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Research article

Larvicidal and histopathology effect of endophytic fungal extracts of *Aspergillus tamarii against Aedes aegypti* and *Culex quinquefasciatus*

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ABSTRACT

Background: Mosquitoes biolarvicides remain the most important method for mosquito control. The previous studies have shown *Aspergillus sp.*-expressed larvicidal properties against mosquito species. The present study evaluated larvicidal and histopathological effect of an endophytic fungus *Aspergillus tamarii* isolated from the-Cactus stem (*Opuntia ficus-indica* Mill).

Method: The molecular identification of isolated *A. tamarii* was done by PCR amplification (5.8s rDNA) using a universal primer (ITS-1 and ITS-2). The secondary metabolites of *A. tamarii* was tested for larvicidal activity against *Aedes aegypti* and *Culex quinquefasciatus*. Larvicidal bioassay of different concentrations (- 100, 300, 500, 800 and 1000 μg/mL) isolated extracts were done according to the modified protocol. Each test included a set of control groups (i.e. DMSO and distilled water). The lethal concentrations (LC_{50} and LC_{90}) were calculated by probit analysis. Experimental monitoring duration was 48 h.

Results: The ethyl acetate extract from *A. tamarii* fungus resulted - excellent mosquitocidal effect against *Ae. aegypti* and *Cx. quinquefasciatus* mosquitoes, with least LC_{50} and LC_{90} values. -After 48 h, the *Ae. aegypti* expressed better results (LC_{50} = 29.10, 18.69, 16.76, 36.78 μg/mL and the LC_{90} = 45.59, 27.66, 27.50, 54.00 μg/mL) followed by *Cx. quinquefaciatus* (LC_{50} = 3.23, 24.99, 11.24, 10.95 μg/mL and the LC_{90} = 8.37, 8.29, 21.36, 20.28 μg/mL). The biochemical level of *A. tamarii* mycelium extract on both larvae was measured and the results shown a dose dependent activity on the level of AchE, α- and β-carboxylesterase assay. Gas Chromatography and Mass Spectroscopy (GC-MS) profile of *A. tamarii* extract reflected three compounds i.e. preg-4-en-3-one, 17. α-hydroxy-17. β-cyano- (7.39%), trans-3-undecene-1,5-diyne (45.77%) and pentane, 1,1,1,5-tetrachloro- (32.16%) which which might had attributed to larvae mortality.

Conclusion: The findings of - present study shows that the use of endophytic *A. tamarii* fungal metabolites for control of dengue and filariasis vectors is promising and needs a semifield and small scale filed trials.

1. Introduction

Mosquitoes play a predominant role as a nuisance insect and vector for transmission of causative agent of dengue, yellow fever, malaria, rift valley and filariasis which greatly affected humans worldwide, particularly in tropical and sub-tropical regions [1, 2]. In recent years, due to the climate change, the mosquitoes are expanding the geographical distribution and vector-borne diseases have been contributed significant social and economic impacts [3, 4]. *Aedes aegypti* (L) (Diptera: Culicidae) is the prime vector of dengue virus that causes dengue and dengue hemorrhagic fevers in all over the world [1, 5, 6] detected with four forms of dengue virus (DEN-1, DEN-2, DEN-3 and DEN-4) from *Ae. aegypti*.

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According to the WHO report of the year 2009, two fifths of the world population are under risk of dengue infection (WHO index) and in the year 2010, 28, 292 cases of infection and 108 deaths were reported in India [7]. Most of the *Aedes* eggs were hatched shortly after flooding [7]. The global expansion of *Ae. aegypti* and the spread of dengue virus are creating major public health issues. *Cx. quinqufasciatus* mosquito is a vector for *Wuchereria bancrofti* which causes the lymphatic filariasis (transmitted by *Culex* and widely distributed in the entire globe [8, 9, 10]. More than 120 million people worldwide are infected with the *Wuchereria bancrofti* causing lymphatic filariasis [11, 12].

Globally, several insecticide classes have been used for fighting against mosquito vectors [2]. Due to the regular and continuous use of these chemicals, the mosquito have developed resistance which makes them better survivors against existing control tools [13]. Fungal derived products are highly toxic to mosquitoes, and reported as having low toxicity against non-target organisms [14]. The use of endophytic fungi and their derived products may be a promising approach to biological control of mosquitoes [15]. Extracellular secondary metabolites from many fungi have been screened for larvicidal activity against mosquitoes [16, 17]. However, fungi are considered a good candidate for bio-control of mosquitoes because of their safety, limited host range and large scale production of secondary metabolites [18]. Endophytic fungi are biologically important microorganisms to produce new pharmaceutical compounds, biological control agents and other useful products [19]. The endophytic fungi are relatively unexplored and could be potential sources of novel natural products for medicine, agriculture, and industry [20]. For example, *Massarina tunicata* [21], *Microsphaeropsis* sp [22], *Arthropsis truncata* [23], freshwater fungus *Massarina tunicata* [24] and marine-derived fungus *Massarina* sp. reported to have best mosquitocidal activity [25]. Metabolites from fungal genera, such as *Metarhizium*, *Trichophyton*, *Chrysosporium* and *Lagenidium*, as well as some *Actinomycetes*, probiotic bacteria and several basidomycetes, have shown potential insecticidal activity [1, 26]. The presence of endophytic fungi implies a symbiotic interaction in all the photosynthetic tissues of vascular plants [27, 28].

The genus *Opuntia* (Cactaceae) consists of 2300 species and have been widely used as ornamental plants, foodstuffs, medicines in arid areas of the world [29, 30]. It is widely distributed in Mexico, - Latin America, South Africa, Australia [31] and the Mediterranean area [32]. *Cactus pear* is found wild in arid and semiarid plateau regions. The fruits and stem are used as green vegetable, used to prepare some value added products such as jam, wine, pickles, body lotions, shampoo, ice cream and salad [33]. It has been used in traditional medicine for cure the number of diseases, including anti-inflammatory effects (Park et al. 1998), inhibition of stomach ulceration [34], neuroprotective effects [35], anticancer [36] and antioxidant [37]. Earlier, Bezerra and colleagues isolated and identified from different endophytic fungi from *Opuntia ficus-indica* named as *Cladosporium cladosporioides, C. sphaerospermum. Acremonium terricola, Monodictys castaneae, Penicillium glandicola, Phoma tropica* and *Tetraploa aristata* [38]. The diversity of the endophytic fungal community associated with *Opuntia* sp was studied and investigated for antifungal potential against phytopathogenic fungal species by Silva-Hughes and others [39]. The previous studies have shown *Aspergillus tamarii* reported as better larvicidal properties against mosquitoes [40]. The present study aimed to isolate, molecular identification of endophytic fungus *Aspergillus tamarii* from *Opuntia ficus-indica* and evaluate its mycelial metabolites to tested against *Ae. aegypti* and *Cx. quinquefasciatus* larvae and their histopathological, biochemical constituents was analysis of 4th instar larvae. In addition, mycelia metabolites to check the toxicity on non-target model organism *Artemia salina* and Zebra fish (*Danio rerio)* embryo.

