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Research Article

A Novel Determination of Trehalase Accumulation with Plant Extracts Against Aedes albopictus from Thiruvarur District of Tamil Nadu

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I N F O

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A B S T R A C T

Aedes albopictus is an important vector in the transmission of dengue and chikungunya. For growth, flight, eclosure, and stress recovery, mosquitoes rely on their stored sugar trehalose. These reserves in mosquitoes are assessed by a key enzyme known as trehalase. A single molecule of trehalose is broken down into two molecules of glucose which is vital for the flight and survival of mosquitoes. The main objective of the study is to find out the correlation between plant extracts and their action on stored trehalose content in mosquitoes. Treatment of lab-grown 3rd and 4th instar larvae with various concentrations (100 ppm, 250 ppm, 500 ppm, and 1000 ppm) of different plants (*Prosopis juliflora, Calotropis porcera, Vitex negundo, Syzygium jambolanum and Azadirachta indica*) crude extract was obtained using both methanol and ethanol as solvent. The larvicidal bioassay was performed and the larval mortality was observed at different time periods. Lethal Concentration (LC) values were predicted using the log-probit analysis. Emerged adults from the larvicidal bioassay were homogenised for the analysis of their trehalose concentration using the modified anthrone-sulfuric acid method. The methanolic extract of Prosopis juliflora had excellent larvicidal efficacy after 72 hrs as well as a massive 23-fold increase of trehalose over control was observed with a 1000 ppm treatment of the extract. Also, a significant increase in trehalose content was noticed in the methanolic extract of V. negundo at 1000 ppm. Further, exploration by GC-MS revealed the active components present in these extracts. One or many of the compounds of the extracts are responsible for the excessive build-up of trehalose in these mosquitoes. Additional docking studies would point out the main chemical compound involved in the desired trehalose accumulation. Pure isolation and optimisation of this chemical would be a revelation in the field of vector control and management.

Keywords: *Aedes albopictus*, Trehalose, Larvicidal bioassay, Anthrone-Sulfuric acid method, GC- MS

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mosquitoes occurred first during the 19th and early 20th centuries when massive human settlementshappened around the islands of the Indian and Pacific Oceans.[1] The second common spreading ofthese mosquitoes started in the late 1970s, mostly favoured by the trade of used tyres across continents. This is also considered as a human-aided mosquito dispersal.[2] In its natural habitat, *Aedes albopictus* uses small shaded bamboo stumps and plant axils as their sites for larval development.[3] The two traits of these mosquitoes include adapting to anthropogenic habitats andhaving hard, long-lived eggs that facilitate their voyage around the world.[4] Although ranked second to *Aedes aegypti* in transmitting dengue, *Aedes albopictus* has now emerged as an important denguevector with the potential to transmit all four serotypes of dengue virus (DENV).[5]

All infected individuals with dengue do not develop severe complications. About 25% of people withDENV infection remain asymptomatic and will only encounter a febrile illness along with minor haematological and biochemical anomalies.[6] According to the World Health Organization (WHO) 1992classification, dengue disease has been classified into dengue fever and haemorrhagic fever.[7] Following this, the new WHO 2007 classification states that the symptomatic individuals as dengue with no major complications and individuals with complications such as plasma leakage, respiratorydistress syndrome, and severe blood loss or dreadful organ damage as severe dengue.[6]

The major insect haemolymph sugar, trehalose, is a non-reducing sugar that is synthesised in the fatbody of insects. Trehalose has been identified as a crucial supplier of Adenine Tri Phosphate (ATP)energy for flight muscles and plays an essential role in the development and storage of glycogen in embryos. The production of trehalose can play a vital role in adapting to nutritional changes as it is essential for survival during fasting and aids body growth when proteins are scarce, highlighting its significance.[8] In order to use stored trehalose, there is a glycosidase enzyme called trehalase (TRE) found in insect tissues. There are two forms of trehalase: soluble trehalase (Tre-1) and membrane-bound trehalase (Tre-2) and both of these forms consist of a signal peptide, two signature motifs, and one glycine-rich region.[9] The hydrolysis of trehalose by trehalase is crucial for several major physiological functions such as chitin synthesis during moulting and larvae thermotolerance.[10]

In recent years, trehalase inhibitors have gained attention as a potential target for the identification of novel insecticides and fungicides due to the biological relevance of trehalose and its processing enzymes in both pathological and physiological states. However, finding environmentally friendly pesticides has been in the limelight for years. *Prosopis juliflora* is a shrub native to Mexico, South America, and the Caribbean.[11] The presence of a variety of metabolites strengthens the scope of*Prosopis* to be an efficient biopesticide.[12] *Calotropis porcera* is an evergreen xerophytic plant naturally found in arid and semi-arid parts of the world. Extracts of *C. porcera* have been used in the past by humans to treat various ailments.[13] Southern parts of both Asia and Africa are home to *Azadirachta indica*, where it is extensively used in folklore medicine. *Vitex negundo* belongs to the Verbanaceae family and is also known as the "five-leaf chase tree" and "nirgundi" in India. There areover 270 species of *Vitex* that have been identified, including shrubs, trees, and plants that grow in temperate, tropical, and subtropical climates. Folk medicines made from the *Vitex* are used in various countries, including India.[14] *Syzygium jambolanum* is also known as "jambolao" in Brazil, "Naval pazham" in India, and "jambolana", "sweet olive", or "java plum" in English-speaking nations. Various studies have shown that derivatives of *Vitex negundo* and *Syzygium jambolanum* present antimicrobial and larvicidal potential.[15,16] This study aims to prove the correlation between theseplants and mosquitoes. Also, the main objective is to observe the effect of these plants on the storedsugar, trehalose.

Materials and Methods

Sample Collection and Maintenance

The mosquito immature was collected by convenient sampling from the sites of the Thiruvarur and Nanilam blocks of the Thiruvarur district. The collected immature was transferred to the labelled containers and transported to the Vector Biology Research Laboratory (VBRL), Department of Biotechnology. The 3:1 ratio of dog biscuit and yeast was provided as feed to the immature and maintained in VBRLat a temperature of 28 ± 2 °C, with a relative moisture content of 80% with a 12 h light-dark cycle. The adult *Aedes* mosquitoes were identified using standard taxonomical keys and they were fed witha 10% glucose pad and soaked resin. The female adult mosquitoes were fed with commercial chicken blood using a membrane feeder. After 3-4 days the *Aedes* eggs were collected by ovitraps. Collectedeggs were stored for further use.

Study Period

July 2022 to September 2022 (Pre-monsoon period).

Chemicals

Trehalose and Anthrone were acquired from SRL (Sisco Research Laboratories Pvt. Ltd, India) chemical

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Plant Collection

Leaves of *Prosopis juliflora* (Common names: Mesquite, Velikaruvai), *Vitex negundo* (Common names: Chastetree, Nocchi), *Calotropis porcera* (Common names: Giant milkweed, Vellerukku), *Syzygium jambolanum* (Common names: Java plum, Jambolan, Naval) and *Azadirachta indica* (Common names: Neem, Vembu) were collected from the campus of Central University of Tamil Nadu. Plants were identified with the aid of Flora: A Compendium of Plant Biodiversity of Central University of Tamil Nadu[17] then the leaves were washed in fresh water and dried in the shade forabout a period of 7–14 days at 25–28 °C.

