International Journal of Mosquito Research

ISSN: **2348-5906**
CODEN: **IJMRK2**
IJMR 2024; 11(1): 175-184

https://www.dipterajournal.com
Received: 26-12-2023
Accepted: 03-02-2024

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Insecticidal Potential of Endophytic Fungal Extracts from *Adhatoda vasica* against Dengue vector, *Aedes aegypti* and Diamondback Moth, *Plutella xylostella*

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DOI: https://doi.org/10.22271/23487941.2024.v11.i1c.759

Abstract
Food production and disease control are two critical challenges that humanity faces in the 21st century. There is an urgent need for eco-friendly control tools for fighting mosquito vectors and agricultural pests. The current study aimed to isolate the fungal strain *Humicola fuscoatra* from the leaves of *Adhatoda vasica* and extract their secondary metabolites using ethyl acetate. The effectiveness of the crude extract against the dengue vector *Aedes aegypti* and the cabbage pest *Plutella xylostella* was then evaluated. The molecular identification of isolated *H. fuscoatra* was accomplished through PCR amplification using a universal primer (ITS-1 and ITS-2). The ethyl acetate extracts of the fungus *H. fuscoatra* exhibited higher toxicity against both insects. The LC_{50} values of ethyl acetate extracts of the fungus *H. fuscoatra* ranged between 132.712 and 450.163 ppm for 1st instar larvae and pupae of *Ae. aegypti*, as well as 287.071 and 656.694 ppm for 1st instar larvae and pupae of *P. xylostella*. The ethyl acetate extracts of the fungus *H. fuscoatra* significantly reduced the longevity and fecundity of *Ae. aegypti* and *P. xylostella* adults. GC-MS analysis indicated the presence of some identified compounds as insecticides in ethyl acetate extracts of fungi such as: Hexadecanoic acid (15.07%), octadecadienoic acid (13.74%), Hexadecanoic acid, methyl ester (12.89%), Hexadecan (6.43%), Octadecan (8.33%) and Phenol, 2,4-bis (1,1-dimethylethyl (2.23%). This study provides valuable insights into the ethyl acetate extracts of the fungus *H. fuscoatra* from plants and their potential in fighting arthropods of medical and agricultural significance. The findings suggest that the tested products could be potentially useful for the development of economically viable pest control approaches.

Keywords: Endophytic fungi, *Aedes aegypti*, *Plutella xylostella*, insecticidal activity, secondary metabolites

1. Introduction
Microbial pest control is a highly effective alternative to chemical insecticides. These biopesticides are highly valued for their ability to degrade naturally, their specificity, and their eco-friendly properties. They serve as effective tools for managing insecticide resistance [1]. The effectiveness of commercially available microbes, including bacteria, fungi, and nematodes, is primarily attributed to the metabolites they produce, which are responsible for their pathogenicity and ability to kill host organisms [2]. The study by Ravindran *et al.* [3] discusses the significant role of entomopathogens as alternatives to chemical pesticides in pest management. These natural insect pathogens have shown great potential for effectively controlling pests.

Fungal metabolites are highly valuable and should be considered as a rich source of synthetically intriguing and exceptionally significant novel lead chemicals for the medical, agricultural, and chemical industries [4]. Over 700 species of fungi are documented as predators of insects; however, only a small number of these species help control the populations of their hosts [5]. Several types of bioactive compounds are released by entomopathogenic fungi. These include polyketides, non-ribosomal peptides, polyketide-peptide hybrid metabolites, and terpenes [6]. Bandani *et al.* [7] and Molnar *et al.* [8] documented the antifeedant and insecticidal activities of some of these secondary metabolites.

Humicola species has significant biotechnological and industrial possibilities. The *Humicola* genus, belonging to the Chaetomiaceae family, is a prolific source of distinct and structurally varied metabolites that exhibit a range of biological functions. Furthermore, *Humicola* species have garnered significant interest due to their notable capacity to generate thermally stable enzymes that possess biotechnological and economic significance [4]. Several secondary metabolites, including fuscoatrol A, 11-epiterpestacin, and β-nitropropionic acid, were extracted from the mycelia of *Humicola fuscoatra* (Traaen) KMM 4629, which is found in association with the Kuril colonial ascidium. These compounds were separated from the ethyl acetate extract. Fuscoatrol A exhibited antimicrobial activity against *Staphylococcus aureus* and *Bacillus subtilis* and demonstrated cytotoxic effects on the developing eggs of the sea urchin *Strongylocentrotus intermedius*. On the other hand, 11-epiterpestacin was able to kill *Staphylococcus aureus* and *B. subtilis*, and β-nitropropionic acid was able to kill *S. aureus* [9].

Food production and disease management are two crucial concerns that humanity faces in the 21st century. These difficulties have been attributed to the growing global human population and the management of pests. Arthropod pests cause significant global crop losses, both before and after harvest, amounting to around 20-50% of the potential yield. Additionally, they are responsible for transmitting several of the world's most serious diseases [10]. Mosquitoes are significant as both bothersome insects and carriers of disease-causing agents such as dengue, yellow fever, malaria, rift valley fever, and filariasis. These diseases have a significant impact on human populations globally, especially in tropical and sub-tropical areas [11]. *Aedes aegypti* mosquitoes serve as the primary carriers of arboviruses, including those responsible for dengue, chikungunya, and Zika diseases [12]. Due to the absence of antiviral medications and efficient vaccinations for these arboviruses, efforts to prevent the spread of these diseases primarily focus on vector management. This involves targeting breeding areas and employing chemical insecticides [13]. Fungal secondary metabolites are numerous bioactive compounds that have the potential to be valuable for mosquito control [14].

