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Antibacterial and mosquitocidal potential of *Fusarium annulatum* (Entomopathogenic Fungi) from Agricultural soil

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Abstract

The current study focused on the secondary metabolites of the fungus *Fusarium* sp. against pathogenic bacteria and vector mosquitoes. The soil samples were collected from 50 different areas of agricultural fields in Cuddalore district. The possessed fungus was isolated and identified using the appropriate methods, and from the categorised isolates, the highest secondary metabolites produced by potential fungi were screened. The highest potential secondary metabolites produced by *Fusarium annulatum* was identified by molecular sequencing method. Then secondary metabolites were produced from mass cultivated fungi and separated by the ethyl acetated solvent technique. In the present study, antibacterial and mosquitocidal activities were performed by collected secondary metabolites against three different mosquito larvae and selected pathogenic bacteria. At a concentration of 150 mg/ml, the extract was discovered to be potentially effective against *Staphylococcus aureus* (19 mm), *Streptococcus pyogenes* (18 mm), *Streptococcus pneumonia* (17mm), *Escherichia coli* (19 mm), *Enterococcus fecalis* (17 mm), *Pseudomonas aeruginosa* (17 mm) and *Proteus* sp (17 mm). The mosquitocidal activity done for the 3 common vectors were *Anopheles stephensi, Aedes aegypti* and *Culex quinquefasciatus*, LC_{50} and LC_{90} values achieved by the Fungi extract were 38.81 µg/ml and 215.17µg/ml for *Ae. aegypti* larvae when compared to *An. stephensi* and *Cx. quinquefasciatus* larvae was relatively more resistance to the fungal extract. The findings support the notion that fungi secondary metabolites are a suitable source of biologically active compounds for drug discovery and the development of biopesticides since they demonstrate that the Fusarium annulatum fungal crude extract exhibits antibacterial and mosquitocidal activities.

Key words : *Fusarium annulatum*, Secondary metabolite, Antibacterial, Mosquitocidal, Drug discovery, Biopesticides.

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Arthropods are harmful carriers of parasites and fatal infections[8]. As carriers of a wide range of dangerous viruses and parasites that affect humans, mosquitoes pose a significant threat to global public health[2]. When a clinically important mosquito feeds on humans, it spreads a variety of diseases. *Aedes aegypti, Aedes albopictus, Anopheles stephensi, and Culex quinquefasciatus* are four mosquito species that are among the major carriers of a variety of diseases, including dengue fever, malaria, and West Nile virus[10]. Global temperature change may hasten the spread of diseases carried by mosquitoes (Lee *et al.,* 2015). Every year, two million people die from diseases spread by mosquitoes (Logeswaran *et al.,* 2019). Controlling mosquito populations is essential to preventing the spread and epidemics of infectious illnesses carried by mosquitoes[8].

Numerous studies have examined techniques for preventing mosquito bites, such as using pesticides or repellents, to decrease the spread of vector-borne diseases[6]. Currently, mosquito vectors important to medicine and veterinary care are primarily controlled by chemical pesticides like organophosphates and pyrethroids. Microbiological control agents, indoor residual spraying, and insecticide-treated bed nets are different methods of mosquito eradication[15]. Smoke from fuels made from biomass and mosquito coils are both contributors to residential air pollution. Smoke from biofuels has been investigated as a potential risk factor for poor pulmonary outcomes[18]. Bio-pesticides offer several advantages over the chemical pesticides *viz.* safety, targeted activity to the coveted pests, operational in lower quantities thereby offering lesser exposure and rapid decomposition[12].

Researchers have recently put much effort into investigating environmentally safe, affordable, biodegradable pesticides for controlling mosquitoes. Insect morbidity is high even under field conditions as a result of entomopathogenic fungi's capacity to bind to the insect by binding to its cuticle and discharging toxins into the hemolymph[22]. According to Pradeep *et al.,*[16], fungi can create a wide range of valuable commodities, including as pharmaceutically active secondary metabolites, industrially essential enzymes, and vibrant colours. About 1000 kinds of fungi are known to infect arthropods, making them the most prevalent diseases in insects[25].

Site information for the soil sample collection :

Soil samples were collected from a range of agricultural fields in the Cuddalore region, near Chidambaram. Chidambaram is 5.97 metres above sea level and is situated at 11° 23' 53.4984''N latitude and 79° 41' 43.2888"E longitude. The average yearly temperature ranges from 94°F (35°C) to 80°F (27°C) and receives 1402.6mm of rainfall.

