Endophytic fungus *Alternaria macrospora*: A promising and eco-friendly source for controlling *Aedes aegypti* and its toxicity assessment on non-targeted organism, zebrafish (*Danio rerio*) embryos

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ABSTRACT

Fungal compounds are believed to be the most efficient method of addressing challenges with pesticide resistance and contamination of the environment. The present study was aimed to evaluate the larvicidal, pupicidal, ovicidal, histopathology profile, antibacterial, cytotoxic effects of cervical cancerous cells and its toxicity assessments using zebrafish embryo (*Danio rerio*). The isolates were confirmed by macroscopic, microscopic and PCR using a universal internal transcribed spacer primer for the molecular identification of the isolated fungus at the species level (GenBank - MN049541). The isolated fungus was first tested for its ability to produce toxic compounds in *Aedes aegpti* larvae in the 1-4th instar. For each instar of determined mosquito larvae, the secondary metabolites were evaluated in different bioassays at different test concentrations (100 to 500 µg/mL). The lethal concentrations of *A. macrospora* mycelial ethyl acetate extract that kill 50% of exposed larvae (LC_{50}) and 90% of exposed larvae (LC_{90}) were found in the first, second, third, and fourth instar larvae of *A. aegypti* (LC_{50} = 96.167, 131.370, 217.595, and 143.864 µg/mL and (LC_{90} = 395.16, 483.692, 882.545, and 655.722 µg/mL). After 120 h of exposure, the ovicidal bioassay's mean (%) was observed. A dose-dependent pattern was found in the enzymatic level of acetylcholinesterase in 4th instar larvae exposed to fungal mycelial metabolites. The treatment of 500 µg/mL of mycelia metabolites produced significant histopathological damage in the 4th instar mosquito larvae. The highest growth inhibition zones were observed during the antibacterial investigation of *Alternaria macrospora* metabolites against *E. coli* (22 mm) and *S. aureus* (20 mm). Also, the detected mycelium metabolites were tested for docking studies to identify their binding ability towards the *E. coli* FimH and *S. aureus* tyrosyl-RNA synthetase protein. The mycelium metabolites with different concentrations were further tested in the *in-vivo* zebrafish model to evaluate developmental toxicity. This is the first report on mosquito larvicidal and pupicidal activity of ethyl acetate metabolites produced by *A. macrospora* species. The study findings demonstrate that *A. macrospora* metabolites might be an ideal, cheap, small, and environmentally suitable source for controlling *A. aegypti* larvae.

Keywords: *Alternaria macrospora, Aedes aegypti*, Biopesticides, Endophytic fungus, Zebrafish embryo,

1. INTRODUCTION

Aedes aegypti is the principal vector of human infections such as Dengue, Chikungunya fever, and Zika virus (Lambrechts, 2023); this species is strongly related to human habitation, and some epidemic patterns of the diseases transmitted by the species are driven by seasonal variations that impact its growth, reproduction, and abundance (Pliego-Pliego et al., 2020). Due to population growth, arboviruses transmitted by *A. aegypti* have become much more widespread during the past 50 years (Ferreira et al., 2023). Dengue causes over 100 million symptomatic illnesses yearly and 10,000 deaths in over 125 countries (Messina et al., 2019). Reducing the vector population is the major approach to control because dengue or other illnesses caused by this vector may be managed (Pliego-Pliego et al., 2020). Generally, organophosphate, carbamate, and pyrethroid insecticides are suggested for use as traditional larvicides due to their low persistence in the environment (Khursheed et al., 2022). The most widely used larvicides are the organophosphorus substances, effective against mosquito larvae and other aquatic insects. Examples include temephos, fenthion, and chlorpyrifos (Okagu et al., 2023). Temephos is the preferred larvicide for controlling *A. aegypti* and *Anopheles* sp., whereas fenthion and chlorpyrifos are used to control *Culex* sp. species (Nithya et al., 2022). The use of synthetic chemical pesticides, insect growth regulators, and chemical repellents for vector control is increasing, and this leads to environmental contamination, adverse effects on non-targeted organisms, and the development of insecticide resistance in mosquito species (Vivekanandhan et al., 2022). This makes environmentally friendly, green pesticides necessary for the control of mosquitoes (Chatterjee et al., 2023). Researchers have found beneficial compounds from biological sources, such as microorganisms and plants, which contain substantial quantities of metabolites and essential oils. The development and research of natural larvicidal products exclusively addresses the utilization of larvicidal oils (Gupta & Gupta, 2022), plant extracts (Kumar et al., 2023; Álvarez Valverde et al., 2022; Ganesan et al., 2023); microbial larvicides act as Bacillus spp., secondary metabolites, or more expensive insect growth regulators as alternatives to synthetic pesticides.

Natural remedies produced from fungus or other biological sources are cost-effective, safe for the environment, and effective in addressing these issues (Narayanankutty et al., 2021). Natural products offer viable substitutes and potential supplies for several biological activities (Singab et al., 2022). Microbes are a prosperous provider of bioactive secondary metabolites and are an opportunity source of mosquito control agents like positive bacterial strains (*Bacillus thuringenesis var. israelensis (Bti)* and *B. sphaericus (Bs)* (da Silva Sa et al., 2023). These bacterial toxins are well-known for their effective against mosquito larvae at a minimal dose and are inert and harmless to non-targeted organisms (Patil et al., 2011). Finding effective and environmentally acceptable bio-larvicides is

essential for managing mosquitoes (John et al., 2022). Fungi-derived secondary metabolites can also be an alternative to artificially formulated pesticides, as they are comparatively cheap and biodegradable (Lorenzen & Anke, 1998). Numerous research has examined the potential application of entomopathogenic fungi genera *Metarhizium*, *Beauveria*, *Coelomomyces*, and *Culicinomyces* in the management of vector-borne illnesses(Kamareddine, 2012; Beltrán & Lozano, 2022).

Endophytes (endo-internal, phytes-flora) are microorganisms dwelling inside other organisms without inflicting any soreness (Elawady et al., 2023). Endophytic fungi asymptomatically live in the inner tissues of flowers, under the epidermis, colonizing the healthy dwelling tissues through quiescent infections (Lanfranco and Fiorilli et al., 2022; Sharma et al., 2022). The endophytic fungal species are hosted in approximately 300,000 terrestrial flowers, each one web hosting one or greater endophytic forms (Mohamed et al., 2023). The endophytic strains were distinct from one-of-a-kind varieties of flora: timber (yew, pine), fodders (clover, alfalfa, sorghum), vegetables, culmination, cereals, and other crops (Lu et al., 2012). They play a critical function in enhancing plant health, particularly as bio-control agents in suppressing plant pest groups like bugs and pathogens (Xiang et al., 2016). Endophytic fungi are known to possess antimicrobials and anticancer compounds like taxol furnishing factors of significance in agriculture, pharmaceutical, medicine, and nanotechnology (Tapwal et al., 2015; Santra & Banerjee, 2022; Joshi et al., 2017). These endophytes are well known for the production of biologically active secondary metabolites namely, terpenoids, alkaloids, steroids, isocoumarins, quinones, phenylpropanoids, lactones, lignans, and phenols (Santos et al., 2015). Endophytic fungal strains of *Pseudotaeniolina globosa* and *Penicillium purpurogenum* are said as larvicides (Seetharaman et al., 2017). *Trichoderma harzianum* is known to control tomato gray mold resulting from *Botrytis cinerea*, without fungicide resistance and is environmentally benign (Pan et al., 2016). The filamentous fungi possess a few advantages: clean to handle; require easy vitamins, have excessive wall-binding capability, and have intracellular metal uptake competence (Araújo et al., 2022).

Alarmingly, more individuals throughout worldwide suffer from health issues carried on by different cancers, microbes that are resistant to drugs, parasitic protozoans, and fungus (Abdelaziz et al., 2023). Drug-resistant diseases are becoming an urgent global issue (Jouda et al., 2016). The emergence of several drug-resistant bacteria increased the demand for the discovery of novel and potent antimicrobial drugs for the management of human diseases (Bacha et al., 2016). Endophytes are a unique source of potentially beneficial medicinal products in the urgent search for stronger and more powerful drugs to help with various diseases challenges (Xing et al., 2011; Toghueo, 2020). Endophytic fungi have the potential to be a source of secondary metabolites that may be used to develop new drugs, which makes them interesting from a biotechnological perspective (Sharma et al.

2016). Several investigations have demonstrated that plant endophytic fungi have antifungal and antibacterial properties (Li et al., 2014; Kalimuthu et al., 2022; Santra et al., 2022). *Escherichia coli* fimbria contain the bacterial adhesin protein FimH, which is responsible for attaching bacteria to their host cells. FimH is therefore important in the development of drugs because it plays a key role in bacterial infections such urinary tract infections (Magala et al., 2020). The production of charged tRNAs by aminoacyl-tRNA synthetases is an essential phase in the production of proteins. An amino acid is transported onto a corresponding tRNA to produce the desired product after being condensed with an ATP molecule to generate a stable aminoacyl-adenylate intermediate. Because the synthetases play such a crucial function, substances that selectively block bacterial aminoacyl-tRNA synthetases might become effective antibacterial drugs (Sahiba et al., 2022). For example, multidrug-resistant *S. aureus* can cause life-threatening infections that need to be addressed in a hospital.

One of the major causes of mortality in the entire world, cancer is on growing, with an alarming increase in the number of people who become diagnosed with different cancers each year. Due to various limitations, existing cancer drugs and treatments are associated with a high death rate (Elghaffar et al., 2022). Cervical cancer is caused mostly by persistent infection with particular strains of human papillomavirus (HPV) and affects women all over the world. The prevention, detection, and treatment of cervical cancer present difficulties in many nations, including India (Chauhan et al., 2020). In this context, eendophytic compounds were evaluated for cytotoxic and antibacterial activities in an attempt to find a safer and innovative medicine utilizing natural products (Katoch et al., 2017). Almost 72% of the extracts exhibited cytotoxic action against one of the investigated cell lines, whereas 39% of the extracts were potent versus all of the tested cell lines with >50% inhibition of growth (Hazalin et al., 2009; Vu et al., 2023). Because of their increased bio compatibility, easy absorption, and improved delivery of medicines at specified target sites.

Alternaria macrospora (Zinm.) is the most important phyto-pathogen responsible for *Alternaria* leaf spot disease in genetic cotton, with regional, national, and worldwide effects (Manpreet Kaur et al., 2015). *Alternaria* spp. are often regarded as pests of agriculture. It is either saprophytes or have developed to become effective phyto-pathogens that cause illnesses in a range of host plants (Thomma, 2003; Guo et al., 2004). More than 70 various kinds of chemicals/toxins are produced by different *Alternaria* sp. (EFSA, 2011; Sharma & Sharma, 2014). *Alternaria* sp. act as various biological properties such as insecticidal activity (Podova et al., 1977; Kaur et al., 2013), biocontrol agents (Ashutosh & Pati, 2011), antibacterial, antioxidant, antiviral and anticancer (Brase et al., 2009; Tsuge et al., 2013; Elghaffar et al., 2022). The *Trichillia* genus (belonging to the family Meliaceae), comprising about 419 plant species, possesses pharmacological and biological propensities (de la Torre-Anzures et al., 2023). *Trichillia connaroides* (Wight and Arn, 1962), a

previous nomenclature named *Heynea trijuga* Roxb, has been used as a traditional remedy in South-East Asia, India, and China (Cazal et al., 2023). It is a small evergreen tree widely distributed in South and East Asia including India, Indonesia, and South China. In recent years, enormous research has been related to the insecticidal and pharmacological efficacy of *Trichillia* sp*.,* which possess biological activities like insect antifeedant, insecticidal, and insect growth regulators, along with anti-microbial, anti-malarial, anti-cancers, antiviral and different pharmacologic uses (de Souza Passos et al., 2022; Kowa et al., 2022).

Additionally, potential compounds for the screening of novel insecticides for controlling *A. aegypti* larvae should exhibit significant efficacy and low hazard to non-target aquatic species. The zebrafish embryotoxicity test has been employed in drug screenings because it makes it feasible to rapidly assess the toxicity of a broad spectrum of compounds. The screening procedure for embryotoxicity used in the assessment of the toxicity of new substances is in accordance with the standard for the OECD 236 guideline and is conducted in multiple well plates (OECD, 2013). The zebrafish is an excellent animal model that is increasingly used for detecting the toxicity and safety of chemicals and for *in-vivo* chemical potency screening (He et al., 2014). Currently, zebrafish screens are used to examine a variety of environmental toxins, pharmaceutical agents, and compound databases over a range of life phases, transgenic and mutant lines, test dosages, and exposure times (Freitas et al., 2023). On the other hand, the zebrafish model shows promise as a practical alternative to the mammalian models presently employed in toxicity testing and assessment (Nishimura et al., 2015). Zebrafish are distinctive for their tiny size, optically clear embryos, quick embryonic development, low care cost, high fertility, and closeness to mammalian systems (Selderslaghs et al., 2013). Besides that, zebrafish larvae can be widely used for genetic and drug tests based on the entire organism (Mamboungou et al., 2022). Thus, we aim to isolate, molecularly characterize, and evaluate the mycelial metabolites of the endophytic fungus *Alternaria macrospora* from *Trichillia connaroides* to determine them against *A. aegypti* larvicidal, ovicidal activity and its estimation of acetylcholinesterase (AChE) activity. Additionally, to assess the ecotoxicological potential of the fungi metabolites produced by *A. macrospora* for aquatic species, we investigated their toxicity in zebrafish embryos (*Danio rerio*). The antimicrobial potential of the fungi metabolites is identified by their amino interaction with the *Escherichia coli* FimH protein and *Staphylococcus aureus* tyrosyl-RNA synthetase protein. On a vertebrate model, we suggest that an endophytic *A. macrospora* fungal extract might be used to control selected mosquito larvae with hard effects.

2. MATERIAL AND METHODS

2.1. Collection of plant material

Fresh leaves of *T. connaroides* were collected from the Botanical Survey of India (BSI) in Yercaud (Latitude*:* 11.7893931; Longitude: 78.216629), Salem (District), Tamil Nadu, India. Dr. D. Natarajan, Assistant Professor, Periyar University, Tamil Nadu, India, performed the taxonomy identification. The receipt for the specimen of the obtained sample was deposited with the Natural Drug Research Laboratory (NDRL), Periyar University, Salem, India (specimen number: PU/DBT/NDRL//2019/09). The collected plant samples were used to isolate endophytes.

2.2. Isolation of fungus from leaves

Fresh and healthy leaves (3-month-old, size-5cm long) of *T. connaroides* were collected and washed thoroughly in distilled water, following the method described by Talukdar et al., (2021). Surface sterilizing (ethanol) of leaves is commonly used in microbiology and plant science prior conducting studies to eliminate surface pollutants such as fungus, bacteria, and other microbes. Subsequently, the surface of each leaf was sterilized with ethanol 70% for 1 min, then a mixture of sodium hypochlorite (to 2%) for 30 min, and again rinsed with ethanol 70% for 30 s. For using sodium hypochlorite to remove any remaining bleach, rinse the leaves several times with sterile distilled water. After that, the leaves were washed three times with sterile distilled water. After surface sterilization, the plant material was dried on sterile blotting paper, and a manual method was used to prepare potato dextrose agar (PDA) (Hi-Media, Mumbai, PDA) medium containing the antibiotic chloramphenicol (at 0.02%). The leaves were chopped to expose the inner surface of the plant (0.5 mm in diameter), placed on a PDA plate, and incubated at 27 ± 2°C for 6 to 7 days. Every day for monitoring on the development of mycelia. Hyphal tips that were emerging from the leaf sections were put on PDA slants, sub cultured, and kept at 4 °C (Tolulope et al., 2015). Inoculated plates were incubated in the biological oxygen demand (B.O.D) at temperature 27 ± 2°C for up to 7 days to further analysis (Santra et al., 2022).