The diamondback moth, scientifically known as *P. xylostella* (Lepidoptera: Plutellidae), is a highly destructive insect that poses a significant threat to cabbage crops. This species, believed to have originated in the Mediterranean region, has subsequently spread globally. This organism has great fertility, possesses the ability to migrate across significant distances, and has a brief life cycle of 14 days at a temperature of 25 °C. According to Gryzwacz *et al.* [15], the anticipated global direct losses and control expenses amount to US$ 1 billion. The economic impact of *P. xylostella* infestation in India is significant, resulting in annual losses exceeding US$16 million. This infection affects a cultivated area of cabbage spanning 501,700 hectares, as reported by Mohan and Gujar [16]. *P. xylostella* was documented as the initial bug to exhibit resistance to the microbial control agent *Bacillus thuringiensis* [17]. There is an urgent need for environmentally friendly control tools.

This study aims to examine the insecticidal properties of secondary metabolites extracted using ethyl acetate from *Humicola* fungal isolates on two insects: the dengue vector, *Aedes aegypti*, and the diamondback moth, *Plutella xylostella*.

2. Materials and Methods

2.1 Isolation of Endophytic fungi

Leaf samples of *Adhatoda vasica* nees were collected from Mercy College Medicinal Garden, located on the Mercy College campus in Palakkad, Kerala, India. The samples were washed three times with tap water, followed by surface sterilisation using the methods described by Zeng *et al.* [18]. In short, the leaves were soaked in 70% ethanol for 2 minutes, then treated with 0.1% mercuric chloride for 4 minutes. Afterwards, the samples were rinsed three times with sterilised distilled water. The samples were cut into 3.0 × 3.0 cm to remove any excess surface chemicals. Afterward, the specimen was sliced into small portions measuring 1.0 × 1.0 cm using a sterile blade. These pieces were then placed in Sabouraud dextrose agar (SDA) medium, which had been supplemented with chloramphenicol (50 µg/mL). The samples were incubated at a temperature of 25 °C ± 2 °C for a duration of two weeks, with daily observations of the plates. The mature mycelia were carefully transferred to fresh SDA plates to obtain a more refined form of fungal isolates [19]. The morphological characteristics of the purified strains were observed on PDA.

2.2 Molecular Identification and Phylogenetic Analysis of Fungal Endophytes

The fungal genomic DNA was extracted from the fresh mycelium of *H. fuscoatra* using the CTAB method described by Gontia *et al.* [20]. The quality of the genomic DNA was assessed using 1% (v/v) agarose gel electrophoresis. The PCR amplification (40 µl) was carried out using universal primers internal transcribed spacer ribosomal DNA. The ITS-1 and ITS-2 primers were used in the experiment. The reaction mixture included 4 µl of the template, 2 µl of each primer (10 µM), 20 µl of Taq PCR mix (Amplicon), and 12 µl of double-distilled water. Afterwards, the amplified regions underwent additional purification using the QIA quick PCR purification kit from QIAGEN. The resulting sequence was then confirmed using a BLAST search. At last, the CLUSTAL W programme was used to perform the multiple sequence alignment. The phylogenetic tree was constructed by aligning the sequences using the neighbor-joining method with 1000 bootstrap replications. The trimmed sequences were then deposited in the NCBI GenBank database [21]. The Clustal W programme [22] was used to perform multiple sequence alignments and calculate similarity. A phylogenetic tree was generated from the ITS sequences using the MEGA-11 programme. The neighbor-joining (NJ) method with 1000 bootstrap resampling replicates was employed for this purpose.

2.3 Extraction of secondary metabolites from isolated endophytic fungus

The effects of secondary metabolites produced by an isolated endophytic fungus. The mature *H. fuscoatra* was cultivated on potato dextrose broth (PDB) and then inoculated into a 500 mL Erlenmeyer conical flask filled with broth. The flask was then incubated at a temperature of 28 °C ± 2 °C for a duration of 14 days. Next, the mycelium was filtered using Whatman No. 1 filter paper. The mycelia were then combined with ethyl acetate solvent for maceration. After 7 days, the mixture was filtered using Whatman No.1 filter paper, and this process was repeated twice. The mycelia extracts were concentrated using a rotary evaporator under reduced pressure. The weight of

extracts was measured after each solvent reached its boiling point [11].

2.4 Rearing of *Ae. aegypti*

The larvae of *Ae. aegypti* were collected from agricultural fields in Palakkad, Kerala. The larvae were relocated to controlled laboratory circumstances with a temperature of 27±2°C, relative humidity ranging from 75% to 85%, and a photoperiod of 14 hours of light followed by 10 hours of darkness. They were then placed in plastic containers measuring 18 × 13 × 4 cm^3, filled with 500 mL of tap water, and left to develop into various larval stages. The larvae were cultivated in the aforementioned plastic containers and provided with a daily diet consisting of a blend of pulverised dog biscuits (Pedigree, USA) and hydrolyzed yeast (Sigma-Aldrich, Germany) at a ratio of 3 parts biscuits to 1 part yeast by weight. The breeding media was replaced on a regular basis, and deceased animals were eliminated. The breeding containers were sealed with muslin cloth to prevent the intrusion of non-indigenous mosquitoes, thus avoiding contamination. For acute toxicity trials, larvae in their first to fourth instars and pupae were taken daily from culture containers and moved to glass beakers filled with 500 mL of de-chlorinated water. Adults of both genders (age: 5 days) were subjected to longevity and fertility experiments.