2. Materials and methods

2.1. Isolation of endophytic fungi

Healthy *Opuntia ficus-indica* stem was collected from Mettur Dam (latitude 11°47′16.66N, longitude 77.48′2.88E), Salem District, Tamilnadu, India and washed three times with tap water –followed by surface sterilization and the methods were described by Zeng and others [41]. Briefly, stems were immersed in 75% ethanol for 2 min, followed by 0.1% mercuric chloride for 4 min and the samples were washed three times with sterilized distilled water. The samples were cut into 3.0 × 3.0 cm to remove excess amount of surface chemicals. Then, it was cut into small pieces (1.0 × 1.0 cm) using a sterile blade and placed in Sabouraud dextrose agar (SDA) medium supplemented with chloromphenicol (50 μg/mL) and incubated (at 25 °C ± 2 °C) for 20 days and the plates were observed daily. The well grown mycelia were picked up and transferred into new SDA plates for further purified form of fungal isolates [42].

2.2. Identification of the endophytic fungus

The endophytic fungal culture was preliminary identified by the visual observation and the mycelium was stained with lacto phenol cotton blue staining. For morphological identification of fungus was based on color, the colony growth nature, the fungal mycelia, conidiophores, and conidia were observed microscopically using a Lobomed microscope [43].

2.3. DNA isolation and PCR amplification

The fungal genomic DNA (5.8s rDNA) was extracted from the fresh mycelium of *A. tamarii* by CTAB method [44]. The genomic DNA quality was evaluated by 1% (v/v) agarose gel electrophoresis. The polymerase chain reaction (PCR) amplification (40 μl) was performed using universal primers internal transcribed spacer ribosomal DNA (ITS rDNA) ITS-1 (forward primer 5′ CTT GGT CAT TTA GAG GAA GTA A 3′) and ITS-2 (reverse primer 5′ TCC TCC GCT TAT TGA TAT GC 3’) prepared and contained 4 μl of the template, 2μl of each primer (10 μM), 20μl of Taq PCR mix (Amplicon), and 12μl of double-distilled water. Subsequently, the amplified regions were further purified using QIA quick PCR purification kit (QIAGEN) and the obtained sequence was ascertained using BLAST search. Finally, the multiple sequence alignment was performed using the CLUSTAL W program. The phylogenetic tree was constructed through aligning the sequences in neighbor-joining method encompassing with 1000 bootstrap replications using MEGA 6.0, and trimmed sequences were deposited in NCBI GenBank database [45].

2.4. Phylogenetic tree

Nucleotide sequences of 5.8s rDNA were edited with Bioedit and aligned by ClustalW [46]. A total of 11, 5.8s rDNA sequences closely related species were used in the phylogenetic analysis, as per the neighbor-joining (NJ) method, and it was carried out using MEGA 5.0 software [47]. The NJ analysis of DNA sequences was done based on the Kimura 2-parameter test. Alignment gaps were treated as missing data. The liability of phylograms was tested by bootstrap analysis with 1,000 replicates using MEGA 5.0.

2.5. Extraction of secondary metabolites from isolated endophytic fungus

The well grown *A. tamarii* cultivated on potato dextrose broth (PDB) and it was inoculated into 500 mL Erlenmeyer conical flask containing broth and incubated at 28 °C ± 2 °C for 14 days. Then, the mycelium was filtered through Whatman No. 1 filter paper, mycelia was added to ethyl acetate solvent for macerating purpose. After 7 days, the mixture was filtered *via.* Whatman No.1 filter paper and the process were repeated as twice. The obtained mycelia extracts were concentrated in a rotary evaporator at reduced pressure. After reaching the boiling point of each solvent, the extracts yield was weighed [48].

2.6. Larvae collection and rearing condition

The larvae of *Ae. aegypti* and *Cx. quinquefasciatus* were collected from agricultural fields, Karuppur Panchayat, Salem District, Tamil Nadu. These collected larvae were transferred in mosquito rearing cages and maintained properly [26 ± 2 °C and 72 ± 5% relative humidity with the light/dark condition (14:10h)]. The I-IVth instars larvae of test mosquitoes were maintained in separate enamel tray [25cm length×15cm width×5cm length]. Each tray contains 200 larvae. Larvae were fed with a diet of finely ground brewer's yeast and dog biscuits (3:1). The larvae were reared in deionized water (at pH 7.0) and the water was added daily for evaporation [49].

2.7. Larvicidal bioassay

Larvicidal bioassay of different concentrations (- 100, 300, 500, 800 and 1000 μg/mL) of the isolated *A. terreus* mycelia extracts were done according to the modified protocol of Deepika et al. [50]. The I-IVth instars larvae (50 Nos) were introduced into a 100-mL glass beaker containing 100 mL of dechlorinated water plus the desired concentrations of mycelium extract and for each concentration, three replicates were maintained. Larval mortality was recorded after 48 h exposure, during the time no food was provided to the larvae. Each test included a set of control groups (i.e. DMSO and distilled water). The lethal concentrations (LC_{50} and LC_{90}) were calculated by probit analysis [51].

2.8. Dose response bioassay

The dose response bioassay of mosquitoes was exposed to the extracts, and the method was adopted as per the modified protocols of World Health Organization [52]. Based on the preliminary screening results, the purified metabolite extracts were subjected to dose–response bioassay for larvicidal activity against *Ae. aegypti* and *Cx. quinquefasciatus* larvae. Four groups (600Nos) of I-IV instars larvae were introduced in 250-mL beakers and exposed to different dosages of metabolites (100, 300, 500, 800 and 1000 μg/mL). The negative control DMSO (Dimethyl sulfoxide) as well as positive control (distilled water) was maintained at the same concentrations. The mortality was recorded at 48 h, after the end of the test. No food was provided to the larvae during the test periods. Larval mortality was reported as an average of four replicates and the mortality percentage was calculated using Abbott's formula [53].

$$\text{Corrected mortality} = \frac{\text{Observed mortality in treatment} - \text{Observed mortality in control}}{100 - \text{Control mortality}} \times 100$$

$$\text{Percentage mortality} = \frac{\text{Number of dead larvae}}{\text{Number of larvae introduced}} \times 100$$

2.9. Preparation of whole body homogenates for enzyme assay

The tested and control (I-IVth instar) larvae of *Ae. aegypti* and *Cx. quinquefasciatus* were washed with double-distilled water, and the adhering water was completely removed from the surface by blotting with tissue paper. The larvae was separately homogenized in eppendrof tubes using a hand Homogenizer by adding 500μl of ice-cold sodium phosphate buffer (20 mM, pH 7.0) for estimating the total proteins and enzymes. The homogenates were centrifuged (8000 × g at 4 °C for 20min) and supernatants used for further analysis. The final homogenates were stored on ice, until the further use [54].

