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Biosynthesis of silver nanoparticles using fungal extract of *Aspergillus* species and their potential mosquito larvicidal property

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Abstract
In this study, the larvicidal potential of microbially generated silver nanoparticles (AgNPs) derived from *Aspergillus* species is examined. AgNPs were subjected to a solution of 1 mM silver nitrate (AgNO3). To verify the synthesis of AgNPs, visual inspection was combined with the use of transmission electron microscopy, X-ray diffraction (XRD), Fourier transform infrared spectroscopy, and UV-Vis spectroscopy. TEM and XRD studies were used to establish the average size of the AgNPs. Significant larvicidal action was demonstrated by biosynthesized AgNPs against vector mosquitoes. Silver nanoparticles were effectively synthesized using *Aspergillus* fungal extract. The produced silver nanoparticles were characterized using FTIR, XRD, TEM, and UV-Vis spectra. The greatest absorption peak in the UV spectrum was located at 420 nm. X-ray diffraction analysis verified that the produced AgNPs were crystalline. The average size was 7-12 nm, according to TEM analysis. The LC50 and LC90 values obtained were 0.173 mg/L and 10.290 mg/L respectively, which concludes that the synthesized silver nanoparticles can be used as a potential larvicide for vector mosquitoes.

Keywords: Biosynthesis, silver nanoparticles, vector-borne disease, biocidal, anti-microbial

1. Introduction
The Culicidae family of mosquitoes is known to spread a number of illnesses, such as yellow fever, dengue fever, chikungunya fever, lymphatic filariasis, malaria, and Japanese encephalitis. The most prevalent and hazardous diseases spread by mosquitoes are malaria, dengue fever, and filariasis, which arise from infections with specific pathogens: Plasmodium spp. transmitted by the Anopheles genus, alphaviruses and flaviviruses from the Aedes genus, and *Wuchereria bancrofti* from the Culex genus. Notably, these diseases are among the leading causes of fatalities in India, claiming millions of lives annually. Approximately 390 million cases of dengue infection have been documented, with an estimated 96 million of those being clinically severe, the annual death toll from Japanese encephalitis, which is around 30,000-50,000, has been steadily increasing on a global scale. (https://www.who.int/news-room /fact-sheets/detail/malaria).
The occurrence of this vector-borne disease is rising at a concerning pace worldwide each year, driven by swift changes in global climate patterns (including precipitation, temperature, and humidity), insufficient water resources, haphazard urban growth, and poor sanitation facilities in numerous nations. Furthermore, vaccines are still under development and in testing stages due to high costs, making the immediate elimination of mosquito breeding sites and the eradication of mosquito larvae through various pesticides like temephos, carbamates, Methoprene, and insect growth regulators the most effective strategies, which remain in use for mosquito control and the management of mosquito-borne diseases. Additionally, chemical pesticides come with numerous drawbacks, such as disrupting nature's ecological balance by harming non-target or beneficial species. These challenges highlight the urgent need to explore alternative control methods that could replace these synthetic insecticides. Consequently, there is a demand for a naturally formulated approach to tackle mosquito resistance to repellents. The term "nano," derived from the Greek word meaning "one billionth of a meter," serves as

the origin for the prefix "nano." A particle size of less than 100 nanometers is classified as a nanoparticle [1].

The use of green synthesized Ag-Nanoparticles for disease vector control is necessary because synthetic nanoparticles, which are less likely to harm the environment, have been identified as a potential substitute for synthetic chemical insecticides with the aid of certain phenolic-based plant extract.

