Acarous calamus L. based nano-bio formulation as an alternative to synthetic fungicides against Aspergillus flavus Link

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Research Article

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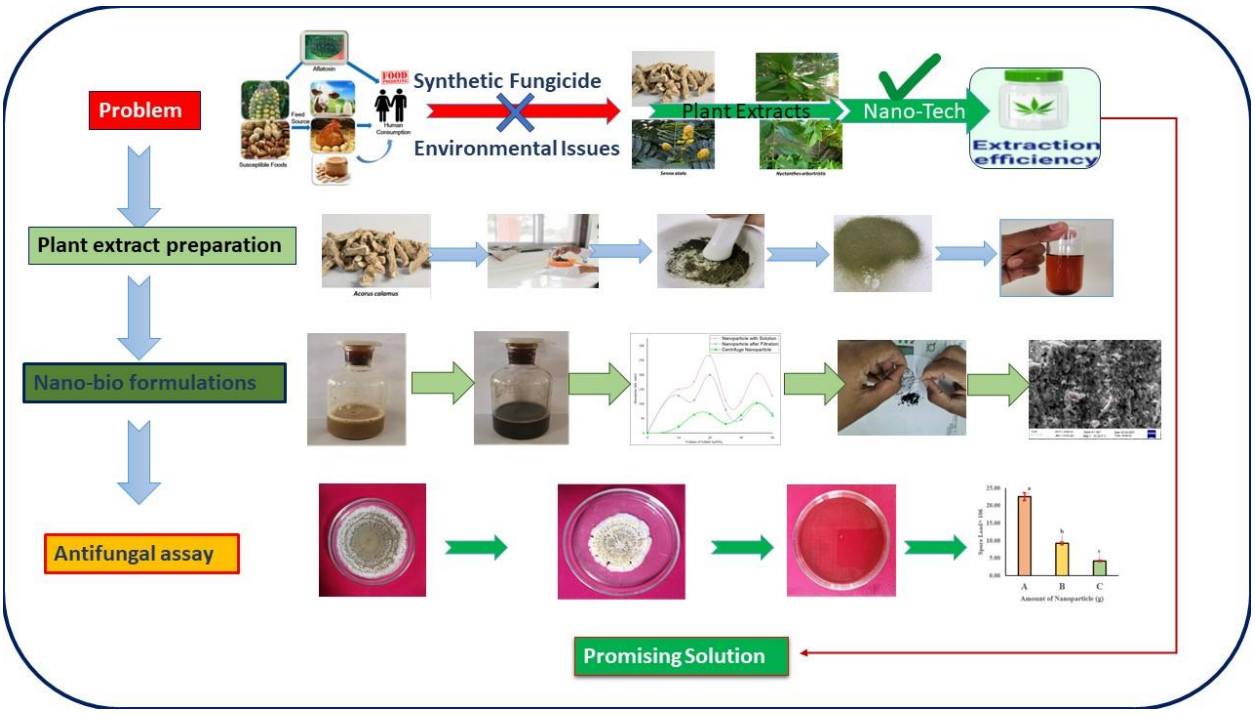
Highlights

- Sweet flag (*Acarous calamus* L.) roots consisting biomolecules are potential to inhibit the growth and reproduction of the Aflatoxins producing *Aspergillus flavus*
- Silver-Nano formulation enhance the efficacy of the biomolecules 50% higher than the ordinary extracts
- Nano formulation reduces the silver nitrate consumption and *Acarous calamus* based AgNPs does not carry silver ions, therefore, no leakages of Ag+ into the environment.

Graphical abstract



Acarous calamus L. based nano-bio formulation as an alternative to synthetic fungicides against *Aspergillus flavus*

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1 Abstract

Carcinogenic Aflatoxins producing *Aspergillus flavus* contaminate food crops and pose health risks to humans and livestock. Synthetic fungicides are commonly used to control *A. flavus*, but they can have negative environmental and health impacts. *Acarous calamus* L., also known as sweet flag, is a plant with potential antifungal properties. Nanotechnology can enhance the effectiveness of plant extracts by increasing their surface area and allowing for better penetration. Current study focused to explore nano-bio formulation of *A.calamus* silver nanoparticle and comparative study of its efficacy on growth inhibition and reproduction of *A. flavus*. All the experiments were conducted in Complete Randomized Design (CRD), and data were subjected ANOVA using SAS 9.1 and Tukey's HSD multiple comparison test was used to determine the best treatment combination at P <0.05. The Jasco V-570 UV-VIS-NIR spectrophotometer results confirmed that the silver nanoparticle was synthesized at the optimum condition of optimum volume was found to as 25 mL of 0.01 mol $L^{(-1)}$ of silver nitrate, optimum time was found as 210 minutes with the absorption peak range of 400 nm - 500 nm. SEM characterization captured the AgNPs size varies from 0.11µm to 2.23µm. AgNPs concentration of 0.02 g inhibit the growth by 50% compared to *A. calamus* water extracts and >90% reduction in spore production reduction at $P < 0.05$. Moreover, growth inhibition percentage and rate spore production of *A. flavus* in Ag-Nano particle inoculated treatments were perfectly corelated with the R^2 value of 0.96 at P <0.01& 0.05. This study concludes Nano-bio formulations show enhanced the efficiency and promise of plant-based solution as a natural fungicide against *A. flavus*. Field research is underway to fully understand the potential of this Nano-bio formulations.

