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Original Article

Anatomical and molecular identification of Culex mosquitoes and comparative larvicidal efficiency of *Aloe vera* and *Cassia fistula* green synthesized silver nanoparticles

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Abstract

Culex mosquitoes are important pathogens carriers to transmit West Nile virus and lymphatic filariasis, so accurate species identification and eco-friendly methods are necessary for proper control measures. The mosquito species (n=2094) collected from 19 different sites across Karachi region, were morphologically segregated intro 3 different genera (Aedes, Anopheles and Culex) by stereomicroscopy, followed by scanning electron microscopy and molecular identification of Culex by PCR. The molecular amplification of PCR product by agarose gel electrophoresis demonstrated a ~740 bp COI fragment and further confirmed the Culex species. The green synthesized silver nanoparticles (Ag NPs) using *Aloe vera* (AV) and *Cassia fistula* (CF) extracts were characterized by UV–Vis, SEM, EDS and FTIR. AV-Ag NPs were found smaller (≈65–79 nm) than CF-Ag NPs (≈80–95 nm) in size. In the larvicidal bioassay, the mortality of fourth-instar *Culex* larvae (n=20 / treatment) was recorded at 16, 32, 64 ppm of different NPs treatments, at 24, 48, 72 h post-treatment compared to the controls. AV-Ag NPs showed significantly ($p<0.05$) greater efficacy (98.30% mortality at 64 ppm at 72 h) than CF-Ag NPs (65.00% at 64 ppm at 72 h). Moreover, the probit regression analysis showed better LC_{50}=17.85 ppm and LC_{90}=42.51 ppm for AV-Ag NPs (72 h) as compared to LC_{50}=36.35 ppm and LC_{90}=361.38 ppm for CF-Ag NPs. Thus, the results demonstrated that *Aloe vera*–mediated Ag NPs are promising, eco-friendly larvicidal candidate for integrated mosquito control.

Keywords: Culex, *Aloe vera*, *Cassia fistula*, Silver nanoparticles, PCR

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Introduction

The Culex is a diverse genus that is distributed at worldwide and is widely known for transmission of medical and veterinary arthropod-borne diseases due to its more than 770 species grouped into 26 subgenera (Bursali and Simsek, 2024). The mosquitoes of genus Culex have considerable epidemiological significance in transmitting medically relevant pathogens like parasites and viruses, thus causing morbidity and mortality in humans and animals (Madhav et al., 2024). There are 26 mosquito species including Aedes (5 species), Culex (8 species), Culiseta (1 species) and Anopheles (12 species) those are involved in the spread of 7 mosquito-borne diseases like 2 parasitic: malaria and filariasis and 5 viral: *West Nile* virus, *Rift Valley* fever virus, Dengue virus, Sindbis virus and Usutu virus infections (Nebbak et al., 2022). Among the mosquito species, the genus *Culex* serve as the primary and most significant vector for these life-threatening parasitic and viral diseases, posing a significant public health concerns at worldwide (Aremu et al., 2024; Moser et al., 2023). With an increased interaction of mosquitoes and humans due to urbanization and climate change, the global outbreaks of Culex-borne diseases have been raised (Duval et al., 2023; Ragab et al., 2025). The outbreaks of Culex-borne *Rift Valley* fever resulted in severe clinical manifestations in humans and also numerous abortions and heavy mortality in small and large ruminants and camels (Kenawy et al., 2018). The disease has resurfaced and experienced a significant increase in the patient numbers during the last two decades (Bostan et al., 2017; Nisa et al., 2025). It is said that the carrier mosquitoes of malarial transmission kill about 2 to 3 million people and infect another 200 million or more every year (Illinois Department of Public Health, 2026).

Rapid urbanization and poor infrastructure in populated cities facilitate mosquito-borne outbreaks by increasing available breeding habitats in stagnant water (Nisa et al., 2025; Khan et al., 2008). In the urban regions, the mosquitoes are usually found to breed at stagnant water sources, in the brackish water and salt containers. There occurs a positive correlation between the temperature and the number of mosquitoes, since warmer environment (up-to 24°C) accelerates the development, shorten the lifecycle and boost-up the reproduction of mosquitoes (Hassan et al., 2016; Ciota et al., 2014). The mosquitoes are usually identified by morphological characteristics but these traditional taxonomic methods are often prone to errors due to high intra-specific variations, overlapping morphological traits and the presence of cryptic species (Dumas et al., 2016). The lack of precise species identification could lead to ineffective vector control strategies, thus emphasizing the need for integrating molecular techniques and PCR-based identification that will provide species-level resolution and allows for genetic analysis of vector populations (Chan et al., 2014; Kayedi et al., 2020). Hence, the molecular characterization through mitochondrial DNA-based phylogenetic analysis (cytochrome c oxidase subunit I, nicotinamide adenine dinucleotide dehydrogenase subunit 4 barcode sequences) are considered as consistent and reliable tool for identification of different species of mosquitoes (Laurito et al., 2017).

In order to combat the diseases caused by these mosquitoes, various chemical (larvicides, insecticides, repellents) and biological (natural predators, microbial larvicides, fungal agents) control measures are adopted to prevent the prevalence of Culex-borne infections (Aamir et al., 2017; Sanei-Dehkordi et al., 2024). However, the mosquitoes are developing resistance to chemical pesticides, hence, the limited effectiveness of biological control methods pose a significant challenge in effective vector management (Punniyakotti et al., 2024). The conventional insecticide-based therapies are becoming less effective because Culex populations are developing resistance due to widespread use of synthetic larvicides (ŞengülDemirak and Canpolat, 2022). Moreover, the chemical pesticides harm the aquatic ecosystems and non-target creatures like birds, honeybees and flies, thus alternate environmental friendly management strategies have to be investigated (Punniyakotti et al., 2024). Although biological management techniques like larvivorous fish including guppies (*Poecilia reticulata*), mosquito fish (*Gambusia affinis*) and predation by native species like *Aplocheilus panchax* provide an environmentally benign substitute (Walshe et al., 2013), however, their effectiveness varies depending on the ecological conditions that restrict their widespread applications (Benelli et al., 2016).