2.3. Morphology identification of *Trichilia connaroides*-4 (TC-4) fungus

The colony and spore's morphological features were examined to determine the TC-4 isolate. According to recognized identification guidelines, the isolates were identified based on their morphological and microscopic features (Cara et al., 2022; Zabouri et al., 2022). The purification procedures used a 5-day-old endophytic fungal culture to evaluate fungi morphology (Ismaiel et al., 2017). The PDA medium was employed for the morphological findings observe their colony surface, granular, powdery, mountainous, growth area, and concentric stripes (Sangeetha & Ashtaputre, 2015). A microscope with a 100× magnification was employed for the microscopic examination

(Lactophenol cotton blue stain (LPCB), Hi-Media, Mumbai) (Manan et al., 2017; Ranganathan & Mahalingam, 2019).

2.4. DNA extraction and polymerase chain reaction (PCR) amplification

Fungal isolates were grown from single conidia and verified by sequencing that the internal transcribed spacer (ITS) region was amplified using the universal primers ITS1 and ITS4 region of the ribosomal DNA. The isolates were grown on PDA for seven days at 28°C for DNA extraction. The mycelia were extracted, lyophilized, and then frozen in liquid nitrogen. The genomic DNA was extracted, according to Suneja et al. (2023). The NanoDrop spectrophotometer 2000 (Thermo Scientific) was used to measure the quantity and quality of the genomic DNA at 260 and 280 nm before the following step.

After that, the ITS region of the isolated DNA was amplified using a PCR (Khalil et al., 2021). Two ITS primers were used, i.e., ITS Primer Forward: *5'*TCCGTAGGTGAACCTGCGG3' and Reverse Primer: *5'* TCCTCCGCTTATTGATATGC 3'. The following were used in the amplification of 25 µL: dNTP to 0.2 mM, DreamTaq 1x buffer (including $MgCl_2$), ITS1 and ITS4 primers to 0.5 pmol, Taq polymerase to 0.125 U, 100 ng of DNA template, and 5.75 µL of NFW. Pre-denaturation at 95°C for 5 min was followed by 35 cycles of denaturation at 95°C for 15 s, annealing at 57°C for 15 s, and extension at 72°C for 45 s during DNA amplification. After 5 min of post-extension at 72°C and 5 min of chilling at 4°C, the PCR process was completed. The PCR products were observed by electrophoresis on 1% agarose gel dissolved in Tris acetate EDTA (TAE) solution. TAE buffer was used for the electrophoresis. First, 2 µL of the DNA sample and 2 µL of loading dye were mixed, and the mixture was then loaded into the agarose gel well. A 4 µL, 1.5 Kb ladder was then placed into one of the gel wells to serve as a marker well once introduced. A voltage of 110V was used to electrophorese the samples for 30 min. The gel was then immersed in ethidium bromide (EtBr) solution for 30 min, followed by a 5-min wash with distilled water. Finally, a UV transilluminator was used to view the electrophoresis products, like Kusumaningrum et al. (2014). Subsequently, the amplified region was purified and sequenced using the QIA Rapid PCR Purification Kit (QIAGEN). Endophyte ITS sequences were compared to a database cataloged in NCBI using BLAST, and phylogenetic relationships were performed by the CLUSTAL X software (Venkateswarulu et al., 2018). The 5.8S rDNA nucleotide sequence was edited with BioEdit software and aligned with CLUSTALW 2.0 software (Hall, 1999). Fourteen sequences were used in a phylogenetic analysis using the neighbor-joining method with 1,000 bootstrap replications based on the Kimura 2-parameter test of the MEGA 6.0 software, according to Tamura et al. (2011).

2.5. Extraction of secondary metabolites

A. macrospora was scale cultivated in a 1000 mL Erlenmeyer flask with 500 mL of PDB. The glass flasks were incubated for 15 days at pH 7.0 and 27 °C, which are the ideal conditions for growth. By using Whatman No. 1 filter paper and washing the fungus mycelial mat more than five times in distilled water, unfavorable broth components have been extracted from the medium. Mycelium was combined with ethyl acetate (500 mL) and transferred to 1000 mL glass beakers containing fungus mat (100 g) for cold extraction for 20 days at 27 °C ±2 °C. After full extraction, Whatman No. 1 filter paper was used to separate the liquid component from the mycelium. The metabolites were obtained by evaporating the solvent using a rotary vacuum evaporator (Hei-VAP series, Heidolph Instruments) at low temperature (40°C–50°C) and reduced pressure after filtration with sterilized cotton filter and Whatman no.1 filter paper, like Ragavendran et al. (2017) and Mahmud et al. (2020).

2.6. Collection of mosquitoes

At the developmental stage, the first to fourth instar larvae, pupae, and eggs of *A. aegypti* were collected from the Indian Council of Medical Research-Centre for Research in Medical Entomology in Madurai, Tamil Nadu, India. The collected larvae, pupae, and eggs were maintained in deionized water at 25 ± 2°C with a photoperiod of 10/14 h day/night. The food contains pedigree biscuits, sucrose, and brewing yeast in a 4:1:1 ratio and is kept at room temperature for 24 h before the start of the experiment, according to WHO (2005).

2.7. 4th instar larvae of the *A. aegypti* metabolite (500µg/mL) based behavioral study

In a larvicidal bioassay, the larvae were closely examined for behavioural alterations (n=300/groups), such as increased wiggling movement, vertical and horizontal activity, and self-biting behaviors. Using a Nikon D-SLR digital camera (Nikon Inc., Japan), indications of larval behaviour were noted and captured on camera. In a similar manner, control as well as treated larvae were both recorded and compared (da Silva et al., 2013).

2.8. Larvicidal and pupicidal bioassays

The experiments, including larval (1st - 4th instars) and pupal stages, were used for larvicidal and pupicidal bioassays according to WHO (1981) protocol with minor modifications. Briefly, fungal metabolites (at 100, 200, 300, 400, and 500 µg/mL) were prepared. Each test larvae and pupae were placed in a 200 mL beaker filled with distilled water. The test solution was dissolved in 1 mL of dimethyl sulfoxide (10% DMSO) and tested against 1st to 4th instar larvae and pupae. The number of dead larvae was counted in 24 h and pupae in 48 h, and the mortality rate was calculated using Abbott's formula (1925) (Equations 1 and 2). A negative control group (10% with distilled water, no

addition of fungal metabolites) was included in each bioassay, and for each concentration, we performed tests five times.

$$\text{Corrected mortality} = \frac{(\text{Observed mortality in treatment} - \text{Observed mortality in control})}{100 - \text{Control mortality}} \times 100 \quad \text{Eq. 1}$$

$$\%\ \text{mortality} = \frac{\text{Number of dead larvae}}{\text{Number of larvae introduced}} \times 100 \quad \text{Eq. 2}$$

2.9. Ovicidal bioassay

The ovicidal activity was determined by measuring the inhibition of egg hatchability. *A. aegypti* oocytes collected from laboratory colonies were cultured in room conditions at 27 ± 2°C. The newly laid eggs were observed with a stereomicroscope to evaluate the survival rate. Approximately 30 viable eggs were collected and exposed to various concentrations (range 100 to 500µg/mL) of mycelial metabolites. The test was repeated five times, and the negative controls were kept separately. Results were recorded by observing egg hatchability 72 h after treatment (Pineda-Cortel et al., 2019; Deepak et al., 2019). Abbott's formula was used to correct the control's zero mortality (Abbott, 1925) (Equation 3).

$$\text{Ovicidal activity} = \frac{\text{Number of unhatched eggs}}{\text{Total number of eggs introduced}} \times 100 \quad \text{Eq. 3}$$

2.10. Acetylcholinesterase assay

To evaluate the possible cholinesterasic effect of the treatments, the total body homogenates of 50th 4th instar *A. aegypti* larvae (100 µL) were spectrophotometrically examined using the Ellman and others (1961) procedure with acetylchiocholine iodide as a substrate (Dahran et al., 2023). *A. aegypti* 4th -instar larvae for testing were extracted with a dropper container at first. Deionized water was used to wash the larvae twice, and filter paper was used to wipe the water off their surfaces. Second, a 1.5 mL centrifuge tube was used to hold the larvae. A crushing rod was then used to mill 0.6 mL of ice-cold, 0.1 M PBS buffer (pH 8.0) in an ice bath for 30 s. Third, using an ultrasonic crusher, the larvae were subjected to ultrasound 20 times at an ambient temperature. In order to remove the remaining pieces of larval tissue, the tube was centrifuged at 4000 rpm for 15 min at 4 °C, and the supernatant was collected to produce the AchE solution that would be evaluated. To determine the total AChE activity in the presence or absence of the mass per unit volume inhibitor,

the concentration of AChE was carried out in two distinct 96-well plates. 25 µL of homogenates were added to 145 µL of Triton/Na phosphate (5 µL of 100% Triton X-100 in 50 µL of 1M sodium phosphate buffer at pH 7.8 and 455 µL of distilled water) and 10 µL of DTNB/Na phosphate (10 mM DTNB in 100 mM sodium phosphate buffer at pH 7.0) and kept in triplicates. All plates were placed at room temperature, with light protection, and spectrophotometric scans for 1 h (at 405 nm).

$$\text{AchE activity inhibition (\%)} = \frac{(\text{Acontrol} - \text{Asample})/\text{Acontrol})}{\text{Acontrol} - \text{Asample} = \text{O. D of control sample}} \text{x100}$$

2.11. Histopathological study

A histopathological analysis of a mosquito sample was performed to detect possible changes in morphological characteristics induced by the reaction of fungal metabolites. The test mosquito's 4th instar larvae (n=20/group) were treated with a metabolite treatment, while the control and treated larvae were fixed with 10% formalin. The tissues were then fixed in paraplast, sectioned (5 µm thick), and dehydrated using an ethyl alcohol series before being cleaned in xylene. Hematoxylin and eosin (H&E staining) were done using the usual section staining method, like Khalifa et al. (2022). Finally, a Labomed light microscope (LX-200) was used to study the midgut region of control and treated larvae (at a magnification of around 40×), and images were taken (Yamany et al., 2022).

2.12. Antibacterial activity

The bacterial strains *Bacillus subtilis* (MTCC 441), *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), and *Salmonella typhi* (MTCC 98) were obtained from the Department of Microbiology at Mohan Kumaramangalam Medical College in Salem, Tamil Nadu, India (Natarajan et al., 2014). All isolates were maintained on a nutrient agar medium (NA). The antibacterial activity of the extracted bioactive metabolites was evaluated by agar well diffusion method, like Maliehe et al. (2022). Each bacterial strain was swabbed evenly onto the individual plates using sterilized cotton swabs. On Mueller Hinton agar (MHA, Hi-Media, Mumbai) plates, 6 mm-diameter wells were produced using gel puncture, and four concentrations (25, 50, 75, and 100 µg/mL) of fungal mycelium metabolites were loaded on the well. The plates were not disturbed for 1 h to achieve uniform diffusion of substances into the agar. The plates were incubated for 24 h at 37°C. Using an antibiotic zone scale, the diameter of the inhibition zone around the wells was measured and calculated in millimeters (mm). Negative (10% DMSO to 20µL, v/v) and positive control (ciprofloxacin to 4 µg/mL). Negative growth zones were only assessed after 24 h to prevent false findings. Each experiment was performed three times, like Sharma et al. (2016).

2.13. Cytotoxicity effects on human cervical cancer cells

HeLa (human cervical cancer) cell strain was obtained from National Centre for Cell Sciences (NCCS) (Pune University, India) and grown in the Eagle's minimal essential medium (EMEM) containing 10% of fetal bovine serum (FBS). Cell cultures were maintained weekly at 5% CO_2, 37°C, 95% air, and 100% relative humidity (RH), and the medium was replenished twice every seven days. Monolayer cells were exfoliated with trypsin-EDTA to prepare a single-cell suspension, and the viable cells were counted on a hemacytometer. The medium was diluted to a final density of 1×10^5 cells/mL using FBS to 5%. After seeding 100 µL of cell suspension per well in 96-well plates with a plating density of 10,000 cells/well, the plates were incubated at 37°C, 5% CO2, 95% air, and 100% RH for cell adhesion. After 24 h, the cells were treated with various concentrations of the mycelial extract. First, they were dissolved in neat DMSO (10%), then diluting the sample aliquots in serum-free medium to twice the desired maximum concentration. Additionally, serial dilutions of up to 4-fold were performed for five concentrations. Aliquots (100 µL) of these variable dilutions were added to the appropriate wells containing 100 µL medium, and the final concentrations required were totaled. After adding the sample, the plates were incubated at 37°C, 5% CO_2, 95% air, and 100% RH for two days. 10% DMSO should be included as control. The sample-free medium served as a control. Chemical 3-[4, 5-Dimethylthiazole-2-yl] 2,5-diphenyltetrazolium bromide (MTT) cleaves the tetrazolium ring with the enzyme succinate dehydrogenase and converts it to insoluble purple formazan. The amount of formazan produced is directly proportional to the number of viable cells, so the reaction was analyzed. After two days of incubation, MTT (15 µL to 5 mg/mL) in phosphate-buffered saline (PBS) (pH 7.4) was added to each well and incubated at 37°C for 4 h. The formed formazan crystals were solubilized in 100 µL DMSO (100%), and the absorbance was measured at 570 nm. Cell viability was calculated using the following formula (Equation 4):

Viability % = Test OD-Control OD/100 Eq. 4

2.14. Gas chromatography and mass spectroscopy (GC-MS) analysis

The GC–MS analysis of fungal mycelial metabolites was performed in electron ionization (EI) mode on GC–MS Perkin Elmer model 680 system (Switzerland). The initial temperature was set up at 70°C for 2 min; oven temperature at 280°C (at the rate of an increase of 5°C/min) was maintained for 9 min. The following were the GC-MS technique operating conditions: TR 5-MS standard non-polar capillary column, 30 Mts, 0.25 mm ID, 0.25 m Film thickness. The mobile phase flow rate (carrier gas: Helium) was fixed at 1.0 mL/min. The temperature program (oven temperature) for the

gas chromatography portion was 40°C elevated to 250°C at 5°C/min. The ionization voltage was kept at 70 eV, and the samples were injected (1µL) in split mode (10:1). The mass spectrum was obtained from 40 to 1000 a.m.u, like Ragavendran & Natarajan (2015). The compounds were identified by comparing their retention time (RT) and mass spectrum using NIST-2008 library database TurboMass ver. 5.8.9.

2.15. Fourier-transformed infrared spectroscopy (FT-IR) analysis

FT-IR spectroscopic studies were performed using an FT-IR (Shimadzu IR Affinity) spectrophotometer with a scanning range of (4000-450 cm^{-1}). The disc was made by mixing potassium bromide (KBr) with fungal mycelial metabolites in a 10:90 weight-to-volume ratio. Clean, dry nitrogen gas was used to clean the detector, increasing the signal level, and removing moisture. The spectrum was then measured once the discs were placed into the spectrophotometer. The online spectroscopic analysis examined the FT-IR spectra, like Rajalakshmi & Mahesh (2014).