Several studies have demonstrated that microorganisms, plant extracts, and fungi can produce nanoparticles via biological pathways [3]. Both pathogenic and nonpathogenic fungi have been reported to biosynthesize nanoparticles of various elements [4]. Using fungal systems, also known as myconanofactories, metal nanoparticles of silver, gold, zirconium, silica, titanium, iron (magnetite), and platinum have been produced. The smaller size (less than 100 nm) of nanoparticles directly correlates with their physical, chemical, and optical properties [5]. Due to their high surface area-to-volume ratio, nanomaterials have distinct physical or medical properties when compared to their bulk counterparts. This ratio increased as their size decreased. Silver nanoparticles (AgNPs), which have excellent antimicrobial activity against bacteria, viruses, and other harmful eukaryotic microorganisms, proved to be the most beneficial among them [6]. *Fusarium oxysporum* is one of many fungal strains that can produce AgNPs (silver nanoparticles) outside of the cell [2], *Aspergillus niger* [7], *Aspergillus fumigatus* (Bhainsa and D'Souza, 2008) [8], *Fusarium* (Basavaraja *et al*., Shaligram *et al*., 2009) [9], *Penicillium brevicompactum* (Balaji *et al*., 2009) [10], *Cladosporium cladosporioides* (Verma *et al*., 2009) [11], and *Aspergillus clavatus* have previously been discussed. Numerous advantages distinguish fungi from other microorganisms. In comparison to plant materials and bacteria, the fungal mycelial mesh can withstand flow pressure, agitation, and other conditions in bioreactors or other chambers. These take very little time to grow, are simple to handle, and are simple to make. Additionally, the nanoparticles that are precipitated outside of the cell lack any unnecessary cellular components, making them suitable for direct application in a variety of fields [1]. In addition, the fungus-derived biomolecules that are coated on the nanoparticles during this synthesis procedure have the potential to enhance stability and confer biological activity. Renata de Lima and Mariana Guilger-Casagrande *et al* went over the studies that used fungi as reducing agents to make silver nanoparticles [12]. Pathogens can be controlled using low-toxicity and biocompatibility silver nanoparticles made from fungi. The application of these nanoparticles as antimicrobials in the fields of agriculture and health opens up new avenues for research in the future.

Although numerous studies on AgNPs' larvicidal activity are available, the precise mechanism underlying this phenomenon is still poorly understood. Even though some authors believed that AgNPs of smaller size could easily penetrate the insect gut wall and bind to the sulfur and phosphorus groups of deoxyribonucleic acid, their normal functions, such as replication, are disrupted, and the molting and physiological processes that cause cell death are disrupted. The primary requirement for the synthesis of AgNPs through the use of AgNO3 is the transfer of an electron from phenolic compounds to the Ag+ ion.

1.1 Morphological damage or changes under NPs

The head, lateral, and caudal antenna hairs of Euphorbia rothiana Linn.-fabricated AgNPs were reduced, and the midgut and caeca epithelium cells of the mosquito larvae were disorganized and disintegrated, indicating cell death [14]. Cuticular damage and hair loss from the antenna, cranium, and upper and lower abdomen were also observed in Aedes aegypti larvae under the influence of Pedalium murex Linn - derived AgNPs (15). Additionally, AgNP penetration-the main cause of death for Aedes aegypti- caused dark blotches inside its body, according to Sap-Iam *et al* [16].

1.2 Physiological and biochemical changes under NPs

AgNPs influence the amounts of proteins, calcein (which is necessary for calcium binding and membrane excitability), lipid droplets, cytokines and reactive oxygen species, membrane potential, and carbohydrates in insect larvae. The treatment of A. albopictus fourth-instar larvae with AgNPs facilitated by salicylic acid and 3,5-dinitrosalicylic acid resulted in a reduction of esterase, total phosphatase protein, and acetylcholinesterase activity (Fouad *et al*. 2018) [17]. Silver nanoparticles disrupt insects' typical body temperature regulation and also impact the usual molting process (Mommaerts *et al*. 2012; Benelli 2016) [18].

2. Material and Methodology

2.1 Culturing of fungal species and Preparation of media

Aspergillus sps. was pre-cultured, and the species was confirmed by Mycologist Dr. Kruti Dave of the Department of Life Science, Ruia College. Potato Dextrose Broth was prepared using 3 potatoes. Medium-sized 3 potatoes were peeled and washed with tap water. It was cut into small pieces and gently boiled in a 150 ml beaker along with water for 30 minutes. The resulting potato broth was filtered using a muslin cloth, and the potatoes were discarded. 3gms of dextrose powder was mixed with filtered potato broth, and volume was made up to 150 ml with D/W. This PD broth was autoclaved.

2.2 Inoculation of the fungus in the media

The next day, a loopful of the *Aspergillus* species was inoculated in the PD broth and then the flask was kept in dark conditions for 15 days. This was done to allow the growth of fungal species in the broth.

Fig 1: PDB broth inoculated with *Aspergillus*

Fig 2: PDB flasks after 20 days of inoculation.

Preparation of fungal extract

The inoculated PDB flask was filtered twice using a Whatman filter paper for the extraction of clear liquid under aseptic conditions.