The use of nanomaterials has increased in the conventional pharmacological formulations like solutions, gels, suspensions and ointments, in the agro-industrial sector due to their distinct properties and has found to enhance the effects of several drug delivery systems (Hussain et al., 2025; Ali et al., 2012). Because of their effective larvicidal effects,

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biodegradability and decreased environmental toxicity, recently the plant-mediated silver nanoparticles (Ag NPs) have demonstrated the potential to replace the traditional larvicides (Dass et al., 2025; Ochieng et al., 2025; Tariq et al., 2025; Idowu et al., 2021). By adding phytochemicals with insecticidal properties, the green synthesis of Ag NPs by using extracts of various parts of plant (flowers, leaves, bark, roots, seeds, fruits) has increased their bioreactivity (Eker et al., 2025; Alharbi et al., 2022). *Aloe Vera* (*Aloe barbadensis miller*, also called as first aid plant or lily of the desert) is quite well-known to boost-up the immune response and in reducing the inflammation and have potent antiviral, antifungal and antibacterial properties, due to presence of natural phyto-chemicals like anthraquinones and flavonoids (Liknaw et al., 2025). Similarly, *Cassia fistula* (also known as golden shower or pudding-pipe tree, purging cassia or Amaltas) is extensively reported for its anti-parasitic, antifungal, antitumor and antioxidant activities and have demonstrated efficient toxicity against cancer and inflammatory cells, since it is also rich in compounds like anthraquinones, phenolics and flavonoids (Abaid et al., 2023). Numerous studies have explored the larvicidal activity of plant-derived Ag NPs against different mosquito vectors, but the direct comparative evaluations of different botanical sources against Culex mosquitoes under identical environmental conditions remain scarce. The Ag NPs prepared by *Aloe vera* extracts were found effective against the malarial vector *Anopheles stephensi* (Dinesh et al., 2015). Similarly, the Ag NPs formed by aqueous extract of *Cassia fistula* fruit pulp have demonstrated significant larvicidal effects in controlling *Aedes albopictus* and *Culex pipienspallens* at 72h of exposure (Fouad et al., 2018). However, the relative effectiveness of these plant-derived nanoparticles has not yet been determined. In this study, we have presented the first comprehensive assessment of *Aloe vera* and *Cassia fistula*-mediated silver NPs for control of Culex mosquito.

Material and Methods

Study areas and period

The study was conducted in the district Karachi (24.8503°N, 67.0325°E) with an average rainfall of up-to 146.5mm during monsoons. A total of 19 sites were selected based on preliminary reports of intensive mosquito density, accessibility and variation in ecological conditions to ensure the collection of representative sampling (Figure 1). The selection criteria included the areas with stagnant water bodies, high vegetation and known mosquito breeding hotspots. All the environmental parameters of various sampling sites / mosquito habitat like temperature, humidity, locality data points (longitude/latitude, elevation) and terrain features were collected and recorded. The geographical coordinates of each sampling site, including latitude, longitude, and elevation, were recorded using a Garmin GPS Map 62S.

A prior permission was obtained from the Institutional Ethical Review Board (IERB) and the animal ethics committee of Shaheed Zulfikar Ali Bhutto Institute of Science and Technology, Karachi for collection and processing of specimen, the use of nanoparticles and to perform different experimental procedures (IERB(12)/SZABIST-KHI(LIFE)/19103105/220044; Dated: 25-05-2022).

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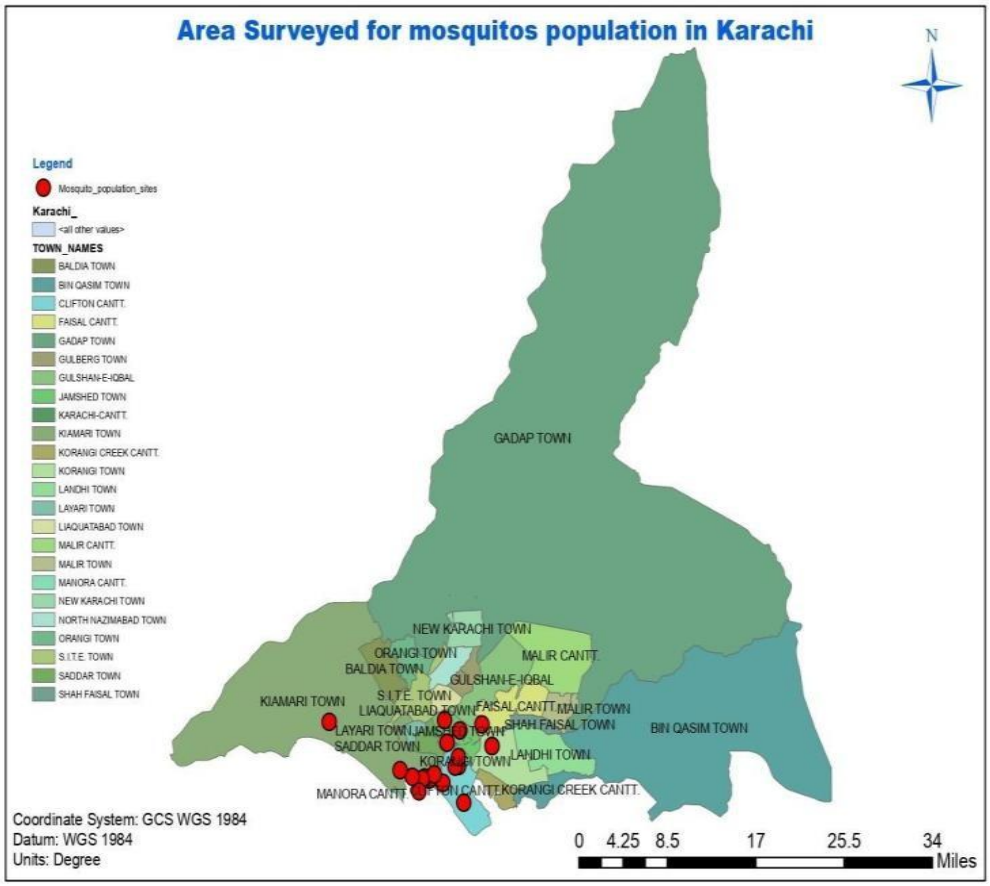

Figure-1. GPS surveillance spots of the study area for collection of different mosquitoes species.