2.10. Acetylcholinesterase inhibition assay

The whole body homogenates of control and treated 4th instar larvae of *Ae. aegypti* and *Cx. quinquefasciatus* (100 μL) was spectrophotometrically analyzed by the method of Ellman and others [55] with acetylchiocholine iodide used as a substrate [56]. The dose of acetylcholinesterase was performed in two separate 96-well plates, assigned to AChE, for estimate the total acetylcholinesterase activity in the presence or absence of the propoxur inhibitor. About 145μl of Triton/Na phosphate (5 mL of 100% Triton X-100 in 50 mL of 1M sodium phosphate buffer at pH 7.8 and 455 mL of distilled water), and 10 μl of DTNB/Na phosphate (10 mM DTNB in 100 mM sodium phosphate buffer at pH 7.0) was added to 25μL of homogenates and maintained in duplicates. All plates were incubated for an hour at room temperature, protected from light, and read spectrophotometrically (at 405 nm).

2.11. Carboxylesterase assay

The α- and β-carboxylesterase assay of *Ae. aegypti* and *Cx. quinquefasciatus* 4th instar larvae was performed by modifyied method of Dauterman et al. [57]. In brief, 100 μl of undiluted and diluted (1:3) homogenates were incubated with 1 mL of 20 mM sodium phosphate buffer (pH 7.0) comprising 250μM of α- and β- naphthyl acetate for 30 min (at 28 °C). To this add, 400μl of freshly prepared 0.3 % Fast blue B in 3.3 % SDS for arrest the enzymatic reaction, and it was allowed to develop color for 15 min (at 28 °C). The optical density (OD) was read spectrophotometrically at 430 nm (α- carboxylesterase) or 588 nm (β-carboxylesterase) against the respective reagent blank.

2.12. Histopathological study

The histopathological study of 4th instar larvae of *Ae. aegypti* and *Cx. quinquefasciatus,* post-exposure to *A. tamarii* mycelia extract was carried out by the method of Sundararajan and Kumari with slight modifications [58]. In brief, treated and control larvae were dehydrated, fixed and sliced by the glass knife with the help of rotary microtome. The sliced larvae was sectioned (at 8 μm thickness) and stained with haematoxylin-eosin (HE stain), with melted paraffin. The observation was made with the microscope (Motic images plus 2.0 ML) connected to a computer and midgut cells of the treated and untreated larvae of mosquitoes tissues were photographed [59]. The larval midgut cells of treated larvae were observed and compared with control.

2.13. Toxicity assay of endophytic fungal mycelium extract against *Artemia salina*

Brine shrimp (*A. salina*) used for testing lethality bioassay of endophytic fungal mycelial extracts. One gram of *A. salina* eggs were hatched in 100mL of artificial seawater with air bubbling and artificial illumination for 24 h. The phototropic *A. salina* (larvae) was collected with a pipette from the lighted side and concentrated in a 7-mL test tube. The bioassay was conducted for determining the 50% lethal concentration (LC_{50}) of the fungal ethyl acetate extracts. For preliminary screening, five different concentrations (10, 20, 30, 40, 50 μg/mL) of culture filtrate crude extracts were tested against 50 larvae of *A. salina* (for each concentration) and the test was repeated in three times. The mortality rate was recorded after 24 h exposure. The concentration of the extract was gradually decreased and the larvae mortality rate was recorded. The highest percentage mortality rates (≥90%) of fungal extracts were

selected. After a series of tests, the fungal extract was tested against *A. salina* larvae to determine the LC_{50} values [59, 60].

2.14. Zebrafish embryo maintenance and acute toxicity test

The maintenance of Zebra fish, egg production and range-finding tests were conducted according to the modified method of Knobel and others [61]. The embryonic acute toxicity test was conducted as per the fish embryo toxicity (FET) test (OECD 2013) with slight modifications. The seven different concentrations of *A. tamarii* mycelia ethyl acetate metabolites (-0, 1.0, 0.5, 0.125 mg/mL, 30, 3.0 and 0.5 μg/mL) were made using reconstituted water, designed on the basis of pre-experiment data. For exposure of zebrafish embryos, 2.0 mL of test solutions and two fertilized eggs were transferred to individual well of a 24-well microtiter plate. Twenty wells in each plate contained seven test concentrations uniformly and the other one well was filled with 2.0 mL of 0.1% dimethyl sulphoxide (DMSO) served as the control. Each test concentration was replicated five times, with 10 embryos per replicate. The exposure metabolites were renewed every 24 h to keep the tested concentration of mycelia metabolite and water quality. The hatching rate, heartbeat count, survival percentage and body length of zebra fish were observed and recorded using an inverted microscope (Nikon TE2000-U).

2.15. Gas chromatography mass spectroscopy analysis of larvicidal potential extract

Gas chromatography (GC-MS) mass spectroscopy analysis of samples were performed using a Perkin Elmer Clarus 500 gas chromatography equipped with a PerkinElmer 1200-l single quadrupole mass spectrometer [62]. A total of 20μL sample was introduced (via an all glass injector working in the split mode) with the carrier gas and a linear velocity of 32 cm/s. The HP-5 fused silica capillary column (Length – 30 m; Film thickness- 25 μm I.D - 0.2 mm) was used in this investigation [63]. The mass spectrometer was operated in the electric impact mode at 70 eV. The ion source and transfer line temperature was kept at 250 °C. Helium was used as the carrier gas at a flow rate of 1 mL/min; the split ratio of 10:1; mass scan 50–600 Da. The oven temperature was programmed as follows: initially at 60 °C for 2 min, rising at 10 °C/min to 300 °C and then held isothermally (6 min) at 300 °C and total run time was 32 min. The mass spectrum was obtained by centroid scan of the mass range from 40 to 1000 amu. The compounds were identified based on the comparison of their retention time (RT) and mass spectrum as well as the literature available in the GC-MS database of Vellore Institute of Technology (VIT), Vellore, Tamilnadu, India.

2.16. Fourier transform infrared spectroscopy (FTIR) analysis

The ethyl acetate extracts of fungal secondary metabolites and the purified sample was dried, powdered and pelleted for FT-IR analysis (Jasco FTIR 5300 spectrophotometer). The sample (1.0 mg) was mixed with 100 mg of KBr (binding agent) using a clean mortar and pestle to make the powder into tablet [47]. The changes in the surface chemical bonding and surface composition was characterized using Fourier Transform Infrared (FT-IR) spectroscopy (Nicolet Avatar series 330) ranged from 400 to 4,000 cm^{-1}. Measurements were carried out to identify the possible bio-molecules responsible for dead mosquito larvae by fungal ethyl acetate extract [64, 65].