Fig 3: Extraction of Fungal extract using Whatman filter paper

2.4 Mycosynthesis of silver nanoparticles from Fungal extract

1mM of AgNO3 solution (1L) Thus, for making 1L of 1mM AgNO3 solution; 0.0169 grams of AgNO3 in 1000ml D/W was taken. Then, for making a 10% solution, 90 ml of AgNO3 solution, along with 10 ml of fungal extract, was taken in a conical flask. This would result in a 10% solution of nanoparticles from fungal extract. (Parashar *et al*. 2009) [19] For making a 5% solution, 95 ml of AgNO3 solution, along with 5 ml of fungal extract was poured into a conical flask using a glass pipette. This resulted in a solution of nanoparticles from a fungal extract of 5%. (Parashar *et al*. 2009) [19]

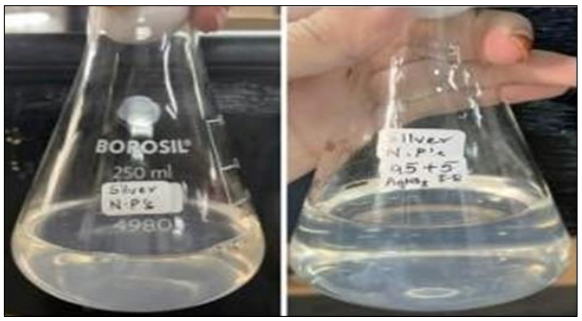

Fig 4: Flask A and B of 10% and 5% AgNP solutions, respectively.

Flask A of 10% and B of 5% AgNP Solution, both were kept in sunlight near the window for around 20-25 minutes (RT). A significant color change from transparent to yellowish brown was observed in both flasks. The color change depicts that the synthesis of nanoparticles has been done. (Parashar *et al*. 2009) [19].

Fig 5: Synthesized AgNPs of 10% and 5%, respectively

Synthesised silver nanoparticles must be stored in cold and dark conditions to avoid photochemical reactions. Both flasks were wrapped in black paper and kept in the refrigerator.

Fig 6: Storage of Ag NPs solution

2.5 Formation of silver nanoparticle powder

The powder was achieved by the process of centrifugation. 10 ml of sample was poured into each centrifuge tube. Centrifugation was done at 12000 rpm for 1 hour. Dark-colored pellets were observed at the bottom. The supernatant was discarded, and the pellets were extracted by adding 1000 microliters of ethanol. The tubes were kept open to allow the alcohol to evaporate. With the help of a small needle, pellets were collected in a Petri plate or small cuvette. The cuvette's mouth was covered with foil, and the whole cuvette was wrapped with black paper completely, kept in dark conditions at 4 degrees Celsius.

Fig 7: Ag NP pellets

Fig 8: Ag NP powder and its storage.

2.6 Characterization of the silver nanoparticles solution and powder

(a) TEM (Transmission Electron Microscopy)

It is carried out at Icon Labs Pvt. Ltd., Sanpada for both 10% and 5% samples. Approximately 10 ml of each sample is required for the analysis (TEM machine- Tecnai G2 Spirit Biotwin 120 kv Tungsten (W) filament, FEI company Camera controller:-Olympus soft imaging solutions).

(b) UV-Visible Spectroscopy (UV-Vis) analysis

UV visible spectroscopy at Ramanathan Labs, Ruia College, Matunga. 5 ml of both samples were required (Shimadzu company UV 1800). The UV range was taken as 300-800 nm with 10 nm intervals.

(c) Fourier Transform Infrared Spectroscopy (FTIR) Spectroscopy analysis

A graph of the substance's infrared light absorbance along the vertical and frequency (wavelength) along the horizontal axes is known as the IR spectrum. The FTIR spectrum was recorded with an FTIR Spectrophotometer after potassium bromide was added to the dried AgNPs powder at 4500 cm−1.

(d) X-ray Diffraction Analysis

The spectrum was recorded by CuKa radiation with a wavelength of 1.54606 A in the 2θ range of 20◦- 80◦. A glass slide was drop-coated with AgNPs solution and air dried, resulting in a thick layer of AgNPs on the slide X-ray diffractometer Shimadzu Xlab 6100.

2.7 Larval Bioassay

A. For setting up the assay following concentrations of the sample were made: 10% sample of AgNPs Control, 3%, and 5% were made with 10 mosquito larvae in each.
B. 5% sample of AgNPs, Control 3%, and 5% were made with 10 mosquito larvae in each. Observation was done for 24 hrs. The mortality rate was calculated using the percentage Mortality formla.