Samples collection and morphological identification

The mosquitoes were collected using standard trapping method including light trap and manual aspirator and the collection was done during peak activity hours (dusk and dawn), to maximize the capture efficiency (Cansado-Utrilla et al., 2020). This systematic approach ensured the inclusion of representative mosquito population for accurate identification and analysis. The collected insects were immediately transported to the laboratory of Centre of Excellence in Science & Applied Technologies, Karachi. The collected specimen of three different types of mosquitoes (Aedes, Anopheles and Culex) were morphologically identified and segregated based on morphological keys and taxonomic characteristics under stereomicroscope (Zeiss, model 1106) at 40X magnification, as described earlier (Wilke et al., 2016; Noundou, 2020; Rueda, 2004; Rozeboom and Komp, 1948). Further confirmation of Culex mosquitoes was done by using a Scanning Electron Microscopy (SEM).

DNA extraction and quantification

A genomic purification kit (Thermo Scientific K032) was used to recover the Culex DNA. The insect was homogenized in 150µl of digestion solution, 20 µL of proteinase K was added in the homogenate, thoroughly vortexed and incubated for 30 minutes at 56°C. Then 500 µL of buffer and 400 µL of 50% ethanol were added, vortexed thoroughly, centrifuged for 3 minutes at 13000 rpm and then transferred to a genomic purification column. To elute the genomic DNA, 200 uL of elution buffer was added. The quantification of DNA was carried out by using NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) at 260 nm (Ali et al., 2014).

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PCR and primer design for COXI gene

For molecular Identification of *Culex* mosquitoes, the Primer 3 tool (https://primer3.ut.ee/) was used to design mitochondrial cytochrome oxidase subunit I (COX1) gene primers (740 bp), as previously reported (Noureldin et al., 2021) and shown as: COI (LCO1490); GGTCAACAAATCATAAAGATATTGG, COI (HCO2198); TAAACTTCAGGGTGACCAAAAAATCA. The DNA ladder of 100 bp (Thermo scientific, SM0243) was served as a reference, to validate the expected fragment length and to ensure the confirmation of specific species of Culex mosquitoes. The PCR procedure was carried out in a solution using Thermo Scientific Master Mix, which consisted of 25µL of ready master mix, 1µL (10pmol) each of reverse and forward primers, 5µL of DNA template and 18µL of nuclease water. The mixture was first denatured to 94°C for 3 minutes then subjected to 30 cycles involving denaturation at 94°C for 1 minute. The primer annealing temperature was maintained at 51°C for half a minute followed by primer extension at 72°C, then for a final extension at 72°C, for 5minutes (Noureldin et al., 2021). The PCR reactions were carried out in triplicate to confirm the reproducibility.

Agarose gel electrophoresis

Agarose gel electrophoresis (Bio-Rad Laboratories Inc. USA) was used to assess the amplified DNA. The amount of 2.5g of agarose (Bio-Helix, Keelung, Taiwan) was dissolved in Tris Acetate EDTA buffer and treated with Ethidium bromide. A positive control was included to validate the amplification of 740 bp COX1 gene, while a negative control (reaction mixture without DNA template) was used to detect any non-specific amplification. The amplified gel was then observed by using Gel Doc system for visualization (Azure Biosystems Inc, CA USA).

Green synthesis and characterization of silver nanoparticles

Aloe vera (AV) and *Cassia fistula* (CF) leaves were collected from the botanical area of the institute, commonly cultivated for medicinal and ornamental purpose. For AV-Ag NPs synthesis, *A. vera* gel was extracted, heated and treated with silver nitrate to synthesize silver nanoparticles according to previously published protocol (Dinesh et al., 2015). Similarly, for preparation of CF-Ag NPs, the leaves of *C. fistula* were dried, dissolved in distilled water, and filtered to obtain the plant extract for nanoparticles synthesis, as previously described (Fouad et al., 2018). The purification of Ag NPs of both plants was done for elemental composition with energy dispersive analysis (EDS) and the possible functional groups were analyzed with the help of Fourier transform-infrared spectroscopy (FTIR). While the size and morphology of NPs were determined through SEM (T100i, Jeol), as described earlier (Fernandes et al., 2025).

Determination of larvicidal activity

Fourth-instar larvae of *Culex* were collected and maintained at 27±2°C, relative humidity of 70±10% and a 12:12 h light-dark cycle. The larvae were divided into different treatment groups (n = 20 / group) with 4 replicates in each group (n = 5 / replicate). Different groups of larvae were exposed to 3 different concentrations of nanoparticles (16ppm, 32 ppm and 64 ppm) of each plant, as determined according to the previous studies showing effective mosquito larvicidal activity of biogenic Ag NPs (Dinesh et al., 2015; Shanmugasundaram and Balagurunathan, 2015; Sutthanont et al., 2019; Wilson et al., 2023). The leaf extracts of *A. vera* and *C. fistula* leaf extracts and $AgNO_3$ solution were used as controls. Each experiment was performed with three replications and the larval mortality was recorded after 24, 48 and 72 hours of the exposure. Since, the mortality in the control group was less than 5%, hence, correction by Abbott's formula was not required (Kendie et al., 2023). LC_{50} and LC_{90} values were calculated using Probit analysis (EPA Probit Analysis Program v1.5).

Statistical analysis

The t-test was performed to specifically reveal the difference between the *Culex* male and female mosquito populations by using the software IBM SPSS Statistics ◦23 (IBM Corp., Armonk, NY, USA). The statistically significant difference was set at $p<0.05$.

Results

Collection and differentiation of mosquitoes

During surveillance, 19 sites were inspected for collection of mosquitoes (Figure 1), wherein the number of mosquitoes were collected ranged from 11 to 376 (Table 1). This suggests a need for broad-scale vector control strategies rather than localized

interventions. The mosquitoes of three different types; Aedes, Anopheles and Culex were identified from the various sites. The *Culex* was found to be the most abundant species (1601; 76.46%) at various sites, since these mosquitoes were collected in higher number than Aedes (488; 23.30%) and Anopheles (5; 0.24%). The male Culex (41.12%) were found to be higher in proportion than females (35.34%) mosquitoes.

Table-1. Geographic distribution of three different mosquito species at different collection sites of Karachi region.