Preparation of Plant Extract

The dried leaves were ground mechanically and the obtained powder was stored in clean storagevials. Plant crude extracts were obtained by the maceration method, with a ratio of solute to solvent as 1:16.[18] 5 g of grounded leaf powder was taken in a 250 ml conical flask and then 80 ml of methanol was added to it and mixed well. The conical flask was cotton-plugged and kept in the shakerfor 24 hours at 100 rpm. In order to get the filtrate, the extract was filtered through a funnel with Whatman No 1 filter paper. Similarly, ethanol was also used as a solvent to prepare ethanolic filtrates. Further,the obtained filtrates were placed in a water bath with a temperature of 47 °C for the evaporation of the solvent. The crude methanolic and ethanolic plant extracts were stored at 4 °C for further use.

Preparation of Stock Solution

A stock solution of 1000 ppm was prepared for all the obtained crude extracts by dissolving 200 mgof crude extract in 2 ml of Dimethyl Sulfoxide (DMSO) volume raised up to 200 ml using distilled water.

Larvicidal Bioassay

The larvicidal bioassay was conducted by standard WHO protocol.[19] All the bioassays were performed under standard temperature, humidity, and light conditions.

About 30 (third or fourth instar) larvae of *Aedes albopictus* were exposed to 100 ppm, 250 ppm, 500 ppm, and 1000 ppm of both methanolic and ethanolic plant extracts. Different concentrations of plant extracts were obtained through serial dilution from the stock solution. The control (1% DMSO with distilled water) had the same 30 larvae of *Aedes albopictus*. Mortality was observed at 24, 48, and 72 hrs time - intervals. All the dead larvae were removed immediately to avoid decomposition. Eachrespective treatment was carried out in three trials.

Abbot's Formula:

$$\text{Percentage of Mortality} = \frac{\%\ \text{test mortality} - \%\ \text{control mortality}}{100 - \%\ \text{control mortality}} \text{ X } 100$$

Trehalose Analysis

After being treated with various plant extracts the alive larvae from the larvicidal bioassays were allowed to develop into adult mosquitoes. Later the adult mosquitoes were anesthetised by refrigeration and stored. Totally 20 mosquitoes of respective concentrations were homogenised with1000 µl of 0.1 M Phosphate buffered saline (PBS) and centrifuged at 1500 rpm for 20 min at 4 °C. The supernatant was taken as the assay solution and water as the blank.

A detailed protocol for the assay is as follows: 100 µl of the assay solution is subjected to a treatment with 150 µl of 0.2 N H_2SO_4 and boiled at 100 °C in a water bath for the degradation of any sucrose orglucose-1-phosphate, and then chilled in ice. Next, 150 µl of 0.6 N NaOH was added and again subjected to heating at 100 °C to get rid of reducing sugars and chilled again. 2 ml of anthronereagent (0.05 g of anthrone per 100 ml of 95% H_2SO_4) was added, and boiled for 10 min, then chilledwith ice.[20] Absorbance was measured at 630 nm using a spectrophotometer (SpectraMax® i3x) at the Central Instrumentation Facility (CIF), Department of Biotechnology, Central University of Tamil Nadu. Trehalose concentration was calculated using a standard trehalose curve and expressed as µM/mg.

GC- MS Analysis

GC-MS analysis was done in a combined 8890 Gas chromatograph system (Agilent: CH- GCMSMS02; 8890 GC System; 7000 GC/TQ) and mass spectrophotometer, equipped with a silica column (5% phenyl methyl siloxane 30.0 m x 250 µm, film thickness 0.25 µm) at Textile Chemistry Division, The South India Textile Research Association (SITRA), Coimbatore. The carrier gas used in the system was Helium gas and the Collison gas was Nitrogen. The column velocity flow was adjusted to 1.0 ml/min. Methanol was utilised as the diluent in the system.

The column temperature started at 50°C and was held for a minute. Then, the temperature was raised to 120°C at a rate of 5°C/min and held for 1 minute. Next, at a rate of 10°C/min, 210°C was held for 1 minute. Finally, the temperature was raised to 280°C at a rate of 10°C/min and was held for 5 minutes. The total elution time was 38 minutes. The relative amount of each component was calculatedby average peak area to that of the total area (Scan range: 30-900m/z). Mass Hunter software was used to obtain the necessary data.

Identification of Compounds

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Unknown compound identification was done with the retention indices and with the interpretation of mass spectrum from the National Institute of Standards and Technology (NIST). This database has about 62,000 patterns of known compounds. The fraction spectra of unknown extracts obtained were compared to that of the standard mass spectra of known compounds stored in the NIST library.

Statistical Analysis

The experiments were set up to a completely randomised design with three replicates. Larval mortality was calculated using Abbot's formula.[21] Lethal concentration (LC) values were calculated using the Log-probit analysis in MS Excel.[22] All the data were processed statistically using the software Jamovi (version: 2.3.18) and the comparison of the mean for each treatment was accomplished by analysis of variance (Two-way ANOVA). Figures were made using MS Excel, error bars represent the standard errors and each bar depicts the mean ± SE of three independent experiments. Asterisks (*)on the bars represent the significant difference ($p < 0.05$).

Results

Larvicidal Activity

Figures 1-5 represent the larval mortality rate against *P. juliflora*, *V. negundo*, *C. porcera*, *S. jambolanum,* and *A. indica* respectively. Similarly, Tables 1-5 provide the mortality percentage of *Aedes albopictus* larvae against both the extracts (methanol and ethanol) of *P. juliflora*, *V. negundo*,

C. porcera, *S. jambolanum,* and *A. indica.* From, Figure 1, it is clear that the methanolic extract of *P.juliflora* has higher larvicidal efficacy than the ethanolic extract. After 72 hrs, 123.45 ppm and 423.60 ppm were the LC50 and LC90 values of *P. juliflora* (methanol) obtained from Log-probit analysis (Table 6). The methanolic extract of *S. jambolanum* had LC50 and LC90 values of 1019.94 and 2004.74 ppm(Table 7). LC50 and LC90 values of *C. porcera* at different time periods (24, 48 and 72 hrs) have beenmentioned in Table 8. 3631.39 and 46735.07 ppm were the LC50 and LC90 values of methanolic extract of *V. negundo* (72 hours) observed from Table 9. Likewise, the LC50 and LC90 values of *A. indica* (ethanolic extract) after a 72 hrs period of bioassay were 1227.61 ppm and 2551.92 ppm, which is lower than that of the methanolic extract *i.e.,* 1387.15 ppm and 7156.51 ppm (Table 10).

Trehalose Content

To explore the effect of plant extracts (*P. juliflora*, *V. negundo*, *C. porcera*, *S. jambolanum* and *A. indica*) of methanol and ethanol on stored trehalose content, all larvae surviving the larvicidal bioassay were allowed to transform into adults. Figure 6 represents the fold increase of trehalose content with respect to control. The activity of the methanolic crude extract of *P. juliflora* in increasing the trehalose content is very significant. In fact, at 1000 ppm concentration of the treatment, a tremendous 23-fold increase of trehalose content was observed over control (Figure 6), and a similar five, six, and nine-fold increase over control was recorded at 100, 250, and 500 ppm concentrations (Figure 6). The trehalose content in adult mosquitoes treated with methanolic extract of *V. negundo* was observed (about 9 folds) at 1000 ppm (Figure 6). No significant trehalose increase was identified in other plant extracts.