3. Results

3.1 UV-Visible spectroscopy

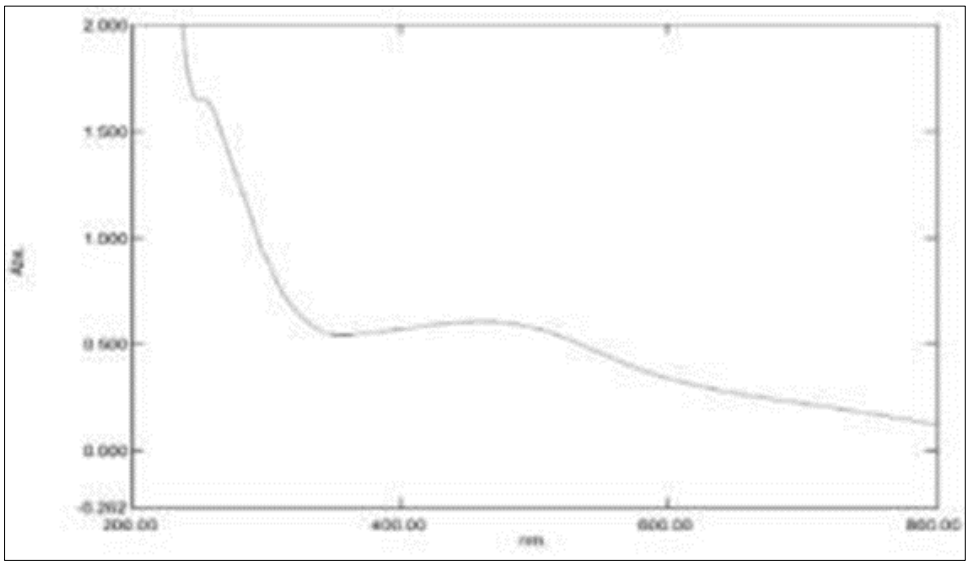

Fig 9: 10% Ag NP graph

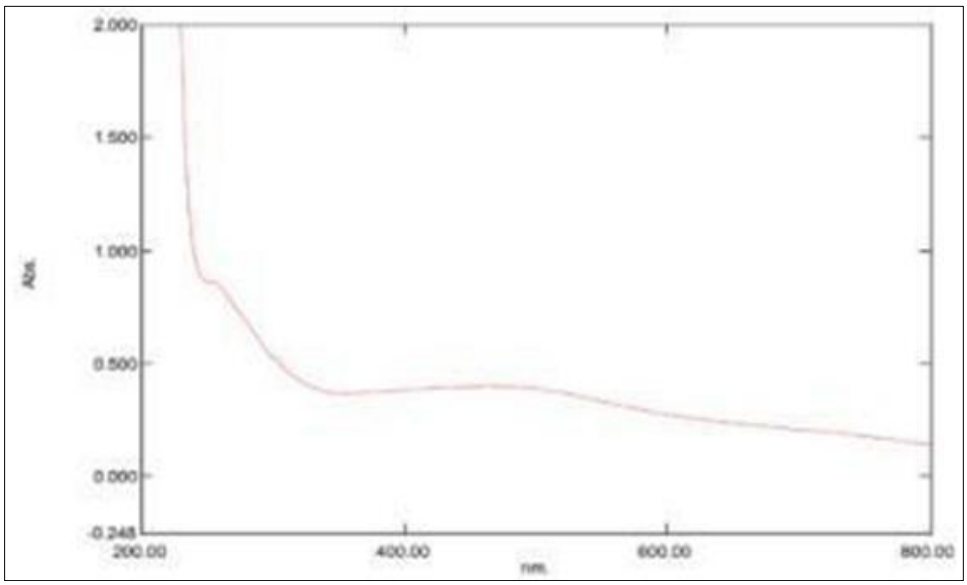

Fig 10: 5% Ag NP graph

A very practical and reliable technique for the initial characterization of produced nanoparticles is UVvis spectroscopy, which is also used to track the synthesis and stability of AgNPs. AgNPs have unique optical characteristics that cause them to interact strongly with specific light wavelengths. Due to the collective oscillation of silver nanoparticle electrons in resonance with the light wave, these free electrons produce an absorption band known as a surface plasmon resonance (SPR).

The chemical environment, particle size, and dielectric medium all influence AgNPs' absorption. *Aspergillus* filtrate treated with silver nitrate solutions had characteristic surface plasmon absorption at 280 and 420 nm in the ultraviolet-visible spectrum. Fungal cell filtrate treated with silver nitrate solution has a high absorbance peak around 420 nm, as Ingle *et al*. demonstrated (2008) [7], which backs up our finding that *Aspergillus* sps is synthesizing nanoparticles by observing absorbance peaks at 420 nm.