	Aedes		**Culex**		**Anopheles**		**Total**
	Male	Female	Male	Female	Male	Female	
Gurumandir	-	1	3	7	-	-	11
DHA Phase-IV	1	1	2	6	1	-	11
DHA Phase-III	51	66	2	2	-	-	121
DHA Phase-II	6	3	30	11	-	-	50
New Cantt	-	-	49	16	-	1	66
PECHS	-	-	50	15	-	-	65
Clifton Star Apartment	1	4	2	5	-	-	12
Clifton block-I	79	148	29	120	-	-	376
Clifton block-II	4	6	116	39	-	-	165
Clifton block-III	-	-	107	116	-	-	223
Clifton block-IV	1	3	77	62	2	-	145
Qayyum abad B area	4	4	24	61	-	-	93
Korangi block-I	-	2	140	28	-	-	170
Korangi block-II	-	-	26	18	-	-	44
Korangi block-III	1	2	43	123	-	1	170
Shireen Jinnah Colony	39	61	142	69	-	-	311
Kemari	-	-	12	10	-	-	22
Kemari Jackson	-	-	7	10	-	-	17
China port	-	-	-	22	-	-	22
Total	187	301	861	740	3	2	2094
Percentage	8.93	14.37	41.12	35.34	0.14	0.10	

The *Culex* were found in higher proportion (1601; 76.46%) than Aedes (488; 23.30%) and Anopheles (5; 0.24%). The male Culex (41.12%) mosquitoes were found higher in number than the females (35.34%).

Anatomical characterization of collected specimen

The scanning electron microscopy revealed distinct morphological and microstructural features of the body of adult Culex mosquito that support the taxonomic identification (Figure 2). The *Culex* adult mosquito was found to have an anterior globular cephalo-thorax and a flexible abdomen that was held under the cephalothorax. The proboscis appears as a slender, elongated feeding organ with a sharply tapered apex and densely distributed cuticular sensilla (Figure 2A, 2B). The compound eye exhibits smooth curvature and tightly packed hexagonal ommatidia arranged in a uniform mosaic pattern (Figure 2A, 2B). The wings displayed elongated scales and marginal fringes with well-defined microtrichia. The adjacent abdominal surface shows overlapping cuticular scales and dense sensory hairs, forming a protective and sensory integumentary surface in adult Culex. The leg is elongated and segmented, with dense arrays of setae and fine spines distributed along the cuticular surface (Figure 2C). The labellar surface shows fine microtrichia and closely packed ridges, while numerous elongated setae arise along the proboscis shaft, reflecting adaptations for host detection and fluid uptake. This configuration is characteristic of Culex species (Figure 2D).

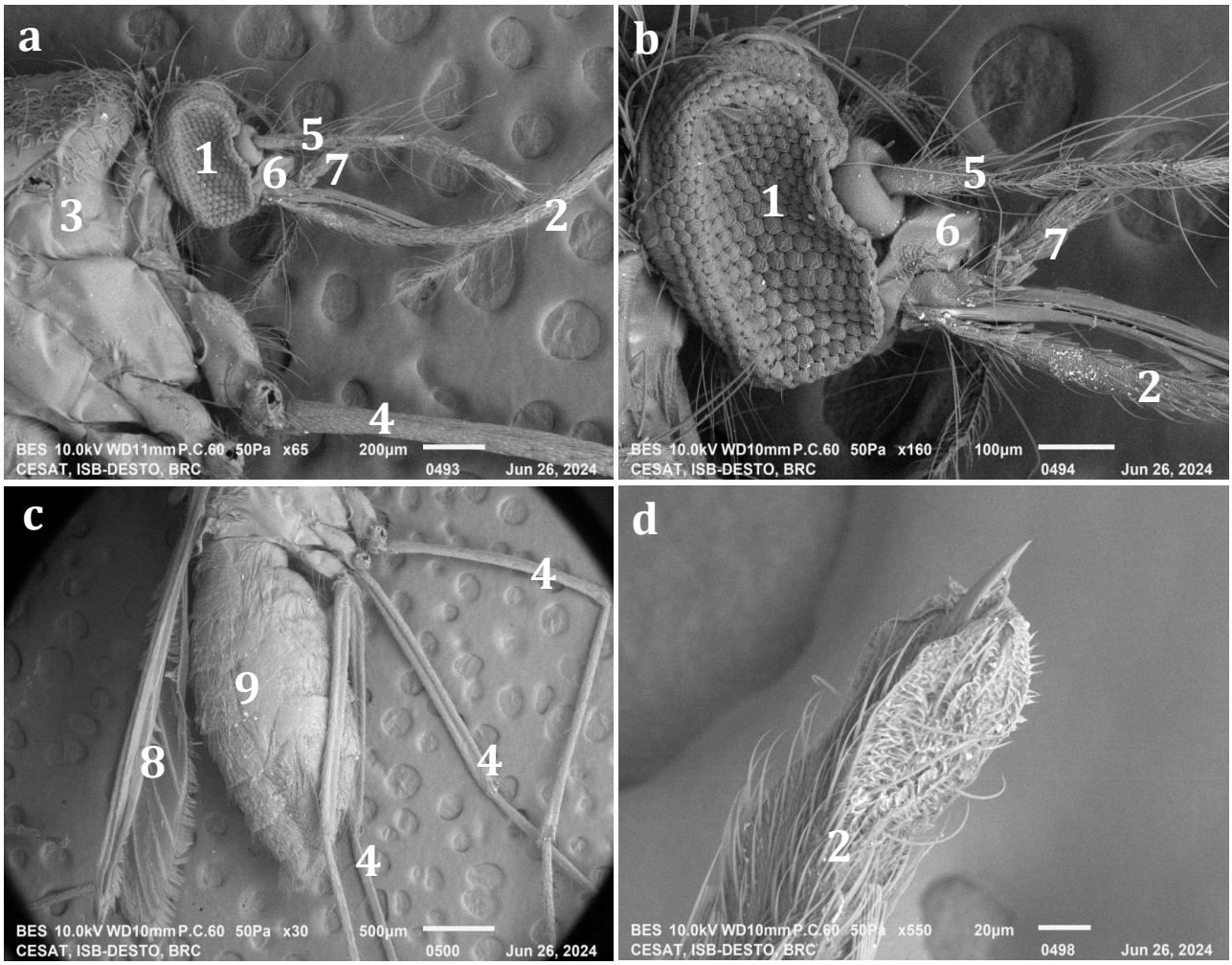

Figure-2. SEM image of Culex mosquito. 1: eye, 2: proboscis, 3: thorax, 4: leg, 5: antenna, 6: clypeus, 7: maxillary palpomere, 8: wing, 9: abdomen. a,c: magnification = 3000×. b,d: magnification = 5000×.