GC-MS analysis

An affirmative response on trehalose content increase in *Ae. albopictus* by *P. juliflora* and *V.negundo* (crude methanol extract) is clear from Figure 6. Hence, these extracts were subjected toGC-MS analysis. Table 11 and 12 exhibits all the chemical constituents of these extracts. 9,12,15- Octadecatrienoic acid, methyl ester,(Z,Z,Z)- with a molecular weight of 292.5 g/mol has a higher component area as well as match factor. Following this, Phytol (Molecular weight (MW)-296.5 g/mol), Methyl stearate (MW- 298.5 g/mol), n-hexadecanoic acid (MW-256.42 g/mol), Tributylamine (MW- 185.35) are the other compounds in the decreasing order of match factor found in methanolic extractof *P. juliflora*. Similarly, components of *V. negundo* includes 1H-Cycloprop[e]azulen-4-ol,decahydr o- 1,1,4,7-tetramethyl-,n-Hexadecanoic acid, Phytol, Benzofuran, 2-methyl-, Caryophyllene and so on (Table 12). Figure 7 provides the chromatogram with peaks of different compounds of methanolic extract of *P. juliflora*. Figure 8 depicts the chromatogram with different peaks representing the various compounds present in the crude extract of *V. negundo*. One or many compounds of this extract are strongly responsible for the effect of accumulation of trehalose in *Ae. albopictus*.

A dose-dependent increase in the mortality rate was observed, with the methanol extract of *P. juliflora* having a higher ppm (parts per million).

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Table 1.Mortality Rate of *Aedes albopictus* Larvae Against Different Concentrations of Methanol and Ethanolic Extracts of *P. juliflora* (72 Hours)

S. No.	Extracts	Total No. of Larvae	Doses (in ppm)	Mortality Rate (%)
1.	Methanol	90	100	57.76
		90	250	81.10
		90	500	93.33
		90	1000	95.57
2.	Ethanol	90	100	30.00
		90	250	31.00
		90	500	56.67
		90	1000	65.57

ppm: parts per million

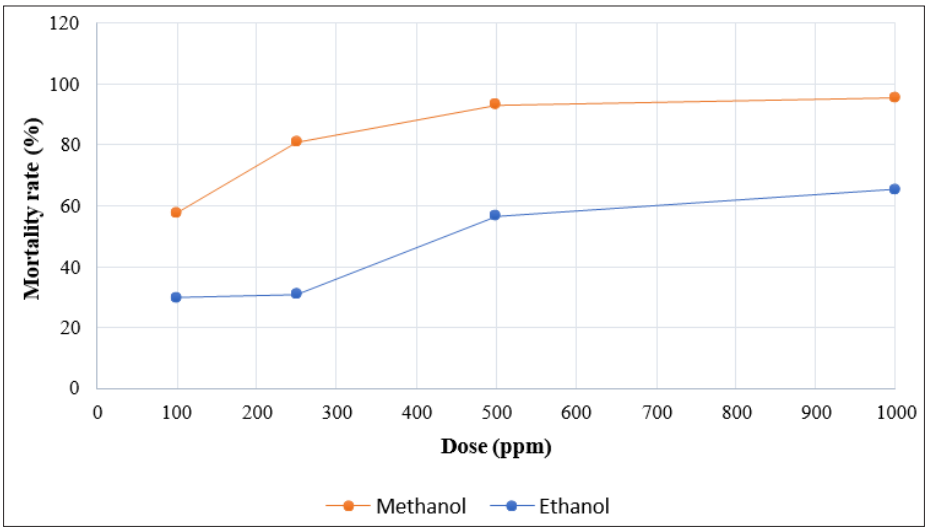

Figure 1.Dose-response Relationship of *Aedes albopictus* Against *P. juliflora* (Methanol andEthanol Extracts)

Table 2.Mortality Rate of *Ae. albopictus* Larvae Against Methanolic and Ethanolic Extract of *V.negundo* (72 Hours)

S. No.	Extract	Total No. of Larvae	Doses (in ppm)	Mortality Rate (%)
1.	Methanol	90	100	3.33
		90	250	6.67
		90	500	10.00
		90	1000	32.23
2.	Ethanol	90	100	4.43
		90	250	10.00
		90	500	10.00
		90	1000	32.23

ppm: parts per million

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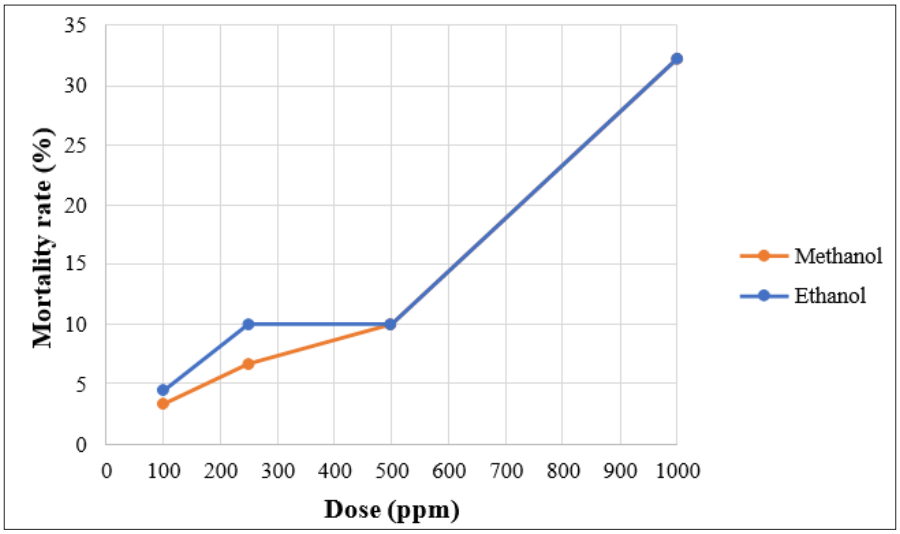

Figure 2.Mortality rate – Dose Graph of *Ae. albopictus* larvae Against Methanolic and Ethanolic Extracts of *V. negundo*

Table 3.Mortality Rate of Aedes albopictus Larvae Against Different Concentrations of Methanol and Ethanolic Extracts of C. porcera (72 Hours)