3.2 Transmission electron microscopy (TEM)

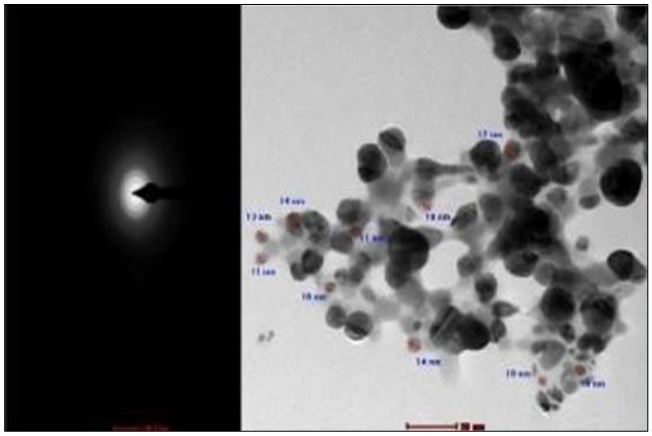

Fig 11: TEM Image of 10% Ag Np solution

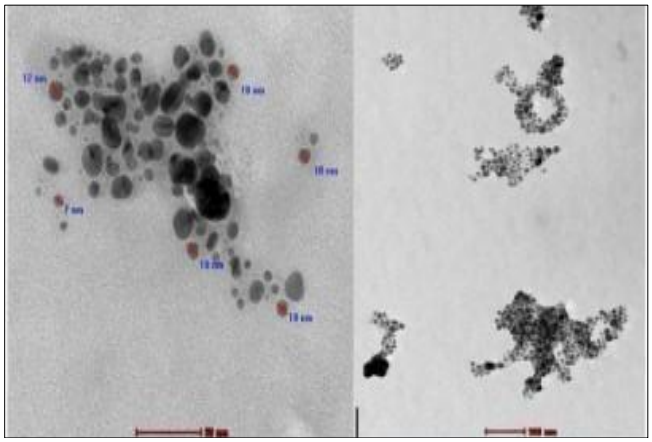

Fig 12: TEM Image of 5% AgNp solution

The transmission electron micrograph data revealed distinct nanoparticle sizes and shapes. AgNPs were uniformly dispersed with some agglomeration, revealing a pattern that was comparable to that of the biosynthesized AgNPs created by Kathiresan *et al*. (2009) [20] as well as Jain *et al*. (2017) [21]. Figure. 11 revealed that the nanoparticles of the 10% sample are spherical with a size range of 10-17 nm. The fig. 12 revealed that the nanoparticles of the 5% sample are spherical with a size range of 7-12nm.

Statistical function	Distance (nm) (10%)	Distance (nm) (5%)
Mean	13.5	9.5
Minimum	10	7
Maximum	17	12

3.3 Fourier-transform infrared spectroscopy (FTIR)

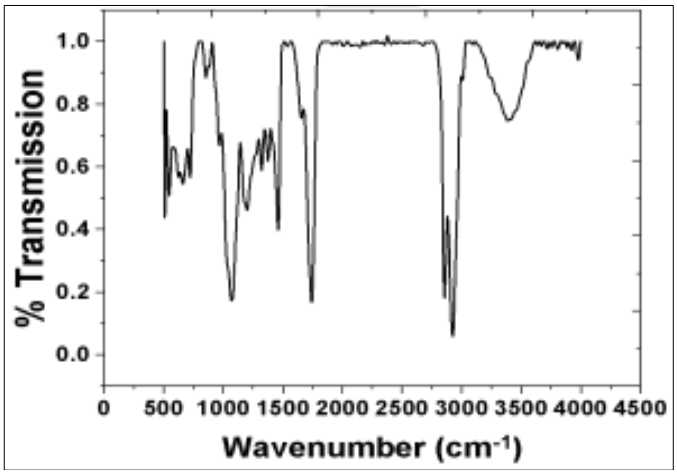

Fig 13: FTIR graph

The representative spectra in the region of 3000 to 450 cm−1 revealed the presence of different functional groups like 3290.73-secondary amide (N-H stretch, H-bonded), 2928.01-alkane (C-H stretching), 2161.23-alkyne (C≡C stretching), 1771.02-anhydride (C=O stretching), 1613.81- alkene (C=C stretching), 1538.60-aromatic (C-C stretching), 1386.45, 1313.48- and 1080.10-primary alcohol (C-O stretching), and 528.55-alkene (=C-H bending), respectively. [22]