Keywords: Aflatoxin, *Aspergillus flavus*, Green synthesis of nanoparticles, Silver nanoparticles

2 Introduction

The plant pathogenic fungal genera *Aspergillus* causes huge economic loss of 25% or more in agriculture production by destroying food crops and producing mycotoxins (1). Aflatoxin is the

secondary poisonous metabolites produced by the key *Aspergillus* species such as *Aspergillus flavus* and *Aspergillus parasiticus* (2) and but some other species, such as *A. nomius*, *A. pseudotamarii*, *A. parvisclerotigenus*, and A. *bombycis* of section *Flavi*; *A. ochraceoroseus* and *A. rambellii* from section *Ochraceorosei*; and *Emericella astellata* and *E. venezuelensis* from *Nidulatans*, have also been reported as aflatoxin producers (3). Ingestion of Aflatoxin contaminated food and feed of more than 20 ppb causes not only severe health issues in humans and livestock, respectively, as it is considered as the structurally related toxic, mutagenic, and carcinogenic substances (4). If *Aspergillus flavus* and *Aspergillus parasiticus* infect the crops, aflatoxin contaminates nuts and even processed nut products like peanuts, peanut butter, and pistachios; cereals like maize, sorghum, and millets; and spices like pepper, chilli, paprika, ginger, cinnamon, and coriander and make them unfit for consumption (5, 6). On the other hand, aflatoxin contaminates milk when animals consume aflatoxin contaminated feed (7, 8). When the environment favorable for pathogen growth they grow well in the grains and produce aflatoxin. In the tropical and sub-tropical regions of the world, the contamination of aflatoxin in rice is high. India, Pakistan, Malaysia, China, South Korea, Philippines, United Arab Emirates, Thailand, and Indonesia have reported aflatoxin contamination in rice grain (9). Aflatoxin contamination in rice, maize, copra, peanuts, lentils, and minor food products such as spices has been recorded in Sri Lanka (10). Moreover, in 2021, the USA reported the pet food and coconut oil imported from Malaysia to Sri Lanka contains aflatoxins (11). Africa is also affected by aflatoxin contamination. Mostly western African people have aflatoxin positive in their blood (5). Aflatoxin *M1*, aflatoxins B-series *B1* and *B2*, aflatoxins G-series *G1* and *G2,* and M-series aflatoxin *M1* are highly significant groupings with regard to food and health safety.

Crops grown in tropical and subtropical countries are highly prone to contaminate aflatoxin, but contaminated planting sources, drought stress, injury, pest infestation, poor management practices, postharvest handling and storage are facilitating immunocompromising factors decide the level of aflatoxin production and contamination in agricultural products (12). Around the globe enactment of strict food safety regulations, prophylactic measures taken prevent pre- and postharvest infection of *Aspergillaus* fungi would be the best precautionary measure to minimize human dietary exposure to aflatoxin (13).

Scientifically proven experiments demonstrated and proved that medicinal plant's extractions can minimize the fungal spore germination, arrest growth, and reproduction of plant pathogenic fungi (14). Therefore, plants which are having rich antifungal compounds can be used for the preparation of bio fungicides to minimize the usage of toxic synthetic fungicides. *Acarous calamus* L. (15)*, N. arbotritis* (16)*, S. atala* (17) and *J. adathoda* (18) are potential and important plants consisting rich source of antifungal compounds. Lack of standardized formulation, poor penetration of due to larger size of active molecules in to the plant, strict legislation, rapid degradation, etc. hinder the farmers' unwillingness to employing natural products as bio-fungicides and the dearth of research in this area limit plant products' broad practical use, even if they are proven viable alternatives to synthetic fungicides (19).

Recent advances in Nano technology application in diversified filed of applied sciences opened a new means of bio-fabrication or green synthesis of plant-mediated nanoproduct using different metals and inert materials called "nanoparticles" (20). Nanoparticles are small in size varies from $1 - 100nm$; vary in shape and chemical composition (21). Compare to their original size of the biomolecules, nano-biomolecules are tiny in size with different shapes such as triangle, spherical, decahedral, quasi-spherical, irregular, irregular spherical, hexagonal, cylindrical, and pyramidal,

therefore, it can bale to deeply penetrate in to plant quickly and spread through the plant system quickly (22, 23). Metallic nanoparticles can be synthesis by using various metals like Silver (Ag), Copper (Cu), Gold (Au), Nickel (Ni), Palladium (Pd), Platinum (Pt), Zinc Oxide (ZNO), and Titanium oxide (TiO) (22). In Agriculture, metallic nanoparticles are being used as slow-release fertilizers, biopesticides, soil remediation and rehabilitation, seed coating, organic acid-base weedicides. Silver has its own antibacterial, antifungal, and antiviral properties. Even though the high dose application of Silver is toxic to the environment, Nano technology provide an option to very minimal leakage of silver when make valuable products, therefore, environmental exposure is negligible. Therefore, Silver nanoparticles are being use for several environmental remediations such as water purification, air disinfection, and wastewater treatment (24) and to inhibit the growth of several fungi such as Aspergillus, Candida, and Saccharomyces (25). Hashem, Saied (26) proved that biosynthesized AgNPs using *Bacillus thuringiensis MAE 6* significantly inhibit the growth of *Aspergillus niger*, *A. terreus*, *A. flavus*, and *A. fumigatus*. The current investigation was aimed to formulate *A. calamus* L. based nano-bio formulation using silver as Nanocoating agent to minimize preharvest Aspergillus contaminations in food commodities.

3 Materials and methodology

3.1 Culturing of pathogen *Aspergillus flavus*

Well characterized (microscopically and using molecular technique) *Aspergillus flavus* mother culture was derived from Microbiology and biotechnology laboratory of the Department of Agricultural Biology and sub-cultured in PDA media under hygienic conditions. Subculture's

virulent characteristics was examined in the healthy groundnut kernels using Koch's postulates (27).

3.2 Extraction of Medicinal plant's extracts using universal solvent

Among the antifungal properties proven medicinal plants such as rhizome of the *Acorus calamus*, leaves of the *Nyctanthes arbortristis, Senna atala*, *Justica adhatoda* tested as per the (28), rhizome of the *Acorus calamus* were collected from the herbal garden maintained at Department of Agricultural Biology as per the literature survey. Plant materials were washed thoroughly with distilled water and surface sterilized using 1 % of NaOCl and again rinsed with running double distilled water to get rid of the impurities. Surface sterilized plant samples were fully dried at 45 ℃ for five days. Dried samples were ground using motor and pestle and electric grinder to make them as fine powder.

