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Synthesis and evaluation of antimicrobial potential of copper nanoparticle against agriculturally important Phytopathogens

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Abstract
Biosynthesis of stable copper nanoparticles was achieved using *Ocimum sanctum* leaf extract. These biosynthesized copper nanoparticles were characterized by using UV-Vis spectroscopy showed absorption of copper nanoparticles at 345nm. FTIR spectrum of copper nanoparticles suggests that copper nanoparticles are surrounded by different organic molecules such as terpenoids, alcohols, ketones, esters, aldehydes and carboxylic acid. TEM analysis showed that the copper nanoparticles were rod, cylindrical and elliptical. The average particle size of copper nanoparticles was 25 nm with total concentration 300.29 particles/frame, 13.53 particles/ml and -14.9 mV zeta potential value.
The maximum antibacterial activity of copper nanoparticles was found against *Xanthomonas axonopodis* pv. *punicae* (17.25±0.95) while *Rhizoctonia solani* (10±0.81) showed minimum activity with 0.01 mg/ml and 0.03mg/ml MIC respectively. The maximum antifungal activity of copper nanoparticles was found against *Alternaria carthami* (18.5±1.7) while the minimum activity was found against *Rhizopus stolonifer* (10.5±0.5). The MIC of the copper nanoparticles against fungus was found to be 0.06 mg/ml for *Alternaria carthami, Aspergillus niger, Fusarium oxysporum* f.sp *udum* and 0.03 mg/ml for *Colletotrichum gloeosporioides, Colletotrichum lindemuthianum,* and *Drechslera sorghicola, Fusarium oxysporum* f.sp. *carthami, Fusarium oxysporum* f.sp. *ciceri, Macrophomina phaseolina, Rhizoctonia bataticola* and *Rhizopus stolonifer.*
Thus, copper nanoparticles can be exploited in the field of agriculture in formulation of various biopesticides, insecticides and ecologically feasible effective management strategy against harmful phytopathogens.

Keywords: Cu nanoparticle, Tulsi, Phytopathogen, UV-Vis, FTIR, TEM, Particle analyzer, Zeta sizer, Antimicrobial activity

Introduction
Development of green nanotechnology is generating interest of researchers toward ecofriendly biosynthesis of nanoparticles. Among the recent advancement in agricultural sciences, metallic nanomaterials play a significant role in crop protection because of its unique physical and chemical properties, huge surface to volume ratio, structural stability and strong affinity to their target (Kumar *et al.*, 2010). Nanoparticles remain bound to the cell wall of pathogen and cuases deformity de to high energy transfer leading to its death. Nanotechnological application in plant pathology targets specific agricultural problems in plant pathogen interactions and provide new perceptions for crop protection. Nanocomposites fulfil the two most important criteria in disease management: efficacy with minimal ecological impact and less toxicity on humans. Copper based fungicides produce highly reactive hydroxyl radicles which can damage lipids, proteins, DNA and other Biomolecules. It plays an important role in disease prevention and treatment of large variety of plants (Borkow and Gabbay, 2005). With the growing demand of pesticide worldwide to control the pathogens and pests, there is an urgent need to tackle the excessive usage of pesticeide and fertilizers by finding alternatives. The potential applications of nanomaterials in crop protection helps in the development of efficient and potential approaches for the management of plant pathogens.

Materials and Methods
Sample collection
The *Ocimum sanctum* leaves were collected from Vilasrao Deshmukh College of Agricultural Biotechnology, Latur (M.S.) campus. Clove and orange were purchased from local market. Copper sulphate was purchased from Hi-media laboratories Mumbai, India.

Preparation of *Ocimum sanctum* leaf Extract
The fresh leaves of tulsi were washed several times with distilled water to remove dust. When the leaves got completely dried, they were chopped into fine pieces.5gm chopped leaves of tulsi were boiled with 50 ml distilled water at 60^{o}C for 1 hour. The extract obtained was filtered through Whatman No.1 filter paper and finally brown extract was collected for further experiment.