Technique for collecting soil samples :

Fifty soil samples in all were gathered from various sites. 20 cm of soil was sampled using a soil core borer, and the soil sample was taken in zippered polyethene bags that had been sterilised with ethanol. They were brought over to the lab. In preparation for future processing, the soils were manually crushed, sieved, and kept at 4 °C [7].

Isolation of fungi from soil sample :

The dilution procedure was used to isolate the fungus. In a conical flask, a soil sample (10g) was combined with 90 ml of sterile water. After shaking for an hour in an oscillator, the soil mixture had been diluted from 10^{-1} to 10^{-3}. To prevent bacterial development, 0.1 ml of the soil solution (10^{-3}) was equally covered with chloramphenicol on Sabouraud dextrose agar (SDA). All plates underwent a week of incubation at room temperature until noticeable fungus growth was detected. Emergent hyphae were transferred to sterile Sabouraud dextrose agar (SDA) plates, where they were then purified. Based on the differences in their morphology, fungal isolates were categorized[14].

Identification of fungi from soil sample:

Cultural characterization :

Fungal isolates were assessed culturally based on the characteristics of their colonies, such as colony color, form, appearance, and growth profile on SDA.

Morphological identification :

LPCB Technique :

The fungal culture was stained with lactophenol cotton blue and observed under a microscope. Using standardized identification guidelines and microscopic characteristics (such as the structure of hyphae, conidia, and conidiophores), fungi were identified[20].

Molecular identification :

After the spores were evenly distributed by vortexing two microliters of Sabouraud dextrose broth (SDB) into tubes, the spores and SDB combinations were added to flasks containing 100 mL of SDB. For two to three days, the flasks were left at room temperature unattended. After being filtered, the mycelium was extracted, lyophilized, and stored at -80°C after being frozen for 30 minutes. For the production of mycelium powder, the mycelium was crushed in liquid nitrogen using a sterile mortar. 20 mg of mycelium powder was used to extract DNA by employing a DNeasy plant micro kit. By electrophoresis on a 0.8% agarose gel and visualisation with ethidium bromide under UV trans-illumination, the DNA amount and quality were assessed.

By using the primers ITS1-F (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), the ITS sequence of ribosomal DNA was amplified by PCR. A final volume of 50 µL, 2 µL of DNA, 0.5 mM of each primer, 150 mM of dNTP, 1 U/µLof Taq DNA polymerase (Promega), and PCR reaction buffer were employed for the PCR amplifications. A beginning denaturation of three minutes at 94°C was subsequent by 35 cycles of one minute at 94°C, one minute at 50°C, and one minute at 72°C, with a final extension of ten minutes at 72°C. The amplified products were examined using 1% agarose gel electrophoresis, and ethidium bromide was used to visualise them under UV trans-illumination. According to the manufacturer's recommendations, an ExoSAPIT kit was used to purify the PCR products. The Big Dye Deoxy Terminator cycle-sequencing kit was used to sequence the purified products in an automated DNA Sequencer.

Mass cultivation :

By soaking sterile distilled water with 0.05% Tween, the spores were collected. Utilising sterile cloth, the media detritus, conidia clumps, and fungal hyphae were removed from this fungus suspension. Hemocytometer spore counts were used to quantify the concentration of the filtered fungal suspension, which was then adjusted to 1×10^7 conidia/ml. In 500 ml Erlenmeyer flasks, 250 ml of Sabouraud dextrose agar (SDA) was used to make the fungus broth. 250 ml of broth culture were injected with 1×10^7 conidia/ml, and the broth was then incubated at 28±2 °C and shaken at 130 rpm for a week.

Secondary metabolite separation :

Selected colonies were inoculated into a 250-mL Erlenmeyer flask that contained 100 mL of the medium to grow the fungus on SDB. The flask was left to incubate for two weeks at 28°C with periodic 150 rpm shaking. After the incubation period, 10% ethyl acetate was added to the fungus' fermentation broth to homogenise it. Ethyl acetate was used as an organic solvent in the solvent extraction process to extract the metabolite. Equal amounts of the solvents were added to the filtrate, which was then thoroughly mixed for 10 minutes and left for 5 minutes to create two transparent, indistinguishable layers. Using a separating funnel, the solvent's upper layer that contained the extracted chemicals was separated. To produce the crude secondary metabolite, the solvent was evaporated, and the resulting chemical was dried in a rotator vacuum evaporator. In order to conduct additional bioassays, the crude extract was then combined with 1 mg/mL dimethyl sulfoxide and kept at 4°C [19].