2.5 Rearing of *P. xylostella*

The larvae and adults of *P. xylostella* were collected from cabbage crops in Palakkad, Kerala, India. In order to facilitate egg laying, a total of 500 mature individuals were confined within a plastic enclosure of 50× 30× 30 cm^3. These individuals were supplied with cabbage leaves from the *Brassica oleracea* L. var. botrytis plant, which belongs to the Brassicaceae family. The eggs were permitted to hatch on *B. oleracea* leaves, and the larvae were kept at a constant temperature of 25±1 °C, with a relative humidity (RH) of 65±5%, under a 16L: 8D photoperiod in a growth chamber. The acute toxicity investigations utilized larval instars I-IV and pupae. Adults of both genders that had recently emerged were subjected to studies to measure their lifespan and reproductive capacity.

2.6 Larvicidal and pupicidal toxicity on *Ae. aegypti*

The culture and maintenance of *Ae. aegypti* mosquitoes were carried out following the protocol outlined by Suresh *et al.* [23]. For the toxicity experiments, a total of 25 *Ae. aegypti* larvae at different larval stages (1^{st}, 2^{nd}, 3^{rd} and 4^{th} instars) and pupae were exposed to various concentrations of fungal metabolites. The exposure lasted for 24 hours in conical flasks filled with 250 mL of distilled H_2O. The concentrations used were 100, 200, 300, 400, and 500 ppm. The experiment was conducted with five replications for each treatment, and control groups were included for comparison. Next, the mortality rate (%) was calculated using the following formula:

$$\text{Mortality (\%)} = \frac{\text{Number of dead individuals}}{\text{Number of treated individuals}} \times 100\ \%$$

2.7 Impact of secondary metabolites of isolated fungal strains on longevity and fecundity of *Ae. aegypti*

Males and females of the mosquito species *Aedes aegypti* were subjected to different concentrations (100, 200, 300, 400 and 500 ppm) of ethyl acetate metabolites derived from the fungus *H. fuscoatra*. The procedure described in Suresh *et al.* [23] was followed to apply the ethyl acetate metabolites of the fungal isolates onto a Whatman no. 1 filter paper (size 12 × 15 cm^2) that lined a glass holding tube (diameter 30 mm, length 60 mm). During the control trials, filter paper was subjected to either an aqueous solution or distilled water. There were no recorded deaths in either control group. For every trial, a total of 20 *Ae. aegypti* mosquitoes were carefully moved into a separate glass container. The mosquitoes were given 1 hour to adjust to the conditions in the tube, and then they were introduced to a test tube that was lined with either treated or control paper for 1 hour. After the exposure period, the mosquitoes were moved into chiffon cages measuring 30 × 30 cm^2. A cotton pad soaked in a 10% (w/v) glucose solution was provided. The adult subjects that received treatment were stored for a duration of 3 days following the blood meal. Subsequently, eggs were retrieved on a daily basis by utilizing small plastic bowls filled with water, which were maintained as ovitraps within the cages. The mean fecundity was determined by dividing the number of eggs laid in the ovitrap by the number of females allowed to mate (n=20). Each adult mosquito's mortality was assessed on a daily basis, and the average longevity of each mosquito was determined.

2.8 Biotoxicity of secondary metabolites of isolated fungal strains against *P. xylostella*

In accordance with the leaf-dip technique described by Sengonca *et al.* [24], the F_2 generation larvae were provided with cabbage leaf discs that had been treated with varying concentrations (100, 200, 300, 400, or 500 ppm) of *H. fuscoatra* ethyl acetate metabolites extract. Following a 24-hour period, the individuals were relocated to new cabbage leaves that had not been treated. The leaves were replaced every 24 hours. The mortality rate was documented 96 hours later. Each treatment was conducted with five duplicates, each containing 10 larvae. The formula of Hardstone *et al.* [25] was used to compute the percentage mortality.

Corrected mortality = (mortality in treatment-mortality in control)/ (100- mortality in control) × 100.

The larvae that remained alive were provided with untreated cabbage leaves as their food source until they entered the pupal stage. Pupal mortality was determined by subtracting the count of emerging adults from the overall number of pupae.

2.9 Impact of secondary metabolites of isolated fungal strains on longevity and fecundity of *P. xylostella*

Male and female *P. xylostella* adults were placed in wooden cages at a 1:1 sex ratio (n=20). The subjects were given a diet consisting of various components, including sucrose, honey, sterile distilled water, and a specific concentration of *H. fuscoatra* ethyl acetate metabolites extract. The diet was controlled without the use of extract. The calculation of mean fecundity involved daily monitoring of the number of eggs laid on five fresh *Brassica olaracea* leaves over a period of four consecutive days. This data was then divided by the number of females that were allowed to mate, which was a total of 20. Every day, we monitored the mortality rate and calculated the average lifespan of each adult *P. xylostella* by adding up their individual lifespans and dividing it by the total number of individuals tested for each treatment.

2.10 Gas chromatography–mass spectrometry (GC–MS) analysis

An Agilent 6890 gas chromatography (Agilent, USA) equipped with a fused silica capillary column PAS-5MS (30 m×0.32 mm×0.25 µm film thickness) was used to analyse the ethyl acetate extracts of *H. fuscoatra*. These extracts showed high efficacy against the dengue vector *Ae. aegypti* and the crop pest *P. xylostella*, as determined by toxicity experiments. The carrier gas used was helium at a flow rate of 1 ml/min in pulsed splitless mode. The solvent delay occurred at 3 minutes, and the injection size was 1 µl. The mass spectrometric detector was used in electron impact ionisation mode with an ionisation voltage of 70 eV, covering the m/z range of 50 to 500. The voltage of the EM was set to 1650 V. The temperature at which ionisation occurred was 230 °C. The programme started with an initial temperature of 60 °C for 2 minutes, and then increased to 280 °C at a rate of 5 °C per minute. The temperatures of the injector and detector were maintained at 250 and 280 °C, respectively. The compounds were identified by comparing their mass spectral patterns with those of the WILEY/NIST mass spectral database.