3.4 X-Ray Diffraction (XRD)

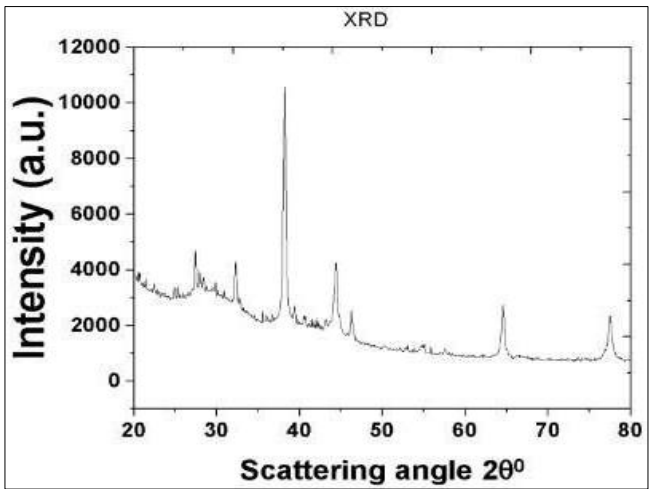

Fig 14: XRD Graph

X-ray diffraction (XRD) is a crucial technique for analyzing crystallinity, particle sizes, and compound identification, while also detecting material impurities. It operates based on Bragg's law and uses elastic scattering of X-rays. For synthesized silver nanoparticles, XRD patterns revealed a face-centered cubic (fcc) structure, with distinct peaks at 15.49° and 15.80° corresponding to the (111), (200), and (220) planes of silver, and a lattice constant of 4.086 Å, aligning with JCPDS values. This analysis confirms the

crystalline nature of the silver nanoparticles and highlights XRD's role in identifying and characterizing materials across various fields, including industrial and forensic applications.

3.5 Larvicidal activity against vector mosquitoes

At a confidence level of 95%, the average larval mortality data were subjected to probit analysis for LC50 and LC90 calculations.

Sample concentration 5 (10%)	% Mortality 90
3 (10%)	90
5 (5%)	80
3 (5%)	70

The larvicidal activity of synthesized Ag NPs showed LC50 values of 0.173 mg/L. The results of the larvicidal activity showed the highest mortality rate in 10% of the sample concentration. All the sample concentrations showed lethal effects, and mortality was positively dose-dependent.

For LC50, X = 0.173 mg/L. This indicates that only 0.173 mg of AgNPs are required for the death of 50% of mosquito larvae. A lower LD50/LC50 value indicates higher acute toxicity. For LC90, X = 10.290 mg/L; this indicates that only 10.290 mg of AgNPs are required for the death of 90% of mosquito larvae. A lower LC90/LD90 value indicates higher acute toxicity.

4. Discussion

The yellowish-brown hue suggests the production of silver nanoparticles, likely resulting from the excitation of surface plasmon vibrations [24]. The distinctive Bragg peaks observed in the XRD pattern further validate their creation, linked to various reducing agents present in the fungal extract that stabilize and facilitate the crystallization of the nanoparticles [25]. FTIR analysis also identified several functional groups, including alkanes, methylenes, alkenes, amines, and carboxylic acids, which could serve as effective reducing agents in the synthesis of silver nanoparticles.

Fungal cell filtrate treated with silver nitrate exhibits a high absorbance peak around 420 nm, supporting our findings that *Aspergillus* biosynthesized silver nanoparticles (AgNPs). These AgNPs were uniformly dispersed with some agglomeration, similar to those produced by Mohamed H. *et al*. [26]. The spectrophotometric evaluation of *Aspergillus* extracts showed a peak at 420 nm, indicative of AgNP production, mirroring surface plasmon vibrations of chemically reduced silver nanoparticles [27]. FTIR analysis identified biomolecules that stabilize the AgNPs. The study noted a 70%-90% mortality rate for *Aspergillus* extract at concentrations of 3 ml and 5 ml, consistent with Kannathasan *et al*. report on *V. negundo* leaves. Green-synthesized AgNPs have shown effectiveness as an eco-friendly larvicide against *C. quinquefasciatus*, addressing vector control challenges in developing nations like India due to climate change and urbanization.

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