2.16. Zebrafish embryo toxicity analysis

The zebrafish embryo/larvae were exposed to different concentrations of mycelium extract (250 µg/mL to 2 mg/mL) to evaluate its toxic range. The exposure period was calculated from 24 hours post fertilization (hpf) to 96 hpf. The survival rate was calculated as the total number of embryos/larvae that survived during the exposure period. The malformation parameters such as scoliosis, yolk sac edema (YSE), and bend tail (BT) were registered, like Malafaia et al. (2020). The heart rate was recorded at 96 hpf and expressed as beat per min. The hatching rate was calculated at the end of 48 hpf, according to Guru et al. (2023) and Priya et al. (2023). The experimental designs in this research article were in accordance with ethical guidelines and approved by Periyar University – Institutional Animal Ethics Committee (IAEC approval No.: PU-IAEC/2018/M1/04).

2.17. *In-silico* toxicity prediction and Autodock studies

The compound identified from the mycelium metabolites was investigated for the computational toxicity analysis using ProTox-II online server (https://tox-new.charite.de/protox_II/). Toxicity concentrations and toxicity classes for particular compounds can be predicted through this tool. The molecular docking studies for the compounds with the target receptor (*Escherichia coli* FimH protein and *Staphylococcus aureus* tyrosyl-RNA synthetase protein) was performed using the Autodock software version 1.5.6. The targeted site of protein was downloaded from the protein data bank (https://www.rcsb.org/). The amino acid interactions between the ligand and receptor were visualized using the Discovery studio visualization (Velayutham et al., 2022).

2.18. Statistical analysis

Data were analyzed using SPSS statistical software (20.0 version). The LC_{50} and LC_{90} values were calculated by Probit analysis with their lower and upper confidence levels (LCL and UCL). The level of significance (p-value) was fixed at $p \leq 0.05$. Probit analysis was used to calculate the median (LC_{50}) and maximum (LC_{90}) lethal concentrations (LCs) limits from this data (Finney, 1971).

3. RESULTS

3.1. Morphology identification of endophytic fungi from *T. connaroides* leaves

The seven-day old isolates of *Alternaria* culture grown on PDA plates were used to study the morphological characters. The colonies of *A. macrospora* were grayish green in color and white with septal hyaline and branched mycelium, solitary, dark brown, straight or slightly curved, oval conidia, with a long-tapered beak. *Alternaria* sp. is based on the colony's diameters and lactophenol cotton blue stain endophytic fungus shown in Figure 1A. Molecular screening provided additional confirmation of this at the 5.8s rDNA species level. According to Venkatesh and Darvin (2016), the morphological features of *A. macrospora* produced results comparable to those of the present study. Researchers reported that conidia measured between 43.4 x 13.3 m and had 1 to 8 transverse septa (Saleem and El-Shahir, 2022).

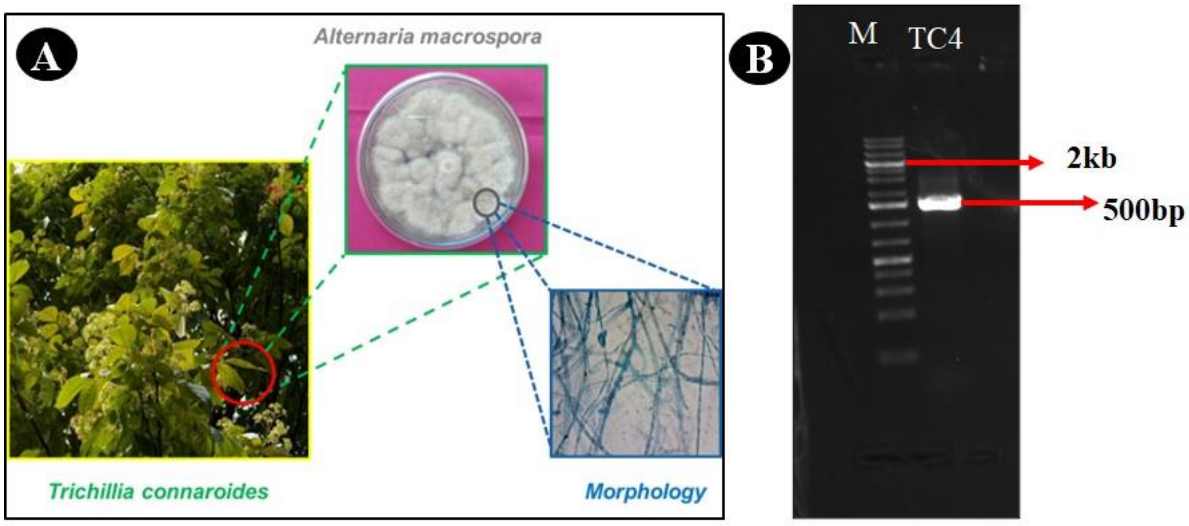

Figure 1. (A) Photography of the plant *Trichillia connaroides*, the fungal species *Alternaria macrospora* isolated and its microscopic view showing morphological characteristics. (B) PCR amplification of isolated genomic DNA at ITS regions (ITS 1 and ITS 4).

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458 3.2. **Molecular identification of isolated culture**

459 The isolated DNA sample was amplified in optimized condition using the universal primer,
460 and the rDNA fragments were shown in Figure 1B. The obtained amplification product was located
461 in the expected ITS region with exactly 552 bp. The obtained RNA sequence of the isolated coding
462 5.8S rDNA gene was deposited in GenBank (NCBI), with accession number of MN049541.
463 Subsequently, based on 5.8S rDNA sequence analysis, the strain showed 99% similarity to the earlier
464 reported *Alternaria* sp. sequence. The phylogenetic tree analysis involved 15 fungal ITS sequences,
465 and phylogenetic tree analyzes were performed in MEGA 11. The ideal tree with the sum of the
466 length of the division = 1.99. The evolutionary history of the isolates interfered with the Neighbour-
467 Joining tree method is shown in Figure 2. The relationship between the taxa of the endophytic fungal
468 isolates and the reference taxa retrieved from GenBank has been shown. The percentage of replicate
469 trees in the bootstrap test (1000 repetitions) where the related taxa grouped were displayed above the
470 branches and the clades established were supported by bootstrap values >50%. The completed
471 database comprises 656 locations. MEGA 11 was used to perform evolutionary studies.

472

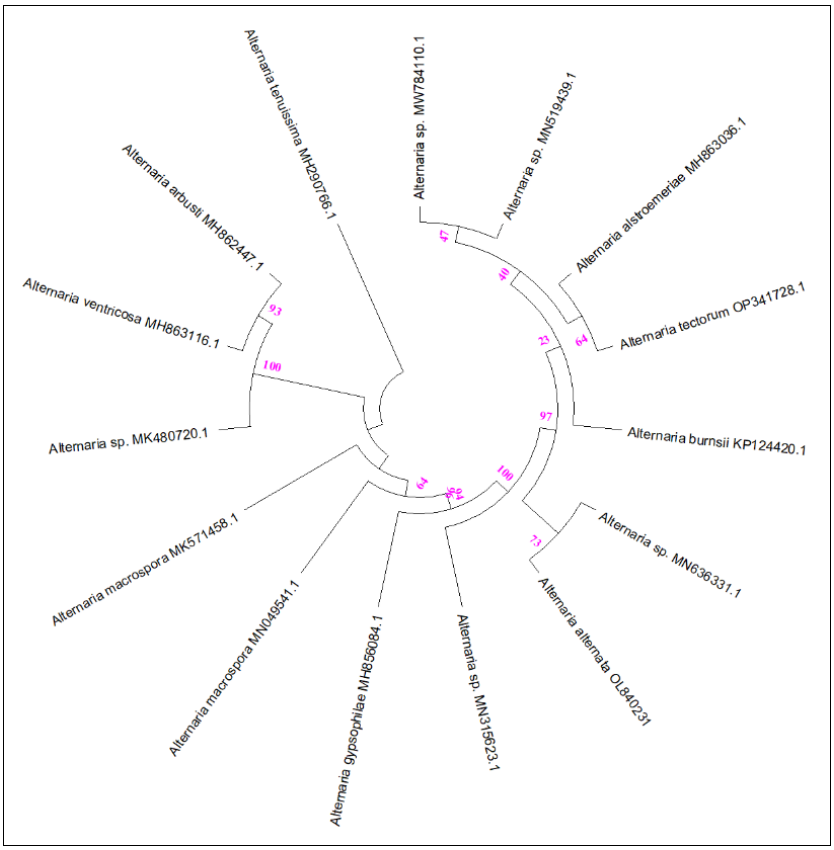

Figure 2. Phylogenetic tree analysis of *A. macrospora*. The percentage of replicates trees in which the associated taxa clustered together in the bootstrap test (1000

replicates) is observed next to the branches.

3.3. Larvicidal activity using endophytic fungi *A. macrospora*

Fungi and fungus-derived compounds are highly toxic to mosquitos but have low toxicity to non-target organisms. Endophytic fungus *A. macrospora* mycelial metabolite was tested for larvicidal activity against 1st - 4th instar larval stages of *Ae. aegypti*. The fungal metabolites exhibited superior toxicity against 1st and 2nd instar larvae of *A. aegypti* after 24 h exposure and reported 99% larval mortality. LC_{50} values of 1st to 4th instar larvae were found to be "96.167, 131.370, 217.595, and 143.864 µg/mL" and "395.16, 483.62, 882.545 and 655.722 µg/mL" (Table 1). Also, the metabolite-treated 1st and 2nd instar larvae revealed a distinct effect at the maximum concentration (500 µg/mL). At the concentration of less than 200 µg/mL from *A. macrospora* mycelial metabolites, mortality rates were slower, but larvae became very sluggish in movement compared to unexposed larvae (control). More than 50% of larval deaths were observed within 8 h. Similarly, in the 3rd and 4th instar larvae, the larval growth and development were arrested and affected. Among larval stages, concentration-dependent growth inhibition was found to be in the order: 4th instar > 3rd instar > 2nd instar > 1st instar.

487 **Table 1.** Larvicidal and pupicidal effects of the *A. macrospora* mycelium metabolites against *A. aegypti*

Larva stages	Concentrations (µg/mL)	Mortality (%) ± SD	LC_{50} (µg/mL) (LCL-UCL)	LC_{90} (µg/mL) (LCL-UCL)	χ^2	Significance
1st instar	Control	0.0±0.0	96.167 (46.786 –140.616)	395.16 (289.20 -635.091)	4.976	0.970
	100	56.67±0.57				
	200	76.67±0.57				
	300	90.0±0.00				
	400	100.0±0.00				
	500	100.0±0.00				
2nd instar	Control	0.0±0.0	131.370 (80.182 –177.849)	483.692 (363.124 - 753.16)	7.973	0.951
	100	46.66±0.57				
	200	66.67±0.57				
	300	83.33±0.57				
	400	100.0±0.00				
	500	100.0±0.00				
3rd instar	Control	0.00±0.00	217.595 (143.640 –288.134)	882.545 (622.50 – 1660.814)	12.73	0.928
	100	36.66±0.57				
	200	53.3±0.57				
	300	76.67±0.57				
	400	93.3±0.57				

	500	100.0±0.00				
4th instar	Control	0.00±0.00	143.864 (82.494 – 199.248)	655.722 (471.998 –1154.365	10.24	0.936
	100	40.0±0.47				
	200	56.67±0.55				
	300	73.3±0.57				
	400	96.67±0.57				
	500	100.0±0.00				
Pupae	Control	0.00±0.00	234.390 (165.957 – 301.777)	739.362 (547.803-1222.998)	14.381	0.954
	100	33.3±0.57				
	200	46.67±0.47				
	300	56.67±0.00				
	400	100.0±0.00				
	500	100.0±0.00				

Note - LC_{50}: lethal concentration required to kill 50% of the population exposed, LC_{90}: lethal concentration required to kill 90% of the population exposed larvae, χ^2 = chi square and SD: standard deviation.

3.4. Pupicidal and ovicidal activity

In pupicidal activity, the percentage of mortality was calculated at different concentrations of mycelia extracts from the endophytic fungus *A. macrospora* against *A. aegypti* (Table 1). The observed LC_{50} and LC_{90} values were 234.390 and 739.362 µg/mL, respectively. The mortality rate was calculated within the first 5 h of exposure, which exhibited 60% mortality. A 100% mortality in the pupae was reported after 48 h. The χ^2 value was significant at $p \leq 0.05$ level. Table 2 shows the percentage of egg hatchability of *A. aegypti* subjected to five different dosages of an *A. macrospora* mycelia metabolite. This exposure had an ovicidal effect, indicating that it affected the capacity of mosquito eggs to develop. The table most likely depicts the metabolite's efficiency in inhibiting the hatching of *A. aegypti* eggs at various doses. The results can be displayed as a dose-response relationship, showing exactly the concentration of the metabolite interacts with the reduction in egg hatchability. No hatchability (100% mortality) was recorded at 500 µg/mL compared to the control group (hatchability rate 96.8 to 100%). Also, LC_{50} and LC_{90} values were calculated as 36.080 and 204.422 µg/mL, respectively.

Table 2. Ovicidal activity of *A. macrospora* endophytic mycelium metabolites against *A. aegypti* eggs.

Concentrations (µg/mL)	Hatchability (%) ± SD	LC_{50} (µg/mL) (LCL-UCL)	LC_{90} (µg/mL) (LCL-UCL)	χ^2	Significance
Control	96.66±1.2	36.080 (2.390 – 77.706)	204.422 (110.541-355.100)	4.422	0.986
100	73.33±0.00				
200	56.66±0.00				
300	43.33±0.57				
400	23.33±0.57				
500	NH				

Note – NH: no hatchability, LC_{50}: lethal concentration required to kill 50% of the population exposed, LC_{90}: lethal concentration required to kill 90% of the population exposed larvae, χ^2 = chi square and SD: standard deviation.

3.5. Behavioural studies

The *A. macrospora* metabolites induced behavioral toxicity on the 4th instar larval stage of *A. aegypti*. The control larval set showed normal behavior in contrast to the

vigorous and restless movement of larvae noticed after 25 min exposure to metabolites. With increased exposure time, the larvae exhibited altered movements, abnormal excitation and restlessness, and forceful self-nibbling of mouthparts and anal papillae, leading to ring-shaped structures. Several larvae exhibited up-down wriggling movements, vibrating movements, and paralytic symptoms (Figure 3).

