Medicinal Plant-Derived Copper Nanoparticles EffectivelyManage Early Blight Disease in Tomato: An *In vitro* Study

ABSTRACT

Aims: Early blight of tomato (*Solanum lycopersicum*L.**)** incited by *Alternaria solani* is an economically important disease. This paper analyses the efficacy of copper nanoparticles (Cu-NPs) as an alternative to its existing management practices that predominantly rely on toxic fungicides which are potentially harmful to humans and the environment.

Study design: The experiment was conducted in a completely randomized block design with 7 treatments *viz*. T_1:Cu-NPs from *tulsi* leaves, T_2:Cu-NPsfrom *neem* leaves, T_3:Cu-NPs from *bael*leaves, T_4: Cu-NPs from chemical A (prepared from 0.2 M of $Cu(NO_3)_2.3H_2O$ + 0.2 M Ascorbic acid), T_5: Cu-NPs from chemical B (0.2 M $Cu(NO_3)_2.3H_2O$ + 0.2 M $CuCl_2$ + 0.4 N NH_4OH), T_6: Carbendazim 50 WP as check and T_7: Control. Each treatment was tested attwo levels of*in vitro* concentrations (i.e.300 and 500 ppm) andthe treatments were replicated thrice.

Place and Duration of Study: Considering the importance of the disease and for developing an effective management strategy, the present study was carried out in Department of Plant Pathology, College of Agriculture, Odisha University of Agriculture and Technology, Bhubaneswar during 2020-21.

Methodology: Stable Cu-NPs were synthesized through three green synthesis methods by using*bael* (*Agel marmelos*L.)*, tulsi*(*Ocimum sanctum* L.), and *neem* (*Azadirachta indica* L.) leaves and two chemical synthesis methods (i.e. chemical A and B).

Results: The size (diameter) of the Cu-NPs from *Agel marmelos, Ocimum sanctum,* and *Azadirachta indica* were 427.4, 1,019, and 246.5 nm, and that from the aqueous solution reduction method and precipitation method were 1,537 and 867.8 nm, respectively. The synthesized Cu-NPs were evaluated against *Alternaria solani*at 300 and 500 ppmwhile Carbendazim 50 WP served as the standard check. The *Azadirachta indica* leaf-derived Cu-NPs at 300 and 500 ppm in the *in vitro* potato dextrose ager (PDA) medium exhibited the maximum mycelial growth inhibition of 60.24% and 75.9%, respectively. The size of Cu-NPs showed an inverse relation with the antifungal efficacy that indicated greater cellular penetration of smaller nanoparticles whereas much lower inhibition of mycelial growth in Carbendazim 50 WP as a check showed the lowest growth inhibition of 39.75% (300 ppm) and 59.03% (500 ppm).

Conclusion:

The results thus indicated higher efficacy of nanoformulations at lower concentrations that can be effectively used for the development of new nano-based plant protection agents against the early blight of tomatoes.

Keywords:*Agel marmelos, Alternaria solani,Azadirachta indica, green synthesis, mycelial growth inhibition,Ocimum sanctum*

1. INTRODUCTION

Tomato (*Lycopersicon esculentum***)**crop is affected by several diseases, of which, early blight incited by *Alternaria solani* is an economically important disease.The existing management of this disease relies predominantly on toxic fungicides that are potentially harmful to humans and the environment. Among the different alternative management technologies, nanotechnology is one of the safe and newly emerging technologies that have not yet been fully explored.The high surface-to-volume ratio of nanoparticles (NPs) due to their smaller size improves their reactivity and potential biochemical activity [1]. It has been observed that NPs efficiently penetratemicrobial cells [2]. Among the various metal-NPs, copper (Cu) has attracted more attention because of its catalytic, optical, electrical, and antifungal/antibacterial properties in the nano-scale range [3, 4]. Earlier studies indicated the efficacy of chemically synthesized copper nanoparticles (Cu-NPs) as a possible antifungal agent [5] however, the literature on the efficacy of green synthesized Cu-NPs is not traceable.