Molecular characterization of collected specimen

The morphological identification of Culex was further confirmed with PCR molecular characterization by using the specific primers of COX1 gene. The presence of distinct 740 bp bands in different samples in the agarose gel confirmed the presence of *Culex* species in the tested specimens (Figure 3).

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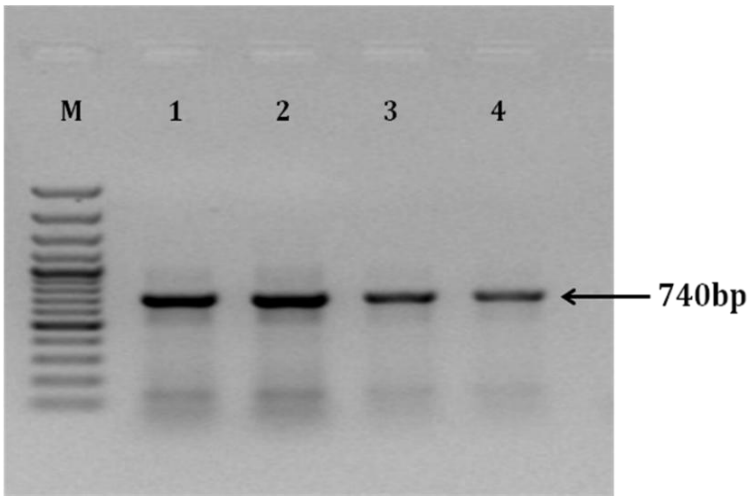

Figure-3. Molecular identification of Culex mosquito using COX1 gene. PCR products were analyzed by agarose gel electrophoresis and the gene expression was found at 740bp in all specimens. M: 100bp molecular ladder, Lane 1: Positive control, Lanes 2 - 4: PCR product of different collected specimens.

Green synthesis and characterization of AV-Ag NPs

The successful green synthesis of Ag NPs using *A. vera* extract was confirmed through spectroscopic analysis. Upon mixing the aqueous *A. vera* extract with silver nitrate ($AgNO_3$) solution, a gradual color change from golden to reddish brown was observed, indicating the reduction of Ag^+ ions to Ag^0 nanoparticles. The characterization of *A. vera* Ag NPs was performed to confirm the successful synthesis and stability of synthesized nanoparticles. UV-Vis spectroscopy revealed a distinct peak at approximately 260 nm (Figure 4A), while typical Ag NPs surface plasmon resonance (SPR) peaks occurred between 380–450 nm, the observed shift suggests dominant absorbance from phytochemical constituents in the extract that might overlap the metallic SPR band. Scanning Electron Microscopy (SEM) analysis showed that AV-Ag NPs exhibited a spherical morphology with an average particle size ranging between 65 to 79 nm (Figure 4B). Energy Dispersive X-ray Spectroscopy (EDS) confirmed the presence of elemental silver, with a strong silver signal at 3 keV, thus further validating the composition of nanoparticles (Figure 4C). In *A. vera*-Ag NPs, absorption bands observed around ~3400 cm^{-1} correspond to O–H stretching vibrations of hydroxyl groups, suggesting the presence of polyphenols and alcohols that act as reducing and capping agents (Figure4D). The peaks in the range of 1600–1650 cm^{-1} represent C=O stretching from amide bonds, likely to be derived from proteins in the plant extract that enhanced the stability of nanoparticles. The additional bands at ~1050–1100 cm^{-1} indicated C–O stretching vibrations, potentially from carbohydrates that also contribute in nanoparticles stabilization (Figure 4D).

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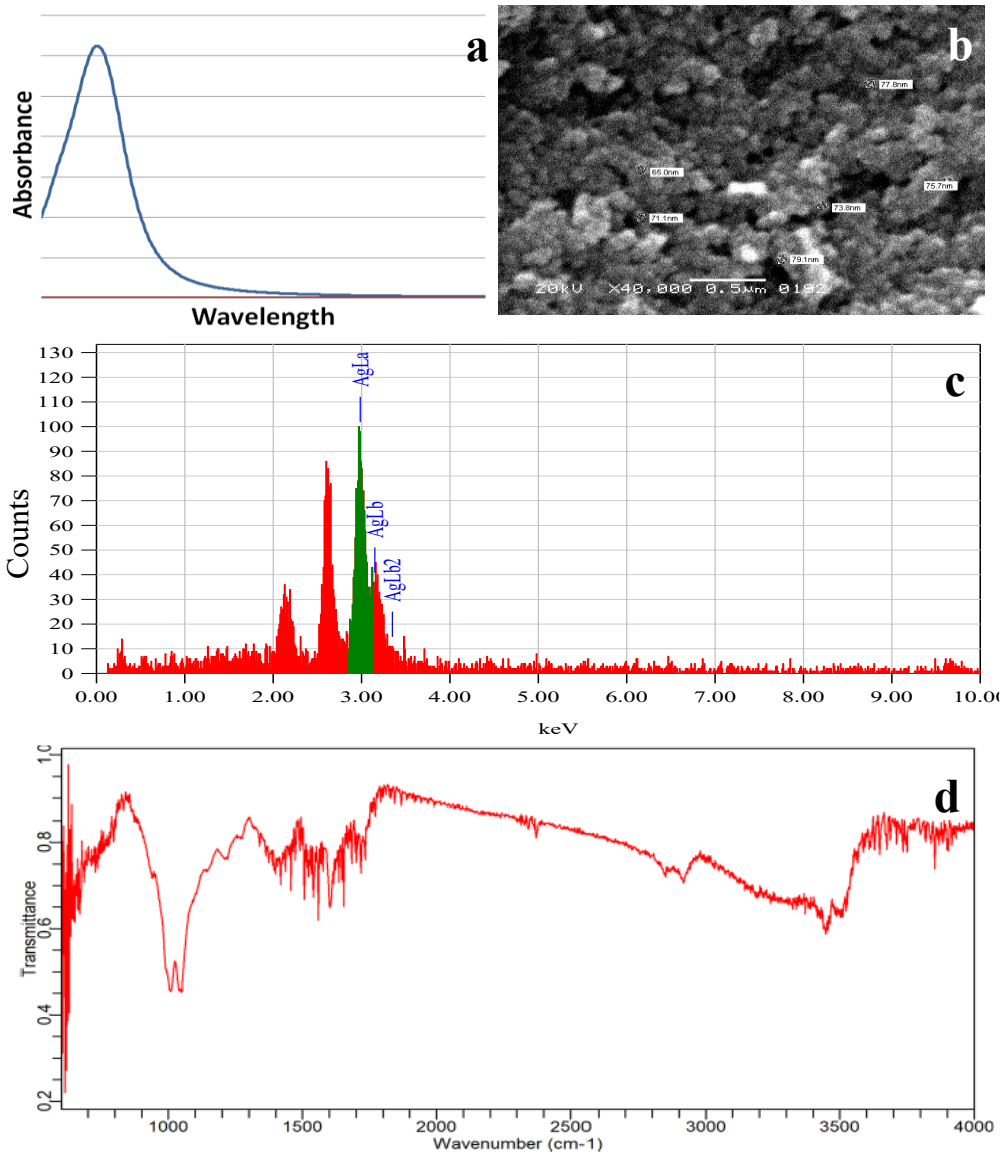