2.17. Statistical analysis

The observed mortality data were corrected using Abbott's formula [66]. The results of larvicidal bioassay were subjected to probit analysis using the IBM SPSS Statistics ver. 20 software (IBM Corp., Armonk, NY, USA) for determining the LC_{50} and LC_{90} values [67]. Enymatic data were expressed as one-way ANOVA performed with Tukey's honest significant difference (HSD) $P<0.05$. Graphs were designed using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, California, USA).

3. Results

3.1. Isolation and identification of endophytic fungus

The isolated endophytic fungal strain was preliminary identified by morphological (color, odor, shape) and microscopic observations (presence of conidial structure and hyphae of morphological features). Based on the colony morphology and lactophenol cotton blue staining results the isolated fungus was identified as *Aspergillus tamari*. The colony morphology shows green color at the first 4 days of growth and shifted to brown or brownish green. The conidiospores are hyphae structures were formed on the medium. The microscopic feature of fungus is very long thick like a bowl shape of conidiospores, surrounded small ball shaped features. The conidia were sub-globose to cylindrical, with an average dimension of 3.11–3.24 μm.

3.2. Molecular identification of isolated DNA by PCR analysis

Molecular identification of isolated *A. tamarii* DNA by PCR amplification using universal primers and 5.8S rDNA sequencing was performed. The isolated DNA fragment was amplified (600bp) and compared with ladder, the universal primers used as Internal Transcribed Spacer ITS-1 (5′ TCC GTA GGT GAA CCT TGC GG 3′) and ITS-2 (5′ TCC TCC GCT TAT TGA TAT GC 3′) (Figure 1 a, b). The ITS region of - *A. tamarii* fungus was sequenced, and the obtained sequences were deposited in the NCBI gen bank data base (GenBank Accession no: MH094278).

3.3. Phylogenetic tree analysis

The evolutionary history of fungal sequences was inferred using the UPGMA method and the percentage of replicate trees were calculated with associated taxa clustered together in the bootstrap test (1000 replicates) (Figure 2). The evolutionary distances were computed by applying the Tajima-Nei method and the analysis were done for totally 11 nucleotide sequences. Evolutionary analyses were conducted in MEGA5 software. The phylogenetic trees of ten phylotypes were constructed to provide the relationship of an individual sequence with closely related species were retrieved from the genbank database. The displayed ITS -1 and ITS - 2 sequences of phylotype (MH094277) was similar to the 100% of *A. oryzae* (KY655350.1), 99% of *A. niger* (KF934482.1) and *A. vesicolor* (EU326209.1), *A. terreus* (EF432562.1), (2–5%) nucleotide sequence differences. However, - the phylogenetic analysis (KY655350.1) results show a higher range of differences with other nucleotide sequences (2–11%).

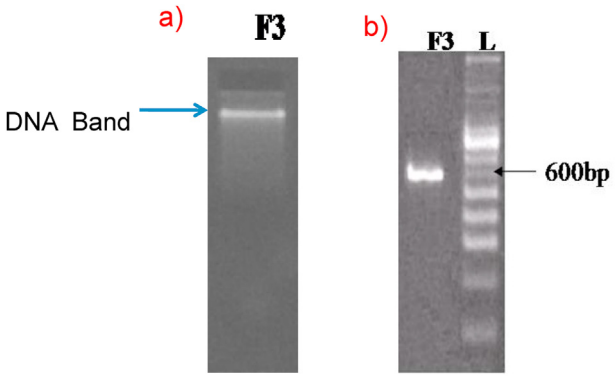

Figure 1. (a) Isolation of *A. tamarii* genomic DNA, (b). PCR amplification of DNA at ITS regions (F3 - ITS 1 and ITS 2).

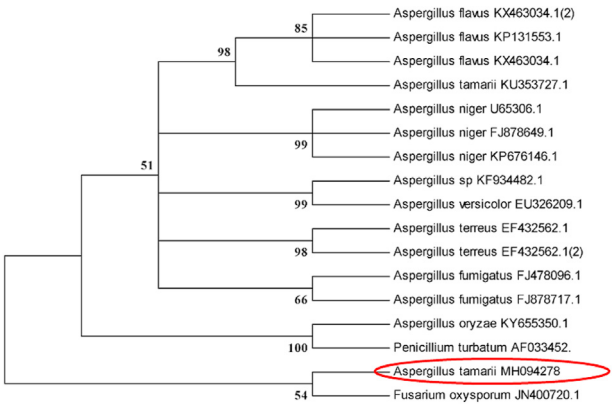

Figure 2. Phylogenetic tree analysis of *A. tamarii*.

3.4. Larvicidal activity

The results of larvicidal assay from the ethyl acetate extracts of the fungus *A. tamarii* tested against larvae instars of *Ae. aegypti* and *Cx. quinquefaciatus* were presented in Tables 1 and 2. The *Ae. aegypti* expressed better LC_{50} and LC_{90} values (LC_{50} = 29.10, 18.69, 16.76, 36.78 μg/mL and the LC_{90} = 45.59, 27.66, 27.50, 54.00 μg/mL) followed by *Cx. quinquefaciatus* (LC_{50} = 3.24, 24.99, 11.24, 10.95 μg/mL, and the LC_{90} = 8.38, 8.29, 21.36, 20.29 μg/mL), respectively. The 1000 μg/mL dose, exhibits maximum mortality and the lower concentration of extracts (100 μg/mL) expressed least mortality. About 97.3 percentage of mortality was noticed in *Ae. aegypti,* at the concentration of 1000 μg/mL. The higher concentration of (1000 μg/mL) ethyl acetate extract from *A. tamarii* was very effective against the 4th instar larvae of *Cx. quinquefaciatus* resulted mortality rates of 76% and 80% respectively (after 48 h) and 100 μg/mL of extracts showed the lowest mortality (28%). Comparatively, *Ae. aegypti* 4th instar larvae had the highest mortality (97%) than *Cx. quinquefasciatus.* The treated larvae exhibited several behavioral changes in the mosquitoes viz. impaired coordination, irregular movements and forceful self-biting (Figure 3b), compared to control group reflected normal behaviors within 15 min of treatment (Figure 3a). These irregular features and orientation symptoms of treated larvae were not stopped and it's becomming more irritated. After treatment, the study was noticed the larval up and down wriggling movements and many of them had vibrating movements (tremors), and paralysis symptoms.

Histopathological profiles of tested *Ae. aegypti* (treated with *A. tamarii* mycelia extracts) showed various histological alterations like damaged and disorganized nerve cord ganglia, gastric caeca, muscles and food column. The epithelium of treated larvae was spoiled and cells were vacuolated but occurred closely in the nuclei (Figure 4a, c**)**. The histopathological lesions of *Cx. quinquefasciatus* was observed (after exposure with *A. tamarii* metabolites) primarily in the midgut of larva, Injury with edema, swelling, deformation of epithelial cells, cells protruding into the lumen, blebbing cells and absent in microvilli. Hyperplasia of the epithelial cells was found in some region of tissues (Figure 4b, d), whereas the control larvae of both mosquitoes showed normal growth and development of cells.