Antibacterial activity :

By using the agar well diffusion method, the antibacterial activity of the fungal secondary metabolitese was assessed against the five Gram-positive and seven Gram-negative human bacterial pathogens were *Staphylococcus aureus, Streptococcus pneumonia, Enterococcus fecalis, Enterobacter* sp., *Klebsiella pneumoniae, Pseudomonas aeruginosa Salmonella paratyphi, Salmonella typhi, Serratia sp., Escherichia coli* and *Proteus* sp.,. Dimethyl sulphoxide (DMSO) served as the negative control, and chloramphenicol served as the positive control. For 24 hours, plates underwent incubation at 37°C.

Mosquitoes larvicidal activity :

The 50 larvae were kept in a 25±2°C, 75 ± 2% relative humidity, and 14:10 (L/D) photoperiod environment. The larvicidal efficacy of the mycelial secondary metabolites against selected mosquitoes was evaluated at doses of 50, 100, 150, 200, and 250 g/ml. Then, using 100 ml of water, different metabolite concentrations were created. The mycelial metabolite was dispersed in 10% DMSO for preparation at a concentration of 1 mg/ml (stock solution, 5 ml), and bioassay experiments were conducted utilising metabolites from mycelium extract at various doses (50, 100, 150, 200, and 250 g/ml). Abbott's formula was used to calculate mortality and survival rates after 24 hours of exposure, and the LC_{50} and LC_{90} values of exposed larvae were calculated using probit analysis. Each experiment's negative control (DMSO-distilled water treatment) was tested three times.

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

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

Data analysis :

The percentages of mortality for *Aedes aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus* larvae were subjected to an analysis of variance (ANOVA). The SPSS 16 programme software was used to do a probit analysis to determine the lethal concentrations (LC_{50} and LC_{90}) needed to kill 50 and 90 of the larvae and pupae 24 hours after treatment.

Isolation and identification of fungal isolates :

The soil samples were used to isolate a total of 10 distinct fungi, which were then purified using a point inoculation approach. The salient characteristics of all the isolates are shown in Table-1 after the fungi were initially recognised using microscopic (40×) morphological parameters and soil fungal recognition by appropriate key manual. The fungi that were isolated have been identified as follows based on the results of the microscopy and morphological features mentioned in Table-1: *Penicillium* sp, *Aspergillus* sp, *Fusarium* sp, *Verticillium* sp, *Alternaria* sp, *Colletotrichum sp, Phoma* sp, *Rhizophus* sp, *Mucor* sp, *Beauveria* sp. In order to obtain a pure culture that could be used for further research, *Fusarium annulatum* was subsequently subcultured in the SDA media because it had generated secondary metabolites with the highest potential. The colony colour, form, texture, and growth pattern of *Fusarium annulatum* isolates provided evidence of their cultural characteristics showed in Figure 1. *Fusarium annulatum* produced copious amounts of white to pinkish-white aerial mycelium on SDA. Macroconidia were fusiform, cylindrical, confined, straight to slightly curved, and had 3-6 septa when observed under a microscope. Chlamydospores weren't discovered. The microconidia, which formed on mono phialides and polyphialides, were generally aseptate, club-shaped, and each had a bent apical cell. The sequences obtained from the samples were aligned and edited using MEGA software version 6. Similarity search was carried out using the aligned sequence against the sequences submitted in NCBI using NCBI-BLAST. The sequence obtained from the sample shows 100% similarity with *Fusarium annulatum.* Sabouraud's dextrose broth medium was developed for mass culturing of *Fusarium annulatum* at 25 °C to separate the secondary metabolites for further bioassays.

