.81	0.01*	0.03*
250		91±5.48	100±0										
125		70±7.91	83±6.71										
62.5		47±10.40	58±11.5										
31.25		22±8.37	36±6.52										
15.625		4±4.18	10±6.12										
Control	Aqueous-ethanol (50:50)	0±0	0±0	y=0.17x+9.17	y=0.16x+21.89	240.18	175.69	475.47	425.69	0.96	0.88	0.002*	0.01*
250		91±4.18	100±0										
125		58±8.37	74±7.42										
62.5		30±3.54	50±7.91										
31.25		21±4.18	32±4.47										
15.625		4±2.24	10±3.54										

Significant at $P<0.05$ level. Value represents mean±SD of five replications. Mortality of the larvae observed after 24 and 48 h. LC_{50}: Lethal concentration brings out 50% mortality; LC_{90}: Lethal Concentration brings out 90% mortality; SD: Standard deviation

Table 7: Larvicidal activity of combination of *Andrographis paniculata* (methanol) + *Tinospora cordifolia* (ethanol) and bioactive fraction of methanolic extract of *Andrographis paniculata* against *Aedes aegypti*

Concentration (ppm)		Mortality (%)		Regression equation		LC_{50} (ppm)		LC_{90} (ppm)		R^2		*P*	
		24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
Control	Combination (50:50)	0±0	0±0	y=0.15x+33.02	y=0.12x+48.11	113.20	15.75	379.87	349.08	0.75	0.60	0.04*	0.11
250		100±0	100±0										
125		94±4.18	100±0										
62.5		66±9.62	83±9.08										
31.25		34±4.20	53±4.50										
15.625		18±4.50	24±6.52										
Control	Active fraction	0±0	0±0	y=0.194x+4.20	y=0.34x+8.25	236.08	122.79	442.26	240.44	0.96	0.95	0.004*	0.004*
25		50±7.91	88±8.37										
12.5		33±5.7	61±12.9										
6.25		19±6.52	32±10.4										
3.125		11±5.48	19±8.94										
1.563		2±2.74	6±4.18										

Significant at $P<0.05$ level. Value represents mean±SD of five replications. Mortality of the larvae observed after 24 and 48 h. LC_{50}: Lethal concentration brings out 50% mortality; LC_{90}: Lethal Concentration brings out 90% mortality; SD: Standard deviation

Table 8: Liquid chromatography-mass spectrometry analysis of molecular mass of 12th bioactive fraction of methanolic extract of *Andrographis paniculata*

Range of $(M - H)^{-1}$ *m/z*	$(M - H)^{-1}$ *m/z* of peaks	Possible compound classes
100-150	113.0, 130.9, 147.1	Flavone, phenolic acid, phenylpropanoids, pyridinecarboxylic acids, phenols, ditepenoids
150-200	170.8	Phenols, ditepenoids
200-250	209.7	Phenols, flavone, phenolic glycosides, phenylpropanoids
250-300	255.0	Phenylpropanoids, flavonoids, phenolic acid, alkaloids, isopalmitic acid
350-400	377.0	Quinic acids, flavonoids, diterpenoids

Extracted ion chromatogram of peaks with retention time 0.268-0.451 min

Table 9: Larvicidal activity of of panicolin and berberine and their combination

Concentration (ppm)		Mortality (%)		Regression equation		LC_{50} (ppm)		LC_{90} (ppm)		R^2		*P*	
		24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
Control	Panicolin	0±0	0±0	y=1.85x+3	y=2.62x+2.5	25.41	18.26	47.03	33.39	0.96	0.85	0.12	0.25
25		42±6.70	64±7.41										
12.5		24±7.42	47±6.71										
6.25		6±2.23	11±2.24										
Control	Berberine	0±0	0±0	y=2.03x+3	Y=3.28x+0.8	23.15	15	42.85	27.19	0.98	0.95	0.04*	0.13
25		47±2.74	79±8.22										
12.5		25±7.91	48±7.58										
6.25		8±2.73	15±5										
Control	Combination (50:50)	0±0	0±0	Y=2.79x+1	Y=3.12x+9.5	17.56	12.98	31.89	25.80	0.93	0.95	0.16	0.24
25		66±5.47	86±5										
12.5		42±2.73	66±4.18										
6.25		11±2.23	24±4.19										

Significant at $P<0.05$ level. Value represents mean±SD of five replications. Mortality of the larvae observed after 24 and 48 h. LC_{50}: Lethal concentration brings out 50% mortality; LC_{90}: Lethal Concentration brings out 90% mortality; SD: Standard deviation

activity of the bioactive compounds present in *A. paniculata, T. cordifolia* and combination study to explore the hopeful path for mosquito control and safeguard the vigorous state of individuals.

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Conflicts of interest

There are no conflicts of interest.

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