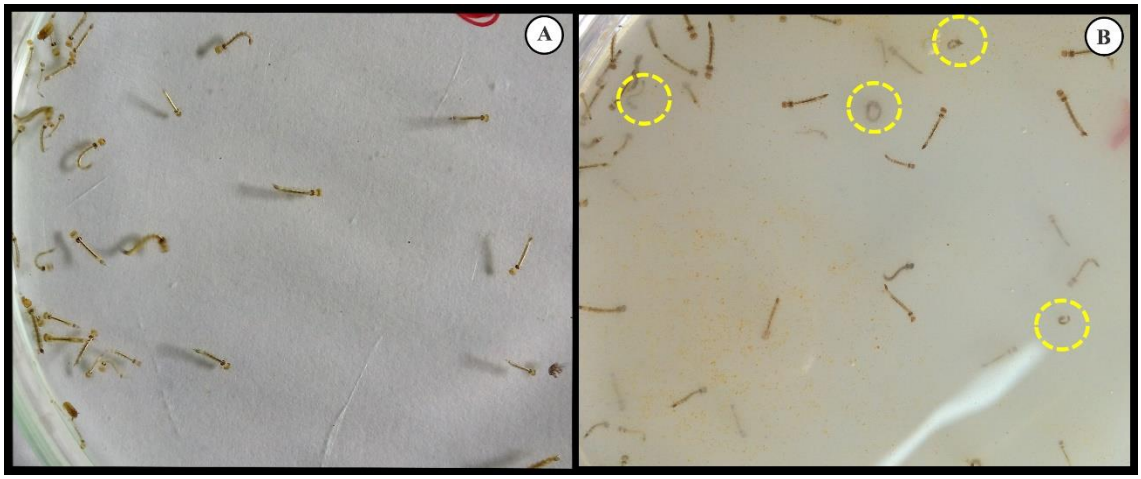

Figure 3. Effectiveness of an ethyl acetate extract of *A. macrospora* as a larvicidal agent against *A. aegypti* after 24 h of exposure. A). Larvae in control; B). Treated larvae (500 µg/mL). Self-biting larvae are indicated by a yellow circle.

3.5. Inhibition of AChE activity analysis

The AChE activity was significantly decreased at fungal mycelial metabolite concentrations of 100 µg/mL, and the highest inhibition was noticed at 500 µg/mL. The enzyme inhibition activity results clearly supported the metabolites' concentration-dependent (Figure 4A). The direction at which the PROBIT plot for A. *macrospora* slopes. The slope of the PROBIT plot is an important factor to consider into account because it exhibits higher effects, i.e., maximum inhibition, at low concentrations, as shown in Figure 4B, due to an elevated slope. However, the high linearity of the fit (r-value= -0.986, R^2 = 0.9721 & p-value ≤ 0.0001) reflects a strong correlation in the concentration dependent inhibition of AChE activity.

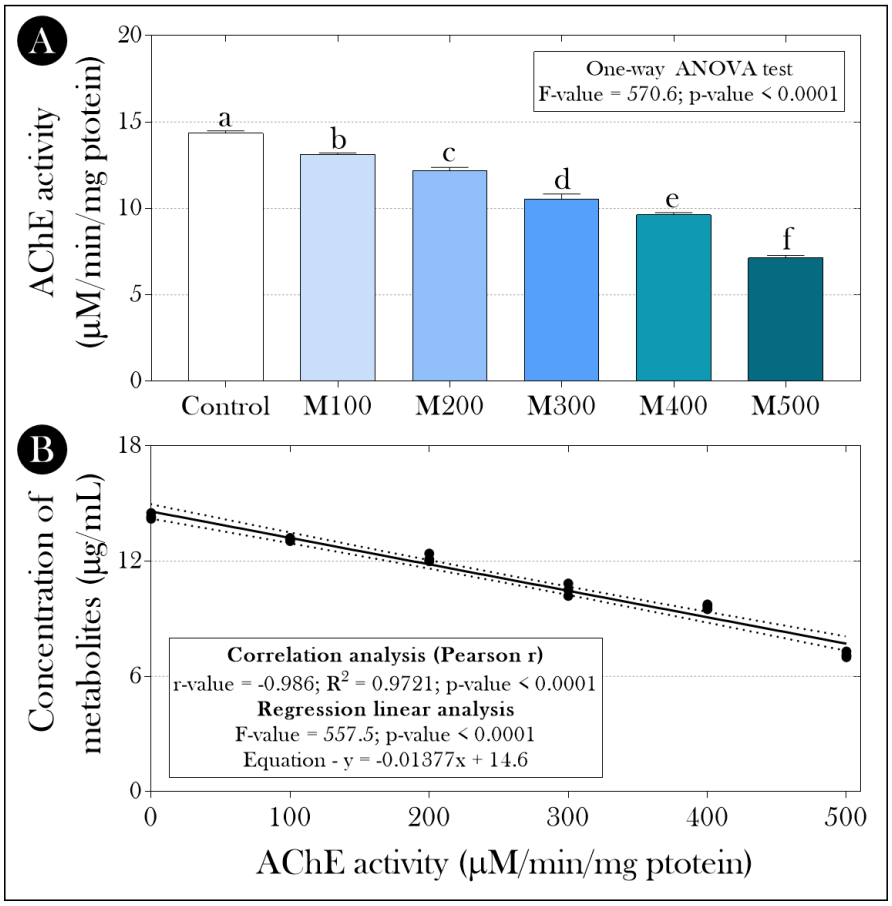

Figure 4. Acetylcholinesterase (AChE) activity in 4th instar *A. aegypti* larvae unexposed or exposed to different concentrations of the endophytic *A. macrospora* metabolites (M). (B) Linear correlation and linear regression analyze (concentrations of fungal metabolites vs. AChE activity). M100 to M500: groups of 4th instar *A. aegypti* larvae exposed to 100 to 500 µg/L of fungal metabolites. Parametric data are presented by the mean + standard deviation. Distinct lowercase letters indicate significant differences between experimental groups. The summaries of the statistical analyzes are displayed next to the graphs.

3.6. Histopathology analysis

The midgut architecture of *A. aegypti* larvae changed after exposure to the fungus mycelium metabolite. When larvae were exposed to mycelium metabolite, it resulted in the lysis of the midgut epithelium layer, vacuolization and significant destruction of the peritrophic membrane, loss, and degeneration of epithelial cells, gastrointestinal tract, midgut, cortex with hyperplasia of gut epithelial cells, ruptured brush border, damaged membranes, and microvilli (Figure 5B). Cellular organelles were severely affected even at a low dose (500µg/mL) treatment with *A. macrospora* metabolites. The control group, the

midgut of the *A. aegypti* larvae had a normal shape; the FB (food bolus), a single layer of digestive cells with well-developed brush borders, epithelial layer, microvilli, cell membranes, and cytoplasm areas in the midgut epithelium (Figure 5A).

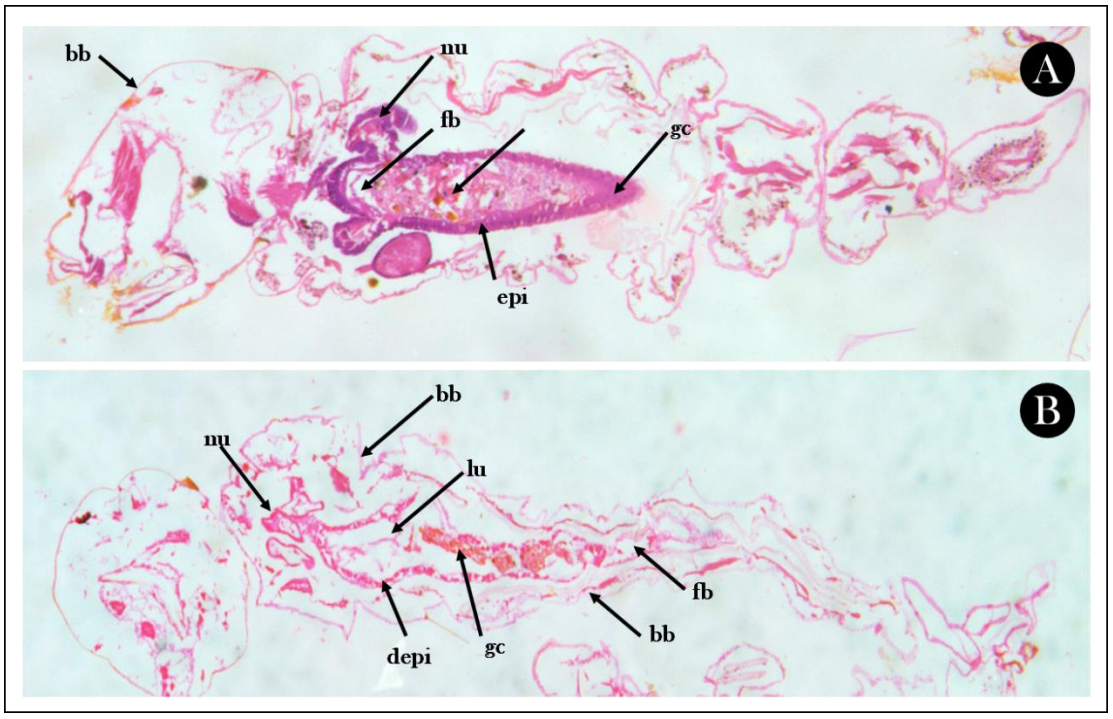

Figure 5. Histopathological profiles of control (A) and (B) 4th instar larvae of *A. aegypti* treated at 500µg/mL concentration of *A. macrospora* metabolite. bb: brush border; nu: nucleus; fb: food bolus; epi: epithelial cells; lu: lumen; depi: degenerative epithelial cells; gc: gastric caeca.

3.7. Antibacterial activity

The antibacterial activity of mycelium metabolites was evaluated against four pathogenic bacterial strains, namely *S. aureus, E. coli, S. typhi,* and *B. subtilis,* with four concentrations (25, 50, 75, and 100 µg/mL). Ciprofloxacin is a standard antibiotic to compare the results with the zone obtained using mycelial metabolites. The maximum inhibitory zone was observed in *E. coli* (22 mm), followed by *S. aureus* (20 mm), *B. subtilis* (19 mm), and an average inhibition zone in *S. typhi* at 100 µg/mL (Figures 7A-E). It shows that the antibacterial activity of mycelial metabolites strongly depends on the concentration of mycelial metabolites present in the reaction mixture, as shown in Figure 6A-D. No inhibition zone was observed in the negative control (DMSO to 10%).

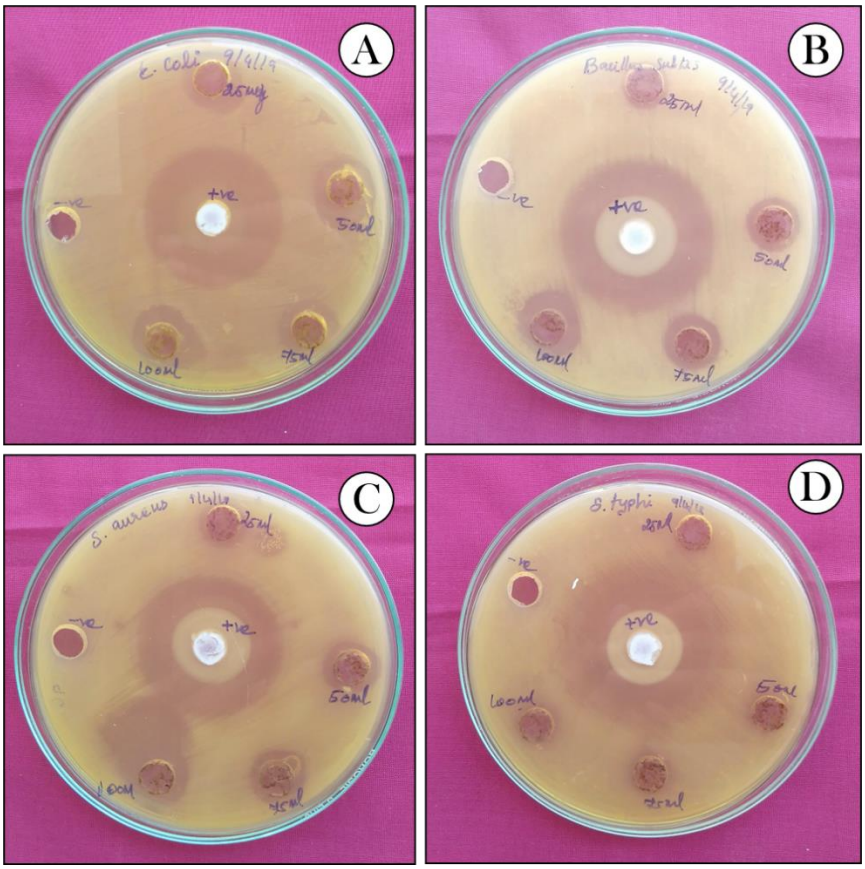

Figure 6. Antibacterial bioassay by agar well diffusion method. A) *E. coli*, B) *B. subtilis*, C) *S. aureus*, D) *S. typhi* zone of inhibition plates. -ve: negative control (only 10% DMSO); +ve: positive control (ciprofloxacin-4 µg/mL); Different concentration of *A. macrospora* metabolites loaded on four different well such as 25, 50, 75 and 100 µg/well.

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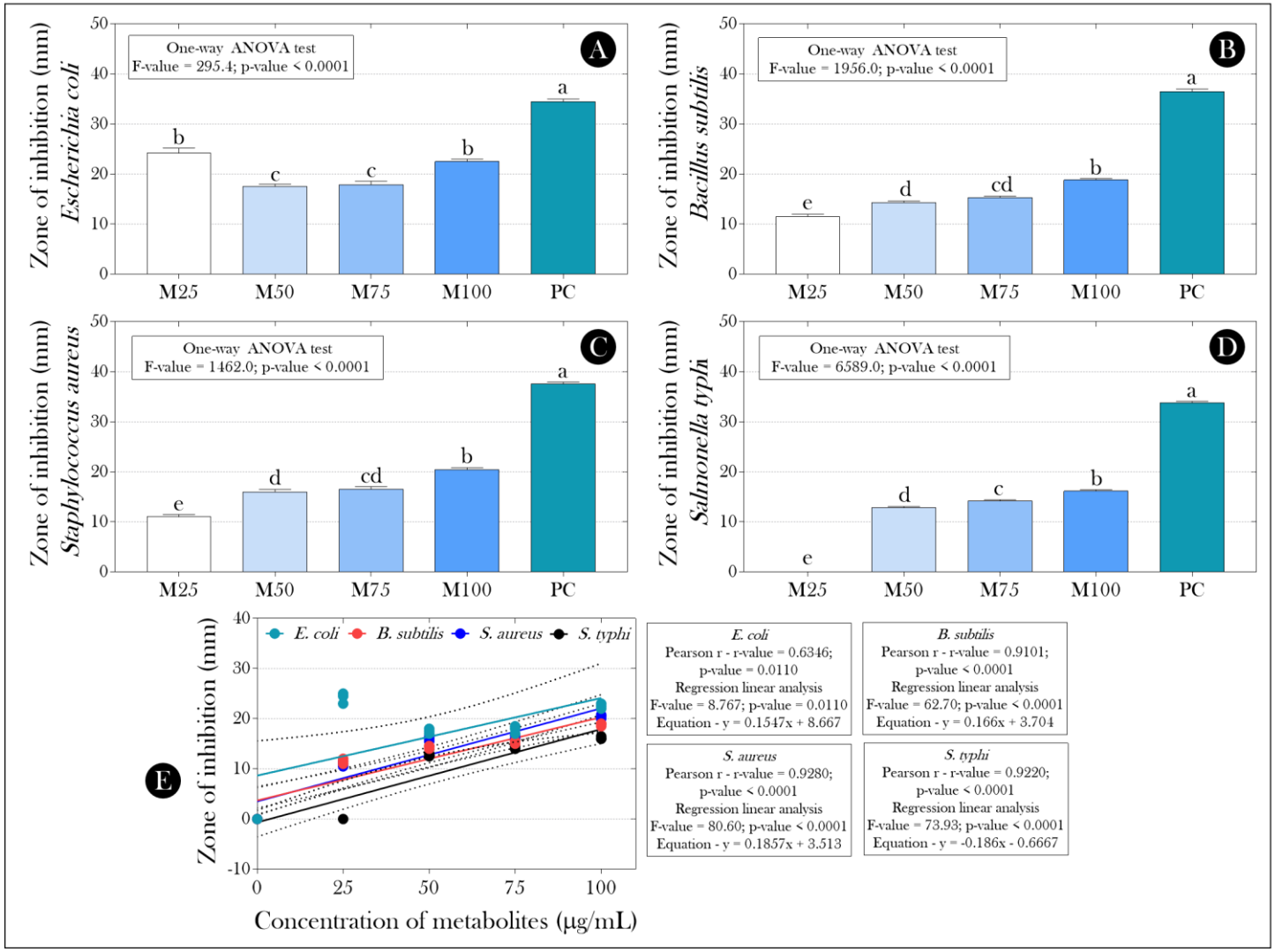

Figure 7. Antibacterial activity (zone of inhibition) of mycelium metabolites evaluated against (A) *Escherichia coli,* (B) *Bacillus subtilis*, (C) *Staphylococcus aureus*, and (D) *Salmonella typhi* exposed to four concentrations (25 to 100 µg/mL). (E) Correlation and linear regression analyze (concentrations of fungal metabolites vs. zone of inhibition). Parametric data are presented by the mean + standard deviation. Distinct lowercase letters indicate significant differences

between experimental groups. The summaries of the statistical analyzes are displayed next to the graphs.