Table-1. Macroscopic and Microscopic characterization of fungi

S. no.	Name of the fungi	Morphological features	Microscopical observation
1.	*Penicillium* sp.	Colonies are usually fast growing, in shades of green, sometimes white, mostly consisting of a dense felt of conidiophores.	Chains of single-celled conidia are produced in basipetal succession from a specialised conidiogenous cell called a phialide.
2.	*Aspergillus* sp.	Greenish-blue with a whitish edge, yellow to brownish on reverse, Yellow-green and growth is white, becoming black later on giving "salt and pepper appearance" which results from darkly pigmented conidia borne in large numbers on conidiophores and reverse turning pale yellow.	The microscopic features included uniseriate aspergilli, columnar conidial heads, flask-shaped vesicles with the phialides covering one half to three quarters of the vesicle, and globose conidia that were finely rough and plain green in color.
3.	*Fusarium* sp.	White, lavender, pink, salmon, or gray colored colonies with velvety or cottony surfaces.	Curved, fusiformed and three septate structures.Macroconidia were least abundant, elongated, cylindrical, slightly.
4.	*Verticillium* sp.	The color is white initially and becomes yellowish, red, pinkish-brown, or green. From the reverse, it is white or brown (rust color).	Septate hyaline hyphae, conidiophores, phialides, and conidia are observed. Conidiophores are hyaline, simple or branched. The branching of the conidiophores occurs in whorls at several levels.
5.	*Alternaria* sp.	Colonies are fast growing, black to olivaceous-black or greyish, and are suede-like to floccose.	Branched acropetal chains (blastocatenate) of multicellular conidia (dictyoconidia) are produced sympodially from simple, sometimes branched, short or elongate conidiophores. Conidia are obclavate, obpyriform, sometimes ovoid or ellipsoidal, often with a short conical or cylindrical beak, pale brown, smooth-walled or verrucose.

6.	*Colletotrichum* sp.	salmon pink-coloured colonies with white margins	Conidia were hyaline, aseptate, broadly elliptical, or cylindrical with rounded ends.
7.	*Phoma* sp.	The color is initially white and later becomes olive grey with an occasional tint of pink. From the reverse, it is dark brown to black. They are flat, spreading, powdery to velvety, and often largely submerged in the medium.	The hyphae are hyaline to brown. Pycnidia are the large, round to pyriform, asexual fruiting bodies. Conidia are unicellular, hyaline, and oval-shaped.
8.	*Rhizophus* sp.	The texture is typically cotton-candy like. From the front, the color of the colony is white initiallyand turns grey to yellowish brown in time. The reverse is white to pale.	The texture is typically cotton-candy like. From the front, the color of the colony is white initially and turns grey to yellowish brown in time. The reverse is white to pale.
9.	*Mucor* sp.	Colonies are very fast growing, cottony to fluffy, white to yellow, becoming dark-grey.	Nonseptate or sparsely septate, broad (6-15 µm) hyphae, sporangiophores, sporangia, and spores are visualized.
10.	*Beauveria* sp.	Grows as a white mould. On most common cultural media, it produces many dry, powdery conidia in distinctive white spore balls. Each spore ball is composed of a cluster of conidiogenous cells.	The hyaline, smooth walled conidia were globose to sub-globose in shape

Figure 1. Macroscopic and microscopic observation of the Fusarium annulatum

Antibacterial activity :

Secondary metabolites of Fusarium annulatum were found to have antibacterial action in the current investigation against common bacterial infections. The ethyl acetate extract was discovered more potent against four of the Gram-positive bacteria Staphylococcus aureus (19 mm), Streptococcus pyogenes (18 mm), Enterococcus fecalis (17mm), Streptococcus pneumonia (17mm) and three of the Gram-negative bacteria Escherichia coli (19mm), Pseudomonas aeruginosa (17mm) and Proteus sp.(17mm) at the concentration of 150 mg/ml. Both the negative control, Dimethyl sulfoxide (DMSO), and the positive control, Ciprofloxacin, had no effect on the investigated bacterial strains. Table-2 displays particular findings of the antibacterial effectiveness of fungi metabolites. The antibacterial activity of secondary metabolites was tested by their zone of inhibition in Figure 2 and 3.

Larvicidal activity :

Larvicidal activity of Fusarium annulatum extract was investigated on An. stephensi, Ae. aegypti and Cx. quinquefaciatus. 24 h post-treatment, LC_{50} values attained by the Fusarium annulatum secondary metabolites were 38.81 ?g/ml for Aedes aegypti larvae compared to Anopheles stephensi and Culex quinquefasciatus larvae which had relatively higher resistance to the fungal extract (LC_{50} 44.79 and 64.89 µg/ml, respectively), LC_{90} detailed in Table-3.