$25g$ of dry powder of sample was taken in a beaker and dissolved with deionized water. That solution $250\ mL$ of each sample was prepared in volumetric flask at the concentration of 0.1×10^3 gdm^{-3}. The prepared solution was kept in the water bath with shaker at 80 ℃ for 30 $minutes$ at $50rpm$. Extracts were filtered using maslin cloths and the filtrate was centrifuged at $3500\ rpm$ for $10\ minutes$. The supernatant was filtered through Whatman *No1* paper and concentrated until reducing the volume 2/5 of the initial volume. The concentration of the final water extracts was $0.25 \times 10^3\ gdm^{-3}$.

3.3 Comparative efficacy testing of bio rational against *Aspergillus flavus*

The repeated poison food technique was performed to screen the best best antifungal activity showing plant extract(s) against *A. flavus*. 15.0 mL of melted PDA was poured into the Petri dishes and mixed with two drops of antibacterial solution and 3.0 mL of *each* treatment (Table 1). 5 mm diameter culture discs from an actively growing region of a 7-day-old stock *A. flavus* culture were transferred to the middle of the treatments and control plates.

Table 1: Treatment combinations tested against *Aspergillus flavus* growth's performance

Treatments	Treatment (3 mL)
T1	Control (Double Distilled Water)
T2	*A. calamus* water extract
T3	0.088 mL, 0.1N $AgNO_3$
T4	0.88 mL, 0.1N $AgNO_3$
T5	8.8 mL, 0.1N $AgNO_3$
T6	0.88 mL,0.01N $AgNO_3$ + 2.12mL, 0.01×103 gdm^{-3} *A.calamus* water extract
T7	2 mL 0.1N $AgNO_3$ + 1mL, 0.25×10^3 gdm^{-3} *A.calamus* water extract
T8	0.02 g synthesized nanoparticles (AgNPs)

The treatments and control without plant extract mix were replicated three times and arranged in Complete Randomized Design (CRD). The entire experimental setup was incubated at 28 ± 2°C and 70 ± 5% RH to facilitate the *A. flavus* growth and development. Mycelia growth was measured every morning from day after inoculation until 10th days of post-inoculation. The percentage of growth inhibition (I) was calculated using the formula given below.

$$\mathbf{I}\,\% = [\frac{(\mathbf{C} - \mathbf{T})}{\mathbf{C}}]\,\boldsymbol{x}\,\mathbf{100}\,\%$$

Where,
I = percentage inhibition of pathogen by botanicals
C = radial growth in control
T = radial growth in the treatment

Among the best inhibition ability exhibited treatment was used for the nanoparticle synthesis.

3.4 Synthesis and optimization of silver nanoparticles using *A. calamus*

As Kanagasabai, Pakeerathan (28) reported $10.0\ mL$ of *A. calamus* water extract was taken and deionized water was added until volume reach $250\ mL$, therefore the concentration was adjusted to $1.0 \times 10^{1}\ g\ dm^{-3}$. $25.0\ mL$ of *A. calamus* the $1.0 \times 10^{1}\ g\ dm^{-3}$ solution was sucked out and poured into a stopped bottle. $10.0\ mL$, $15.0\ mL$, $20.0\ mL$, $25.0\ mL$, $30.0\ mL$, $35.0\ mL$, and $40.0\ mL$ of $0.01\ mol\ L^{-1}\ AgNO_3$ was added into solution and kept in mechanical shaker for $210\ minutes$ at $250\ rpm$ constant speed. Then UV-Visible study was conducted for each sample and volume of $AgNO_3$ was optimized.

The optimum volume of $AgNO_3$ was added into $25\ mL$ of $1.0 \times 10^{1}\ g\ dm^{-3}$ concentrations of *A. calamus* solution in 5 stopped bottles. The prepared solution was kept in the mechanical shaker and a UV-Visible spectrophotometer study using Jasco V-570 UV-VIS-NIR spectrophotometer was conducted at $150, 180, 210, 240$ and $270\ minutes$' interval. A graph was plotted using origin software. Using the graph optimum time required to form nanoparticles with an optimum volume of $1.0 \times 10^{-2}\ mol\ L^{-1} AgNO_3$ was determined.

In order to compare the various absorbance for the synthesized Silver nanoparticle with various sorption times and volumes of added $AgNO_3$, and to ease the analysis of this direct difference, one can simply consider the overall integrated absorbance graph (as a function of wavelength). This is computed by integrating the absorbance between the two points, when the pure *A.calamus* water extract absorbance increases and the Ag nanoparticle absorbance decreases to zero (as shown in

Figure 1). And from here on, this integrated value will be referred to as the "amount of Ag nanoparticles"

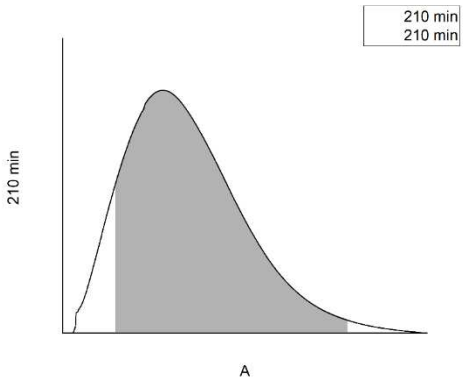

Figure 1: Integrating absorption profiles (shaded area) for the amount of absorbance on 210 min recorded as a function of wavelength.

The amount of produced nanoparticles is directly proportional to the absorbance of the synthesized Ag Nanoparticle (based on the Beer-Lambert law). The amount of synthesized silver nanoparticle is calculated according to the following equation:

$$An = As - Ac$$

where An = number of synthesized nanoparticles

As = amount of nanoparticles in the solution

Ac = amount of nanoparticles after centrifugation

The amount of synthesized silver nanoparticles (An) is used as a tool to compare many absorption profiles quantitatively in the following sections. The quantity of Ag nanoparticle absorption was determined at different periods in order to look into the ideal sorption time. Where the number of synthesized nanoparticles (An) is high at that point is considered the optimum time for nanoparticle synthesis. The quantity of absorption for the Ag nanoparticles was tested at different volumes of

silver nitrate in order to explore the ideal volume of silver nitrate. Where the number of synthesized nanoparticles (An) is high at that point of volume measured as optimum volume.