S. No.	Extract	Total No. of Larvae	Doses (in ppm)	Mortality Rate (%)
1.	Methanol	90	100	0.00
		90	250	4.33
		90	500	12.23
		90	1000	30.00
2.	Ethanol	90	100	0.00
		90	250	0.00
		90	500	7.77
		90	1000	15.56

ppm: parts per million

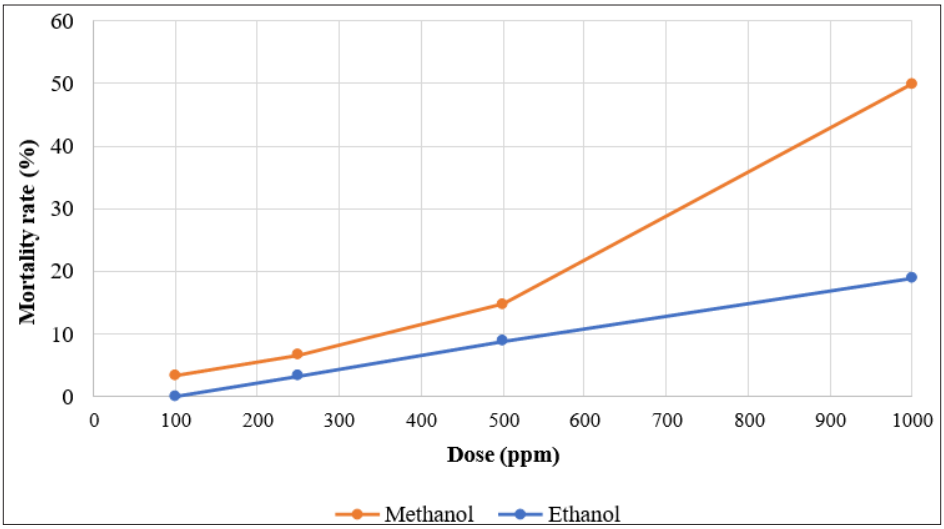

Figure 3.Dose-response Relationship of *Aedes albopictus* Larvae Against Methanol andEthanol Extracts of *C. porcera*

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Table 4.Mortality Rate of *Ae. albopictus* Larvae Against Methanolic and Ethanolic Extracts of *S. jambolanum* (72 Hours)

S. No.	Extract	Total No. of Larvae	Doses (in ppm)	Mortality Rate (%)
1.	Methanol	90	100	0.00
		90	250	4.33
		90	500	12.23
		90	1000	30.00
2.	Ethanol	90	100	0.00
		90	250	0.00
		90	500	7.77
		90	1000	15.56

ppm: parts per million

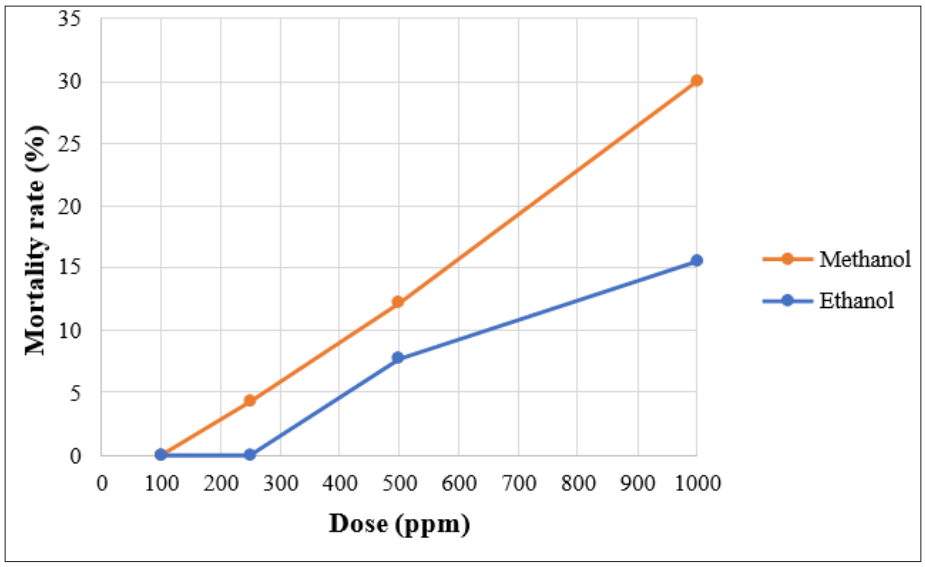

Figure 4.Mortality Rate – Dose Graph of *Ae. albopictus* Larvae Against Methanolic and Ethanolic Extracts of *S. jambolanum*

The larvicidal efficacy of the methanolic extract observed was higher than the ethanolic extract.

The mortality rate gradually increased with respect to the dose concentration in both the methanolicand ethanolic extracts. A minute difference between mortalities against methanolic and ethanolic extracts is noted only at 100

The mortality rate against ethanolic extract of *V. negundo* is slightly greater than the methanolic extract at 100 ppm and 250 ppm. Further above 500 ppm, the mortality rate observed was the samefor both extracts.

A gradual increase in mortality rate was observed in both extracts. A steep increase in the mortalityrate of methanolic extract was observed from 500 ppm to 1000 ppm.

Although a gradual increase in the mortality rate of both extracts was observed. The methanolic extract has a higher larvicidal activity compared to that of ethanolic extract.

No mortality was noted for 100 ppm in larvae treated with methanolic extract and for 100 ppm and250 ppm in larvae treated with ethanolic extract.

In this graph, the mortality rate was gradually increased against the methanolic extract of *S. jambolanum* and against ethanolic extract the mortality rate was observed from 250 ppm.

The mortality rate of methanolic extract observed at the beginning was slightly higher. At 1000 ppmboth extracts exhibit the same mortality rate.

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Table 5.Mortality Rate of *Aedes albopictus* Larvae Against Different Concentrations of Methanol and Ethanolic Extracts of *A. indica* (72 Hours)

S. No.	Extract	Total No. of Larvae	Doses (in ppm)	Mortality Rate (%)
1.	Methanol	90	100	1.10
		90	250	3.33
		90	500	11.10
		90	1000	20.00
2.	Ethanol	90	100	0.00
		90	250	1.10
		90	500	4.43
		90	1000	20.00

ppm: parts per million

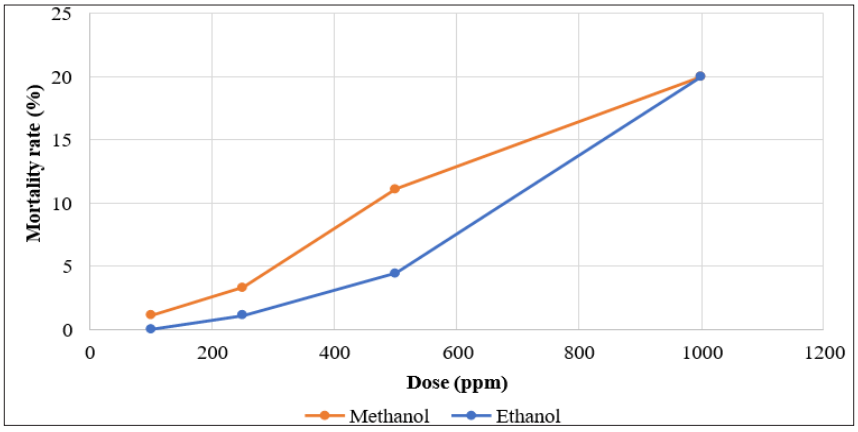

Figure 5.Dose-response Relationship of Aedes albopictus Larvae Against Methanol and Ethanol Extracts of A. indica

Table 6.Log-probit and Regression Analysis of the Larvicidal Activity of *P. juliflora* Against *Ae. albopictus* Larvae

S. No.	Extract	Period of Bioassay (Hour)	LC50 Value (in ppm)	LC90 Value (in ppm)	Regression Equations	R^2 Value
1.	Methanol	24	219.8567374	874.4350604	Y = 2.1348X	0.998
		48	171.2925145	639.1097029	Y = 2.2384X	0.996
		72	123.4596781	423.5996338	Y = 2.3906X	0.997
2.	Ethanol	24	19706.84418	2938738.74	Y = 0.5889X + 2.4709	0.901
		48	934.2410402	12469.02801	Y = 1.1374X + 1.6214	0.941
		72	425.4129958	7507.987173	Y = 1.0267X + 2.301	0.855

LC: Lethal concentration; R^2: Coefficient of regression; ppm: parts per million

The coefficient of determination (R^2) is key to analyse how well the outcome of a statistical model ispredicted. The highest possible R^2 is 1, thus values closer to one are accurate predictions.