The development of green NP production technologies is becoming an important branch of nanoscience[6].The use of *Azadirachta indica*[7], *amla*[8], mangosteen [9], and *Chenopodium album*leaf [10] has already been reported for the synthesis of NPs. Synthesis of Cu-NPsfrom *Hibiscus rosa-sinensis*and*Ocimumsantnum*(leaf extract), *Syzygiumaromaticum* (cloves), andlemon (fruit extract), *Vitis vinifera*, *Eucalyptus*, *Cassia alata*, *Centella asiatica*, *Malva sylvestris*, etc. have been reported by earlier researchers [11].Theirfindings revealthe role of biomolecules present in plant extract such as proteins, phenols, and flavonoids in reducing ions to the nanoscale and capping NPs[12] (Arya 2010).Among the different established chemical methods to synthesize the NPs viz. reduction method, colloidal method, sonochemical method, etc., the use of ascorbic acid (vitamin C) as a reduction and capping agent in the chemical reduction of Cu salts is novel and environment-friendly[13].

Although earlier researchers have synthesized NPs from different metals, including Cu, reports on the green synthesis of Cu-NPs and its use in controlling the early blight of tomatoes are lacking. In this experiment, our objective was to synthesize and characterize Cu-NPs from three easily available medicinal plants viz. *Agel marmelos*, *Ocimum sanctum,* and *Azadirachta indica*andstudy their efficacyagainst *Alternaria solani disease* of tomato *in vitro*in comparison with two chemically synthesized Cu-NPs.

2. MATERIALS AND METHODS

Fresh leaves of *Agel marmelos,Ocimum sanctum*,and *Azadirachta indica*were collected fromthe campus of the Odisha University of Agriculture and Technology (O.U.A.T.), Bhubaneswar. Analytical grade copper nitrate, ammonium hydroxide, and copper chloride were purchased from HIMEDIA laboratories Mumbai, India, and the deionized (DI) water from the Central Instrumentation Facility (CIF), O.U.A.T. was used for this experiment.

2.1 Collection, Isolation, Purification, and Identification of the Causal Organism

2.1.1 Collection of the Diseased Samples

The diseased leaves of tomatoes showing typical early blight symptoms were collected in zipper polythene bags from the infected tomato field of the All India Coordinated Research Project (A.I.C.R.P.) on vegetable science, O.U.A.T. during *rabi* (winter) 2020-21 and were brought into the laboratory for isolation of the pathogenfor further studies.

2.1.2 Isolation and Purification

Potato Dextrose Agar (PDA) medium was used for isolating, culturing, and sub-culturing the microorganisms. It was prepared in the laboratory using potato (200 g), dextrose (20 g), and agar-agar (20 g) in 1,000 mL DI water, and the pH of the media was adjusted to 6 to 6.5[14]. Commercially available potato fromthe local market was used whereas dextrose and agar-agar were used from HIMEDIA Pvt. Ltd (Mumbai, India). The isolation of fungus was done on the PDA mediumunder aseptic conditions from the leaves of tomato (cv. BT-2) having spotted appearance and incubated at room conditions (28 ± 1^0C). The purification of culture (fungus) was done by the single spore technique[15,16].The pure culture was maintained on PDA slants at 28 ±1 ^{0}C for further investigations.

2.1.3 Identification of the Fungus

Identification of the fungus was done fromcultural and morphological characteristicsof the fungus underthe PDA medium. The microscopic examination of the mycelia and conidia of the pathogen in the culture medium indicated the development of light grey colored mycelia which then changed to dark brown and black showing a white margin after 7 days of incubation at 28 ± 1 ^{0}C. The whole upper surface of the colony turned black when the medium was completely covered by the fungus on the 15thday of incubation (Figure1). Morphological examination of the fungusunder a trinocular microscope revealed large dark-coloredmuri form conidia (having both horizontal and vertical septa) (Figure1 and 2). Some of the conidia appeared in form of chains (Figure3). Studies on morphological characteristics of different isolates of *Alternaria solani* have shown variation with respect to mycelial width, conidial size, and beak length, and septation. The mycelial length of conidia varied from 13.5 to 60.9 µm with an average length of 35.3 µm and the mycelial width varied from 7.7 to 22.6 µm with an average width of 12.8 µm. The number of horizontal septa varied from 3 to 10 and the number of vertical septa varied from 0 to 3.The fungus was identified as *Alternaria solani*according to available literature[17, 18, 19].