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3.8. Cytotoxic effects on HeLa cells

The cytotoxic activity of fungal endophytes was tested by MTT assay on the HeLa cell line. Cells were exposed to variable concentrations of fungal metabolites (50-300 µg/mL), and changes in cell shape were observed under an inverted microscope. The IC_{50} value of *A. macrospora* mycelial metabolites was 118.49 µg/mL. When the concentration of mycelial metabolites was increased to 18.75, 37.75, 75, 150, and 300 µg/mL, the percentage of cell inhibition was increased to 10.38 + 1.5, 21.64 + 1.0, 37.66 + 0.5, 56.27 + 1.0 and 68.18 + 1.0%, after 24 h of incubation, evidencing a concentration-dependent effect (Figure 8A-B). In the present work, the morphological alterations of the normal cells were usually short and round. Still, the exposed cells exhibited noticeable morphological changes like cell shrinkage, decreased cell volume, membrane blebbing, loss of adhesion, and apoptotic body formation (Figure 8C-H).

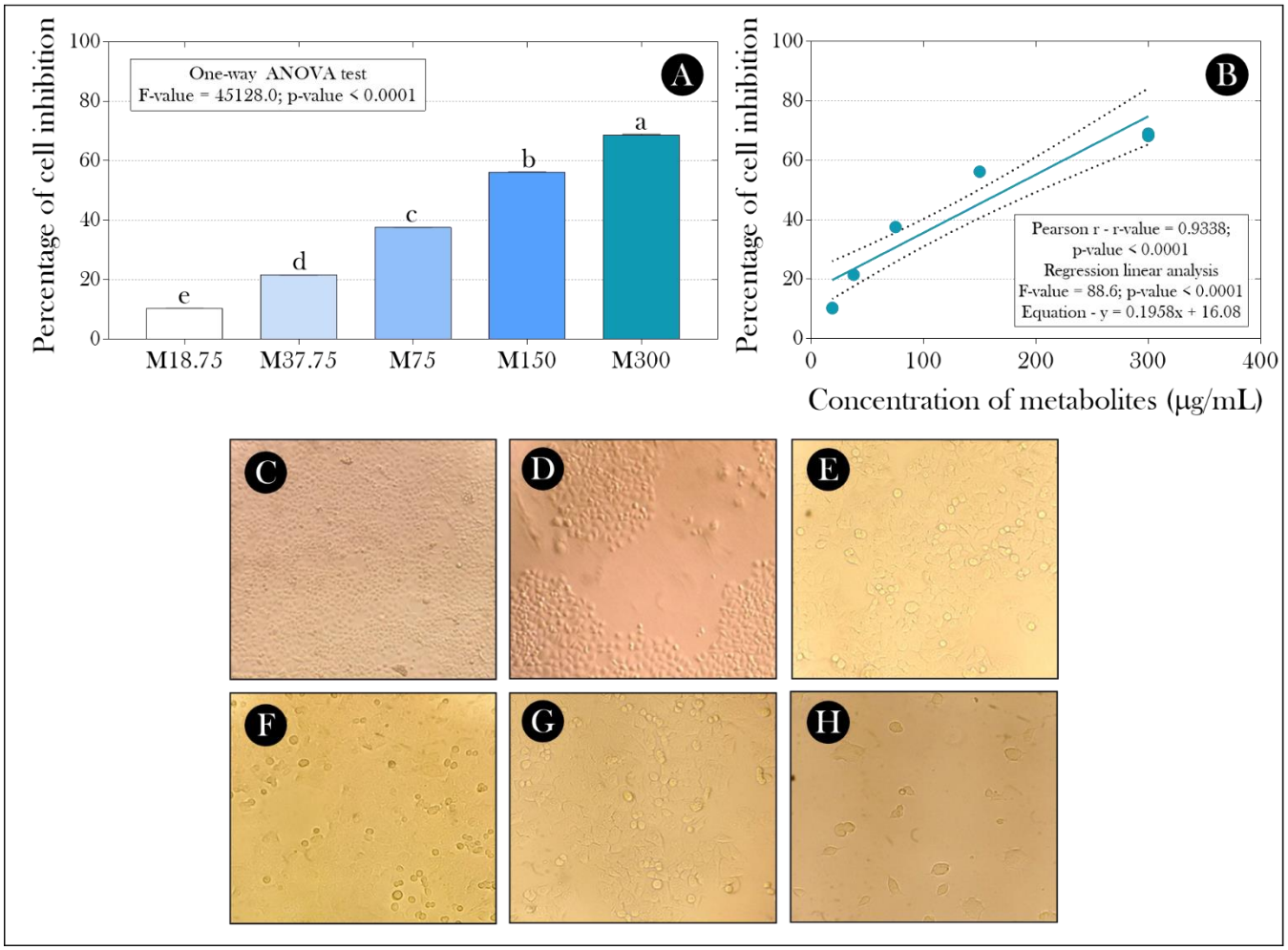

Figure 8. (A) Percentage of HeLa cell line inhibition exposed to different concentrations of the endophytic *A. macrospora* metabolites (M) (18.75 to 300 µg/mL). (B) Correlation and linear regression analyze (concentrations of fungal metabolites vs. percentage of HeLa cell line inhibition). Parametric data are presented by the mean + standard deviation. Distinct lowercase letters indicate significant differences between experimental groups. The summaries of the statistical analyzes are displayed next to the graphs. (C-H) Imagens representatives of the (C) HeLa control cell line

and exposed to mycelial metabolites to (D) 18.75 μg/mL, (E) 37.57 μg/mL, (F) 75 μg/mL, (G) 150 μg/mL and (H) 300 μg/mL.

3.9. GC–MS analysis

GC-MS analysis of *A. macrospora* fungal metabolites produced some bioactive compounds. The compounds were identified based on NIST databases by their comparisons to actual mass spectral data (Figure 9A and Table 3). Here, a total of seven compounds were identified: phenol, 3,5-bis(1,1-dimethylethyl) (4.572%), 3-isopropoxy-1,1,1,7,7,7- hexamethyl-3,5,5-tris (trimethylsiloxy) tetrasiloxane (4.453%), dihydroartemisinin, 10-o-(t-butyloxy (8.33%), cyclohexanol, 4-ethenyl-4-methyl-3-(1-methylethenyl) (15.284%), oleic acid (22.093%), eicosanoic acid (9.407%), ether, 6-bromo-1-ethyloctyl methyl. The chemical structure of these compounds is depicted in Figure 9B.

3.10. FT-IR analysis

The FT-IR spectrum (4000-400/cm) of mycelial metabolites of *A. macrospora* confirmed the presence of various functional groups of different organic compounds (Figure 9C and Table 4). The sharp and strong spectrums indicated bands at 3317.54, 2924.09, and 2854.65 cm^{-1} were assigned to O-H and C-H stretching vibrations of alcoholic, alkynes, and alkyl groups. The broad peak at 1710.86 cm^{-1} corresponds to the C=O stretching of carboxylic acid present in the sample. The medium band at 1656.85 cm^{-1} can be described as C=C stretching vibration, indicating the presence of alkenes groups. The strong band could be assigned at 1305.81 cm^{-1}, 1452.40 cm^{-1} corresponding to C-H bend, and C-F stretch provides alkyl and halide groups. A peak at 1232.51 cm-1 denotes the alkyl halides (C-F stretch). The absorption bands have exhibited the presence of the ethers group at 1259.52 cm^{-1} (=C-O-C stretch). The peak at 1163.08 demonstrated the presence of esters (O=C-O-C stretch). The medium peak at 1099.93 showed the presence of alcohol (C-O stretch) groups. The strong 844.82 and 800.46 cm^{-1} peaks indicate the alkyl halides (C-Cl stretch). A peak at 698.23 cm^{-1} revealed the alkenes (=C–H bend).

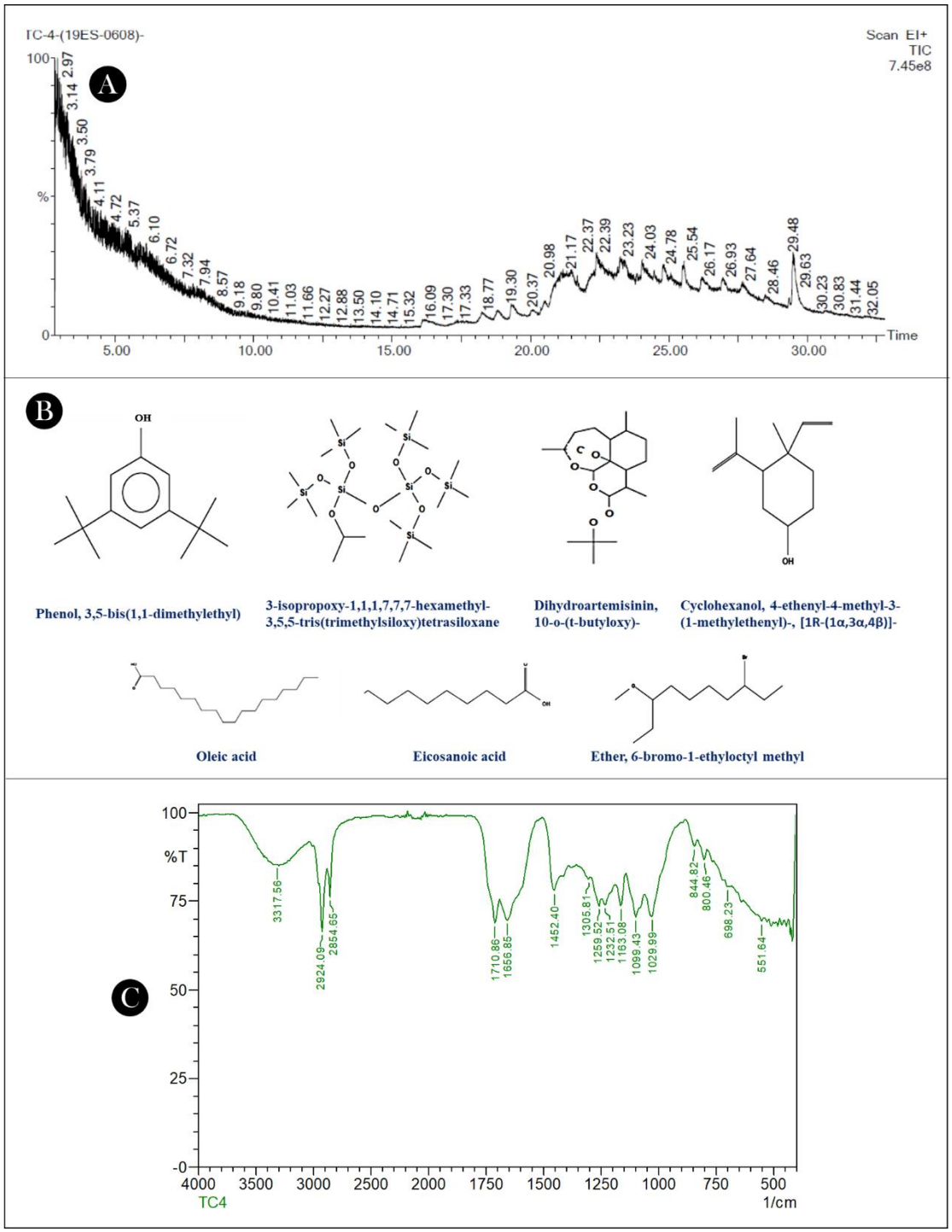

Figure 9. (A) Gas chromatography and mass spectroscopy (GC-MS) analysis, (B) chemical structures, and (C).Fourier-transformed infrared spectroscopy (FT-IR) spectrum of endophytic *A. macrospora* metabolites.

604 **Table 3.** Identification of secondary metabolites from ethyl acetate metabolites of *A. macrospora* by Gas chromatography and mass spectroscopy
605 (GC-MS) analysis.

S. no	Compound names	R_T	Area (%)	Molecular formula	Molecular weight	Biological activity	References
1.	Phenol, 3,5-bis(1,1-dimethylethyl)	29.48	4.572	$C_{14}H_{22}O$	206	Antimicrobial activity	Fabio Granados et al., (2016)
2.	3-isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris(trimethylsiloxy)tetrasiloxane	25.54	4.453	$C_{18}H_{52}O_7Si_7$	76	Antimicrobial activity	Gupta et al., (2017)
3.	Dihydroartemisinin, 10-o-(t-butyloxy)-	26.93	1.794	$C_{19}H_{32}O_6$	56	Anticancer activity	Chan et al., (2013)
4.	Cyclohexanol, 4-ethenyl-4-methyl-3-(1-methylethenyl)-, [1R-(1α,3α,4β)]-	23.23	15.284	$C_{12}H_{20}O$	180	-	-
5.	Oleic acid	22.37	22.093	$C_{18}H_{34}O_2$	282	Larvicidal & Antioxidant activities	Wei et al. (2016) and Fontana et al. (2013)
6.	Eicosanoic acid	21.17	9.047	$C_{20}H_{40}O_2$	312	Larvicidal and Antioxidant activities	Silva et al., (2016)
7.	Ether, 6-bromo-1-ethyloctyl methyl	24.78	4.182	$C_{11}H_{23}OBr$	250	-	-

606
607

608 **Table 4.** Identification of functional groups from ethyl acetate metabolites of *A.*
609 *macrospora* by Fourier-transformed infrared spectroscopy (FT-IR).