Table-2 Antibacterial activity of Fungi crude extract

Concentration of fungal extract (mg/ml) and Zone of inhibition (mm)					
Bacterial pathogens	50	75	100	150	Ciprofloxacin (100µg/ml)
S. aureus	9±0.3	11±0.4	15±0.5	19±0.6	21±0.3
S. pyogenes	8±0.5	10±0.5	12±0.3	18±0.5	20±0.3
E. fecalis	9±0.3	12±0.6	14±0.4	17±0.3	19±0.3
Enterobacter sp.	8±0.4	10±0.4	12±0.5	15±0.2	18±0.4
S. pneumoniae	10±0.3	13±0.3	15±0.7	16±0.3	20±0.5
P. aeruginosa	8±0.2	10±0.2	13±0.4	17±0.5	21±0.2
K. pneumoniae	9±0.4	11±0.5	13±0.4	15±0.4	19±0.5
S. paratyphi	7±0.6	9±0.3	12±0.4	16±0.6	18±0.4
S. typhi	8±0.5	10±0.3	12±0.2	15±0.2	20±0.3
Serratia sp.	10±0.5	12±0.5	13±0.2	15±0.3	21±0.4
E. coli	7±0.5	10±0.3	12±0.5	19±0.5	20±0.3
Proteus sp.	8±0.5	11±0.2	14±0.6	17±0.7	20±0.6

Mean±SD, Positive control-Ciprofloxacin and Negative control-DM

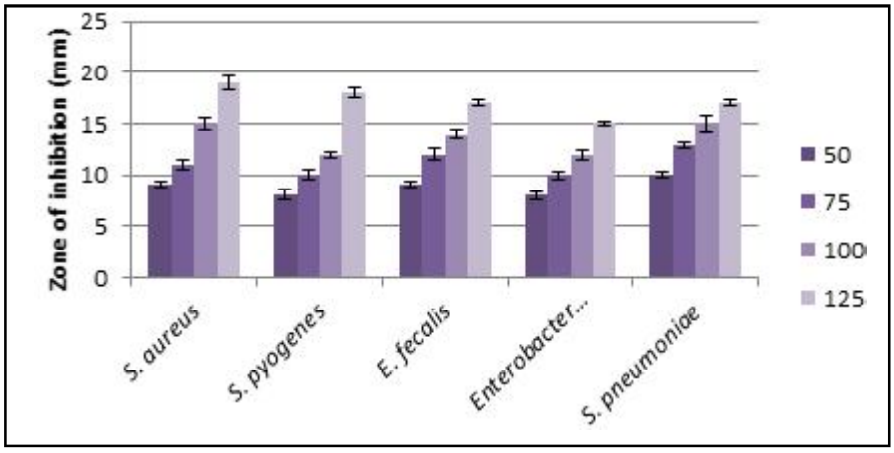

Figure 2. A bar diagram showing the antibacterial activity of Gram-positive bacteria in various concentrations

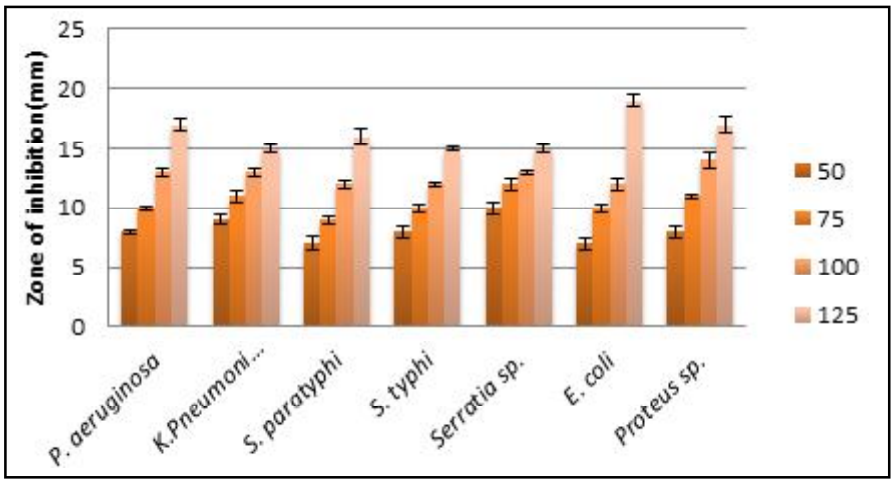

Figure 3. A bar diagram showing the antibacterial activity of Gram-Negative bacteria in various concentrations