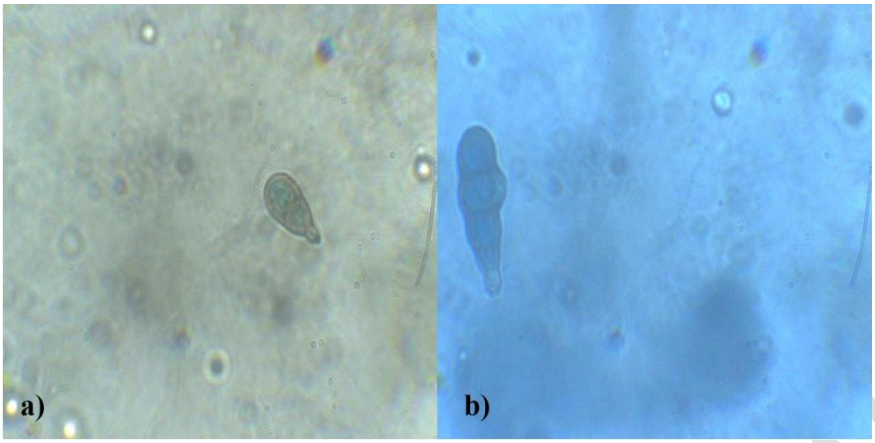

Figure 1.Microphotographs of a) mycelium mat and b) conidia of *Alternaria solani* at 40X

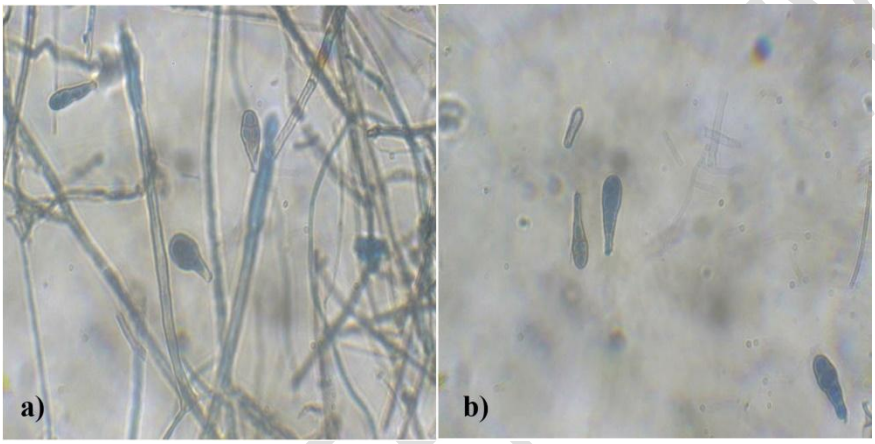

Figure 2. Microphotographs of conidia of *Alternaria solani* at 100X

Figure 3.Microphotographs of conidia in chains of *Alernariasolani* at 40X

The pathogenicity of *Alternaria solani*was tested by its artificial inoculation by following standard procedure (Koch's postulates). The test was conducted by inoculation of the pathogen into the leaves of healthy tomato plants grown in five earthen pots (30 cm diameter) that were filled in with sterilized soil and FYM in the ratio of 3:1. The tomato seedlings (cv. BT 2) in earthen pots were covered with a plastic sheet to avoid any air-borne infection inside the glasshouse. The surface of the leaves was sterilized with 0.1% mercuric chloride solution and then washed thoroughly with sterile distilled water. Thirty days old plants were used for inoculation purposes. Spore suspension @ 2×10^4 spores mL^{-1} was spray inoculated in the evening hours with the help of an atomizer. The plants in the control pot were sprayed only with sterilized distilled water. The pots along with the tomato plants were covered with polyethylene bags for 48 hours to induce high humid environment. Intermittent observations on

the disease development were recorded to monitor the spread of the disease from the stage of its initiation. Typical symptoms of early blight disease appeared on leaves 15 days after inoculation. The symptoms produced after inoculation were conclusively similar with the symptoms appeared on the diseased specimen. The pathogen was again isolated from the diseased leaves and was compared with the initial culture of the pathogen for similar morphological characters.

Initially, the symptoms of this disease comprised minute yellow dot-like circular bright yellow-colored spots on leaves. After that, spots enlarged and transformed into brown to dark brown necrotic spots having a bright yellow halo and less prominent concentric rings. Spots further intermingled and surrounded a larger area of the leaf that indicated *Alternaria solani* as the causal organism for the early blight of tomatoes by applying Koch's postulates[20].

The pure culture was then maintained on PDA slants and was kept at 28 ± 1 °C for further investigation. Subsequent, subculturing of isolates was done once in 15 days on PDA slants and was preserved in a refrigerator at 4 ^{0}C for further experimental use.