3.5. Acetylcholinesterase inhibition assay

The level of AchE activity on the 4th instar larvae of *Cx. quinquefasciatus* and *Ae. aegypti* was gradually decreased at 48h treatment. The exposure to fungal metabolites significantly inhibited the larval AchE activity. At100μg/mL concentration of metabolites treated *Ae. aegypti* larvae expressed the highest level of AchE, followed by *Cx. quinquefasciatus*. Similarly, the higher concentration (500 μg/mL), the AchE activity was gradually declined in test larvae based on metabolite concentrations. Overall, the obtained results revealed the enzyme expression was mainly dose dependent (Supplementary Figure 1 Acetylcholinesterase activity of *Cx. quinquefasciatus* and *Ae. aegypti*).

3.6. Carboxylesterase assays

The carboxylesterase profile of 4th instar larvae of *Ae. aegypti* and *Cx. quinquefasciatus* was observed with exposure to the different

Table 1. Larvicidal activity of endophytic fungus *A. tamarii* against different instars larvae of *Ae. aegypti*.

Larva stages	Concentrations (μg/mL)	48-h mortality(%)±SD	LC_{50}(μg/mL) (LCL-UCL)	LC_{90}(μg/mL) (LCL-UCL)	χ^2
First instar	Control	0.0 ± 0.0	29.10 (4.24–66.80)	45.59 (9.004–92.55)	24.62n.s
	100	33.33 ± 1.0			
	300	40.00 ± 1.0			
	500	57.77 ± 0.5			
	800	72.22 ± 1.5			
	1000	90.00 ± 1.0			
Second instar	Control	0.0 ± 0.0	18.69 (7.167–34.06)	27.66 (11.941–46.90)	15.37n.s
	100	64.44 ± 1.5			
	300	76.66 ± 1.0			
	500	82.22 ± 1.5			
	800	91.11 ± 1.5			
	1000	92.22 ± 0.5			
Third instar	Control	0.0±0.0	16.76 (0.57–51.97)	27.50 (1.557–72.72)	32.19n.s
	100	44.44 ± 1.5			
	300	48.88 ± 0.5			
	500	53.33 ± 1.0			
	800	74.44 ±2.5			
	1000	95.55 ± 1.1			
Fourth instar	Control	0.0 ± 0.0	36.78 (6.91–77.14)	54.00 (13.038–102.44)	29.71n.s
	100	33.33 ± 1.0			
	300	40.00 ± 1.0			
	500	56.66 ± 1.0			
	800	73.33 ± 1.0			
	1000	97.33 ± 1.0			

Control (deionized water) - No mortality.
LC_{50}– Lethal concentration that kills 50% of the exposed larvae, LC_{90}– Lethal concentration that kills 90% of the exposed larvae, LCL = Lower confidence Limit, UCL = Upper confidence Limit, *d.f* degree of freedom, χ^2– Chi-square values are significant at $P < 0.05$ level. Mean value of five replicates. n.s- not significant.

Table 2. Larvicidal activity of endophytic fungus *A. tamarii* against different instars larvae of *Cx. quinquefasciatus*.

Larva stages	Concentrations (μg/mL)	48-h mortality(%)±SD	LC_{50} (μg/mL) (LCL-UCL)	LC_{90} (μg/mL) (LCL-UCL)	χ^2
First instar	Control	0.0 ± 0.0	3.23 (0.01–17.88)	8.37 (0.12–33.76)	7.97n.s
	100	28.88 ± 0.5			
	300	36.66 ± 1.0			
	500	44.44 ± 0.5			
	800	50.00 ± 1.0			
	1000	66.66 ± 1.0			
Second instar	Control	0.0 ± 0.0	24.99 (6.30–51.93)	43.53 (14.08–79.68)	8.29n.s
	100	26.66 ±1.0			
	300	36.66 ± 1.0			
	500	48.88 ± 0.5			
	800	56.66 ± 1.0			
	1000	73.33 ± 1.0			
Third instar	Control	0.0 ± 0.0	11.24 (1.51–30.01)	21.26 (4.00–48.38)	8.57n.s
	100	33.33 ± 1.0			
	300	41.11 ±0.5			
	500	48.88 ± 0.5			
	800	65.55 ± 1.1			
	1000	76.66 ± 1.0			
Fourth instar	Control	0.0 ± 0.0	10.95 (1.61–8.62)	20.28 (4.07–45.60)	12.46
	100	37.77 ± 0.5			
	300	45.55 ± 0.5			
	500	53.33 ± 1.0			
	800	70.00 ± 1.0			
	1000	80.00 ± 1.0			

Control (deionized water) - No mortality.
LC_{50} – Lethal concentration that kills 50% of the exposed larvae, LC_{90} – Lethal concentration that kills 90% of the exposed larvae, LCL = Lower confidence Limit, UCL = Upper confidence Limit, *d.f.* degree of freedom, χ^2 – Chi-square values are significant at $P < 0.05$ level. Mean value of five replicates. n.s- not significances.

concentrations of *A. tamarii* metabolites (100, 200,300, 400, and 500 μg/mL) for 48 h. The gradual decrease in the level of α- and β-carboxylesterase was observed during the normal development and maintained an equal level, even after 48 h exposure (Supplementary Figure 2a α-Carboxylesterase activity of *Ae. aegypti* & *Cx. quinquefasciatus*). The α-carboxylesterase activity level was decreased steadily during the development of the larvae (Supplementary Figure2b β-Carboxylesterase activity of *Ae. aegypti* & *Cx. quinquefasciatus*), and it reaches the lowest level (after 48 h treatment).

3.7. Biotoxicity assay of Artemia salina and zebrafish embryo

Biotoxicity assay of metabolites with *A. salina* of brine shrimp (suitable test aquatic organism) was carried out to measure the toxicity level against non-targeted organisms. The LC_{50} and LC_{90} values of the ethyl acetate extract from *A. tamarii* strain based on the brine shrimp lethality bioassay (Table 3). The better LC_{50} and LC_{90} values of *A. terreus* mycelia extract against *A. salina* larvae treatments were noted [38.483, 49.710 μg/mL (48 h)]. The swimming behaviour was significantly altered in