2.11 Data analysis

The acute toxicity data for both insect species were analyzed using a two-way ANOVA, considering the targeted instar and the tested dose as factors. The mortality data underwent probit analysis. The calculation of LC_{50} and LC_{90} was performed using the method described by Finney [26]. The longevity and fecundity data were analysed using a two-way ANOVA, considering two factors: the treatment and the dose. Data analysis was conducted using the SPSS Statistical Software Package version 17.0. A significance level of $p<0.05$ was employed to determine the differences between values.

3. Results and Discussion

3.1 Isolation of endophytic fungi

The isolates of endophytic fungi were obtained from the leaves of *Adhatoda vasica* in the presence of 13 isolates. The presence of endophytic fungi in plants is widespread, as they could colonize various parts of the plant [27]. The plant can be infiltrated by endophytic fungi through stomata, lenticels, natural wounds, roots, or sprouting radicles. The fungus colonizes the plant at the point of entry and then spreads throughout all sections of the plant through the xylem. Abundant endophytic fungus can be found in both temperate and tropical rain forests, as noted by Khiralla *et al.* [28]. It is worth noting that over 300,000 plant species have been found to harbour endophytic fungi. This study aims to explore the insecticidal properties of secondary metabolites extracted by ethyl acetate from *Humicola* fungal isolates. The focus is on their effects on the Dengue vector, *Aedes aegypti*, and the diamondback moth, *Plutella xylostella*.

3.2 Morphological identification of endophytic fungi

The morphological identification revealed that 13 isolates of endophytic fungi from *A. vasica* leaves were classified under the genus *Humicola* (Figures 1a–c). *Humicola* is a widely distributed species of fungus that has been found in various environments, such as wood, soil litter, grass, forest soil, sea water, and compos. The morphology of results in the production of numerous small conidia, which enables them to disperse over long distances and enhance their population [29]. The genus *Humicola* exhibits distinct characteristics, including a dark green colony, spherical to semi-spherical conidia, and elongated, unbranched, and thick-walled conidiophores [30].

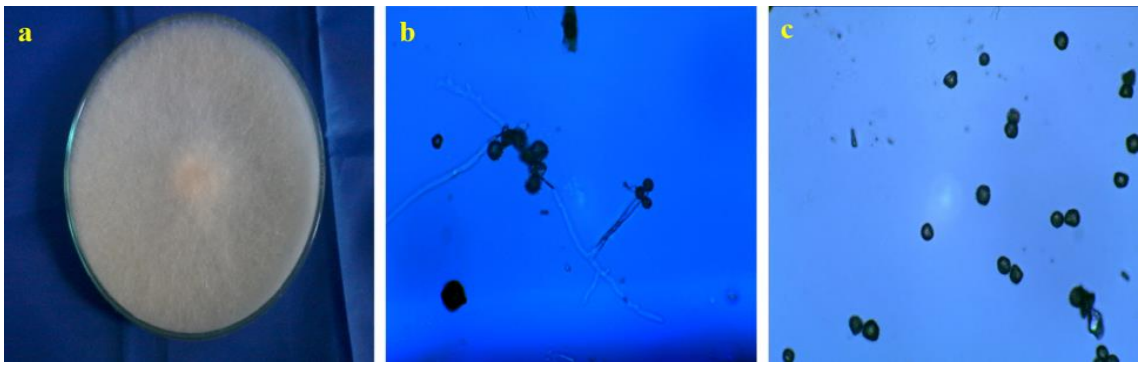

Fig 1: Morphology of endophytic fungi from *Adhatoda vasica* leaves *Humicola* (a to c)

3.3 Molecular identification of isolated DNA by PCR analysis

The identification of isolated *H. fuscoatra* DNA was achieved through PCR amplification using universal primers and subsequent sequencing of the 18S rRNA. The DNA fragment was amplified (600 bp) and compared with a ladder. The amplification was done using universal primers, namely Internal Transcribed Spacer ITS-1 (forward primer 5' TCCGTAGGTGAACCTGCGG 3') and ITS-2 (reverse primer 5' TCCTCCGCTTATTGATATGC 3') (Figure 2). The ITS region of the *H. fuscoatra* fungus was sequenced, and the obtained sequences were analyzed in the NCBI gen bank database.