S. No	Wavenumbers (cm^{-1})	Vibration	Visible intensity	Functional groups
1	3317.54	O-H stretching	Sharp	Alcohols or phenols
2	2924.09	C-H stretching	Strong	Alkenes/Alkyls
3	2854.65	C-H stretching	Strong	Alkenes/Alkyls
4	1710.86	C=O stretching	Broad	Carboxylic acids
5	1656.85	C=C Stretching	Medium	Alkenes
6	1452.40	C-H Bend	Strong	Alkanes/Alkyls
7	1305.81	C-F Stretch	Strong	Alkyl/Halides
8	1259.52	=C-O-C Sym stretch	Medium	Ethers
9	1232.51	C-F Stretch	Strong	Alkyls/Halides
10	1163.08	O=C-O-C Stretch	Strong	Esters
11	1099.93	C-O Stretch	Medium	Alcohols
12	844.82	C-Cl Stretch	Strong	Alkyls Halides
13	698.23	=C-H Bend	Broad	Alkynes

610

611 ### 3.11. Toxicity effect on zebrafish embryo/larvae

612 The zebrafish embryos/larvae were tested with different concentrations of
613 mycelium extract. The concentrations from 250 µg/mL to 1 mg/mL were found to be
614 non-toxic in most of the biomarkers evaluated (Figure 10). But the higher 2 mg/mL
615 concentration was toxic (Figure 10). A significant difference in survival (63%), hatching
616 (86%), and heartbeat rate (142 beats/min) was observed in the 2 mg/mL treatment group
617 (Figure 10A-C, respectively). The malformation was observed in the 96 hpf larvae with
618 YSE and BT in animals exposed to endophytic *A. macrospora* metabolites to 2 mg/mL
619 (Figure 11).

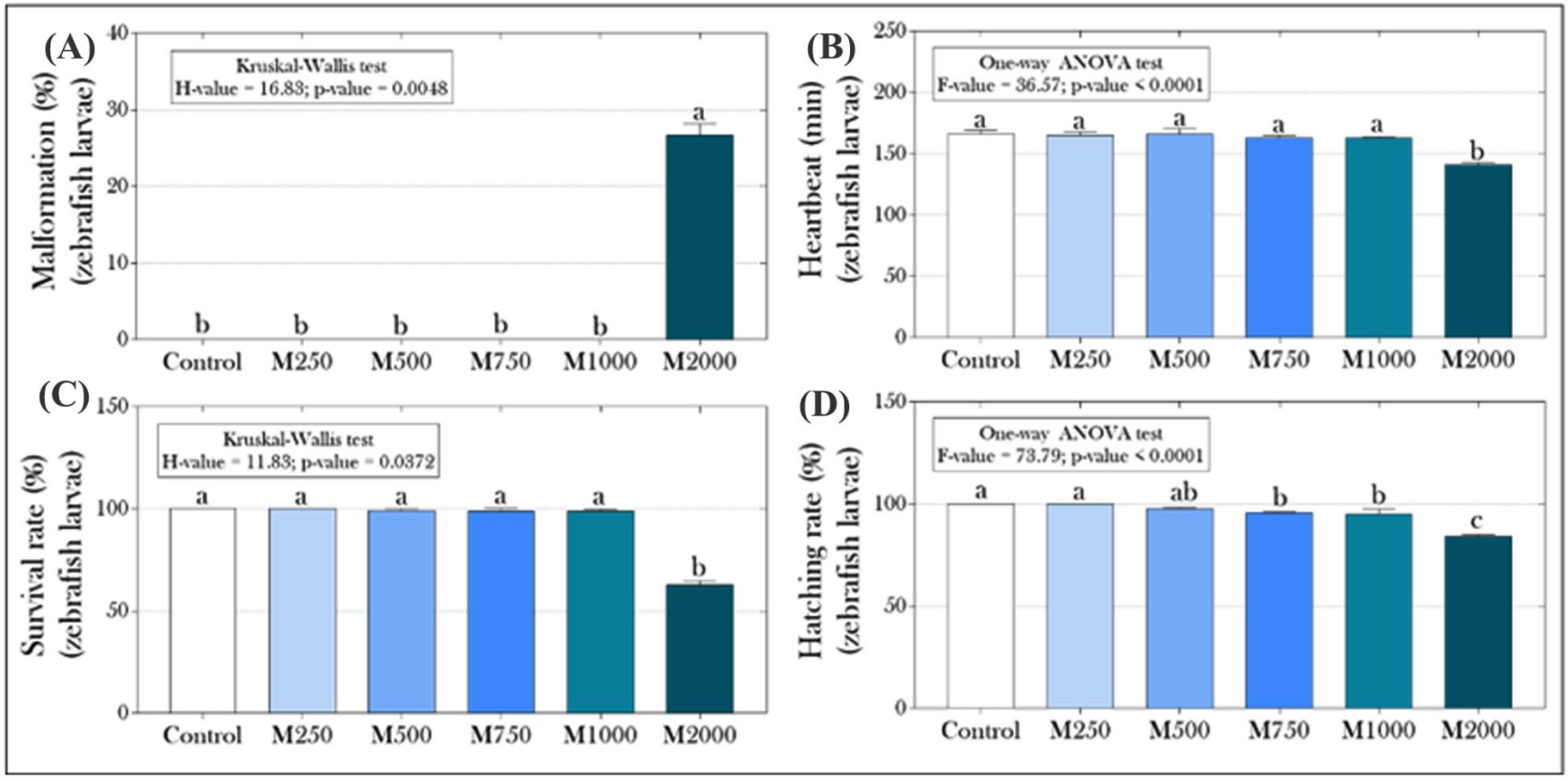

Figure 10. (A) Malformation rate (%), (B) heartbeat rate, (C) survival rate, and (D) hatching rate of zebrafish (*Danio rerio*) larvae unexposed or exposed to different concentrations of the endophytic *A. macrospora* metabolites (M) (250 to 2000 µL). Parametric data are presented by the mean + standard deviation, whereas non-parametric data are presented by the median and interquartile range. Distinct lowercase letters indicate

significant differences between experimental groups. The summaries of the statistical analyzes are displayed next to the graphs

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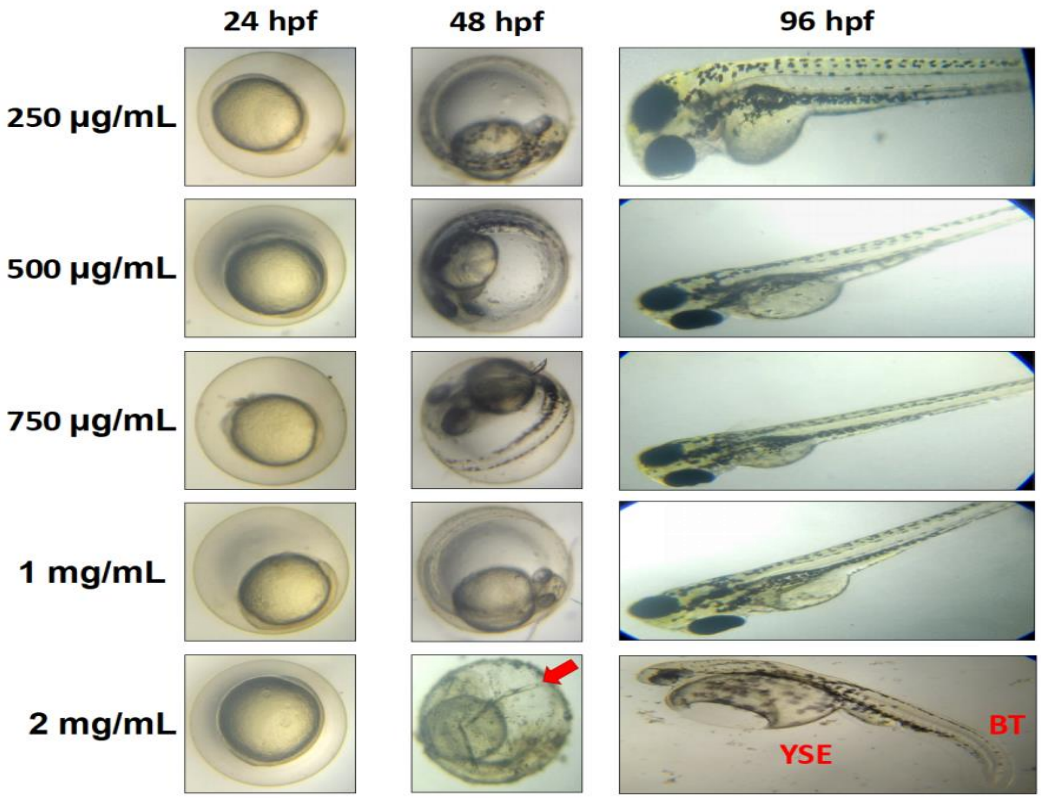

Figure 11. Representative images of eggs, embryos and larvae of zebrafish (*Danio rerio*) larvae exposed to different concentrations of the endophytic *A. macrospora* metabolites (M) (250 to 2000 µL) after 24, 48, and 96 hpf. YSE: yolk sac edema and BT: bend tail.

621

622 **3.12. *In-silico* analysis of compounds on toxicity and binding affinity**

623 The fungi metabolites identified from the GS-MS are investigated for toxicity
624 prediction. A decrease in toxicity class and LD50 (mg/kg) value indicates the toxic
625 condition of the compounds. In our study, the reported compounds have shown non-
626 toxic conditions except oleic acid. The oleic acid was less harmful, with a toxicity of class
627 2 and CL_{50} of 48 mg/mL (Table 5). The antibacterial potential of compounds can be
628 identified through their interaction ability with the pathogen receptor. Based on the
629 binding affinity value, the antimicrobial compounds are identified. In our results, the
630 fungi metabolites are docked with targets of *E. coli* FimH protein and *S. aureus* tyrosyl-
631 RNA synthetase protein. Among the metabolites, dihydroartemisinin, 10-o-(t-butyloxy)-
632 showed higher binding affinity in both the targets of *E. coli* FimH protein (-9.45 kcal/mol)
633 and *S. aureus* tyrosyl-RNA synthetase protein (-9.84 kcal/mol) (Figure 12A-G).
634 Dihydroartemisinin, 10-o-(t-butyloxy)- showed the amino acid interaction of TYR in *E.*

635 *coli* FimH protein and LYS, GLY, and ASP in *S. aureus* tyrosyl-RNA synthetase protein
636 (Table 6 &Figure 13A-G).
637

638 **Table 5.** *In-silico* analysis: toxicity profile of the compounds from ProTox online server

Compound	Predicted LD_{50} (mg/kg)	Predicted toxicity class	Water partition coefficient (logP)	Molecular refractivity	Topological polar surface area	Toxicity
Phenol, 3,5-bis(1,1-dimethylethyl)	965	4	18.24	255.86	65.57	Non-toxic
3-isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris(trimethylsiloxy)tetrasiloxane	24134	6	6.56	151.36	54.61	Non-toxic
Dihydroartemisinin, 10-o-(t-butyloxy)-	567	4	3.94	91.62	55.38	Non-toxic
Cyclohexanol, 4-ethenyl-4-methyl-3-(1-methylethenyl)-, [1R-(1α,3α,4β)]-	5300	5	4.75	70.42	0	Non-toxic
Oleic acid	48	2	6.11	89.94	37.3	Less toxic
Eicosanoic acid	900	4	7.11	100.03	37.3	Non-toxic
Ether, 6-bromo-1-ethyloctyl methyl	5000	5	4.15	63.95	9.23	Non-toxic

639

640

641

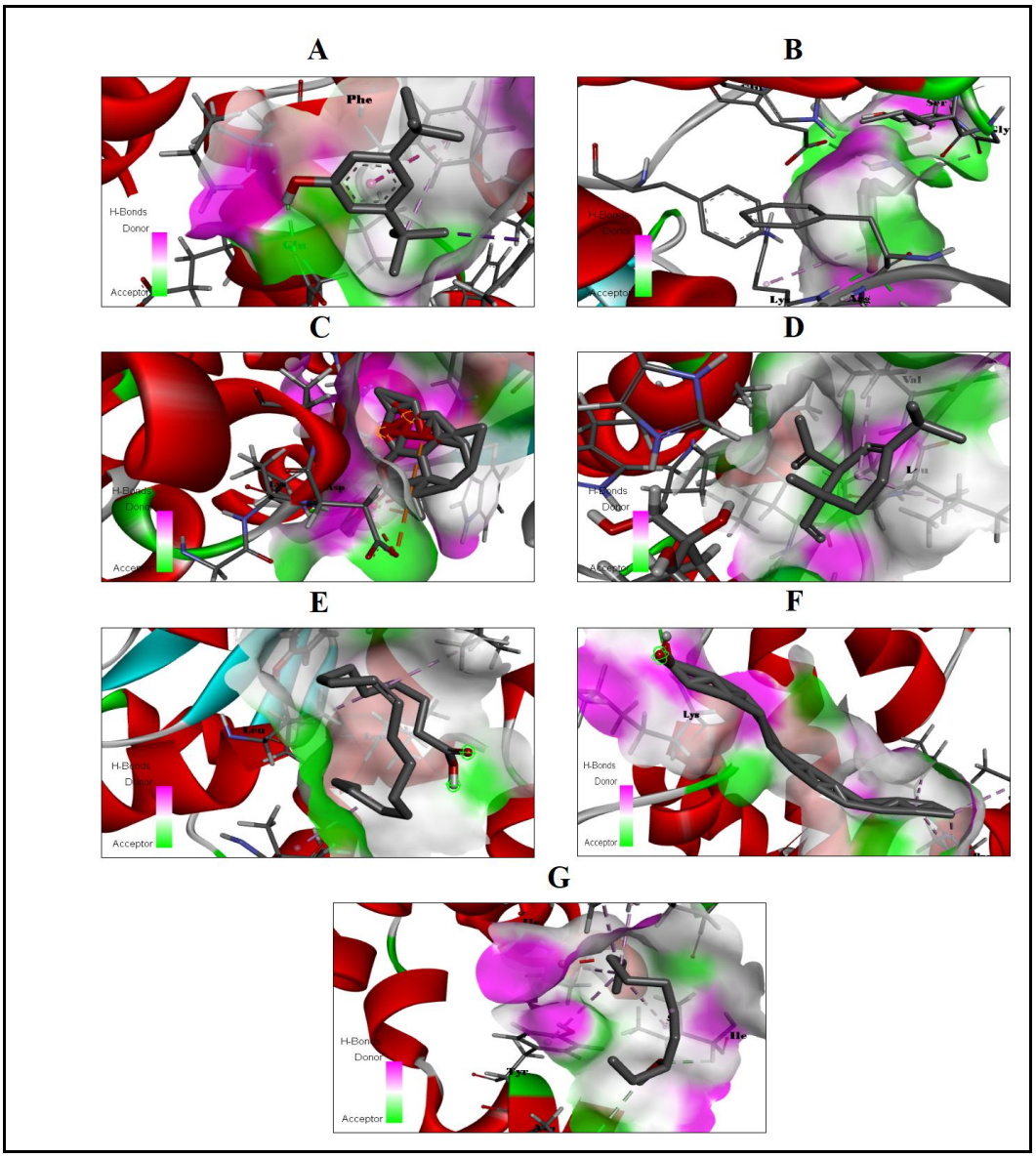

Figure 12. 3D interaction of different ligands with the targeted receptor of *Staphylococcus aureus* tyrosyl-RNA synthetase protein. (A) Phenol, 3,5-bis(1,1-dimethylethyl) (**B**) 3-isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris(trimethylsiloxy)tetrasiloxane, (**C**) dihydroartemisinin, 10-o-(t-butyloxy)-(**D**) cyclohexanol, 4-ethenyl-4-methyl-3-(1-methylethenyl)-, [1R-(1α,3α,4β)], (E) oleic acid, (F) eicosanoic acid, and (**G**) ether, 6-bromo-1-ethyloctyl.