3.5 Mass production of *A. calamus* based silver nanoparticles and Ultra structure characterization using SEM

The amount of $AgNO_3$ required for the optimum synthesis of nanoparticles was calculated based on the optimization. $4 \times 10^{-3}\ mols$ of $AgNO_3$ were used to synthesize *A. calamus* nanoparticles. For that $4.2468\ g$ of $AgNO_3$ granules were taken in a volumetric flask and it was volume up to $100\ mL$ with distilled water to prepare $2.5 \times 10^{-1}\ mol\ L^{-1}\ AgNO_3$. $20\ mL$ of $2.5 \times 10^{2}\ g\ dm^{-3}$ water extract of *A. calamus* was taken, and $16\ mL$ of $2.5 \times 10^{-1}\ mol\ L^{-1}$, of $AgNO_3$ was added to the *A. calamus* extract. Then it was kept in a mechanical shaker for $180\ minutes$. Thoroughly shaken solution was centrifuged in TOMY Hybrid refrigerated centrifuge CAX-571 at $10{,}000\ rpm$ for $20\ minutes$. The supernatant was discarded and the precipitate was collected, and washed with distilled water. Same procedure repeated until got clear supernatant solution. The pellet was collected from the bottom of the centrifuge tube and placed into the oven at 110 ℃ until get the constant weight of nanoparticles. Then, these well dried nanoparticles were allowed to cool down at room temperature and kept it in the desiccator.

The ultra structure of AgNPs was captured using high performance Scanning Electron Microscopy (ZEISS EVO LS15, Germany).

3.6 In-Vitro reproductive efficacy testing of green synthesized nanoparticles against *A. flavus*

Synthesized AgNPs and other treatment combinations mentioned in Table 1 were prepared tested their efficiency in controlling *A. flavus* reproduction using spread plate PDA medium for fourteen days. After the fourteen days, the spore count was determined using a hemocytometer [HBG Germany (Bright Line)] using serial dilution procedure.

3.7 Experimental design and statistical analysis

All the experiments were conducted in Complete Randomized Design (CRD). Collected data were subjected to ANOVA using SAS 9.1 and Tukey's HSD multiple comparison test was administrated to identify the best treatment combination at $P < 0.05$.

4 Results and Discussion

4.1 Confirmation of *A.calamus* Nano particle formation

Visual observation

In all experiments, the addition of *A.calamus* extract into the stopped bottle containing an aqueous solution of silver nitrate exhibited the colour changes from yellowish to reddish-brown. In addition to different concentrations of silver nitrate solution to aqueous leaf extracts keeping its concentration of 25 mL ($1.0 \times 10^1\ g\ dm^{-3}$) constant, the colour of the solution changed from faint light to yellowish-brown and finally to the colloidal brown indicating the formation of silver nanoparticles.

Different parameters were optimized including the concentration of silver nitrate, and time which had been identified as factors affecting the yields of silver nanoparticles. Silver nanoparticles were synthesized at different concentrations of silver nitrate such as 10-40 mL of using 0.01 mol $L^{(-1)}$of

silver nitrate were analyzed by UV spectroscopy. The optimum volume was found to as 25ml of 0.01 mol $L^{(-1)}$ of silver nitrate. Likewise, optimum time was found as 210 minutes (Figure 2).

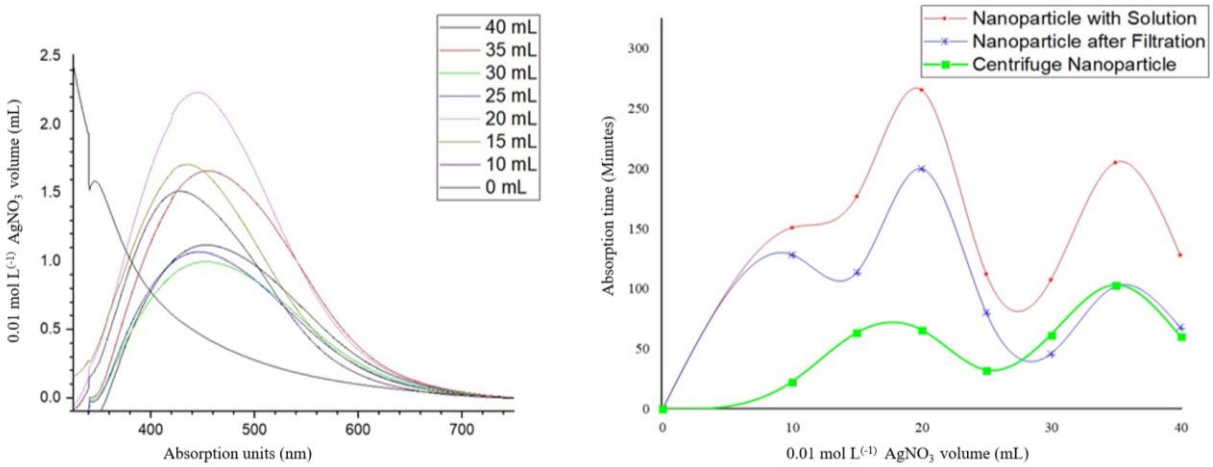

Figure 2: UV-Visible study for volume and time optimization for Silver Nanoparticles synthesis

The colour of the plant extract with silver nitrate solution in the stopped bottle turns from light yellow to brown after 210 minutes of incubation. The formation of Silver nanoparticles exhibit dark brown colour in an aqueous solution. This notable observation demonstrates that extracellular processes are involved in the reduction of Ag ions. The development of a dark brown color in the *Acorus calamus* aqueous extract solution is a surefire sign that silver nanoparticles are formed in the reaction mixture. The current finding are tally with the findings of Chawla and Sathaye (29). Similarly, Vijayaraghavan, Nalini (30) reported that silver nanoparticles of clove showed colour change from colorless to pale red.