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Table 7.Log-probit Analysis and Regression Analysis of Larvicidal Activity of *S. jambolanum* Against *Ae. albopictus* Larvae

S. No.	Extracts	Period of Bioassay (Hour)	LC50 Value (ppm)	LC90 Value (ppm)	Regression Equations	R^2 Value
1.	Methanol	24	3322.36	7598.97	Y = 3.5624X -7.5448	0.819
		48	1209.32	2428.65	Y = 4.2269X -8.0296	0.928
		72	1019.94	2004.74	Y = 1.0267X + 2.301	0.877
2.	Ethanol	24	24666.66	87892.83	Y = 2.3195X -5.1875	0.551
		48	2440.86	5158.79	Y = 3.9384X -8.3415	0.819
		72	1562.88	2954.18	Y = 4.6291X -9.785	0.807

LC: Lethal concentration; R^2: Regression coefficient; ppm: parts per million

The coefficient of determination (R2) is key to analyse how well the outcome of a statistical model is predicted. The highest possible R2 is 1, thus values closer to one are accurate predictions.

Table 8.Log-probit and Regression Analysis of the Larvicidal Activity of *C. porcera* Against *Ae. albopictus* Larvae

S. No.	Extract	Periodof Bioassay (Hour)	LC50 Value (in ppm)	LC90 Value(in ppm)	Regression Equations	R^2 Value
1.	Methanol	24	14864.46881	91968.87218	Y = 1.6172X - 1.7472	0.182
		48	2521.727522	14691.47145	Y = 1.6724X - 0.689	0.802
		72	1387.15656	7156.510792	Y = 1.7963X - 0.6442	0.900
2.	Ethanol	24	3322.356379	7598.966807	Y = 3.5624x -7.5448	0.819
		48	2828.018287	7466.027609	Y = 3.036x -5.4787	0.780
		72	1227.610184	2551.923278	Y = 4.0276x -7.4415	0.854

LC: Lethal concentration; R2: Coefficient of regression; ppm: parts per million

The coefficient of determination (R2) is key to analyse how well the outcome of a statistical model is predicted. The highest possible R2 is 1, thus values closer to one are accurate predictions.

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Table 9.Log-probit and Regression Analysis of the Larvicidal Activity of *V. negundo* Against *Ae. albopictus* Larvae

S. No.	Extract	Period of Bioassay (Hour)	LC50 Value (ppm)	LC90 Value (ppm)	Regression Equations	R^2 Value
1.	Methanol	24	20948.25	267523.23	y = 1.1571x	0.993
		48	4214.68	35702.95	y = 1.3794x	0.998
		72	2499.73	18524.82	y = 1.4715x	0.998
2.	Ethanol	24	3322.36	7598.97	y = 0.5889x +2.4709	0.82
		48	17363.12	321425.19	y = 1.1374x +1.6214	0.668
		72	3631.39	46735.07	y = 1.0267x +2.301	0.859

LC: Lethal concentration; R^2: Regression coefficient; ppm: parts per million

The coefficient of determination (R2) is key to analyse how well the outcome of a statistical model is predicted. The highest possible R2 is 1, thus values closer to one are accurate predictions.

Table 10.Log-probit and Regression Analysis of the Larvicidal Activity of *A. indica* Against *Ae. albopictus* Larvae

S. No.	Extract	Period of Bioassay (Hour)	LC50 Value (in ppm)	LC90 Value (in ppm)	Regression Equations	R^2 Value
1.	Methanol	24	Nil	Nil	Nil	Nil
		48	2229.425271	4612.850814	Y = 4.0535X - 8.5719	0.810
		72	3488.855054	23604.19002	Y = 1.5416X - 0.4614	0.984
2.	Ethanol	24	Nil	Nil	Nil	Nil
		48	1652.926111	3621.944048	Y = 3.7571x -7.0913	0.905
		72	1376.497123	2857.432708	Y = 4.0353x -7.6659	0.929

LC: Lethal concentration; R^2: Coefficient of regression; ppm: parts per million

The coefficient of determination (R^2) is key to analyse how well the outcome of a statistical model ispredicted. The highest possible R^2 is 1, thus values closer to one are accurate predictions.

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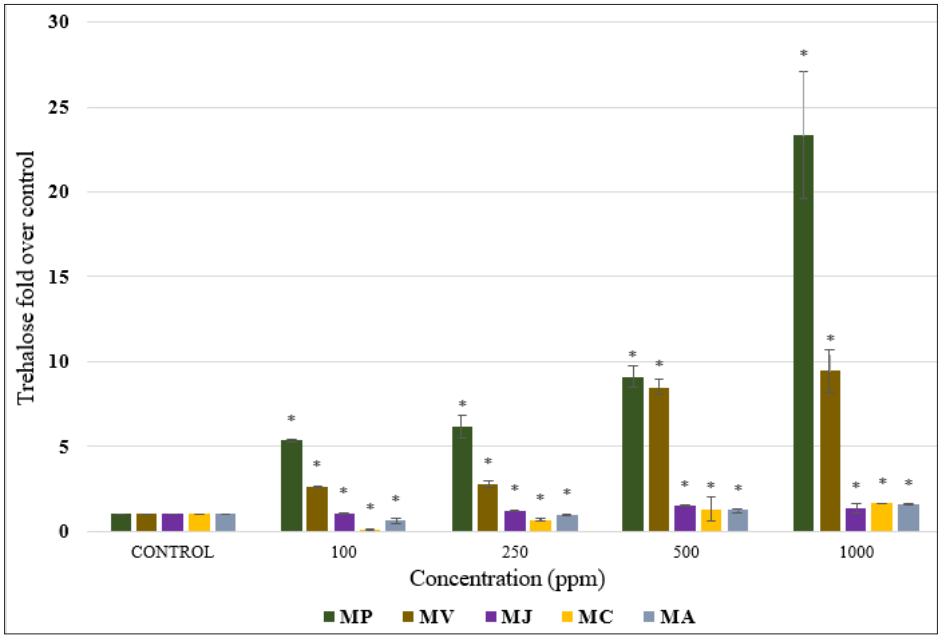