642

643

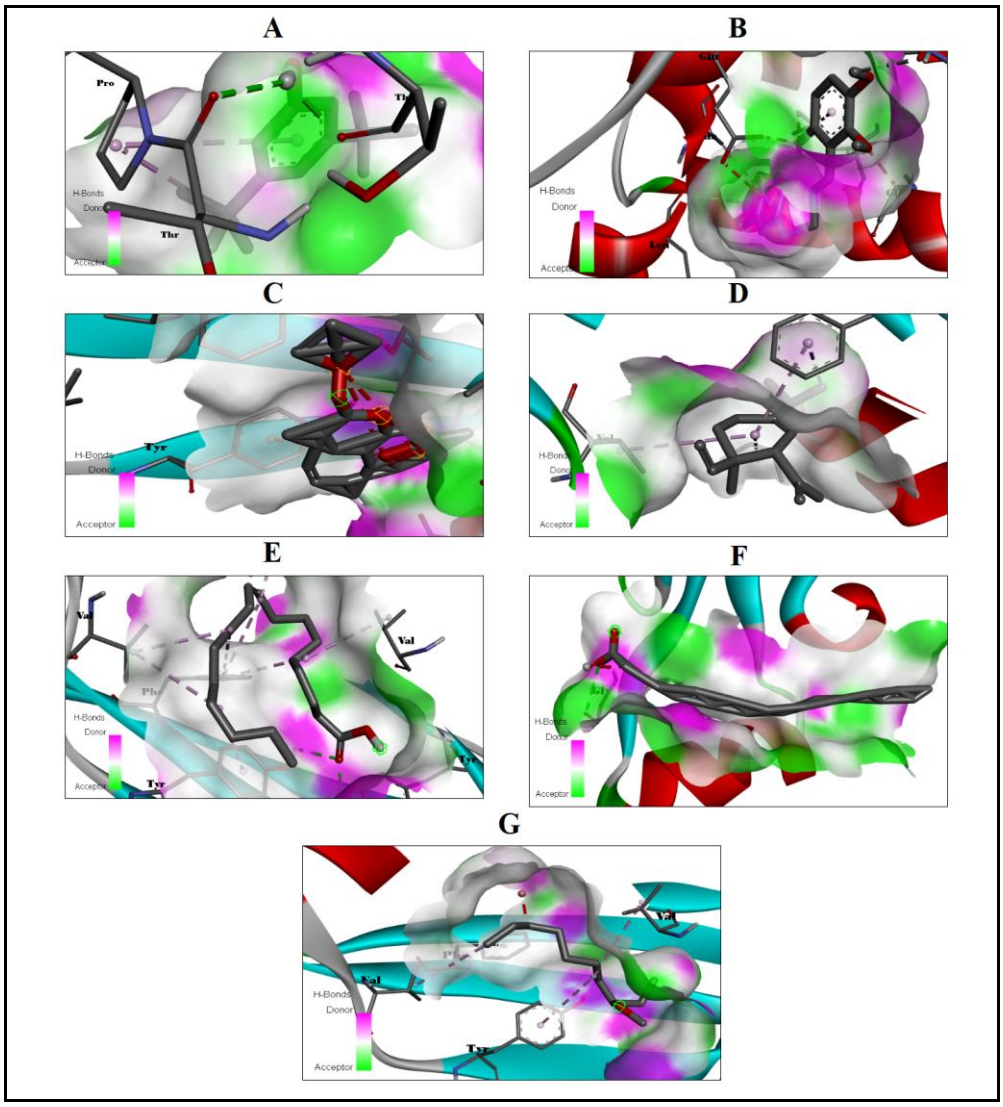

Figure 13. 3D interaction of different ligands with the targeted receptor of *Escherichia coli* FimH protein. (**A**) Phenol, 3,5-bis(1,1-dimethylethyl), (**B**) 3-isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris(trimethylsiloxy)tetrasiloxane, (**C**) Dihydroartemisinin, 10-o-(t-butyloxy)- (**D**) Cyclohexanol, 4-ethenyl-4-methyl-3- (**E**) Oleic acid, (**F**) Eicosanoic acid, and (**G**) Ether, 6-bromo-1-ethyloctyl methyl.

644

645 **Table 6.** Molecular docking scores of selected compounds against and the protein (PDB ID:4XO8 & PDB ID: 1jij)

Compound	Binding affinity (PDB ID:2jvu)	Binding affinity (PDB ID: 1jij)	Amino acid interaction	Amino acid interaction
Phenol, 3,5-bis(1,1-dimethylethyl)	-5.49 kcal/mol	-5.93 kcal/mol	THR, PRO and THR	PHE, PHE, and GLU
3-isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris(trimethylsiloxy)tetrasiloxane	-3.23 kcal/mol	-3.94 kcal/mol	GLU, GLU, LEU, and ALA	SER, GLY, ARG, LYS, and GLU
Dihydroartemisinin, 10-o-(t-butyloxy)-	-9.45 kcal/mol	-9.84 kcal/mol	TYR	LYS, GLY, and ASP
Cyclohexanol, 4-ethenyl-4-methyl-3-(1-methylethenyl)-, [1R-(1α,3α,4β)]- [1R-(1α,3α,4β)]-	-5.81 kcal/mol	-6.03 kcal/mol	SER, SER, ASP, ALA, PRO, VAL, ASP, TRP, and GLU	LEU
Oleic acid	-3.72 kcal/mol	-5.81 kcal/mol	TYR, VAL, ILE, PHE, and TYR	ILE, LEU, ILE, and LEU
Eicosanoic acid	-1.59 kcal/mol	-1.7 kcal/mol	ALA, PHE, and VAL	GLY, LYS, LEU, PRO, and ILE
Ether, 6-bromo-1-ethyloctyl methyl	-3.96 kcal/mol	-4.04 kcal/mol	PHE, VAL, TYR, and VAL	TRP, HIS, ILE, TYR, ASP, and ILE

646 Note – THR: Threonine, PRO: Proline, GLU: Glutamic acid, LEU: Leucine, ALA: Alanine, TYR: Tyrosine, SER: Serine, ASP: Asparagine, VAL:
647 Valine, TRP: Tryptophan, ILE: Isoleucine, PHE: Phenylalanine, HIS: Histidine, LYS: Lysine, GLY: Glycine, and ARG: Arginine.
648

4. DISCUSSION

In the current study, the endophytic *A. macrospora* was isolated from the leaves of *T. connaroides* and identified based on fungal morphology, microscopic features, and molecular analysis. In previous reports, the endophytes *Aspergillus* sp., *Cladosporium* sp., *Fusarium* sp., *Phomopsis* sp., *Colletotrichum* sp., *Alternaria* sp., *Diaporthe* sp., and *Trichoderma* sp. were identified based on colony morphology and molecular analysis (Mbilu et al., 2018). Similarly, endophytes *Gliocladium solani* and *Penicillium melinii* from *Artemisia scoparia* with white colonies have been reported (Nisa et al., 2018). In our study, the endophyte *A. macrospora* showed whitish colonies. In the context of this study, the colony morphology of *Alternaria officinalis*, previously isolated by the study group, has been shown to produce wool-like pale gray septal hyphae with linear and curved conidia arranged in linear and bifurcated chains (Ranganathan & Mahalingam, 2019). In this study, *A. macrospora* colonies were grayish green in color and were septal, vitreous, straight, and branched mycelia. In addition, molecular phylogenetic analysis of the 5.8s DNA ITS (500 bp) sequence confirmed that the isolated strain was *A. macrospora*. Using a combined approach of morphological and molecular confirmations, similar reports on the phylogenetic identities and relationships of endophytic fungi isolated from *Artemisia indica*, *A. capillaris*, and *A. lactiflora* plant species have been previously documented (Huang et al., 2009).

Different groups of endophytic fungi are known to be pioneering producers of bioactive molecules with different configurations and structural arrangements (Schuller et al., 2022). It is a known fact that various organic compounds derived from endophytes have biological activities such as antibacterial, antifungal, antitumor, antioxidant, anti-inflammatory, and larval control (Selvi, 2014). Several endophytes from various medicinal plants have contributed to the fight against emerging infectious diseases including HIV, influenza, malaria, Zika virus, Dengue and foot and mouth disease (Sharma et al., 2016; Kim & Ahn, 2017). These chemicals are produced by *Alternaria* spp. and are effective against insect pest *Spodoptera litura* (Kaur et al., 2019). Some researchers have therefore attempted to link the anti-insect properties of pure or semi-purified extracts or chemicals (Singh et al., 2012; Yang et al., 2012). The study reveals that the ethyl acetate mycelial metabolites of *A. macrospora* exhibit a significant mortality rate of *A. aegypti* larvae (500µg/mL) and showed sensitivity to chemicals present in the mycelial extract. This may support the research to develop new mosquito repellents based on bioactive compounds from fungi on behalf of chemical larvicides (Abutaha et al., 2015). It was found that most

683 endophytes have excellent larval control activity. Thus, *C. ptroselinum* for *Culex pipiens*
684 larvae showed a clear LC_{50} value of 152.94 µg/mL (Khater & Shalaby, 2008). A novel
685 isoquinoline from *F. moniliforme* against *A. aegypti* and *A. stephensi* showed LC_{50} of
686 237.0-276.4 ppm (Pradeep et al., 2015). In this study, LC_{50} and LC_{90} values were found to
687 be 96.167 to 143.864 µg/mL and 395.16 to 655.77 µg/mL, respectively, for the 1st to 4th
688 instar larvae of *A. aegypti*. The values shown indicate the concentration of the tested
689 substance or treatment needed to kill, in percentage terms (either 50% or 90%), the *A.*
690 *aegypti* larvae at the respective larval instar. The study most likely attempted to determine
691 if a specific medicine or therapy was effective in controlling or becoming eliminate of
692 these mosquito larvae. Similar studies have reported on the mosquito and larval-killing
693 properties of the fungus *A. terreus* against *A. stephensi* (LC_{50}=97.410, 102.551, 29.802,
694 and 8.907; LC_{90}=767.957, 552.546, 535.474, and 195.677 µg/mL) and *A. aegypti*
695 (LC_{50}=83.541, 84.418, 80.407, and 95.926; LC_{90}=515.464, 443.167, 387.910, and 473.998
696 µg/mL). (Ragavendran and Natarajan 2015).
697
698 Vyas et al. (2007) found 100% mortality when the metabolites of the fungus
699 *Lagenidium giganteum* were treated with 1st instar larvae of *A. aegypti*, *C. quinquefasciatus*
700 and *An. stephensi*. In our study, 500 µg/mL *A. macrospora* extract showed 100%
701 mortality in 1st instar larvae. Fungal metabolites or spores can disrupt the cuticle's integrity
702 and pigmentation on the outside while also altering lipid, carbohydrate, and protein
703 metabolism on the inside, impairing insect growth and reproduction, and triggering
704 immunological reactions (Vivekanandhan et al., 2020). Before metabolites attach to
705 phosphorus from DNA or sulfur from proteins and cause denaturation of organelles and
706 enzymes in insects, it is known that fungal metabolites puncture the exoskeleton (Masi et
707 al., 2017) and reach the intracellular region. Reactive oxygen species (ROS) production is
708 one of the most severe cellular consequences brought on by metabolite exposure, and
709 three basic processes have been put out to explain how this happens in living organisms.
710 Metabolites can be internalized into the cell by (1) endocytosis; (2) formation of the
711 endocytotic vesicles; and (3) release of compounds/substances from vesicles into the cell.
712 The primary causes of ROS production by metabolites include the following: parameters
713 involved with the physicochemical characteristics (size, shape, properties, and surface
714 chemistry); interactions with the mitochondria; and the relationship to NADPH oxidase.
715 These components trigger the production of ROS and all of its aftereffects, which include
716 cellular membrane damage, mutations in DNA, cell cycle arrest, and changes in apoptosis

(Tunçsoy, 2018). Dharumadurai et al. (2010) have reported 23 isolates of actinomycetes, in which *Streptomyces* sp. showed remarkable activity against *Anopheles* sp. Wherein, the present study the LC_{50} and LC_{90} values for mycelial metabolites from *A. macrospora* ranged from 234.390 to 739.362 µg/mL with the impact on pupal development of *A. aegypti*. A similar report by Murugan et al. (2011) stated the insecticidal and pupicidal effects of *M. anisopliae* against the malaria mosquito *A. stephensi*. Ragavendran et al. (2017) analyzed the insecticidal and pupicidal effects of the entomopathogenic fungus *B. bassiana* against three targeted mosquitoes. The *A. macrospora* extract also showed promising ovicidal activity due to the bioactive metabolites present in the extract. Al-Mekhlafi (2018) has reported on the ovicidal activity and histopathological changes induced by the *Carum copticum* extract against *C. pipiens*. In our study, 100% ovicidal activity was observed in *A. aegypti* and LC_{50} was 36.08 µg/mL. The rate of hatching was found to be inversely proportional to the concentration of the extract. Reegan et al. (2013) reported an LC_{50} value of 62.5 ppm of *Clionacelata* extract for eggs of *C. quinquefasciatus* and *A. aegypti*. Martínez et al. (2007) have screened the ethanol extracts of five sponges, *Ircinia campana*, *Amphimedon compressa, Agelas sventres*, *Topsentia ophiraphidites*, and *Svenzea zeai*, were screened against larvae and eggs of *C. quinquefasciatus* and *A. aegypti*.

The increase in resistance is a crucial concern because arthropod-borne diseases are addressed mainly by insecticide-based vector activity (Gan et al., 2021). Southeast Asia is noticing an increase in insecticide resistance, continuing the worldwide trend of other types of resistance (WHO, 2023). Natural ingredients may be used as potential insecticides to manage disease vectors due to their accessibility and safety, which would minimize the number of synthetic pesticides (Lengai et al., 2020). Insects exposed to fungal-based biopesticides may experience upregulation or downregulation in their whole-body protein levels (Li et al., 2019; Parthiban et al., 2021). The findings strengthen the probability that *A. aegypti* mortality may be connected to the inhibition of enzyme activity by *A. macrospora*.