UV Spectroscopic confirmation

The UV spectroscopic study shows that nanoparticles synthesized in the range between 400nm to 500nm. Absorbance peck was centered around $380 - 650\ nm$ for synthesized nanoparticles from *Acorus calamus*, but based on silver nitrate volume the peck of the nanoparticle varied. Pipriya

and Tiwari (31) reported that the extracellular biosynthesis of AgNPs from in the *Acorus calamus* aqueous extract solution exhibited a maximum absorbance in the wave length 420nm-430nm whereas (29) reported that green synthesis AgNPs is observed in-between 425nm-435nm. The bio-fabricated AgNPs using Citrus aurantifolia fruit peel extract was displayed the characteristics of a surface plasmon resonance band peak from 360–405 nm (32). Ag nanoparticle properties appear at a wavelength range of 400–600 nm and often peaks at the absorption of 450-462 nm regions which are close to the characteristics surface plasmon resonance (SPR) wavelength of metallic silver (33, 34). Further, Melkamu and Bitew (20) confirmed that the absorption peaks of AgNPs varied with the concentration of AgNO3. Highest absorption with slightly lower wavelength which indicates the highest amount of AgNPs being synthesized.

SEM characterization

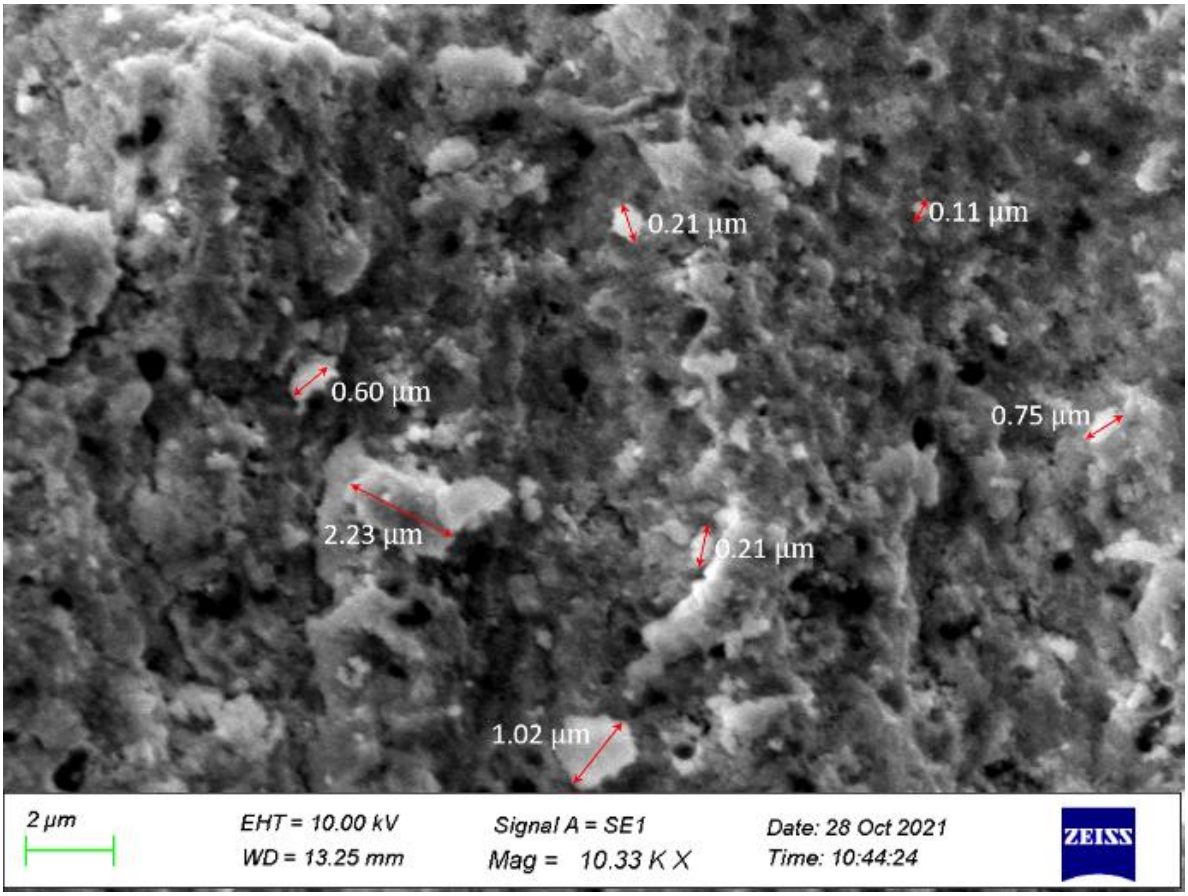

Figure 3: SEM Observation of synthesized AgNP

The particles size and the pores size of the AgNPs were studied using scanning electron microscope (Fig. 3). Figure shows the SEM image of the green synthesized AgNP. Particles size varies from 0.11µm to 2.33µm at the 10.33KX magnification level. Synthesized silver nanoparticles vary in different shape and size. Studies mentioned face centered cubic structure, had three shapes and ranged in size from 70 nm up to 600 nm. Spheres were between 70 nm and 250 nm; cubes were between 80 nm and 300 nm and triangular plates were between 80 nm and 600 nm. Spore size of the particles range from 0.35-0.41 µm.

Sarkar, Kumbhakar (34) reported by the silver nanoparticles size lies down in the range of 40-160 nm and size range may vary as per the morphology of the raw material used for the nano synthesis. Accumulated silver nanoparticles in the spherical shape (33). Mustapha, Ithnin (32) reported that

polydisperse, spherical shaped, and smooth surface nanoparticles with an average size of 24.023 nm when observe high resolution transmission electron microscope (HR-TEM) and field emission scan electron microscope (FESEM). The current findings on the formation of *A. calamus* AgNPs have been validated and supported by all previous studies.

4.2 Antifungal assay

Growth inhibition efficacy of *A.flavus* in eight different treatments show that 97.78% and 98.89% of growth of *A.flavus* suppressed by 8.8 mL,0.1N AgNO3 and 0.02g AgNPs in 3mL water with highly significant different ($P \leq 0.001$) compare to other treatments (Fig 4 and 5) even after 14 days of incubation. The growth suppression rate was increased with increasing concentration of AgNPs whereas the growth suppression was significantly low (T3=37.78%) at water based A.*calamus* extract than nano formulated A.*calamus* extract at ($P \leq 0.05$). When Nano particle concentration was $0.02\ g$, the growth was 50% minimal and highly significant when compare to *A. calamus* water extracts at $P < 0.05$ (Fig 5 H&G). Findings conclude that Nanoparticles suppress the growth of the *A.flavus* effectively than a direct plant extract.