2.2 Synthesis of Cu-NPs

2.2.1 Green synthesis

The Cu-NPs were synthesized in the laboratory from *bael* (*Agel marmelos*L.) *tulsi* (*Ocimum sanctum* L.) and*neem* (*Azadirachta indica* L.) leaves independently following green chemistry method. The synthesis was a three-stage process in which 50 g of fresh leaves of each plant were collected separately from the campus and washed thoroughly with the deionized water. The leaves were then dried under shade at room temperature for 2 hours to remove excess water. Any further excess water clinging to the leaf surface was then removed gently by using blotting paper. The air-dried fresh leaves were then macerated separately with deionized water by using a mortar and pestle. The macerated leaf substrates of each plant were then transferred into a 500 mL glass beaker into which 400 mL of DI water was added. The mixture was then kept on a hot plate at 80 °C for about 1 hour. The contents were then filtered into another 500 mL beaker by using Whatman No.1 filter paper.

In the other two beakers, solutions of 0.2 M $Cu(NO_3)_2.3H_2O$ and 0.2 N NH_4OH were prepared separately by using DI water. The volume of 0.2 M $Cu(NO_3)_2.3H_2O$ solution used for capping and reducing the leaf extract was four times (1,200 mL) of the leaf extract (300 mL). The beaker containing the filtered leafextract was kept on a hot plate with a magnetic stirrer at 60 °C and at 1,000 rpm and into which, 0.2 M $Cu(NO_3)_2.3H_2O$ solution was added dropwise from a burette. From another burette, 0.2 N NH_4OH solution was added to the beaker containing leaf extract to make the media alkaline and to facilitate NP synthesis. The change in color of the leaf extract to pine green in *Agel marmelos*, chocolate brown in *Ocimum sanctum*, and coffee brown in *neem* indicated the formation of Cu-NPs. The suspended media were then centrifuged at 10,000 rpm for 20 minutes. The supernatant was discarded and the substrate at the bottom was collected which was then washed and centrifuged thrice for purification. The purified materials mixed with DI sterile water were stored separately at 4 °C in a refrigerator for characterization and *in-vitro* application.

2.2.2 Chemical Synthesis

The Cu-NPs were also synthesized chemically in the laboratory by following the two procedures viz. aqueous solution reduction method [21] and the precipitation method [22]. In the aqueous solution reduction method, (0.2 M of $Cu(NO_3)_2.3H_2O$ solution was prepared in a beaker in 200 mL of DI water. In another beaker, Ascorbic acid solution of 0.2 M was prepared in 100 mL DI water and was filled into a burette. The $Cu(NO_3)_2.3H_2O$ solution was kept on a hot plate with a magnetic stirrer at 60 °C and 800 rpm; into which 0.2 M Ascorbic acid solution was added drop-wise. The color of the medium changed from blue to green and then to peanut brown. Then the mixture was heated in a microwave oven at 100 °C till black color appeared which indicated the formation of Cu-NPs. The suspended medium was then centrifuged at 10,000 rpm for 20 minutes and the supernatant was discarded. The substrate at the bottom was then collected and centrifuged thrice with DI sterile water for purification. The collected material thus obtained finally was then stored at 4 °C in a refrigerator by mixing with DI sterile water.

In the precipitation method, 250 mL of 0.2 M $Cu(NO_3)_2.3H_2O$ solution was prepared in a beaker by using DI sterile water and 250 mL of 0.2 M $CuCl_2$ solution was prepared in another beaker. Subsequently, both the solutions were mixed on a hot plate with a magnetic stirrer at 60 °C and 600-800 rpm. Into the beaker, 0.4 N NH_4OH solution was added dropwise to maintain the alkalinity of the

medium (pH >10). The endpoint was noticed with the color change from blue to turbid light blue and on further heating at 60 °C, the color changed to dark brown that indicated the formation of Cu-NPs. The suspended media was centrifuged and stored in a refrigerator like the above aqueous solution reduction method. The synthesized CuNPs were characterized by a Dynamic Light Scattering (DLS) analyzer (Zetasizer PSA 1190).

2.3 Evaluation of Cu-NPs

In the first set of experiments, the Cu-NPs so obtained through green and chemical synthesis were tested at 300 and 500 ppm for evaluating their efficacy *in vitro* against *Alternariasolani*along with fungicide treated check i.e.Carbendazim 50 WP @ 300 and 500 ppm using 'poisoned food technique' [23]. The investigation was implemented under CRD with three replications. The observation of the radial growth (mm) was recorded from 24 hours of the incubation at 28 ± 1 ^{0}C till the attainment of the complete growth of the test pathogen in the control plates. The percent growth inhibition (PGI) over control was estimated from the formulae given below by Vincent [24].