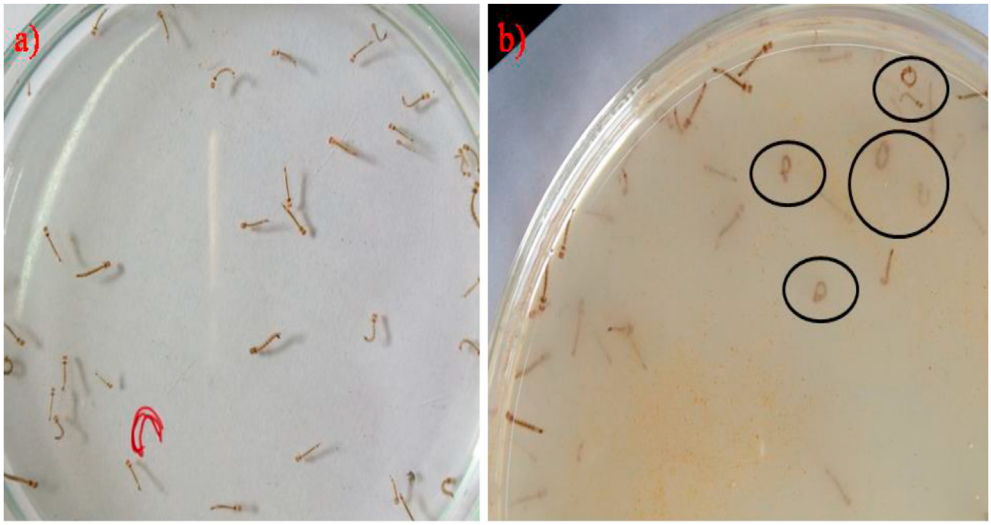

Figure 3. Larvicidal efficacy of *A. tamarii* ethyl acetate extract against *Ae. aegypti* and *Cx. quinequefasicatus* (after 48 h of exposure periods). a). Control larvae; b). Treated larvae. Black circle indicates 'self-biting larvae'.

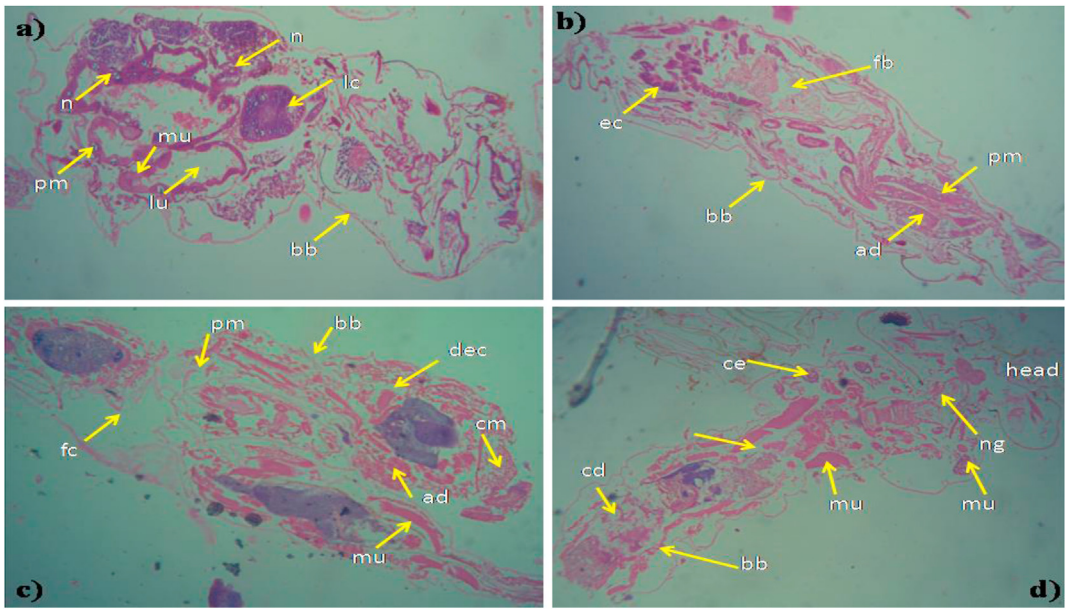

Figure 4. Histopathology profile of 4th instar larvae of *Ae. aegypti* a). Control, c). Treated and *Cx. quinquefasciatus* b). Control, d) treated *A. tamarii* ethyl acetate extract. (Larval tissues showing vacuolated gut epithelium cells (ec), gut lumen (lu), adipose tissue (ad) muscles (mu) nucleus (nu), brush border (bb), peritropic membrane (pm), food column (fc), degenerative epithelial cells (dec), broken cells (ce), destroyed cells (cd), malformed cells (cm) and nerve ganglia cells (ng)).

Table 3. GC-MS analysis of mycelia ethyl acetate extracts of *A. tamarii.*

S. no	Rt	Area	Area%	Molecular weight /formula	Compound name
1.	19.340	9,069,465.0	7.399	313/$C_{20}H_{27}O_2N$	Preg-4-en-3-one, 17.alpha.-hydroxy-17.beta.-cyano-
2.	20.190	56,109,004.0	45.774	146/$C_{11}H_{14}$	Trans-3-undecene-1,5-diyne
3.	21.221	39,430,720.0	32.167	208/$C_5H_8C_{l4}$	Pentane, 1,1,1,5-tetrachloro-

A. salina larvae exposed to *A. tamarii* metabolite at maximum concentration (50 μg/mL) and the following changes were observed, namely disrupt and affect their movement, feeding, which resulted mainly in the decreased swimming ability (Supplementary Figure 3c&d). Intestinal enlargement and loss of antennae and deformation of antennae (Treated)). In control experiments, clearly stated that all the concentrations of extracts haven't induces any lethal effect on *A. salina* and adult at 24 h exposure (Supplementary Figure 3a&b). Morphological changes of *A. salina* exposed to *A. tamarii* mycelium ethyl acetate extract. *A. salina* did not expose to extract (Control)). From the results, the percentage of mortality rate was extensively increased which corresponds to the concentration of metabolites and exposure periods.

The acute toxicity assay of zebrafish embryo was treated with *A. tamarii* mycelia ethyl acetate extract, after 96 hpf hatched embryo. The body length was significantly reduced based on doses of mycelia extract compared to control groups (Supplementary Figure 4) Zebra fish embryos exposed to different concentrations of *A. tamarii* extract (0.5 3.0, 30 μg/mL and 0.125, 0.5, 1.0 mg/mL) at 72hrs (Ssupplementary Figure 5a Body length). The hatching rate of zebrafish embryos was noticed as 97% (after 72 h in the untreated group) and it was 90% declined at 1 mg/mL concentration compared to control and 0.1% DMSO groups (Supplementary Figure 5c Heart-beat counts). While, exposure with mycelia metabolites haven't induce any deformities in the pericardial region of matured embryo. Furthermore, the heart beat count was remarkably decreased in larvae from all tested groups than control (Supplementary Figure.5b Hatch rate) and the reduced heart beat rate was mainly noticed depends in a concentration-dependent manner (5%, 10%, 15% 20% 25% and 30% in the 0.5 3.0, 30 μg/mL and 0.125, 0.5, 1.0 mg/mL). There was no malformation in the pericardial, tail, intestine and ear region of embryos (Supplementary Figure 5d Percentage of survival) in both treated and control groups.