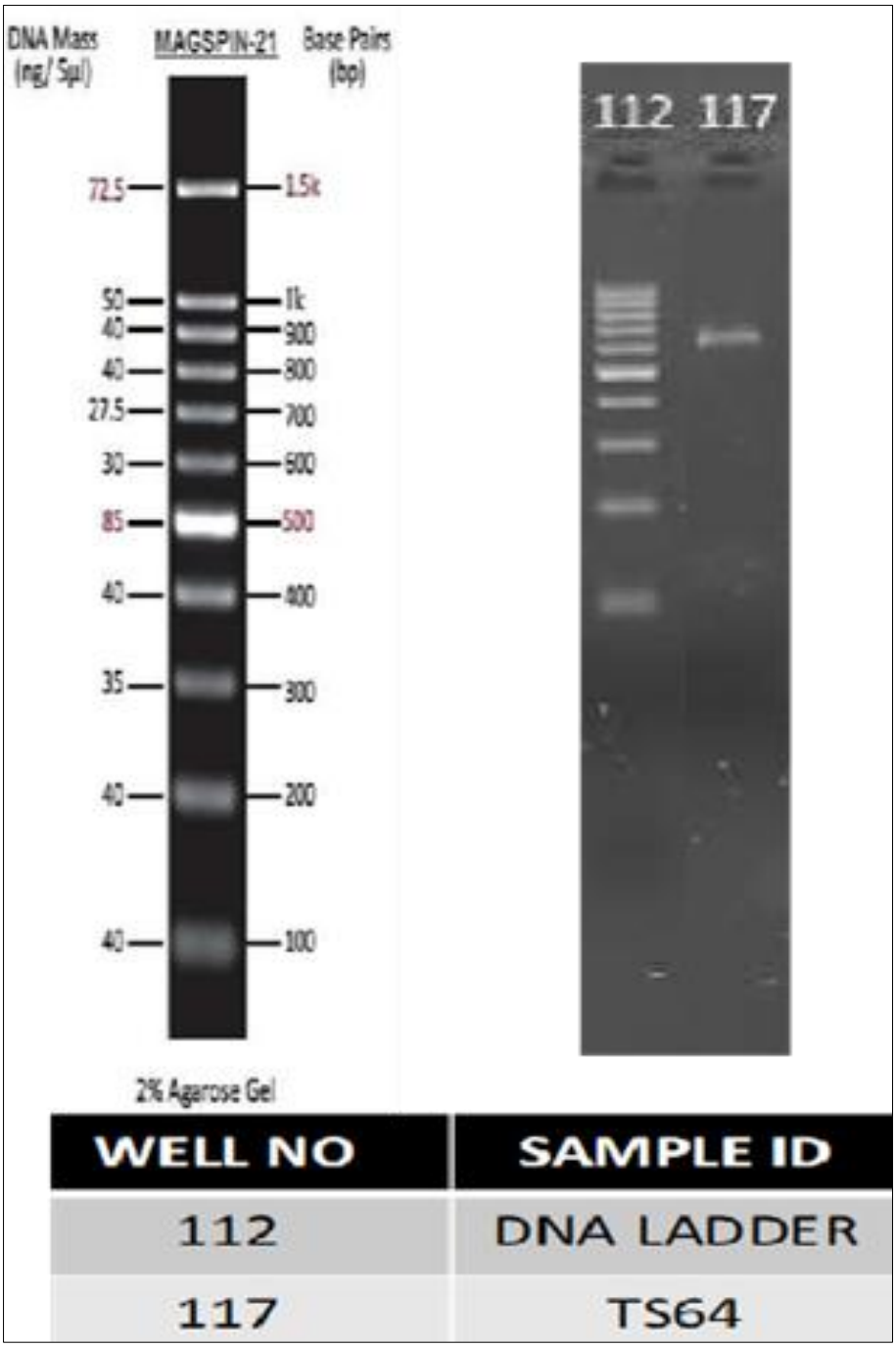

Fig 2: DNA and PCR amplification of DNA at ITS regions

3.4 Phylogenetic tree analysis

The evolutionary branch of fungal sequences was deduced utilizing the UPGMA method, and the proportion of replicate trees comprised of taxa that were grouped together in the bootstrap test (1000 replicates) was computed (Figure 3). Utilizing the Tajima-Nei method, the evolutionary distances were calculated for a total of eleven nucleotide sequences. Molecular analyses were performed using the MEGA5 software. We constructed phylogenetic trees for ten phylotypes using data retrieved from the GenBank database to establish the relationship between a specific sequence and closely related species. The *H. fuscoatra* strain's ITS sequences were similar to 88% of the *Humicola* Species strain and 100% of the *Humicola sardinia* strain (OP597922). Evidence of inadequately structured conidiogenous apparatus in other medically significant fungi [31, 32] clearly attributes this phenomenon to *Humicola* species, including cryptic taxa. Although *Humicola* contains over 20 species, the most isolated species from the environment is *H. fuscoatra*. These fungi are notably cellulolytic in nature [33, 34].