PGI = (DC-DT) x 100 / DC

Where,

DC = Mean diameter (mm) of the mycelial colony in control

DT = Mean diameter (mm) of the mycelial colony in the treated set

The overall concentration of synthesized Cu-NPs was calculated by taking 10 mL of a liquid suspension of Cu-NPs in an empty petri dish and by drying the liquid in a hot air oven to get its powder form.

Concentration of liquid suspension (g mL^{-1}) = $(W_3 – W_1) / (W_2 – W_1)$

Where,

W_1 = Weight of empty petri dish (g)

W_2 = Weight of petri dish after adding 10 mL of liquid suspension (g)

W_3 = Weight of petri dish after drying of liquid to powder form (g)

Then convert this g mL^{-1} of liquid suspension to mg L^{-1} which gives the concentration of entire Cu-NP suspension in ppm.

3. RESULTS AND DISCUSSION

The characterization of Cu-NPs by a zetasizer (PSA 1190) revealed the smallest size (246.5 nm) from *neem* leaves followed by *Agel marmelos* (427.4 nm) while *Ocimum sanctum*leaves produced the largest NPs (1,019 nm). In between two chemical methods, the precipitation method produced smaller Cu-NPs (867.8 nm) than the aqueous solution reduction method (1,537 nm) (Table 1 and Figure4a to 4e). The neem leaf derived Cu-NPs remained deflocculated for more the entire experimental period whereas both the chemically synthesized NPs settled down within an hour of sonication. The settling down of *Ocimum sanctum* and *Agel marmelos* leaf-derived Cu-NPs were in the next order.

Table 1. Average particle size of Cu-NPs in aqueous medium

Sl. No.	Cu-NPs synthesized from	Average particle size (nm)
1	*Neem* leaves	246.5
2	*Tulsi* leaves	1,019
3	*Bael* leaves	427.4
4	Aqueous solution reduction method	1,537
5	Precipitation method	867.8

We evaluated Cu-NPs against *Alternaria solani* to observe their inhibitory effects on mycelial growth (Table 2). Among the seven treatments (T_1 to T_7), Cu-NPs synthesized from *neem* leaves at 500 ppm proved to be the most effective with 75.9% growth inhibition of *A. solani*followed *Agel marmelos* leaves (73.49%). The mycelial growth with the Cu-NPs from *neem* leaves was also the lowest among all other treatments at 300 ppm (33 mm) and 500 ppm (20 mm) concentrations whereas the aqueous solution reduction method showed the lowest growth inhibition of 40.96% and 62.65% at 300 and 500 ppm, respectively (Figure5 and 6). The mycelial growth at 500 ppm in Cu-NPs from *neem* leaves was 41.18% milder than Carbendazim 50 WP and at 300 ppm, it was 34% milder. Whereas, *Agel marmelos* leaf-derived Cu-NPs had manifested 28% higher inhibition than Carbendazim 50 WP at 300 ppm and 35.29% at 500 ppm. *Ocimum sanctum*leaf-derived Cu-NPs at 300 and 500 ppm resulted in 14% and 11.76% higher mycelial growth inhibition over the corresponding application of Carbendazim 50 WP.

Table 2. Comparative *in vitro* inhibitory efficacy of Cu-NPs at different concentrations

against *Alternaria solani*

Treatment symbols	Treatment details	Mycelial growth (mm) at		Growth inhibition (%) at	
		300 ppm	**500 ppm**	**300 ppm**	**500 ppm**
T_1	Cu-NPs from *neem* leaves	33	20	60.24	75.9
T_2	Cu-NPs from *tulsi* leaves	43	30	48.19	63.85
T_3	Cu-NPs from *bael* leaves	36	22	56.62	73.49
T_4	Cu-NPs by aqueous solution reduction method	49	31	40.96	62.65
T_5	Cu-NPs by precipitation method	42	29	49.39	65.06
T_6	Carbendazim 50 WP	50	34	39.75	59.03
T_7	Control (no treatment)	83	83	-	-
S.Em (±)		0.69	0.655	-	-
C.D. (5%)		2.113	2.005	-	-