Figure 6.Trehalose Fold Increase Over Control Group

MP: Methanolic extract of *P. juliflora*; MV: Methanolic extract of *Vitex negundo*; MJ: Methanolic extract of *Syzygium jambolanum*; MC: Methanolic extract of *C. porcera*; MA: Methanolic extract of *A. indica*; ppm: parts per million

Table 11.GC-MS Analysis of Chemical Constituents in *P. juliflora* (Methanolic Extract)

S. No.	RetentionTime	Compounds	MolecularFormula	Molecular Weight (ing/mol)	MatchFactor
1.	3.7417	5-Methyl-4-hexene-1-yl acetate	C9H16O2	156.22	83.0
2.	12.7636	Tributylamine	C12H27N	185.35	94.4
3.	13.8765	3-Pyridinecarbonitrile, 1,4-dihydro-1-methyl-	C7H8N2	120.15	86.2
4.	16.3933	Phenol, 5-ethenyl-2-methoxy-	C9H10O2	150.17	91.9
5.	21.5903	Phenol, 4-ethenyl-2,6-dimethoxy-	C10H12O3	180.2	90.9
6.	24.4322	6-Hydroxy-4,4,7a- trimethyl-5,6,7,7a- tetrahydrobenzofuran-2(4H)-one	C11H16O3	196.24	80.7
7.	25.1221	Myo-Inositol, 4-C-methyl-	C7H14O6	194.18	81.4
8.	25.5530	Phthalic acid, 6-ethyl-3-octyl butyl ester	C22H34O4	362.5	81.2
9.	26.2204	Hexadecanoic acid, methyl ester	C17H34O2	270.5	94.3
10.	26.6778	n-Hexadecanoic acid	C16H32O2	256.42	94.6
11.	28.3150	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	C19H34O2	294.5	90.2

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12.	28.3958	9,12,15-Octadecatrienoic acid, methyl ester,(Z,Z,Z)-	C19H32O2	292.5	96.7
13.	28.5220	Phytol	C20H40O	296.5	94.9
14.	28.6529	Methyl stearate	C19H38O2	298.5	94.7
15.	28.8255	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	C18H30O2	278.4	91.1
16.	29.0385	Octadecanoic acid	C18H36O2	284.5	84.8
17.	32.5843	dl-.alpha.-Tocopherol	C29H50O2	430.7	86.7
18.	32.7868	d-Proline, N- methoxycarbonyl-, heptyl ester	C14H25NO4	271.35	80.7

Table 12.List of Compounds of Methanolic Extract of *V. negundo* Analysed by GC-MS

S. No.	Component RT	Compound Name	Molecular Weight	Formula	Match Factor
1.	3.3788	Propanoic acid, 2-oxo-, methyl ester	102.09	C4H6O3	87.9
2.	5.6325	Proline, 2-methyl-5-oxo-, methyl ester	157.17	C7H11NO3	87.4
3.	5.6329	1,2-Cyclopentanedione	98.1	C5H6O2	91.5
4.	6.9982	Phenol	94.11	C6H6O	88.4
5.	7.2667	Propanoic acid, anhydride	130.14	C6H10O3	80.3
6.	9.3133	Benzofuran	118.13	C8H6O	91.7
7.	12.2431	Benzofuran, 2-methyl-	132.16	C9H8O	93.4
8.	17.7238	2-Propenoic acid, 3-phenyl-, methyl ester, (E)-	162.18	C10H10O2	83.9
9.	18.9673	Caryophyllene	204.35	C15H24	93.0
10.	22.0480	1H-Cycloprop[e]azulen-4-ol, decahydro-1,1,4,7- tetramethyl-,[1aR-(1a.alpha.,4.beta.,4a.beta.,7 .alpha.,7a.beta.,7b.alpha.)]-	222.37	C15H26O	97.2
11.	25.0101	7-(2-Hydroxypropan-2-yl)- 1,4a-dimethyldecahydronaphthal en-1-ol	240.38	C15H28O2	86.6
12.	25.1215	Neophytadiene	278.5	C20H38	90.3
13.	25.5997	1-Penten-3-one, 1-(2,6,6-trimethyl-1-cyclohexen-1-yl)-	206.32	C14H22O	80.7
14.	25.8503	Bicyclo[9.3.1]pentadeca- 3,7-dien-12-ol,4,8,12,15,15-pentamethyl-, [1R-(1R*,3E,7E,11R*,12R*)]-	290.5	C20H34O	80.4
15.	26.1856	1-Naphthalenone, 1,2,3,4,4a,7,8,8a-octahydro- 2,4a,5,8a-tetramethyl	206.32	C14H22O	83.9
16.	26.6776	n-Hexadecanoic acid	256.42	C16H32O2	95.4

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17.	26.7743	(S,E)-8,12,15,15-Tetramethyl-4- methylenebicyclo[9.3.1] pent adeca-7,11-diene	272.5	C20H32	82.6
18.	27.0647	(13R)-13-Methoxylabda-7,14-diene	304.5	C21H36O	87.4
19.	27.1291	1-Naphthalenepropanol, .alpha.-ethenyldecahydro-.alpha.,5,5,8a-tetramethyl-2-methylene-, [1S- [1.alpha.(R*),4a.beta.,8a.alp ha.]]-	290.5	C20H34O	92.2
20.	27.5546	Kolavenol acetate	332.5	C22H36O2	86.9
21.	27.5559	Kolavenol	290.5	C20H34O	84.9
22.	28.2391	1-Naphthalenepropanol, .alpha.-ethenyldecahydro-2- hydroxy-.alpha.,2,5,5,8a- pentamethyl-, [1R-[1.alpha.(R*),2.beta.,4a.beta .,8a.alpha.]]-	308.5	C20H36O2	82.5
23.	28.3962	9,12,15-Octadecatrienoic acid, methyl ester,(Z,Z,Z)-	292.5	C19H32O2	90.6
24.	28.5232	Phytol	296.5	C20H40O	94.8
25.	28.8300	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	278.4	C18H30O2	91.9
26.	30.8104	1-Phenanthrenemethanol, 1,2,3,4,4a,9,10,10a-octahydro-1,4a-dimethyl-7-(1-methylethyl)-, [1R- (1.alpha.,4a.beta.,10a.alpha.)]-	286.5	C20H30O	83.6
27.	31.3987	Vitexifolin D	322.4	C19H30O4	84.0
28.	35.5845	Squalene	410.7	C30H50	85.7

RT: Retention time

This graph represents the trehalose content increase in folds with respect to the control group. Error bars represent the standard error (SE) and each bar represents the mean ± SE of three experiments. The asterisk represents a significant difference ($p < 0.05$) from the control group with no extract treatment. Two-way ANOVA was used to find the statistical significance.

This graph represents the peaks of different chemical compounds present in the methanolic extract of *P. juliflora*.

The retention peak depicted in this graph aids in analysing the various chemical constituents of theextract.