Figure-4. Physicochemical characterization of AV-Ag NPs. (a) UV–Vis spectra: a strong band near ~260 nm arises from plant biomolecules, (b) SEM micrographs showing predominantly near-spherical particles (scale bar: 0.5 µm; 20 kV), (c) EDX spectra confirming silver as the major element with C and O from capping phytochemicals, (d) FTIR spectra indicating O–H, C=O (amide I), C–N and C–O–C functional groups.

Green synthesis and characterization of CF-Ag NPs

C. fistula extract was used to reduce the silver ions and producing stable silver nanoparticles. A color change from pale yellow to dark brown on addition of silver nitrate ($AgNO_3$) confirmed the reduction of Ag^+ ions to Ag^0 nanoparticles. UV-Vis spectroscopy displayed a prominent peak at 260 nm, indicating the successful formation of nanoparticles (Figure 5A). SEM analysis revealed that the size of CF-Ag-NPs ranged from 80 to 95 nm (Figure 5B). EDS analysis detected a strong silver signal at 3 keV, confirming the elemental composition purity of the synthesized nanoparticles (Figure 5C). FTIR analysis of *C. fistula* Ag-NPs revealed strong O–H and C=O peaks, alongside aromatic C=C stretching (~1500 cm^{-1}) and alkane C–H stretching (~2920 cm^{-1}), indicating the involvement of phenolic compounds, flavonoids and other phytochemicals in the reduction process (Figure 5D).

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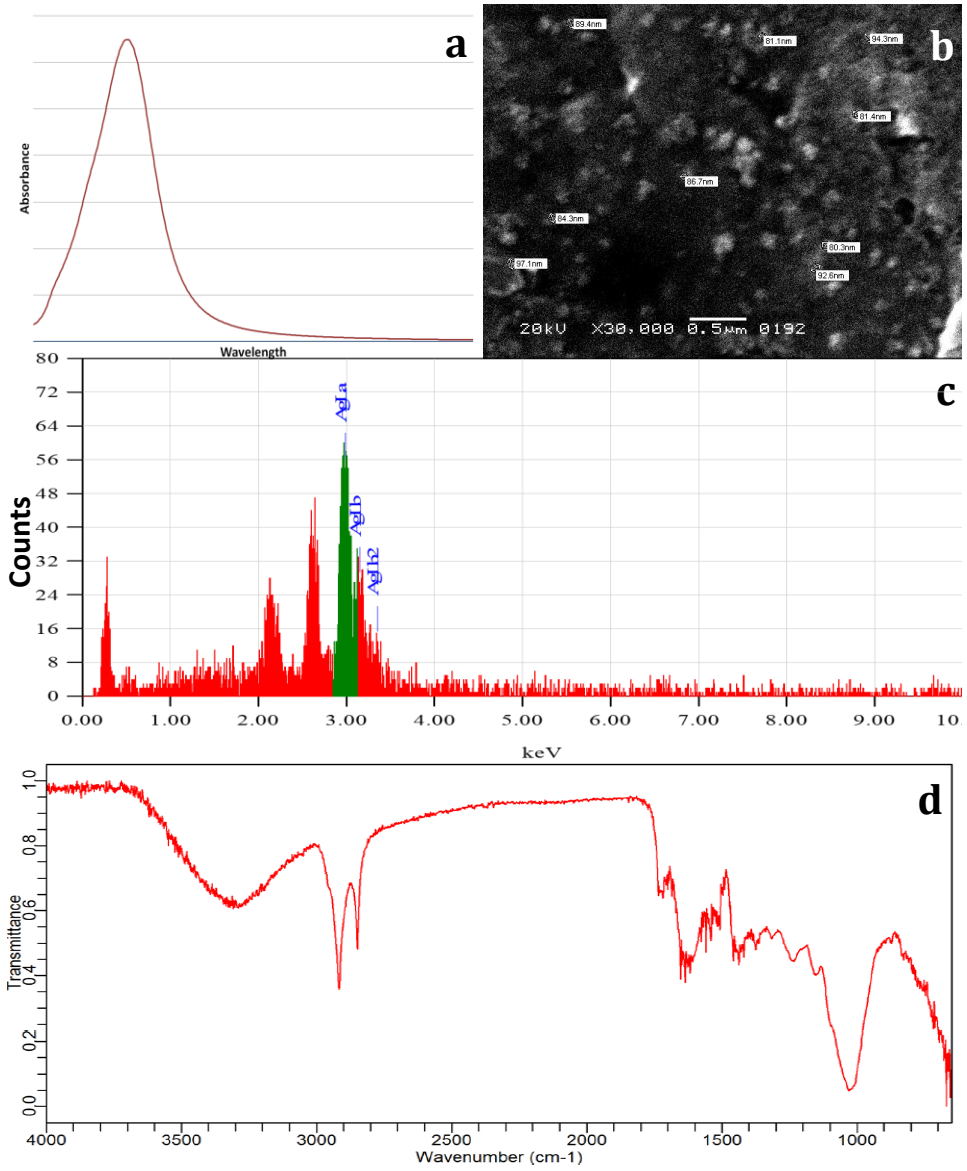

Figure-5. Physicochemical characterization of CF-AgNP. (a) UV-Vis spectra of absorbance at 260 nm, (b) SEM showing NPs in the range of 80-95 nm, (c) EDX spectrum revealed strong signal at 3 keV, confirming the purity of synthesized NPs and (d) FTIR peaks of Ag NPs in 1500 cm^{-1} range.