An ongoing study of soil fungi for potential antibacterial and mosquito-repelling properties. The study found that the secondary metabolite produced by the fungus *Fusarium annulatum* had antibacterial and mosquitocidal properties. The fungus extract likely had highly mosquitocidal effects on *Aedes aegypti*. In comparable manner, *Fusarium endophyticum* with death rates of 70.2%, 83.2%, and 70.5% against cotton bollworm *Helicoverpa armigera* first instar larvae, *Fusarium sambucinum* shown exceptional larvicidal activity[24]. Several common insect pests are affected by the entomopathogens *Beauveria bassiana* and *Trichoderma harzianum*. *Beauveria bassiana* and *Trichoderma harzianum* had LC_{50} values

Table-3. Larvicidal Activity of mosquitoes

Mosquitoes species	Concentration (ppm)	Mortality (%)	LC_{50} (ppm)	LC_{90} (ppm)	χ^2
Anopheles stephensi	50 100 150 200 250	45 55 72 83 95	44.79	139.07	1.67
Aedes aegypti	50 100 150 200 250	60 64 74 85 99	38.81	215.17	8.62
Culex quinquefasciatus	50 100 150 200 250	35 44 66 74 85	64.89	172.72	0.577

(Significant at P<0.05level)

of 575 and 3238 ppm and 226 and 389 ppm, respectively, against *Spodoptera littoralis,* according to Abdullah *et al*.,[1].

Bai *et al*.,[5] isolated the endophytic fungus *Cladosporium* sp. JS1-2 from the mangrove *Ceriops tagal*, inhibited the growth of newly hatched *Helicoverpa armigera* Hubner larvae with IC50 values between 100 and 150 mg/mL. *Talaromyces pinophilus* endophytic Strain discovered on strawberry trees. According to Vitale *et al.*,[21], the main metabolite generated by this strain had harmful effects on the pea aphid *Acyrthosiphon pisum. Lecanicillium attenuatum,* an entomopathogenic fungus, exhibits significant levels of JH antagonist (JHAN) activity in both *Aedes albopictus* and *Plutella xylostella*[23]. The Trichoderma hamatum FB10 strain produced a bioactive metabolite that successfully stymied bacterial and fungal development. The secondary metabolite synthesized by *Trichoderma hamatum* FB10 showed nematicidal activity against *Meloidogyne incognita* eggs. When the extract was given nematode treatment, egg hatch inhibition was 78 2.6% and the juvenile stage death rate was 89 2.5% [4].

According to the investigation of Akpotu *et al*.,[3], the novel bioactive chemical produced by endophytic fungi associated with Catharanthus roseus exhibited antibacterial and antifungal activity with minimum inhibitory concentrations ranging from 0.0625 to 1 mg/mL. Leylaie and Zafari[13] examined the four species of *Trichoderma asperellum, T. brevicompactum,*

T. koningiopsis, and *T. longibrachiatum* exhibited essential antimicrobial bioactivity against the human pathogenic bacteria *Staphylococcus aureus* and *Escherichia coli* as well as the plant pathogenic bacteria *Ralstonia solanacearum* and *Clavibacter michiganensis*. *Aspergillus allahabadii* bioactive secondary metabolites were shown effective larvicidal and antibacterial properties[17]. Ibrahim *et al*.,[11] evaluated the bacterial and antifungal characteristics of Fusarithioamide B from Fusarium chlamydosporium against various microbial strains using a disc diffusion experiment. The secondary metabolites of *Fusarium annulatum* were also demonstrated in the current investigation to be efficient against the bacterial pathogens and mosquito population.

In the modern world, drug discovery is a constant problem. The traditional approaches are progressively losing their efficacy. Therefore, a change must be made to something fresh, original, natural, and obviously sustainable. A number of fungi that produce novel compounds can be found in the soil. The molecular identification of *Fusarium annulatum,* which was isolated from an agricultural soil sample, revealed that it had the strongest antagonistic properties towards bacterial pathogens and significant mosquito vectors. Further molecular research may aid in the investigation of innovative drugs and biopesticides.

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