Our research found that the AChE activity of the 4th instar larvae exposed to fungus metabolites were significantly lower than those of the control larvae. Similarly, Ragavendran et al. (2019) observed that treatment with *Penicillium* sp. metabolites decreased the AChE activity in *A. ae*gypti and *C. quinquefasciatus* larvae. AChE is a neurotransmitter molecule that hydrolyses acetylcholine into acetate and choline (Rajagopalan et al., 2023). Pre-synaptic neurons constantly release acetylcholine into the

751 synaptic cleft, interacting with receptor/ligand-gated sodium channels, enabling the
752 sodium ions to transport rapidly through this channel (Oni et al., 2019). One of the
753 fundamental resistance mechanisms in insect pests, AChE change, has been identified to
754 be resistant to carbamate and organophosphorus (Ghosh et al., 2012). Fungal active
755 molecules may bind to and decrease the activity of the enzyme AChE, which destroys
756 acetylcholine, a key neurotransmitter in the central nervous system of insects (Rants'o et
757 al., 2022). AChE inhibitory properties of fungal metabolites have been reported (Zibaee
758 et al., 2009; Abraham et al., 2015; Santra et al., 2022). The result that larvae died after
759 being exposed to fungus mycelial metabolites demonstrated that the chemical in the
760 metabolites might interfere with the cholinergic connection and interrupt the network of
761 exonic communication, inhibiting the release of nerve signals. Thus, the lethal effect may
762 result from a build-up of the neurotransmitter acetylcholine at synaptic junctions,
763 disrupting coordination between the nerve and muscle connections (Jankowska et al.,
764 2018). According to an earlier finding, AChE inhibitors slow larvae growth, reduce the
765 hatching of treated eggs, and negatively impact insect behavior (Al-Massarani et al., 2019).
766 To the best of our knowledge, this is the first data available for the
767 histopathological study of *A. macrospora* mycelial metabolites against targeted
768 mosquitoes. The present work has demonstrated that tested mycelial metabolites led to
769 histological changes in the midgut component of *A. aegypti* larvae, including food bolus,
770 epithelial cells, microvilli, and gastric caeca. In the midgut, there were irregular shapes for
771 the epithelial cells, microvilli, and Epithelium and breaks in the food bolus and
772 peritrophic membrane. According to a recent study, 4th instar *A. stephensi, A. aegypti* and
773 *C. pipiens* larvae treated with the entomopathogenic fungus *Metarhizium*
774 *anisopliae* displayed numerous histological alterations and malformations in body tissues.
775 They affected various body parts, particularly the cuticle, adipose cells, and midgut
776 region, compared to untreated larvae (Vivekanandhan et al., 2022). Likewise, Abutaha et
777 al. (2015) observed that *Aspergillus sydowi* metabolites were administered against *A.*
778 *caspius* caused disintegration of the peritrophic membrane, cytoplasmic vacuolization,
779 and malformations of cellular microvilli in the larvae pathological pattern.
780 Many secondary metabolites produced by plant endophytic and pathogenic fungi
781 are significant for toxicity and interaction with other pathogens (Narayanan & Glick,
782 2022). Some of these natural products can display strong biocidal activity against human
783 pathogenic microorganisms due to their broad-spectrum action (Jakubczyk & Dussart,
784 2020). The discovery and production of medicines for treating human diseases depend

heavily on natural products, and the microbial environment is a significant source of new bioactive substances (Cruz et al., 2020). The pharmacological diversity of *Alternaria* fungus, such as the production of phytotoxins and mycotoxins, is also well documented (Lou et al., 2013; Tsuge et al., 2013). Over the past few decades, at least 268 biomolecules with phytotoxic, cytotoxic, and antibacterial activities have been discovered in *Alternaria* fungus (Brase et al., 2009; Dalinova et al., 2020; Al Mousa et al., 2021). The antibacterial activity of mycelial extracts has been reported in many studies (Sharma et al., 2016). Santos et al. (2015) have reported the endophytes from the leaves of *I. suffruticosa* against bacterial pathogens such as *B. subtilis* and *E. coli*. In our study, it was found that *A. macrospora* mycelium metabolites strongly inhibit *E. coli* (22 mm), *B. subtilis* (19 mm), *S. aureus* (20 mm), and *S. typhi* (16 mm). Thus, in this study, the fungal metabolites may be caused by bacteriostatic action on the test bacteria by disrupting their cell membranes. The test bacteria were inhibited as the outcome of the respiratory chain of the bacteria being inactivated, which also affected the oxidative phosphorylation and tempered the generation of energy route of the bacteria (Maliehe et al., 2022). Thus, it may be stated that the extract suppressed the test bacterium by obstructing the respiratory chain dehydrogenase function. The findings are consistent with the research of Gomaa, (2017), which found that antimicrobial drugs might work by interfering with the function of bacterial respiratory cycle dehydrogenase.

These outcomes agree with the findings of Maliehe et al. (2022), which showed that the ethyl acetate extract inhibited *S. aureus* and *E. coli* by puncturing their cell membranes. Makut and Owolewa (2011) reported the inhibitory activity of the endophytes *A. flavus* and *A. niger* against *S. aureus* and *E. coli*. Petit et al. (2009) have studied the activities of *Penicillium* sp. against *Streptococcus pyogenes*, *S. typhimurium,* and *B. cereus*. Premjanu et al. (2016) have noticed the largest inhibitory zone against *Malassezia pachydermis* from *A. niger*. In a crude extract of *Alternaria sp.*, the compound oleic acid was detected with antifungal properties against phytopathogenic species of *Moniliophthora perniciosa* and *P. ultimum* (Walters et al., 2004). In agreement with the earlier findings, the oleic acid compound in the mycelial extract of *A. macrospora* exhibited excellent antibacterial activity against clinical pathogens such as *E. coli* and *S. aureus* (Zhang et al., 2023).

The present study also showed that the cytotoxic activity of mycelium metabolites against HeLa cells was tested by MTT assay. The fungal metabolites showed 68% cytotoxicity at 300 µg/mL concentration and were significantly active against cancer cells (IC_{50} value 118.49 µg/mL). Likewise, Kumari et al. (2018) have reported the cytotoxic activity of the endophyte *Talaromyces purpureogenus* against HeLa and MCF7 cell lines. Itsuki et al. (2007) reported that the cytotoxic effect was exhibited in several phenolic metabolites from *Alternaria*. L5178Y mouse lymphoma cells. Similarly, Artika et al. (2017) have reported the anticancer activity of the endophyte *Phomopsis sp.* against an MCF7 cell line with an IC_{50} of 19.2 µg/mL. Huang et al. (2012) reported that the Alterporriol L, a bianthraquinone derivative found in the marine fungus *Alternaria* sp. ZJ9-6B inhibited the MDA-MB-435 breast cancer cells from proliferating and growing by damaging their mitochondria. Earlier, Ragavendran and Natarajan (2015) investigated the mycelial metabolites of *A. terrus* and its components, showing that the chemical (phenol, 3,5bis1,1 dimethylethyl) had more potent insecticidal and pupicidal effects on *C. quinquefasciatus*, *An. stephensi* and *A. aegypti*. In support, the presently revealed compound phenol, 3,5-bis 1,1-dimethylethyl (4.57%) from the *A. macrospora* might exert antifungal activity. Likely, Sharma et al. (2016) have evaluated the bioactive secondary metabolites isolated from the endophyte *Pestalotiopsis neglecta* contained oleic acid as similar to the GC-MS of *A. macrospora* metabolite, i.e., oleic acid (22.093%) with a higher peak than other compounds. The GC-MS analysis of *A. macrospora* metabolites results clearly show that seven major chemical constituents namely phenol, 3,5-bis(1,1-dimethylethyl), 3-isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris(trimethylsiloxy)tetrasiloxane, ether, 6-bromo-1-ethyloctyl methyl, dihydroartemisinin, 10-o-(t-butyloxy)-, oleic acid and eicosanoic acid. Among the chemical constituents, oleic acid and eicosanoic acid as major chemical ingredients found in the fungal metabolites, so these chemical constituents may be involved in mosquito larvicidal activity. Earlier research found that, *B. bassiana, A. tamarii,* and *A. terreus* secondary metabolites/essential oils contain similar kinds of organic chemical constituents with larvicidal and pupicidal activities against targeted mosquitoes (Ragavendran and Natarajan, 2015; Ragavendran et al., 2017; Baskar et al., 2020). The GC-MS analysis of the endophyte *Curvularia aeria* contained eicosene (7.93%), which is quite similar to the peak area (9.047%) of the presently revealed icosanoic acid from *A. macrospora* (Sahani and Thakur, 2019). FT-IR is commonly used to identify chemical constituents' functional groups and helps elucidate compounds (Starlin et al. 2012). Our FT-IR results showed

852 the presence of phenolic compounds, alkenes, and amines based on wavenumber, peak
853 assignment, and vibration mode. Similarly, CH stretches of alkene groups in ethyl acetate
854 extracts of the endophytes *Chaetomium globosum*, *Penicillium janthinellum*, and
855 *Cladosporium tenuissimum* were reported from an earlier study at 2855.08 cm^{-1} (Kanjana
856 et al. 2019). The mycelium extract of *A. macrospora* also confirmed the corresponding
857 alcohols, carboxylic acids, alkene, and other minor proportions. These functional groups
858 may be involved in mosquito larvicidal, cytotoxicity, and antibacterial activity. Similarly,
859 Ragavendran et al. (2019) and Vivekanandhan et al. (2018) stated that the *Penicillium* sp.
860 and *B. bassiana*-28 derived metabolites have similar functional groups, respectively.
861 Furthermore, the main peaks of 3317.54 and 2924.09 cm^{-1} were assigned to the functional
862 groups of alkyl (= CH stretch), which resemble intense peaks (3300.20 and 2924.09 cm^{-1})
863 representing aromatic and alkene-based CH stretches from the crude extract of *C.*
864 *gloeosporioides* (Rabha et al. 2015). However, more studies on identifying active
865 compounds and semi-field trials are necessary before suggesting the active ingredients of
866 microbial metabolites for establishing eco-friendly mosquito vector control strategies.
867 Zebrafish models are used for drug screening and toxicity analysis due to their
868 genetic similarity, short life cycle, and cost-effectiveness. Using zebrafish embryos/larvae,
869 the toxicity range of the bioactive compounds or plant extract can be predicted (Siddhu et
870 al., 2022). To use microbial metabolites as eco-friendly mosquito vectors, their extract
871 toxicity range was tested in the zebrafish model. The toxic exposure to the zebrafish
872 larvae causes the development of malformation. In the meantime, the increased extract
873 concentration affects the survival, heart rate, and hatching rate of the embryos/larvae as
874 well as causes BT and YSE development in the larvae. Likewise, Cao et al. (2016)
875 detected physiological and morphological changes in zebrafish treated with cyhalofop-
876 butyl, as well as a decreased rate of embryonic development, delayed autonomous
877 movement, a decreased pulse, and embryos with shortened body lengths (at higher
878 doses). Similarly, Abutaha et al. (2015) reported that *Cochliobolus spicifer* endophytic
879 fungal extract produces minor anomalies or deformities in zebrafish embryos. The latest
880 report by Taher et al. (2022) on endophytic *Phyllosticta fallopiae* molecules found
881 teratogenic and low toxicity in zebrafish models, with a concentration-dependent
882 correlation.
883 Further, the *in-silico* toxicity analysis was performed for each microbial metabolite
884 to identify its toxic nature. All the metabolites showed a non-toxic condition except oleic
885 acid. Even oleic acid was less harmful, which may be why they have not demonstrated

toxic effects in zebrafish developmental toxicity analysis. The *in-silico* and *in-vivo* toxicity analysis confirmed that these metabolites are not toxic and can be used as eco-friendly vectors. The mycelium extract was tested for antimicrobial activity, and they showed better results. But their specific mechanisms of interaction with pathogen receptors are not known. So that through the molecular docking analysis, metabolites binding affinity towards the target of *E. coli* FimH protein and *S. aureus* tyrosyl-RNA synthetase protein was performed. Bacteria translocation to the intestinal epithelium causes the life-threatening disease of sepsis (Sun et al., 2022). FimH helps the *E. coli* with translocation so that compound that interacts and acts as an inhibitor helps prevent the infection (Poole et al., 2017). *S. aureus* tyrosyl-RNA synthetase protein is mainly involved in converting the covaleny amino acid to tRNA (Elkolli et al., 2023). The compound that specifically binds to this receptor helps inhibit the protein synthesis process (Athar et al., 2020). In our findings, all the metabolites showed good binding affinity and amino interaction toward the target protein. Among the metabolites, dihydroartemisinin, 10-o-(t-butyloxy)- led the better binding affinity in both the targeted receptor. This could be the reason for the effective antibacterial activity of mycelium extract against the tested pathogens.

5. CONCLUSION

Overall, this study shed light on the endophytic fungus *A. macrospora* isolated from the medicinal plant *T. connaroides* based on conventional morphological (colony morphology and conidia structure) and molecular (ITS region amplification) approaches. In conclusion, *A. macrospora* fungal metabolite produced 100% mortality in larvae of *A. aegypti*, after 24 h exposure. They were followed by the 100% of mortality of pupae observed at the concentration of mycelium metabolite (500 µg/mL). Hatchability is significantly inhibited at 500 µg/mL of the metabolites of *A. macrospora*. Fungus metabolites significantly reduced AChE enzyme activity in the larvae. The control group histological study confirmed that the midgut epithelium is made up of a single layer of digesting cells with well-developed cytoplasmic and brush borders. Severe damage to the treated group included the hindgut, vacuolated cells, particularly damaged epithelium larvae, collapse of the epithelial barrier and exterior cuticle, and internal membranes. In addition, four different pathogens were used to evaluate the antibacterial properties of four different mycelia metabolite concentrations (25, 50, 75, and 100 µg/mL). *E. coli* exhibited the broadest zone of growth inhibition (22 mm), followed by *S. aureus* (20 mm), *B. subtilis* (19 mm), and *S. typhi* (14 mm). Furthermore, the fungal constituents

resulted in the IC_{50} value of 118 µg/mL on HeLa cells using MTT assay, indicating that the metabolites are cytotoxic. GC-MS analysis showed the presence of potential secondary metabolites, namely cyclohexanol, 4-ethenyl-4-methyl-3-(1-methylethenyl) (15.284%), eicosanoic acid (9.047%), ether, 6-bromo-1 ethyloctyl methyl (4.182%) and oleic acid (22.093%). Further, the functional groups were identified from the *A. macrospora* using the FT-IR spectrum. Meanwhile, the *in-silico* and *in-vivo* zebrafish larvae toxicity prediction confirmed that they are non-toxic. Finally, the metabolite's antibacterial potential was confirmed by interacting with specific *E. coli* and *S. aureus* receptors. The results clearly indicate that these metabolites can be used as antimicrobial agents with lesser toxic effects.

6. ACKNOWLEDGEMENTS

The authors thanked the Periyar University administration for providing the laboratory space needed to conduct this research. The DST-FIST [SR/FIST/LSIS-673/2016], a substantial award to the Biotechnology Department, is gratefully acknowledged by the authors of Periyar University for their assistance in boosting the instrumentation infrastructure. Furthermore, the authors thank the National Council for Scientific and Technological Development for the financial support. Malafaia G. holds a productivity scholarship from CNPq (Proc. #308854/2021-7). Liverpool John Moores University supported the work.

7. AUTHOR CONTRIBUTIONS

CR and CK: planned the research, conducted the experiments, shared chemicals, analyzed the tools, and wrote the manuscript. CR **and DN**: conceptualization, methodology, and performed the embryotoxicity experiments. **CR, GM, and CK**: writing, reviewing, and editing. IN, TC, VM, MK, and GM revised the manuscript All authors have read and decided on the published version of the manuscript.

8. DECLARATION OF COMPETING INTEREST

We validated that there were no known conflicts of interest related to this work, and there was no significant financial backing that might have affected how it turned out. We also guarantee that all identified writers have read, reviewed, and authorized the manuscript and that no other individuals who meet the requirements for authorship but

are not mentioned have contributed. The integrity of the work has been guaranteed with proper attention.

9. ETHICAL ASPECTS

All experimental methods were carried out in accordance with the ethical guidelines for using animals in research, and special care was taken to minimize the animals' suffering and external causes of tension, pain, and discomfort. The quantity of animals used in the present study is within the range required to generate valid scientific data. No research involving human subjects was referenced in this paper by any authors.

10. AVAILABILITY OF DATA AND MATERIALS

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

11. CONSENT TO PARTICIPATE

Not applicable.

12. CONSENT TO PUBLISH

Not applicable.

13. REFERENCES

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