Larvicidal activity of green synthesized Ag NPs

The results demonstrated the efficacy and toxicity of different concentrations of nanoparticles against the Culex larvae. The maximum mortality of 4th stage larvae of *Culex* mosquito was observed at 72 hours of treatment for both AV-Ag and CF-Ag NPs (Table 2). For AV-Ag NPs, the mortality rates observed were 31.65%, 88.32% and 98.30% at concentrations of 16, 32 and 64 ppm, respectively, at 72 hours of treatment. While the mortality rates observed for CF-Ag NPs were 13.36%, 46.65%, and 60.00% at concentrations of 16, 32 and 64 ppm, respectively, at72 hours of treatment. Thus, the AV-Ag NPs demonstrated an almost complete larvicidal activity of 98.30% as compared to 65.00% by CF-Ag NPs. The control treatments (C1 – C3) containing only *Aloe vera* extract (C1), $AgNO_3$ solution (C2) or only *Cassia fistula* extract (C3) exhibited a negligible larval mortality at 72 hours of treatment (Table 2). Thus, there observed a significant difference in the larvicidal efficacy of silver nanoparticles of both plant extracts,wherein the nanoparticles of *A. vera* consistently exhibited a

significantly ($p<0.05$) higher larvicidal activity than *C. fistula* nanoparticles at every condition (Table 2). These green synthesized nanoparticles demonstrated the larvicidal efficiency in a dose dependant manner against the mosquito larvae that was enhanced with an increase in the concentration of the NPs and duration of the treatment.

Table-2. The larvicidal activity of AV-Ag NPs and CF-Ag NPs against 4th stage larvae of Culex mosquitoes.

Ag NPs (ppm) of extract	Time (hours)	Mortality (Mean ± SD)		Mortality (%)		Mean difference	*p*-value
		AV	CF	AV	CF		
16	24	2.33±0.27	0.30±0.07	11.65	1.50	10.15	0.035*
16	48	4.66±0.41	1.33±0.14	23.30	6.65	16.65	0.021*
16	72	6.33±0.62	2.66±0.42	31.65	13.36	18.29	0.019*
32	24	12.44±1.47	5.33±0.62	62.23	26.65	35.58	0.002**
32	48	15.66±2.24	8.66±0.71	78.36	43.32	35.04	0.004**
32	72	**17.66±1.94**	9.33±0.56	**88.32**	46.65	41.67	0.001**
64	24	18.33±2.07	10.66±1.47	91.65	53.30	38.35	0.001**
64	48	19.33±1.76	12.33±0.96	96.65	61.65	35.00	0.003**
64	72	**19.66±0.47**	13.00±1.24	**98.30**	65.00	33.30	0.002**
64 (C1)	72	-	0.33±0.04	-	0	-	-
64 (C2)	72	-	0.66±0.09	-	3.33	-	-
64 (C3)	72	-	0.33±0.07	-	0	-	-

The nanoparticles of *A. vera* exhibited a significantly ($p<0.05$) higher larvicidal activity than *C. fistula* nanoparticles at all condition. The larvicidal efficiency of respective green Ag NPs was enhanced with an increase in the concentration of NPs and duration of treatment. C1: *A. vera* (AV) extract only, C2: $AgNO_3$ solution, C3: *C. fistula* (CF) extract only. $p<0.05$ was considered as statistically significant level.

LC_{50} and LC_{90} determination

The probit analysis was performed on larval mortality data at 72 hours for Ag NPs formed of *A. vera* and *C. fistula* extracts. The calculated LC_{50} for AV-Ag NPs was 17.85 ppm (95% CI: 14.2–21.5), with an LC_{90} of 42.51 ppm (95% CI: 37.0–49.8). In contrast, CF-Ag NPs showed LC_{50} of 36.35 ppm (95% CI: 29.8–43.9) and a significantly higher LC_{90} of 361.38 ppm (95% CI: 310.5–429.6). These results confirmed that AV-Ag NPs have greater toxic profile against *Culex* larvae than CF-Ag nanoparticles (Table 3).

Table-3. LC_{50} and LC_{90} values of AV and CF-synthesized Ag NPs against *Culex* larvae (72 h post-treatment) by probit regression analysis.

Nanoparticle Type	LC_{50} (ppm)	LC_{90} (ppm)
Aloe vera–Ag NPs	17.85	42.51
Cassia fistula–Ag NPs	36.35	361.38

The lower LC_{50} and LC_{90} values of AV-Ag NPs estimated by probit analysis demonstrated their better toxicity activity than CF-Ag NPs at 72 hours.

Discussion

The traditional identification of different insects usually relies on morphological characteristics but these methods are usually prone to various errors. Comparatively, although the molecular techniques offer proper identification and greater precision but still are under-utilized in the local surveillance programs. Hence, PCR based molecular characterization of Culex species has been employed

in the current study for accurate identification of the investigated insects. The chemical insecticides used for the control of mosquitoes usually pose environmental and health risks, thus, it has been a great need for alternative, eco-friendly plant based approaches with limited health hazards. In the modern times, the essential oils and extracts from various parts of plants offer promising alternatives to the synthetic drugs (Bilal et al., 2024; Aslam et al., 2021, 2023a, 2023b). Similarly, the molecular level analysis is essential for taxonomic accuracy and practical public health applications, thus, requires the translation of these findings into observable ecological indicators to empower the community-led vector control strategies using commercially available green synthesized Ag NPs larvicides. The current study provides a bridge between these gaps by integrating the morphological and molecular techniques for accurate species identification along with evaluating the efficacy of biogenic silver nanoparticles synthesized with herbal extracts, as a sustainable larvicidal agent.