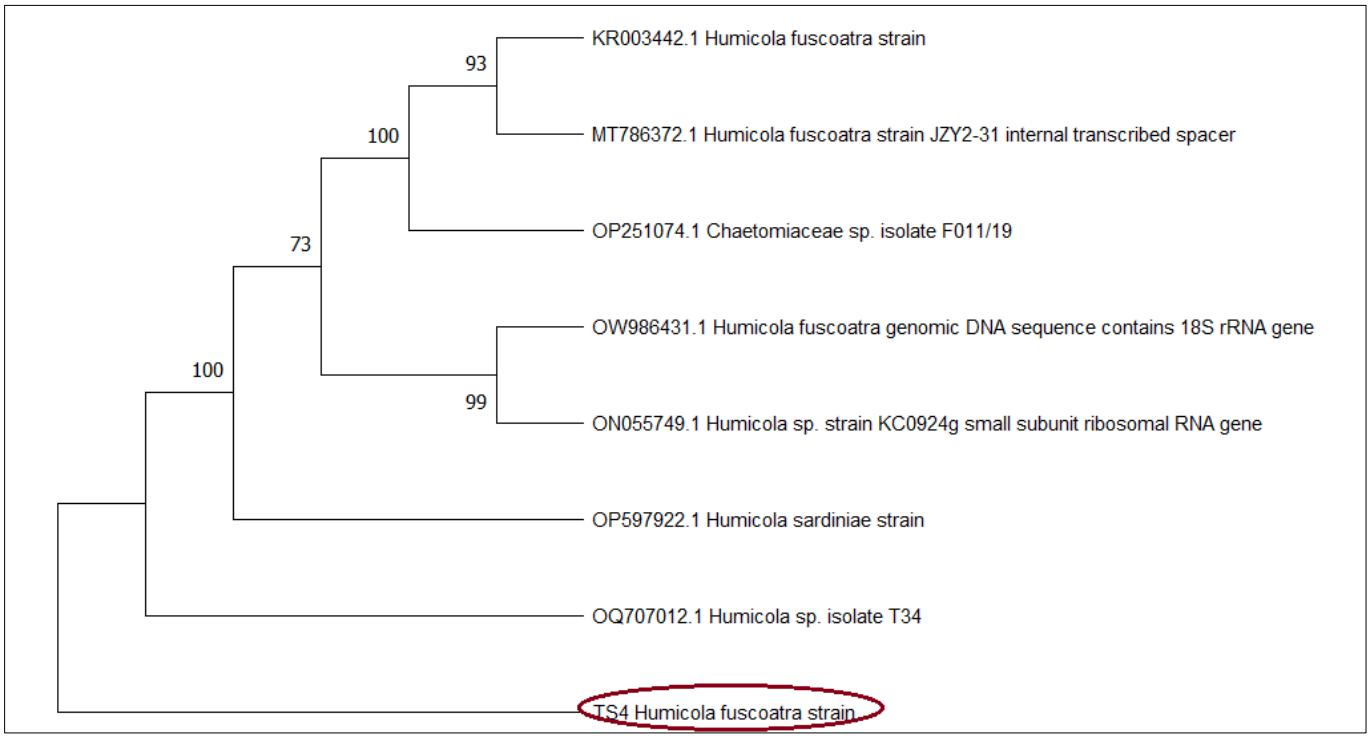

Fig 3: Phylogenetic tree analysis of *Humicola fuscoatra*

3.5 Biotoxicity against *Ae. aegypti*

The ethyl acetate extracts of the fungus *H. fuscoatra* exhibited toxicity towards larval instars (I–IV) and pupae of *Ae. aegypti* in acute toxicity experiments. These were the LC_{50} values: 132.71 ppm for I instar, 192.82 ppm for II, 272.25 ppm for III, 372.98 ppm for IV, and 450.163 ppm for pupae (Table 1). The toxicity results indicated that the concentration of ethyl acetate extracts derived from the fungus *H. fuscoatra* was proportional to the mortality rate. A prior investigation documented the lethal impact of the *Paecilomyces* fungus on the larval instars of significant vector species. Notably, the fungus had no effect on non-target organisms, and its extracellular metabolites exhibited biological stability. These findings provide a promising alternative to larvicides based on mycelium and conidia [35]. Similarly, Abutaha *et al.* [36] found that the endophytic fungus *Cochliobolus spicifer* had a big effect on the death of third-instar larvae of Culex and Aedes sp., which is similar to what we found in this study.

3.6 Biotoxicity against *P. xylostella*

The ethyl acetate extracts derived from the fungus *H. fuscoatra* exhibited toxicity against the larvae and pupae of *P. xylostella*. The LC_{50} values for the substances were as follows: 287.07 ppm for (I), 344.80 ppm for (II), 412.65 ppm for (III), 467.82 ppm for (IV), and 656.69 ppm for the pupa (Table 2). Researchers have identified several additional plants as potential sources of metabolites harmful to the diamondback moth. In their study, Wang *et al.* [37] discussed the ability of entomopathogenic fungi to produce a wide variety of bioactive molecules, referred to as secondary metabolic compounds. Fungal metabolites have demonstrated insecticidal properties and the potential to serve as biopesticides. Batta [38] found that introducing *M. anisopliae* to *Brassica napus* plants using foliar spray resulted in a mortality rate of 63.3% for *P. xylostella* larvae after 4 weeks of endophytic establishment. In their 2016 study, Gautam *et al.* [39] found that *P. xylostella* larvae did not survive when exposed to leaves sprayed with *B. bassiana*. This was attributed to the generation of certain volatile chemicals resulting from the interaction between *B. bassiana* and the host plant. Interestingly, no fungal infection was seen in the deceased larvae. In this study, no fungal infection was detected in the deceased larvae.

Table 1: Larval and pupal toxicity of Ethyl acetate extracts of the fungus *H. fuscoatra* against the dengue vector, *Ae. Aegypti*

Larval and pupal stage	Larval and pupal mortality (%) (Mean ± S.D) Concentration (ppm)					LC_{50} (LC_{90})	95% Confidence Limit LC_{50} (LC_{90})		Regression equation	χ^2 (*d.f.*=3)
	100	200	300	400	500		Lower	Upper		
Larva I	49.0 ±0.8	55.4±1.6	76.5±1.5	84.5±1.3	95.8±0.0	132.712 (441.342)	81.703 (397.463)	169.384 (505.752)	*x*= 0.004 *y*= -0.551	3.328
Larva II	41.5±1.0	48.6±1.0	61.1±1.1	73.5±2.2	86.1±1.2	192.823 (593.016)	140.271 (520.490)	231.622 (712.366)	*x*= 0.003 *y*= -0.617	1.358
Larva III	33.2±0.4	41.4±1.4	50.4±0.8	64.2±2.0	75.0±1.6	272.251 (731.005)	226.958 (626.436)	313.019 (916.702)	*x*=0.003 *y*= -0.761	0.518
Larva IV	25.6±1.7	33.0±1.8	41.8±2.0	51.5±1.4	63.9±1.2	372.982 (884.493)	327.362 (737.534)	434.291 (1167.372)	*x*= 0.003 *y*= -0.934	0.226
Pupae	18.7±1.0	27.4±1.0	34.4±1.4	45.7±1.5	54.7±2.0	450.163 (961.951)	396.583 (795.661)	537.478 (1288.294)	*x*= 0.003 *y*= -1.127	0.106

The larval mortalities are expressed as mean ± SD of five replicates. Nil mortality was observed in the control. Within a column means followed by the same letter(s) are not significantly different at 5% level by Duncan's multiple range test. LFL - Lower Fiducial Limit; UFL - Upper Fiducial Limit. x^2, Chi-square value.