Synthesis of Copper Nanoparticles
For the synthesis of copper nanoparticles, 50 ml of tulsi leaf/orange peel/clove extract was mixed with 50 ml aqueous solution of 1mM copper sulphate (1:1 ratio of plant extract and copper solution) and stirred continuously for 2 min at 30^{o}C. Reduction takes place rapidly which is indicated by the change in colour of the solution. The mixture was incubated at room temperature overnight. Mixture was centrifuged at 3500 rpm for 10min to get copper nanoparticles. The nanoparticles were washed and dried at room temperature.

Antimicrobial activity of copper nanoparticles

The antimicrobial activity of the nanoparticles was tested against a three bacterial pathogens *viz*., *Rhizoctonia solani*, *Xanthomonas axonopodis* pv. *Citri*, *Xanthomonas axonopodis* pv. *Punicae*, and eleven fungal pathogens *viz*., *Alternaria carthami*, *Aspergillus niger*, *Colletotrichum gloeosporioides*, *Colletotrichum lindemuthianum*, *Drechslera sorghicola*, *Fusarium oxysporum* f.sp. *carthami*, *Fusarium oxysporum* f.sp. *ciceri*, *Fusarium oxysporum* f.sp *udum*, *Macrophomina phaseolina*, *Rhizoctonia bataticola* and *Rhizopus stolonifer*.

$1x10^4$ cfu/ml bacterial culture and $1x10^5$ spore/ml fungal spore suspension were spread on nutrient and potato dextrose agar media respectively. The disc were soaked in copper nanoparticle solution and placed on media. Similarly, copper sulphate and plant extract solutions were soaked on disc to measure the control activity. Zone of inhibition was observed after 24 hours in bacterial and 3-4 days in fungal cultures.

Results and Discussion

Synthesis of copper nanoparticles

Green synthesis of copper nanoparticles was achieved in aqueous solution using plant extract as reducing agent. When plant extract was mixed with copper sulphate solution, the colour of aqueous solution was changed immediately within 10 min, which turns dark brown within 24 hours (Fig 1) indicated the formation of copper nanoparticles.

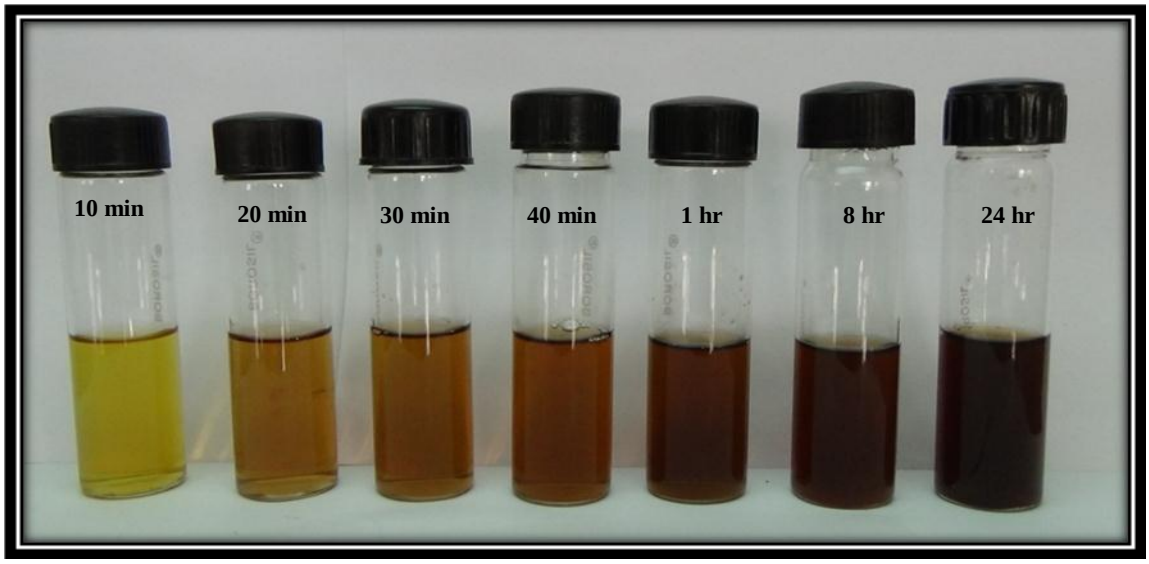


Fig 1: Change in coloration of plant extract at different time intervals.