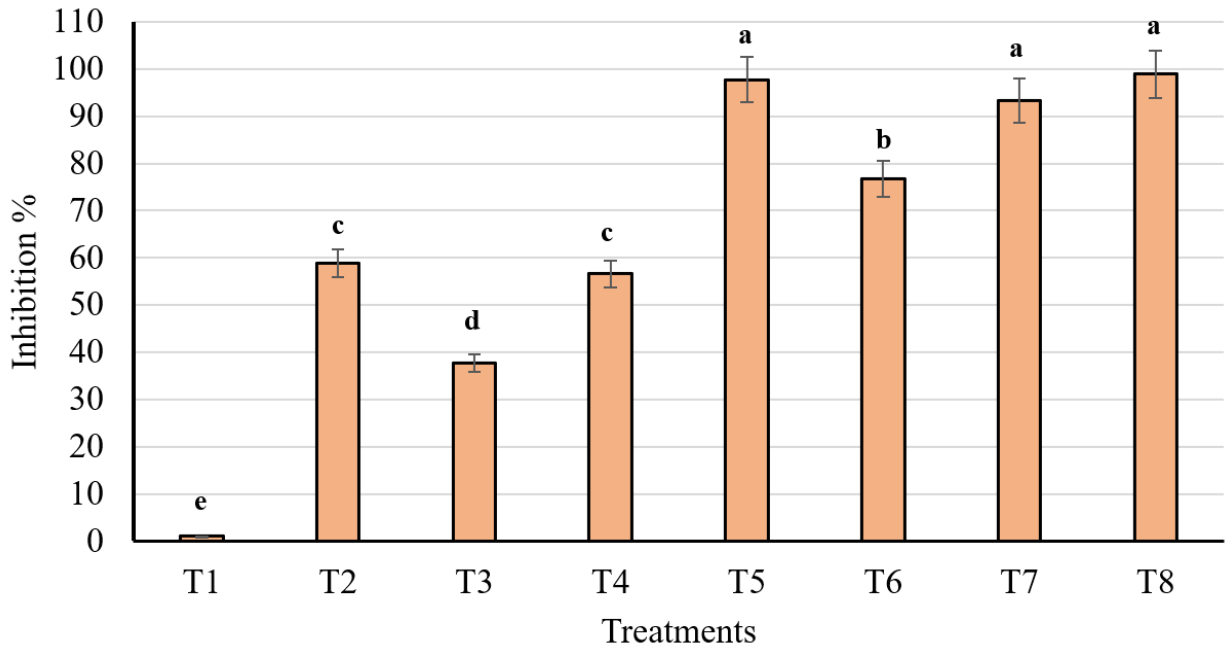

Figure 4: Growth inhibition of *A. flavus* by different treatments T1: Control; T2: *A.calamus* water extract; T3: 0.088 mL,0.1N AgNO3; T4: 0.88 mL, 0.1N $AgNO_3$; T5: 8.8 mL,0.1N $AgNO_3$; T6: 0.88 mL,0.01N $AgNO_3$ + 2.12 mL, 0.01×103 gdm^{-3} *A. calamus* water extract; T7: 2 mL 0.1N $AgNO_3$ + 1 mL, 0.25×10^3 gdm^{-3} *A. calamus* extract and T8: 0.02g AgNPs in 3mL water 7 days of post inoculation