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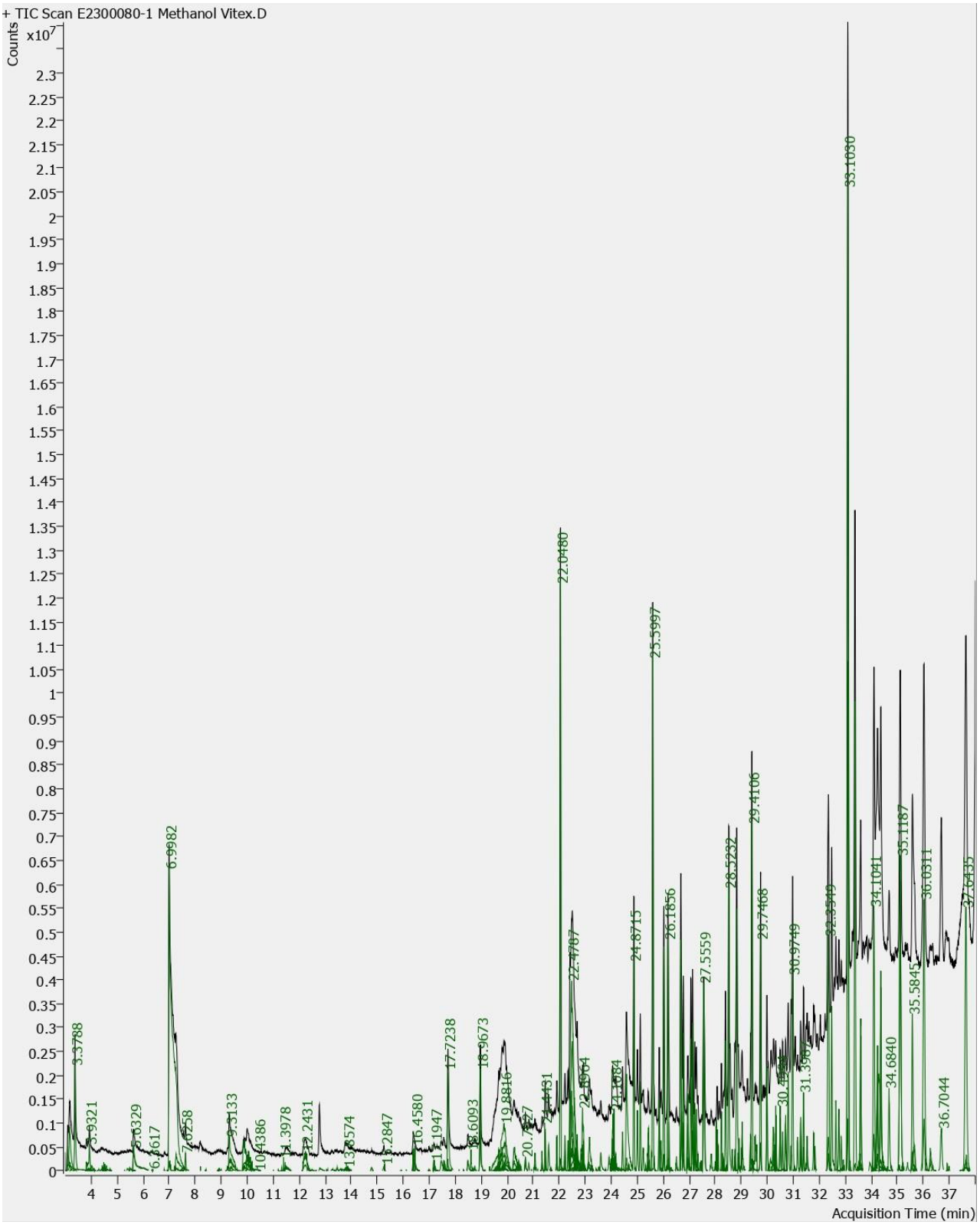

Figure 7.GC-MS Chromatogram

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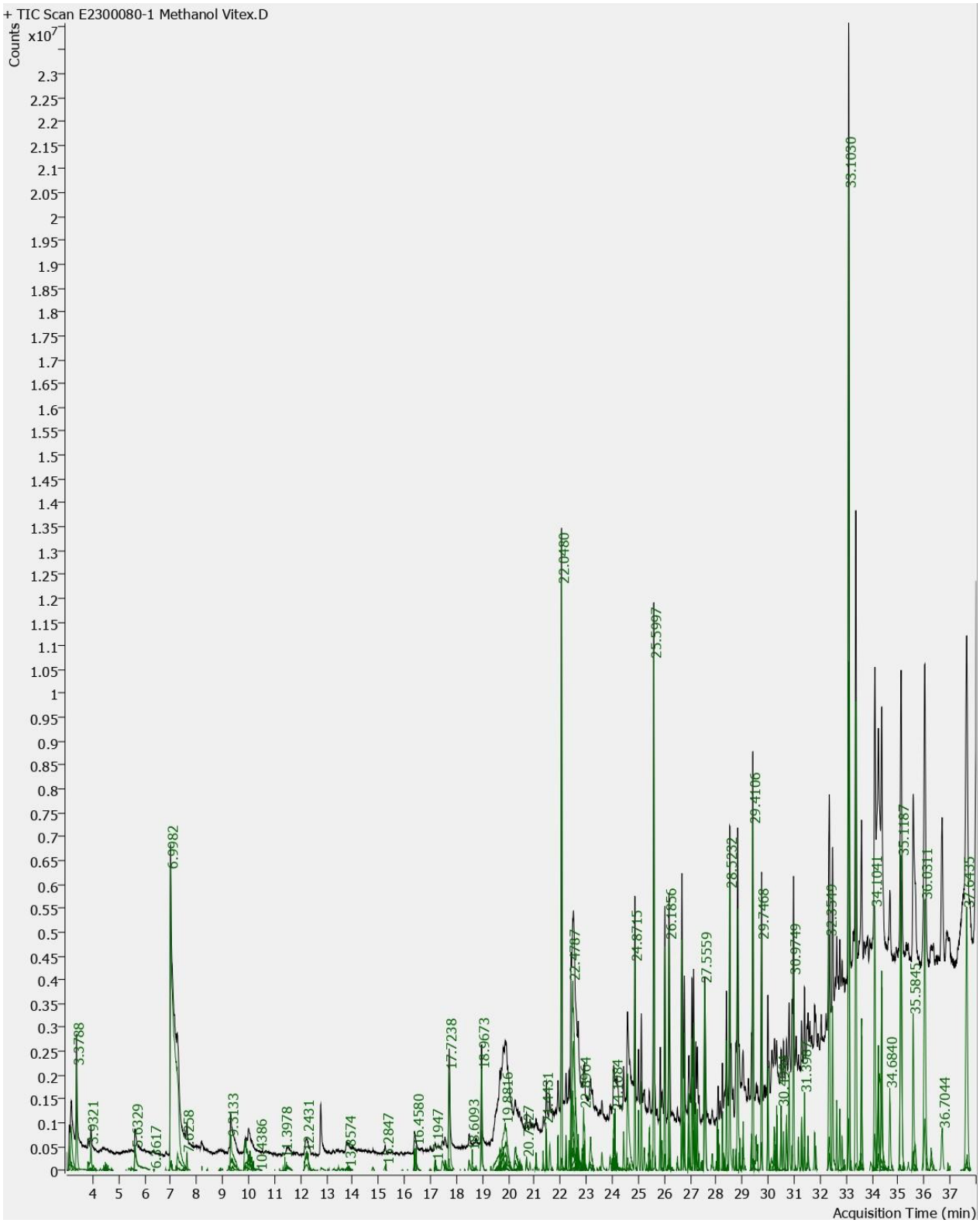