3.8. Gas chromatograpy mass spectroscopy analysis of A. tamarii

The chemical profile of *A. tamarii* mycelia ethyl acetate extract was characterized and identified by GC-MS analysis. The interpretation of mass spectra of GC-MS was performed using the database of National Institute Standard and Technology (NIST) and based on the active principles with their retention time (Rt), molecular formula, molecular weight and percentage (area %) (Table 4). Totally three major compounds were identified from the metabolites i.e. Preg-4-en-3-one, 17.Alpha.-hydroxy-17.Beta.-Cyano- (7.39%), Trans-3-undecene-1,5-Diyne (45.77%) and Pentane, 1, 1, 1, 5-tetrachloro- (32.16%), respectively.

3.9. Fourier transform infrared spectroscopy analysis

The FTIR spectra value of *A. tamarii* mycelial extract reflected many functional groups and peaks at 3428.81, 2926.45, 2857.02, 1632.45, 1383.68, 1251.58, 1078.01, 1039.44 and 619.038 cm^{-1}, respectively (Table 5). The FTIR spectrum of the *A. tamarii* extracts show, bands at 3428.81 cm^{-1} along with an intense broad absorbance. The band peaks at 3428.81cm^{-1} characteristics of O–H stretching vibrations of the functional group in alkanes. The 2926.45 cm^{-1} band can be assigned to aromatic C–H stretching vibrations and the characteristic of the functional group in alkanes. The peak at 2857.02cm^{-1} is probably attributable to the C–H stretching vibrations and the functional group in alkanes. The band at 1632.45 cm^{-1} developed for N–H bending, and was found in primary amines. The presence of sharp peak at 1383.68 $cm^{-1}$was assigned to X = C=Y stretching vibrations, and functional group in allenes or ketenes compounds. The peak at 1251.58 cm^{-1} explains the stretching of C–N bonds of aliphatic amines in the extract. The peak at1078.01 cm^{-1} confirmed the C–N stretching of dissociated in functional group of

Table 4. GC-MS analysis of mycelial ethyl acetate extracts of *A. tamarii.*

S. no	Rt	Area	Area%	Molecular weight /formula	Compound name
1.	19.340	9,069,465.0	7.399	313/$C_{20}H_{27}O_2N$	Preg-4-en-3-one, 17.alpha.-hydroxy-17.beta.-cyano-
2.	20.190	56,109,004.0	45.774	146/$C_{11}H_{14}$	Trans-3-undecene-1,5-diyne
3.	21.221	39,430,720.0	32.167	208/$C_5H_8C_{l4}$	Pentane, 1,1,1,5-tetrachloro-

aliphatic amines compounds. A broad intense band at 1039.44 cm^{-1} in the spectra can be assigned to the C–N stretching frequency of aliphatic amines. The peak at 619.038 cm^{-1} resulted from the bending bands of C–H and alkanes functional group.

4. Discussion

The findings of this study have revealed that, endophytic fungi are biologically important microorganisms to produce new pharmaceutical compounds, bio-control agents and other useful products [68]. The isolated fungus was identified based on the colony morphology and microscopic features. These conventional and routine method most popular classical methods but, it is believed that these are time consuming and not sufficient to characterize the different fungal species, due to their intra and inter specific morphological feature [69, 70]. Recently, the molecular identification of fungal isolates is a rapid and quick method which requires minimal handling and able to distinguish even morphologically, similar fungal species [71, 72]. Therefore, molecular identification of fungus was carried out for this study using fungal universal primers ITS-1 and ITS-2. In the present study, we have isolated and identified as *A. tamarii,* based on morphology, staining (LPCB) and molecular analysis. Similarly, Silva-Hughes and others reported the endophyte fungi (*viz Biscogniauxia*, *Cryptococcus* and the species *Cladosporium, Asperulatum*) isolated from several plants [39]. Previously, Naik reported that, *A. caelatus* was differenciated from *A. tamarii* based on colony color, yellow pigments in the agar on CZ slants, conidiophore structure and mycotoxin profile [73]. Similarly, Wilson and colleagues isolated the endophytic fungus *A. tamarii* from the *Arachis hypogaea* and *Zea mays* [74].

PCR amplification was performed from DNA of *A. tamarii* run on the agarose gels electrophoresis. The amplified products were visualized on 1% agarose gel to confirm the presence of single band. Previously, El Khy and others reported that the PCR's amplification of all fungal strains from using the primer pair IGS-F/IGS-R [75]. The present study observed *A. tamarii* DNA was amplified and yielded 674 bp size amplicons and no additional or nonspecific bands. Consequently, Ezra and others reported ITS, amplified by PCR using the primer ITS1/ITS4 and the obtained amplicons were verified by electrophoresis on 1.2% agarose gels, then sequenced with the corresponding primers used for their amplification [76]. Finally, the nucleotide sequences of the ITS, were blasted against GenBank using the NCBI/BLAST tool. Colloca and team reported that the universal fungal primers (ITS1 and ITS2) found efficiently in amplifying the culture independent DNA isolated from fungal strains. On the basis of the morphological characterization, it was very difficult to identify all the isolates upto their species level. Further, molecular level identification of the isolates were done by large ribosomal rDNA gene sequencing. The evolutionary history of the sequence was inferred using the Tajima-Nei method and the phylogenetic tree analysis involved 17 nucleotide sequences [77, 78]. According to Tamura and colleagues stated that, the Neighbor joining method is a good one for analyzing the evolutionary history of fungal isolates [47]. Currently, Tam and team studied the phylogenetic analysis of ITS (336 nucleotide positions) of the 11 clinical isolates, three reference strains, and other closely related species [79].

We tested the larvicidal effect of the ethyl acetate extract of *A. tamarii* against the 1st to 4th instar larvae of *Ae. aegypti* and *Cx. quinquefasciatus* resulted these extract have found very active against *Ae. aegypti* (LC_{50} values of 29.10, 18.69, 16.76, 36.78 μg/μL, LC_{90} = 45.59, 27.66, 27.50, 54.00 μg/μL and value for LC_{50} = 3.23, 24.99, 11.24, 10.95 μg/μL, and the LC_{90} = 8.37, 8.29, 21.26, 20.28 μg/mL) than other extracts. Previous study reported the killing effect of *Paecilomyces* fungal against the larval instars of important vector species and the lack of effect on non-target organisms, as well as the biological stability of extracellular metabolites, promising alternative to mycelium-and conidial based larvicides [80]. Likewise, Abutaha and others reported that, an endophytic fungus *Cochliobolus spicifer* against 3rd instar larvae of *Culex* and *Aedes* sp had great mortality effects similarly to what was observed in our current study [27].