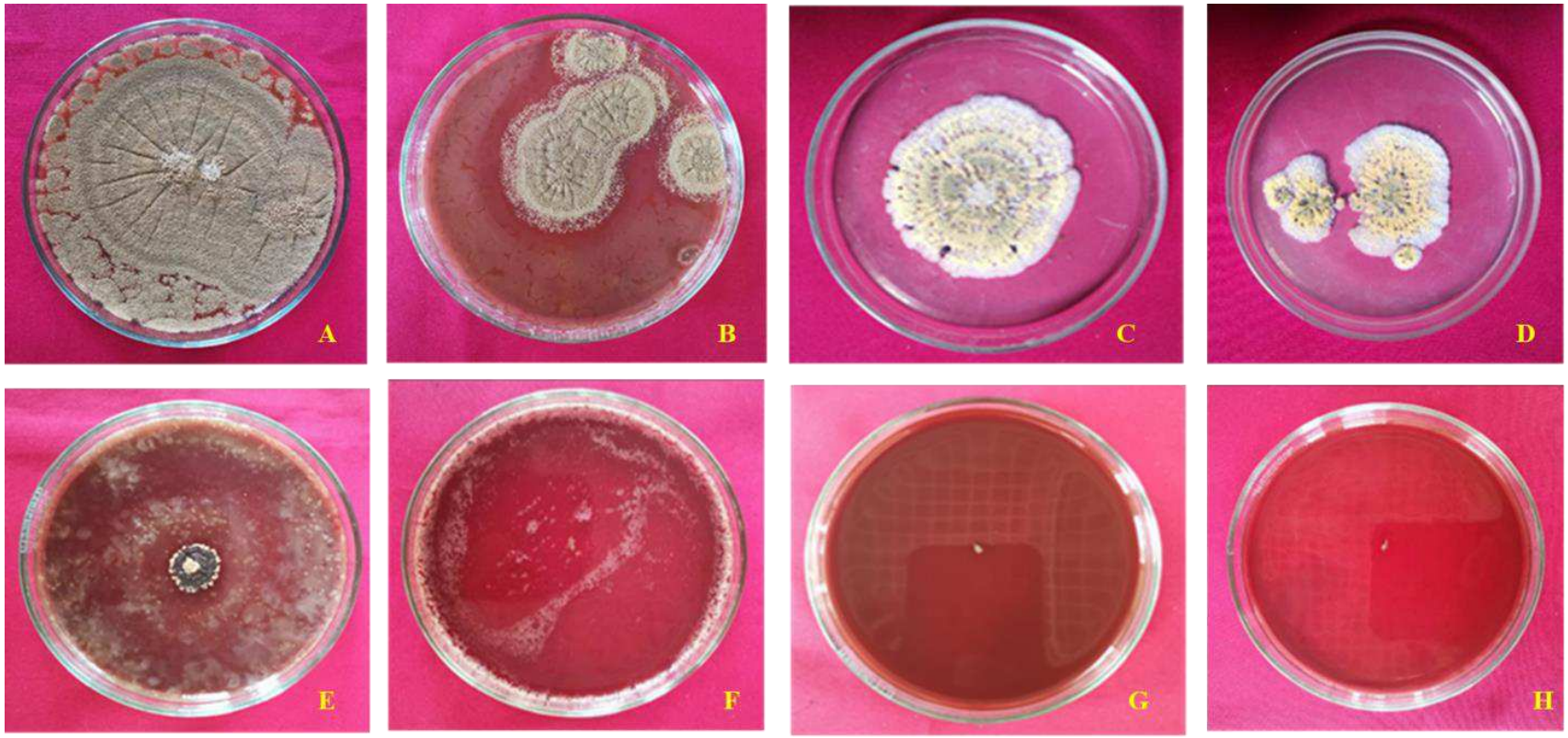

Figure 5: Mycelial growth of A.flavus in different herbal extract A: Control; B: *A.calamus* water extract; C: 0.088 mL,0.1N AgNO3; D: 0.88 mL, 0.1N $AgNO_3$; E: 8.8 mL,0.1N $AgNO_3$; F: 0.88 mL,0.01N $AgNO_3$ + 2.12 mL, 0.01×103 gdm^{-3} *A. calamus* water extract; G: 2 mL 0.1N $AgNO_3$ + 1 mL, 0.25×10^3 gdm^{-3} *A. calamus* extract and H: 0.02g AgNPs in 3mL water after 7 days of post inoculation

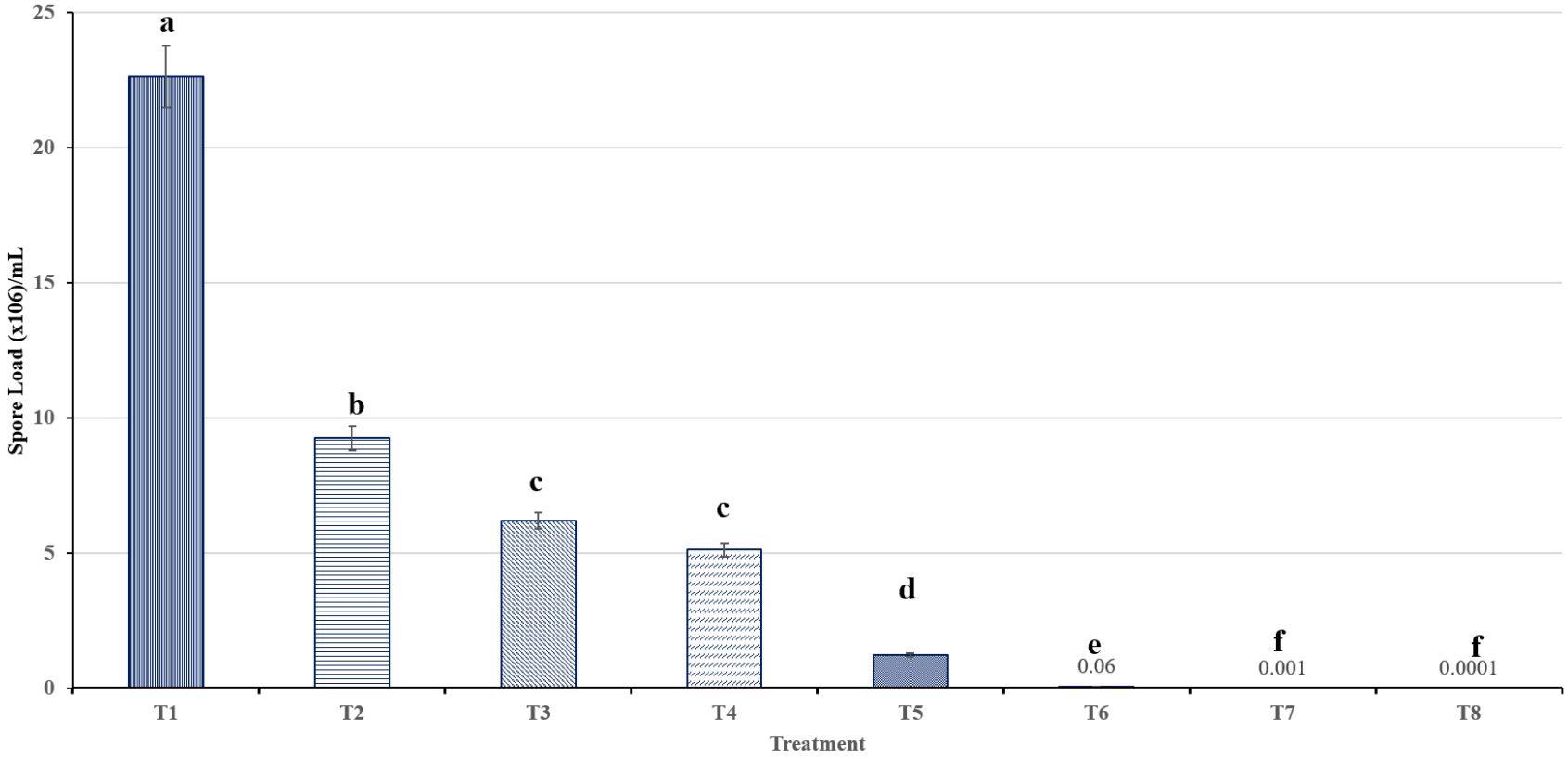

Figure 6 Reproduction efficacy of A. flavus Spore formation (Spore load) in T1: Control; T2: A.calamus water extract; T3: 0.088 mL,0.1N AgNO3; T4: 0.88 mL, 0.1N AgNO3; T5: 8.8 mL,0.1N AgNO3; T6: 0.88 mL,0.01N AgNO3 + 2.12 mL, 0.01×103 gdm-3 A. calamus water extract; T7: 2 mL 0.1N AgNO3 + 1 mL, 0.25×103 gdm-3 A. calamus extract and T8: 0.02g AgNPs in 3mL water in 14 days of post inoculation