Figure 8.Chromatogram for GC-MS Analysis of *V. negundo* (Methanolic Extract)

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Discussion

In many regions of the world, several plants are used for their mosquito larvicidal properties. The evaluation of readily available plants to control mosquito vectors will lead to reduced reliance on expensive and imported goods and inspire regional efforts to improve the public health system.[23] The numerous biological effects of plant extracts on insects may be due to the presence of different phytochemicals that may work alone or together. Due to the synergistic activity of these compounds, natural insecticides are biodegradable, which reduces the long-term environmental implications of using synthetic insecticides.[24]

Our larvicidal bioassay has revealed 123.45 ppm and 423.60 ppm as the LC50 and LC90 values of the methanolic extract of *P. juliflora* against *Ae. albopictus* larvae. Earlier studies conducted by Tyagi et al., 2015 have shown the larvicidal activity of *P. juliflora* (methanol extract) against *Ae. aegypti* larvae with an LC50 value of 126.79 ppm and an LC90 value of 457.32 ppm.[25] The same extract against *Anopheles subpictus* larvae had 39.19 ppm and 175.24 ppm (LC50 and LC90 values) and also for *Culex quinquefasciatus* larvae had LC50 and LC90 values of 59.37 ppm and 243.20 ppm respectively. Similarly, Yadav, 2015 has shown the LC50 and LC90 values against *Ae. albopictus* were 0.44 g/l (440.50 ppm) and 1.85 g/l (1852.11 ppm).[26]

From our study, the ethanolic extract of *C. porcera* had 1227.61 ppm and 2551.92 ppm as their LC50 and LC90 values, versus 3rd and 4th instar larvae of *Ae. albopictus*. Contrastingly, the study conducted by Elimam et al., 2009 has shown LC50 and LC90 values of leaf extract of *C. porcera* treated against the 3rd and 4th instar larvae of *Anopheles arabiensis* and *Culex quinquefasciatus* as 454.99 ppm, 1224.62 ppm and 264.85 ppm, 769.13 ppm.[27]

The larvicidal efficacy of ethanolic extract of *A. indica* against *Ae. albopictus* recorded by our study was 1376.50 ppm and 2857.43 ppm (LC50 and LC90 values). In contrast to this, the study by Ayinde et al., 2020 reported the LC50 and LC90 values of neem oil against *Anopheles gambiae* as 723.257 ppm and 1971.51 ppm.[28] The larvicidal study of neem fruit extract performed by Batabyal et al., 2009 presented the LC50 value as 74.04 ppm against *Culex quinquefasciatus*.[29]

In our study, the LC50 and LC90 values of *V. negundo* (methanolic) extract against *Aedes albopictus* were 2499.73 and 18524.82 ppm respectively. Earlier studies conducted on the larvicidal activity of the essential oil of *Vitex negundo* (methanolic) against *Aedes aegypti* by Chandrasekaran et al., 2019 showed the LC50 and LC90 values as 50.86 and 73.12 ppm respectively.[30] The methanolic extract of *V. negundo* has good larvicidal activity against *Ae. aegypti* with an LC50 value of 211.34 ppm.[31] We analysed the larvicidal effect of methanolic extract of *S. jambolanum* against *Ae. albopictus* and the LC50 and LC90 values were 1019.94 and 2004.74 ppm respectively. In a previous study conducted by Kanthammal et al., 2018, the LC50 and LC90 values showed by methanolic seed extract of *Syzygium cumini* (*S. jambolanum*) against *Ae. aegypti* as 196.771 and 190.960 ppm respectively.[32] The larvicidal activity of petroleum ether extract of *Eugenia jambolana* (*S. jambolanum*) against *Ae. aegypti* showed LC50 and LC90 values of 40.97 and 83.29 ppm respectively.[33]

The trehalose content analysis was carried out using the modified anthrone-sulfuric acid method adopted by Li, 2014. Minor changes in the assay protocol were made considering the physiology and anatomy of mosquitoes. Various factors play a crucial role in the accumulation and depletion of trehalose in mosquitoes.[34] An outrageous 23-fold increase in trehalose content was recorded over the control mosquitoes when treated against the methanolic crude extract of *P. juliflora*. Similarly, the mosquitoes treated with extract of *V. negundo* also showed a 9-fold increase over the control mosquito group. The study on *Spodoptera litura* pupae by Asano et al., 1990 using validoxylamine A as a trehalase inhibitor has proved no adult emergence due to the excessive build-up of unutilised trehalose.[35] Although other plant extracts show a slight trehalose content increase over control mosquitoes, this cannot be accounted as a significant change in trehalose due to the varying nature of natural trehalose content in mosquitoes because of constant flight and various other environmental factors. Only methanolic extract of *P. juliflora* and *V. negundo* has shown a profound effect on the accumulation of trehalose. A recent study conducted by Zhong et al., 2023 proved that decreased trehalase enzyme activity from 76.2% to 45.3% using ZK-PI-5 and ZK-PI-9 compounds (Trehalase inhibitors) against *Spodoptera frugiperda* larvae.[36] Conventional insecticide includes polychlorinated compounds, organophosphorus compounds, carbamate compounds, and pyrethroids. Besides the beneficial pest control activity, their effects on non-target organisms and the contamination of natural resources pose an alarming threat to the environment. Extensive studies have been conducted by various researchers to identify and optimise green insecticides.[25,28,37]

The compounds of both methanolic leaf extracts of *P. juliflora* and *V. negundo* were identified using GC-MS. Among all the chemical compounds obtained from GC-MS, only compounds with the highest match factor and component area were considered potential insecticides. Further docking analysis of these compounds against trehalase would pinpoint the active compound responsible for this desirable effect. Elaborate studies based on this purified compound in the near future would play a

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vital role in vector control and management. Also, this compound could potentially be an effective and eco-friendly alternative to the existing conventional pesticides.

Conclusion

The larvicidal efficacy of *P. juliflora*, *V. negundo*, *C. porcera*, *S. jambolanum* and *A. indica* against *Ae. albopictus* was observed in this study. Their respective LC50 and LC90 values were analysed using log-probit analysis. Further, emerged adults out of the larvicidal bioassay were subjected to trehalose content analysis by the modified anthrone-sulfuric acid method. Finally, the component analysis of trehalose accumulating plant extract was done using GC-MS.

In conclusion, the present study has identified that *P. juliflora* had both larvicidal as well as trehalose accumulating activity in *Ae. albopictus*. Correspondingly, *V. negundo* elucidated only trehalose accumulating nature in *Ae. albopictus*. Docking studies of one or more compounds of the methanolic plant extracts will provide a bright future prospect. Also, the vast presence of *P. juliflora* and *V. negundo* across India would provide easy access to obtain the desired compound from them. A future study, focusing more on the efficacy of the active compound stand-alone or along with already-known green pesticides would definitely be a pathbreaker in the field of pesticides.

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Ethical Approval: Not Applicable

Conflict of Interest: None

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