To the best of our knowledge, this is the first information on hand for histopathological analysis of *A. tamarii* mycelium ethyl acetate extracts against targeted mosquitoes. In this study, histopathological alterations of treated mosquitoes were observed in the midgut including edema, swelling, and the elongation of epithelial cells. Moreover, cells protruding into the food lumen and lacking microvilli were also found in some areas. Recently the conducted study observed the entomopathogenic fungus *Beauveria bassiana* treated with 4th instar larvae of *Cx. pipiens* showed many histological alterations and malformations in body, tissues and affected the different parts of the body, especially the cuticle, adipose cells, and midgut region compared to untreated larvae [81]. Interestingly, Ragavendran et al. reported that the midgut cells of *Ae. aegypti* and *Cx. quinquefasciatus* (4th instar larvae) had enlargement in the gut lumen, decreased intercellular contents and degeneration of nuclei, after treating with *Penicillium daleae* mycelium metabolites [59, 82]. Similarly, Abutaha and other scientists reported the *Aspergillus sydowi* metabolites treated against *Ae. caspius* and the histopathology profile of larvae showed disruption of the peritrophic membrane, cytoplasmic vacuolization and deformities of cellular microvilli [27].

The biotoxicity assay of ethyl acetate extract of *A. tamarii* metabolites were tested on non-target organism *A. salina* was compared with control

Table 5. FT-IR analysis of mycelial ethyl acetate extract of *A. tamarii.*

Observed Wave numbers cm^{-1}	Peak Assignment	Vibration mode	Functional groups
3428.81	O-H Stretching	Strong	Alcohols(or)phenols
2926.45	C-H Stretching	Medium	Alkanes
2857.02	C-H Stretching	Medium	Alkanes
1632.45	N-H bending	Medium	Primary amines
1383.68	X=C=Y	Medium	Allenes (or)ketenes
1251.58	C-N- Stretching	Strong	Aromatic amines
1078.01	C-N- Stretching	Medium	Aliphatic amines
1039.44	C-N- Stretching	Medium	Aliphatic amines
619.038	C-H bending	Broad	Alkanes

groups, there was no death in untreated group. Recently, Ragavendran and his group studied the *Penicillium daleae* mycelium metabolite treated with *Artemia nauplii* and its showed morphological changes in body structures (i.e., intestine enlargement, eye formation, outer shell malformations and loss of antennae) [59]. The study done in Brazil reported the bioassays of the isolates and combinations of compounds from endophytic fungus [83]. Likewise, Diana et al. performed the *Fusarium* isolates (No: 24) showed various levels of toxicity on the brine shrimp, due to the presence of other toxins such as fusaproliferin and beauvericin compounds [84]. The zebrafish embryo is a best research model organism for toxicological studies of metabolites and nanoparticles due to its size, transparent, easy to maintain, quick embryogenesis and constant reproduction [85, 86]. The present study *A. tamarii* mycelia ethyl acetate extract tested with zebrafish embryo acute toxicity assay of after 96 hpf hatched embryo. We noticed the reduced body length, heart beat count, survival percentage and body length due to the doses of mycelia extract in the treated group of mosquitoes than control. Another study conducted isolated *Bacillus licheniformis* bio-surfactant tested on its toxicity using zebrafish embryo showed well developed healthy head, sculptured brain, yolk sac, tail and eyes [87]. Similarly, Abutaha and others reported the *Cochliobolus spicifer* endophytic fungal extract tested against zebrafish (*D. rerio*) embryo could not induce any malformations or deformities in the treated groups [27]. Likewise, a study conducted in china reported the camphor extract tested against *D. rerio* embryo, at higher concentration altered the embryo morphological deformities, delayed hatching, yolk sac edema, pigmentation and pericardial edema [88].

Esterases are the key enzymes that are responsible for the resistance mechanism against synthetic insecticides in mosquitoes [89]. *A. tamarii* metabolites treated with larvae of *Ae. aegypti* and *Cx. quinquefasciatus* resulted in the decreased level of acetylcholinesterase enzyme level than control [90]. The level of α- and β-carboxylesterase activity was gradually decreased during the development of the larvae tested after 48 h. Similarly, Lija-Escaline et al., reported the level of α- and β-carboxylesterase activity was significantly decreased during the development of the fourth instar larvae [91]. Exposure to metabolites had shown maximum inhibition of carboxylesterase activity of larvae. Likewise, Vasantha-Srinivasan et al. reported the level of α- and β-carboxylesterase was significantly altered by the activity of metabolites [92].

Three major - compounds were identified in the ethyl acetate extract of *A. tamarii* by GC-MS analysis and named as preg-4-en-3-one, 17.alpha.-hydroxy-17.beta.-cyano-Trans-3-Undecene-1,5-diyne pentane, 1,1,1,5-tetrachloro- pentane, 1,1,1,5-tetrachloro. Similarly, Abdel-Hady et al. reported the identification of some other compounds from the same extract of the *A. tamarii* fungus by GC-MS [25, 93]. Similarly, Kaul and others identified bioactive compounds extracted from *Periconia atropurpurea*, an endophyte from *Xylopia aromatica* [94]. The recent study in India has isolated bioactive secondary metabolites from the endophytic fungus *Pestalotiopsis neglecta* from leaves of *Cupressus torulosa* and its derived taxol compound showed various biological activities [95]. The functional group of identified compounds from *A. tamarii* mycelial extract was characterized using FTIR based on its peak values. The peak at 1251.58 cm^{-1}, 1078.01 cm^{-1} and 1039.44cm^{-1} represents the C–N stretching vibration of medium, aliphatic and aromatic compounds. The peak at 2926.45 $cm^{-1}$and 2857.02 cm^{-1} designated as the C–H stretching in alkanes group. The peak at 3428.81 cm^{-1} indicates the vibration mode form strong and O–H stretching the functional group is alcohols or phenols. Finally, two peaks at 1383.68 cm^{-1} and 619.038 cm^{-1} show the presence of X = C=Y and C–H bending the functional group in allenes and alkynes. The outcome of results were comparable with results of that found by Abuntaha and others who studied the IR analysis of *Cochliobolus specifer* revealed as a peak at 1745 cm^{-1} assigned to the C=O stretching vibration [27]. Likewise, another study reported that, the FTIR analysis of *A. tamarii* extract show peak value at 2925 cm^{-1} corresponds to the functional group of alcohols or phenols [96].

5. Conclusion

The present study have shown that, the isolated and identified *Aspergillus tamarii,* from the stem of *O. ficus-indica* showed a high larvicidal effect against mosquitoes. The efficacy shown could be promising stages to complement the existing tools in vector control toolbox for malaria vector. The outcome of present study suggests *A. tamarii* fungal extracts are more potent, selective, biodegradable and natural mosquito larvicidal agents and can be used as tools for the developing eco-friendly larvicides. Further, the fungal metabolites treated with zebrafish (*D. rerio*) embryos shown significantly reduced body length and heartbeat count due to the doses of mycelia extract compared to control groups.

Declarations

Author contribution statement

Kannan Baskar: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Ragavendran Chinnasamy, Karthika Pandy, Manigandan Venkatesan, Prakash Joy Sebastian, Murugesan Subban, Adelina Thomas: Performed the experiments; Wrote the paper.

Eliningaya J. Kweka: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Natarajan Devarajan: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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