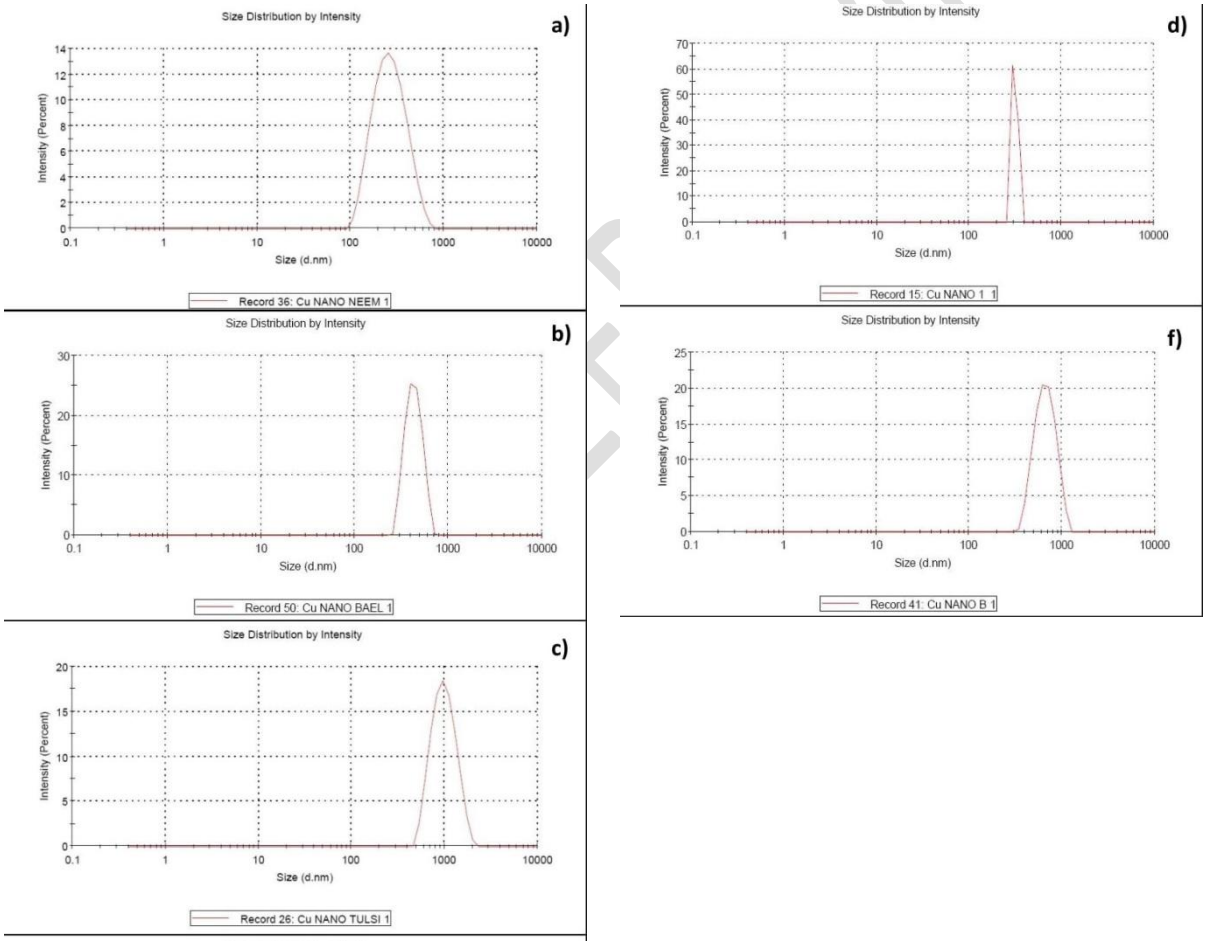

Figure 4.DLS particle size analysis of Cu-NP formulation prepared from a) *neem* leaf extract, b) *Agel marmelos leaf* extract, c) *Ocimum sanctum*leaf extract, d) aqueous solution reduction method, and e)precipitation method