Figure 6 shows that reproduction efficacy of *A. flavus* vis spore production in different amounts of synthesized silver nanoparticles present culture media. When the concentration of nanoparticles increased in the PDA media, the production of spores *A. flavus* was declined significantly at $P < 0.05$ (Fig 6). In control (T1) spore load was 2.263×10^7 spore/mL and highly significant $P < 0.01$ in comparison to other all treatments compared. where as in 2 mL 0.1N AgNO3 + 1 mL, 0.25×103 gdm-3 A. calamus extract, 0.02g AgNPs incorporated PDA spore amount were, 0.001×10^6, 0.0001×10^6 Spore Load /mL, respectively. This is actually >90% reduction in spore production reduction. Moreover, growth inhibition percentage and rate spore production of *A. flavus* in Ag-Nano particle inoculated treatments were perfectly corelated with the R^2 value of 0.96 at $P < 0.01$& 0.05

4.3 Qualitative analysis of presence of silver ion in different formulations

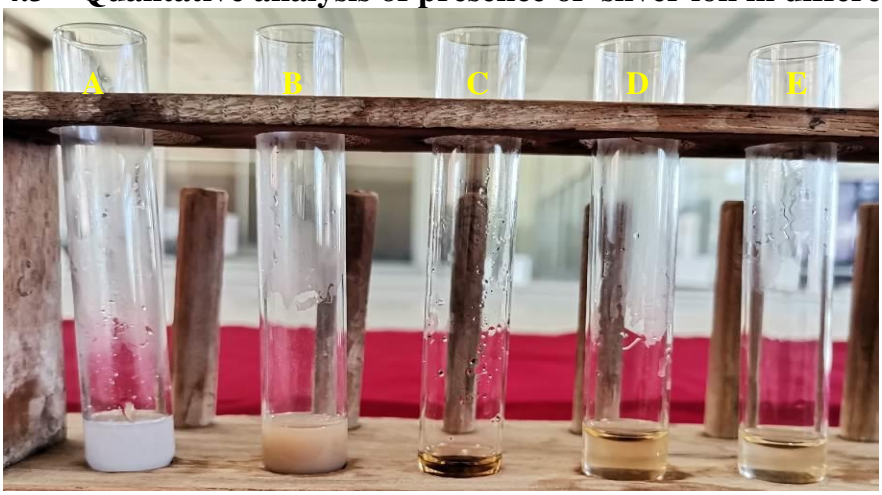

Figure 7 *Confirmatory assay of presence of Silvar ion in the extract while adding con HCl to the (A) silver nitrate, (B) supernatant obtained from the filtration of nanoparticles after centrifuged, (C) AgNPs alone, (D) AgNPs incorporated PDA, and (E) Control (PDA)]*

When add concentrated HCl to the silver ions white colour precipitate was observed (Fig 7). Whereas adding con HCl to other all treatments (C-E), there was no white precipitate. Further,

observation obtained from the supernatant (B) shows the presence of silver ions in the solution. Moreover, silver ions didn't form after added nanoparticles in the PDA media too. Therefore, all silver nitrate that was added to the extract did not use for the formation of nanoparticles. This assay confirms that in the nanoparticles silver ions are not in the highly toxic leachable Sivar form.

Variety of active ingredients present in many under-utilized plants are proved to be arresting the growth and reproduction of many fungal pathogens that are causing deleterious effect on plants like mycotoxin producing *Aspergillus* spp (26, 35). But the physical properties like large particle size, low penetration capability are limiting the functional efficacy of these biomolecules. Because of the novel physiochemical properties of having a high surface-to-volume ratio, nanoparticles (NPs) are thought to be better suitable candidates for performance-oriented applications, particularly in pest management (36).

Using Nano technology, particle size reduction and penetration capability of biomolecules could be engineered easily using metals like Ag, Ni, Ti, Cu and Zinc and the green-bio synthesis of Ag-NPs by virtue of their bio-active ingredients from plant parts is more facile, economical, hypo-allergic, eco-friendly, less laborious and less hazardous than other metals as Ag has its own antifungal properties too (16, 37). The current investigation successfully examined the synthesized & characterized Ag-NPs using plant extracts from *A.calamus*, and its efficacy against carcinogenic aflatoxin producing *A. flavus's* growth inhibition and reproduction retardation.

The current findings report that the AgNPs coated biomolecules are effective than ordinary molecules, therefore, the possibility to complement fungicides with silver nanoparticles to control growth and aflatoxin formation (38). Silver nanoparticles inhibit the metabolic activity and colony forming unit of *A. flavus,* therefore growth and reproduction are restricted (39). Dobias and

Bernier-Latmani (40) reported that leach of the Ag+ from the AgNPs are comparably less, and will take longer time depending on the size oxidative potential and temperature variation of the environmental (41).

5 Conclusion

The current experiment identified best conditions for synthesis of A. *calamus* AgNPs were 25 mL of 0.01 mol $L^{(-1)}$ of $AgNO_3$, 210 minutes, and an absorption peak range of 400 nm - 500 nm. The synthesized AgNPs size range 0.11µm to 2.23µm. At a concentration of 0.02 g, AgNPs decrease growth by 50% and reduce reproduction by over 90%. This study suggests that nano-bio formulations improve the efficacy and potential of plant-based solutions as natural fungicides against *A. flavus*. Detailed filed experiments are undergoing to recommend the current invention in the real field scenario.

Compliance with ethical standards

Author's contribution: KP and GS conceived the research idea; VK conducted the experiments; VK and KP wrote the manuscript; GS and KP edited the manuscript.

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