The configuration of proboscis as observed by electron microscopy is characteristic of Culex and considerably differs from the comparatively compact sensillar organization observed in Aedes and the distinct proboscis sensilla pattern reported in Anopheles, which are associated with different feeding specializations (Chapman, 2013). The compound eye exhibits a tight hexagonal ommatidial packaging that provides a broad visual field essential for host and habitat detection (Rocha et al., 2015). The observed leg microtrichia and spine arrangements correspond to culicid characteristics and provide additional morphological markers for genus-level identification (Harbach, 2024). Collectively by electron microscopy, the proboscis, ocular, wing, abdominal, and leg microstructures demonstrate a coherent suite of traits consistent with Culex taxonomy and allow clear differentiation from related mosquito genera (Clements, 1992). Moreover, the molecular analysis further confirmed the identification of *Culex* mosquitoes by specific molecular markers, by PCR amplification of COX1 gene. These finding support an accurate molecular level identification of *Culex* species since several studies employed the COX1 barcoding (Demari-Silva et al., 2011; Chan et al., 2014; Reidenbach et al., 2009).

The NPs of *A. vera*-Ag exhibited smaller and more uniform spherical particles (65nm to 79nm) compared to *C. fistula*-Ag NPs (80nm to 97nm) that highlight that former NPs possess well-defined physical and chemical properties, making them highly stable and effective for biological applications as like mosquito larvicidal activity. Since, the particle uniformity is critical for consistent interaction with larval cuticles and penetration into the peritrophic membrane and due to this morphological advantage of AV-Ag NPs demonstrated the observed better larvicial results as compared to CF-Ag NPs. EDX-confirmed the silver core coupled with these surface chemistries and FTIR spectra of *A. vera*-Ag NPs revealed prominent peaks corresponding to hydroxyl (–OH), amide (–CONH) and carbonyl (C=O) functional groups, suggesting the involvement of phenolic compounds, proteins and polysaccharides in the nanoparticles stabilization. These biomolecules not only cap the nanoparticles, prevent the aggregation but also enhance their larvicidal activity through synergistic oxidative stress mechanisms (Siddiqi et al., 2018). The distinct peaks of aromatic C=C and alkene C–H groups in both extracts indicated the presence of flavonoids and terpenoids those are known to demonstrate promising larvicidal activity against Culex (Pushpanathan et al., 2006) and Anopheles (Jeyabalan et al., 2023). In the same context, Ag NPs formed by *Azadirachta indica*, *Moringa oleifera* and *Coleus aromaticus* demonstrated significant toxicity against larvae of *Culex quinquefasciatus* (Aremu et al., 2023), *Anopheles gambiae* (Idowu et al., 2021) and *Aedes aegypti* (Dass et al., 2025), respectively, The green synthesized Ag NPs using both plant extracts demonstrated a significantly higher toxicity compared to negligible mortality exhibited by *Aloe vera* (C1) or *Cassia fistula* (C3) alone or minute toxic effects of $AgNO_3$ solution (C2). This enhanced larvicidal activity is driven by superior bioavailability and toxicity due to reduction of $AgNO_3$ by plant extracts as was confirmed by the color changed to dark brown. Hence, it could be suggested that the phytochemicals like terpenoids, phenols and alkaloids of different parts of plants could be efficiently used against various harmful arthropods by incorporating in the form of nanoparticles (Aslam et al., 2025; Rahmani, 2015).

The better LC_{50} and LC_{90} values derived from probit regression provide strong evidence for a better larvicidal potential of Aloe vera (LC_{50}=17.85 ppm) synthesized NPs than *Cassia fistula* (LC_{50}= 36.35 ppm). Thus, *Aloe vera* could be used as a promising biogenic material for nanoparticles synthesis with better dosage optimization in vector control programs. In the same context, *Azadirachta indica*-synthesized Ag NPs demonstrated the mortality rate of >90% of *C.*

quinquefasciatus at a concentration of 50 ppm at Osun State Nigeria (Aremu et al., 2023), similar to the observed high potency of *A. vera*-Ag NPs (98.30%) in the current study. Similarly, polymeric curcumin nanocapsules exerted an effective larvicidal activity at 48 h of treatment (LC_{50}=1.95 mg/L, LC_{90}=6.46 mg/L) against 4th instar larvae of *Aedes albopictus* and thus demonstrated their efficient potential as safe, food-grade and biodegradable alternate for chemical larvicides (Ahmed et al., 2021). In contrast, *C. fistula*-Ag NPs here showed lower efficacy (65% mortality at 64 ppm), aligning with reports from India wherein *C. fistula*-based nanoparticles achieved moderate larvicidal activity (George et al., 2023), suggesting that phytochemicals composition and nanoparticles morphology can vary with plant origin and conditions of nanoformulation. Moreover, different types of phytochemicals also influence the NPs formation, shape and stability that ultimately improve their antimicrobial and therapeutic effects and hence, their incorporation in the biomedical applications.

Conclusions

The current study cumulated the morphological and molecular approaches for accurate confirmation and characterization of Culex mosquito species. The evaluation of green-synthesized Ag NPs from *A. vera* and *C. Fistula* demonstrated their promising larvicidal potential, wherein lower LC_{50} and LC_{90} values of *A. vera*-based NPs exhibited significantly higher larvicidal efficacy than NPs formed of *C. fistula* in a dose- and duration of exposure dependent manner. These findings suggested that *A. vera*-synthesized Ag NPs could be better served as eco-friendly alternative to conventional insecticides for mosquito control. Moreover, it has been suggested that field validations under real environmental conditions and potentially incorporating slow-release formulation optimization will further enhance the practical applicability of these biogenic nanoparticles, to enhance persistence and cost-effectiveness in integrated vector management programs.

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Data Availability Statement

All the data related with the study are included in the manuscript.

Contribution of Authors

Ullah S, Kayani HA: Conceived and designed the study; Ullah S, Naz S, Fatima R, Haq F: Collected the data, Kayani HA, Ali HM, Rehman HMS, Aslam J: Analyzed and discussed the results; Ullah S, Naz S: Synthesis and characterization of nanoparticles; Fatima R: Conducted the morphological identification studies, Yousaf MZ: Conducted the molecular identification studies, Ullah S, Ali HM, Ahmed HA, Rehman HMS, Sajid N: Wrote and revised the manuscript.

All authors read and approved the final draft of the manuscript.

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