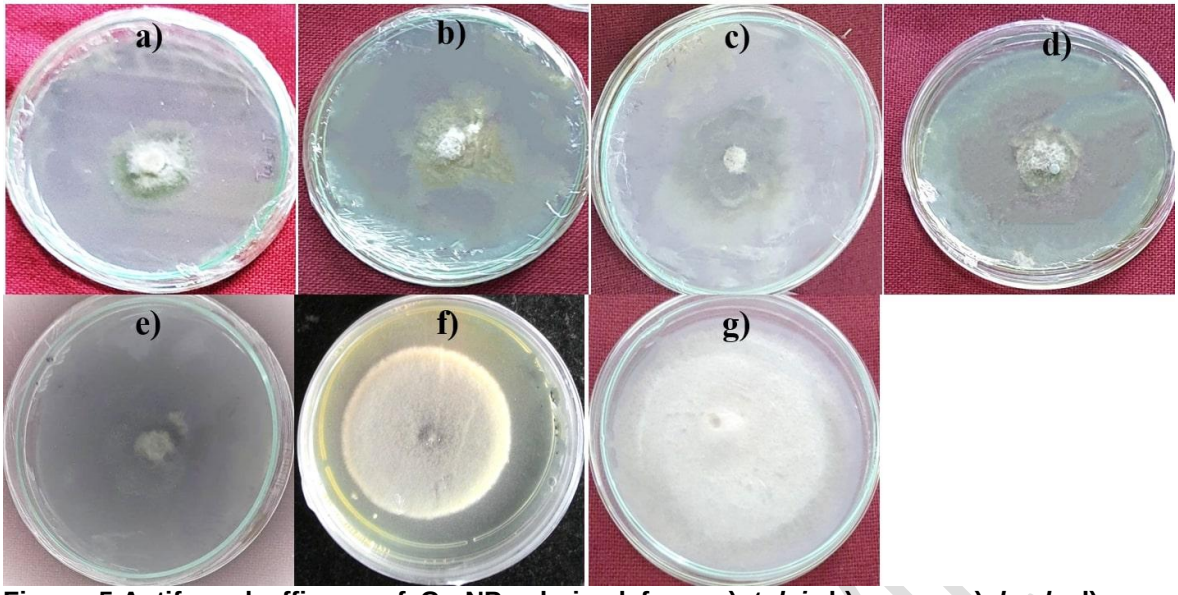

Figure 5.Antifungal efficacy of Cu-NPs derived from a) *tulsi*, b) *neem*, c) *bael*, d) aquous solution of reduction method, e) precipitation method, and f) Carbendazim 50 WP and g) control against *Alternaria solani* at 300 ppm on PDA

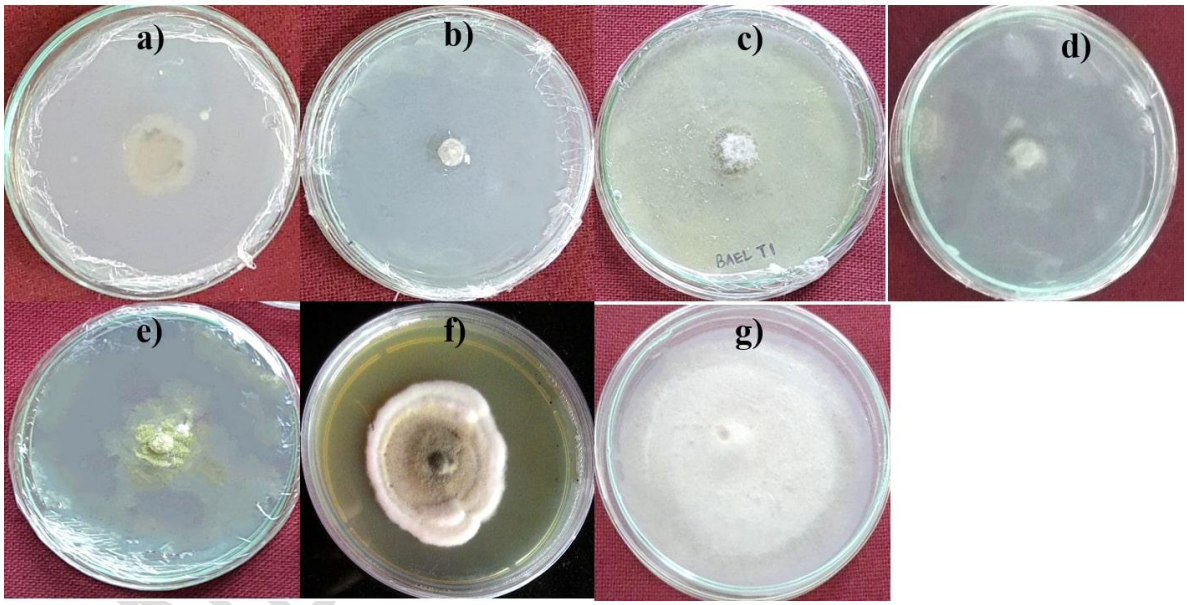

Figure 6.Antifungal efficacy of Cu-NPs derived from a) *Ocimum sanctum*, b) neem, c) *Agel marmelos*, d) aquous solution of reduction method, e) precipitation method, and f) Carbendazim 50 WP and g) control against *Alternaria solani* at 500 ppm on PDA

In between two chemical methods, the precipitation method showed relatively better fungal growth inhibition than the aqueous solution reduction method compared to the Carbendazim application at both concentration levels but the degree of inhibition was far below the corresponding levels of Cu-NPs from *neem* and *Agel marmelos*.