Table 2: Larval and pupal toxicity of Ethyl acetate extracts of the fungus *H. fuscoatra* against the *P. xylostella*

Larval and pupal stage	Larval and pupal mortality (%) (Mean ± S.D) Concentration (ppm)					LC_{50} (LC_{90})	95% Confidence Limit LC_{50} (LC_{90})		Regression equation	χ^2 (*d. f.*=3)
	100	200	300	400	500		Lower	Upper		
Larva I	21.1±1.58	32.8±2.40	48.6±2.70	67.3±2.54	89.5±2.28	287.071 (548.934)	261.962 (503.202)	311.696 (612.699)	*x*= 0.005 *y*= -1.405	3.456
Larva II	18.6±2.91	28.2±1.58	43.8±3.78	56.5±3.27	73.4±2.07	344.803 (683.796)	314.073 (609.276)	379.431 (798.626)	*x*= 0.004 *y*= -1.304	0.319
Larva III	15.4±2.86	23.6±5.31	39.4±4.39	45.9±4.39	61.4±3.57	412.656 (810.587)	373.498 (702.401)	466.244 (992.961)	*x*=0.003 *y*= -1.329	0.870
Larva IV	9.5±2.07	18.4±5.31	32.2±4.87	39.7±4.0	53.4±4.15	467.824 (848.767)	423.837 (734.980)	533.041 (1041.255)	*x*= 0.003 *y*= -1.574	0.903
Pupae	2.2±2.38	7.6±3.04	15.4±2.73	21.4±3.56	28.5±4.56	656.694 (980.716)	570.496 (816.826)	822.415 (1308.849)	*x*= 0.003 *y*= -2.101	1.640

The larval mortalities are expressed as mean ± SD of five replicates. Nil mortality was observed in the control. Within a column means followed by the same letter(s) are not significantly different at 5% level by Duncan's multiple range test. LFL - Lower Fiducial Limit; UFL - Upper Fiducial Limit. x^2, Chi-square value. *Significant at $p< 0.05$ level, n. s. = not significant (α = 0.05).

3.7 Impact of ethyl acetate extracts of the fungus *H. fuscoatra* on *Ae. aegypti* longevity and fecundity

The study found that the adult longevity and fecundity of *Ae. aegypti* were significantly reduced after being treated with ethyl acetate extracts of the fungus *H. fuscoatra* (Table 3). The longevity of male subjects decreased to 6.2 days when treated with a 500-ppm concentration of ethyl acetate extracts from the fungus *H. fuscoatra*, compared to a control group with a longevity of 14.74 days. Similarly, the longevity of female subjects decreased to 13.4 days under the same treatment, compared to a control group with a longevity of 25.68 days. The treatment with ethyl acetate extracts of the fungus *H. fuscoatra* also resulted in a reduction in female fecundity. The treatment groups at different concentrations resulted in a range of 147.6 to 111.0 eggs recorded, while the control group had 150 eggs recorded (Table 3).

Table 3: Impact of ethyl acetate extracts of the fungus *H. fuscoatra* on longevity and fecundity of the dengue vector *Ae. aegypti*

Treatment (ppm)	Adult longevity		Fecundity
	Male	Female	
Control	14.74±1.58	25.68±1.48	159.4±1.04
100	11.4±2.40	23.2±2.91	147.6±1.60
200	10.2±1.34	20.8±3.63	137.8±1.38
300	9.2±1.92	17.6±1.34	128±1.54
400	7.2±1.58	15.6±2.19	116.6±1.63
500	6.2±1.58	13.4±2.30	111±1.84

Values followed by the same letter (s) are not significantly different (Tukey's HSD, α=0.05).

Similarly, results were noted in *An. gambiae* that were sprayed with solutions containing *B. bassiana* spores; females that were exposed to the spores laid an approximate 16% fewer eggs compared to noninfected females [40]. Mouatcho *et al*. [41] found that *M. anisopliae* and *B. bassiana* caused a comparable reduction in the number of eggs laid per female *Anopheles funestus* over the course of three gonotrophic cycles. Darbro *et al*. [42] found that exposure to *B. bassiana* for 24 hours reduced the fecundity of *Ae. aegypti* females by 39%. In a similar fashion, exposure to *M. anisopliae* spores reduced the reproductive capacity of *Ae. albopictus* by 42%, as measured by the mean number of eggs laid, in comparison with uninfected females [43].

3.8 Impact of ethyl acetate extracts of the fungus *H. fuscoatra* on *P. xylostella* longevity and fecundity

The effectiveness of ethyl acetate extracts derived from the fungus *H. fuscoatra* in reducing adult longevity and fecundity in the diamond-back moth *P. xylostella* was demonstrated in Table 4. Following treatment with ethyl acetate extracts of the fungus *H. fuscoatra* at a concentration of 500 ppm, male longevity decreased to 5.4 days, while the control group maintained 14.68 days. Similarly, treatment with ethyl acetate extracts of the fungus *H. fuscoatra* reduced female longevity to 11.4 days, while the control group maintained 26.62 days. The treatment with ethyl acetate extracts of the fungus *H. fuscoatra* significantly reduced fecundity; the control group provided 870 eggs, whereas the treatment groups produced 157.8 eggs at 100 ppm, 200 ppm, 300 ppm, and 200 ppm, respectively (Table 4). This study is the first to assess the effects of ethyl acetate extracts derived from the fungus *H. fuscoatra* on the longevity and fecundity of moth parasites. Further evidence supports the concept that extracts of endophytic fungi isolates are deleterious to crop pests.

Table 4: Impact of ethyl acetate extracts of the fungus *H. fuscoatra* on longevity and fecundity of the crop pest *Plutella xylostella*

Treatment (ppm)	Adult longevity		Fecundity
	Male	Female	
Control	14.68±1.58	26.62±1.48	870.86±2.04
5	12.4±2.15	24.8±1.91	762.8±2.60
10	10.4±1.60	21.8±1.32	540.8±2.84
15	8.2±1.92	18.6±2.20	420.56±2.84
20	7.2±1.60	14.6±2.07	290.2±1.90
25	5.4±1.58	11.4±3.01	157.86±2.12

Values followed by the same letter(s) are not significantly different (Tukey's HSD, α=0.05)

Previous studies have shown that the presence of endophytic fungi in plants can effectively decrease the feeding and oviposition of insect pests. For example, research on bean plants demonstrated a reduction in the bean stem maggot population [44]. Similarly, in wheat plants, the presence of endophytic fungi led to a decrease in the population of cotton leafworms [45]. The presence of fungal endophytes in plants can lead to a reduction in insect pests, possibly because of the production of secondary metabolites by these fungi. Previous studies have reported that plants colonized with endophytic fungi are less favourable to insects and indirectly impact the fitness of pests. Our findings align with previous studies that have demonstrated the detrimental effects of endophytic fungi on the reproductive potential and lifespan of insects [46]. The negative effects observed may be attributed to secondary metabolites or the activation of a systemic response in the colonized plants [47].

3.9 GC–MS analysis

The compounds found in the ethyl acetate extracts of *H. fuscoatra* are listed in Tables (5). Through the analysis of *H. fuscoatra* ethyl acetate extracts, a total of 24 different compounds were identified, accounting for 100% of the extract (Table 3). The primary components identified in the extract included hexadecanoic acid (15.07%), octadecadienoic acid (13.74%), hexadecanoic acid, methyl ester (12.89%), hexadecan (6.43%), octadecan (8.33%), and phenol, 2,4-bis(1,1-dimethylethyl) (2.23%). Previous studies have mentioned the major compounds found in ethyl acetate extracts of fungi, which have shown potential as larvicidal, nematicidal, pesticide, and insectifuge agents [48, 49].

Table 5: GC–MS analysis of methanol extract of the endophytic fungi *H. fuscoatra.*

R. Time	Area	Area%	Height	Height%	Name	Base m/z
7.049	6962260	5.05	7284196	6.88	DODECANE	57.10
9.409	6407156	4.88	4564705	4.31	4-NITROBENZALDEHYDE	51.05
10.157	11341065	3.38	5424243	5.12	1-PENTADECENE	41.05
10.306	6148964	13.74	19961991	18.85	Octadecanoic acid	57.10
11.942	7495631	2.23	3070905	2.90	PHENOL, 2,4-BIS(1,1-DIMETHYLETHYL)-	191.15
12.229	1819255	0.54	276056	0.26	Benzoic acid, 4-ethoxy-, ethyl ester	121.10
12.767	9617273	2.86	971984	0.92	DODECANOIC ACID	60.05
13.244	14311001	4.26	6146854	5.80	Cetene	41.05
13.375	43293118	12.89	17627838	16.64	Hexadecanoic acid, methyl ester	57.10
15.568	8233090	2.45	716210	0.68	TETRADECANOIC ACID	43.10
16.058	0761235	3.20	4983902	4.71	1-Nonadecene	43.05
16.163	7991322	8.33	10406207	9.82	OCTADECANE	57.10
17.573	4339990	1.29	1659587	1.57	7,9-DITERT-BUTYL-1-OXASPIRO [4.5] DECA-6,9-DIENE-2,8-DIONE	57.10
17.742	1463293	0.44	559547	0.53	HEPTADECANE	74.05
17.942	1973765	0.59	250767	0.24	Nonadecane, 9-methyl-	43.10
18.319	0637790	15.07	7644236	7.22	HEXADECANOIC ACID	43.05
18.908	2026115	0.60	488166	0.46	OCTYLCYCLODECANE	55.05
20.297	1131583	0.34	269809	0.25	NONADECANE, 9-METHYL-	57.10
20.618	1593325	6.43	4655426	4.40	HEXADECANE	43.05
20.925	11630896	3.46	1552896	1.47	1-Docosene	43.05
21.211	1180790	0.35	309155	0.29	(1-PROPYLNONYL) CYCLOHEXANE #	83.15
22.808	6406264	4.88	4417006	4.17	INDOLIZINE	70.05
23.272	6967864	2.07	2107340	1.99	ACETAMIDE, 2,2-DICHLORO-N- [2-HYDROXY-1-(HYDROXYMETHYL)-2-(4-NITROPHENYL) ETHYL]-, [R	117.05
24.898	2194517	0.65	570256	0.54	Hexacosane	57.10
	5927562	100.00	105919282	100.00		

4. Conclusion

The study demonstrates that *Humicola fuscoatra*, isolated and identified from the leaves of *Adhatoda vasica*, exhibits a significant larvicidal effect on the dengue vector *Ae. aegypti* and the crop pest *P. xylostella*. This research significantly contributes to our understanding of the toxic effects of ethyl acetate extracts from the fungus *H. fuscoatra* on medically and agriculturally significant invertebrates. The findings suggest that these tested products have the potential to be developed into more advanced and safer pest control solutions. The study highlighted the practical application of ethyl acetate extracts from the fungus *H. fuscoatra* in integrated pest management programmes.

5. Acknowledgement

Authors have no competing Acknowledgements.

6. Funding Statements

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

7. Declaration of Competing Interest: The author (s) declare

(s) that there is no conflict of interest regarding the publication of this paper.

8. Abbreviations

- **CTAB:** Cetyltrimethylammonium bromide.
- **DNA:** Deoxyribonucleic acid.
- **PCR:** polymerase chain reaction.
- **ANOVA:** Analysis of variance.
- **RH:** relative humidity.

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