A strong relationship between the fungal mycelial growth and the size of NPs was noticed through Pearson's linear correlation studies that depicted significantly higher coefficients at 300 ppm ($r=0.996$) and 500 ppm ($r=0.93$) of Cu-NPs at a 5% level of significance ($P \leq 0.05$). The generalized linear model between the size (diameter) of Cu-NPs and fungal mycelial growth indicated a positive relation whereas the relationship of the former was negative with the growth inhibition.

The deteriorating cell wall altering the functioning of cell organelle ultimately kills the pathogens and controls a variety of fungal and bacterial infections by the use of Cu-NPs [25]. We and other researchers have found antifungal effects of Cu-NPs as their size plays an important role in such antimicrobial activities. For example, smaller NPs with a larger surface-to-volume ratio provides

greater interaction with the pathogen, and hence, a significant growth inhibition has been reported by [26] in *neem*. Greater concentration and smaller size of Cu-NPs exhibited the most harmful effects, and when compared to unexposed control cultures, Cu-NPs exerted substantial harmful impacts at all sizes as examined by [27]. In this experiment, we observed the lowest mycelial growth and the maximum inhibition of *Alternaria solani*from *neem* leaves at 500 ppm while the aqueous solution reduction method showed the lowest growth inhibition. Shende et al. [28] noticed potential antimicrobial activities by using Cu-NPs from *Ocimum sanctum* leaf extract but in this experiment we observed the efficacy of *Ocimum sanctum*-derived NPs was next to *neem* and *Agel marmelos*. The smaller size of neem leaf-derived Cu-NPs could be attributed to enhanced bioactivity of biological reducing agents such as flavones, organic acids, ketones, amides, aldehydes, etc. as reported by [29]. In a similar note, Ismail *et al*. [30] have also reported the effectiveness of silver NPs against the pathogenic fungus *Alternaria solani* under *in vitro* conditions. The detrimental effects of silver NPs on conidial germination have been reported by Lamsa*et al*. [31]. Both copper and silver NPs damage the hyphae of *Alternaria alternata*[31]. Due to the smallest size, the *neem*-derived NPs remain suspended over a longer period compared to other Cu-NPs as a result of which the farmers can spray the nanoformulations uniformly without any need for frequent deflocculation or sonication.

4. CONCLUSION

The present results revealed that smaller Cu-NPs exhibited higher antifungal efficacy compared to larger ones at both 300 and 500 ppm concentrations that indicating higher penetrability of the smaller Cu-NPs into the fungal cells inhibiting growth and cell wall synthesis of the *Alternaria solani*. Whereas Carbendazim 50 WP, which was used as a check, exhibited much lower inhibition of mycelial growth compared to the Cu-NPs at the corresponding concentrations. Raghupathi*et al*. [32] and Naqvi *et al*. [33] have reported size-dependent zinc oxide NP-induced bacterial growth inhibition in *Staphylococcus aureus*. Higher bacterial (*Azotobatervinelandii*) and fungal (*Fusarium oxysporum*) growth inhibitions were achieved with the smaller size of silver NPs and at their higher concentrations[34, 35]. The results from the present investigation also corroborated the findings of earlier researchers who have advocated for a strong positive relationship between the particle size of the metallic nanoformulations and growth inhibitory effects in different fungi and bacteria. We also believe that Cu-NPs irrespective of their methods of synthesis, either green or chemical, have significant growth inhibitory effects on the *in vitro* fungal mycelia growth of *Alternaria solani* causing early blight of tomatoes whereas their efficacy is size and dose dependent. As green synthesis of Cu-NPs from *neem* leaves resulted in their smallest size which ultimately had the maximum mycelial growth inhibitory effect and the nanoformulation remained suspended for longer period, hence further efforts for developing an environment-friendly neem leaf-derived nano-fungicide could safeguard against hazardous synthetic fungicides.

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