Characterization of copper nanoparticles

The synthesized copper nanoparticles were characterized by using UV-Vis spectroscopy, FTIR, TEM, Particle size analyzer (LM-20) and Malvern zeta sizer.

UV-Vis spectrophotometer

The synthesized copper nanoparticles were characterized by using UV-Vis spectrophotometer and showed maximum absorbance (2.15) at 345nm by using tulsi leaf extract as reducing agent (fig.1)

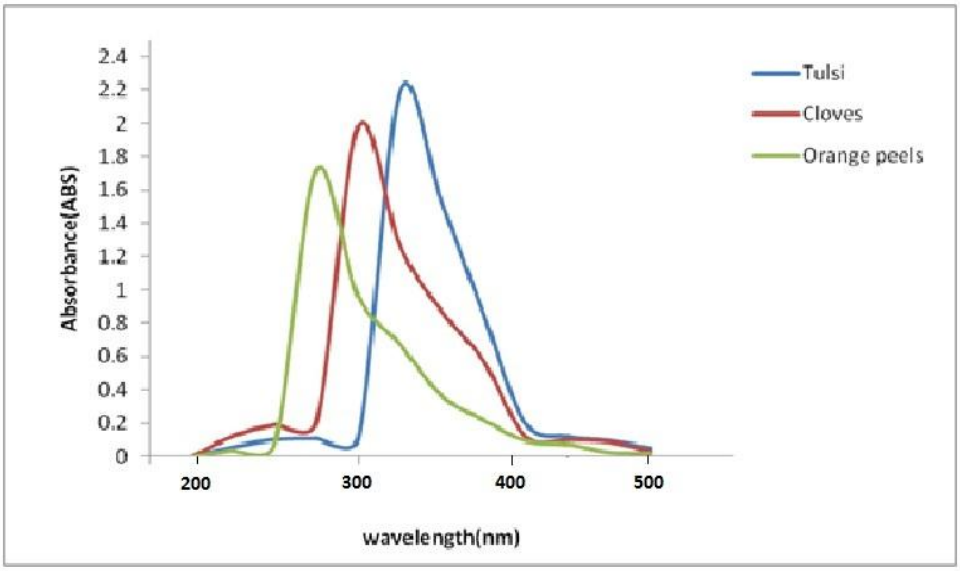


Fig 2: UV-Visible absorption spectra of biosynthesized copper nanoparticles**.**

Fourier Transform Infra-Red Spectroscopy (FTIR) analysis

The FTIR spectra of copper nanoparticles are shown in figure-2. The following peak were observed in spectrum and the band at 3407·06 cm^{-1} is assigned to the O-H stretching of H-bonded alcohol and phenols. The band at 2926·89 cm^{-1} attributed to O-H stretching of carboxylic acids. The band at 1628·67 cm^{-1} corresponds to the NO_2 stretching of Nitro compound and presence of amide I and II, which arises due to the carbonyl stretch and -N-H- stretch vibration in the amide linkages of the proteins. The band at 1423.14 cm^{-1} is related to the C-C stretching of aromatic ring structure, scissoring and bending of alkanes. The band at 1081.91 cm^{-1} is related to the C-O stretching of alcohol, ether, esters, carboxylic acid. The peak at 601.86, 437.73, 437.91 cm^{-1} indicated fingerprint region is complicated by the large number of different vibrations that occur here. FTIR spectrum of copper nanoparticles suggested that copper nanoparticles were surrounded by different organic molecules such as terpenoids, alcohols, ketones, aldehydes and carboxylic acid.

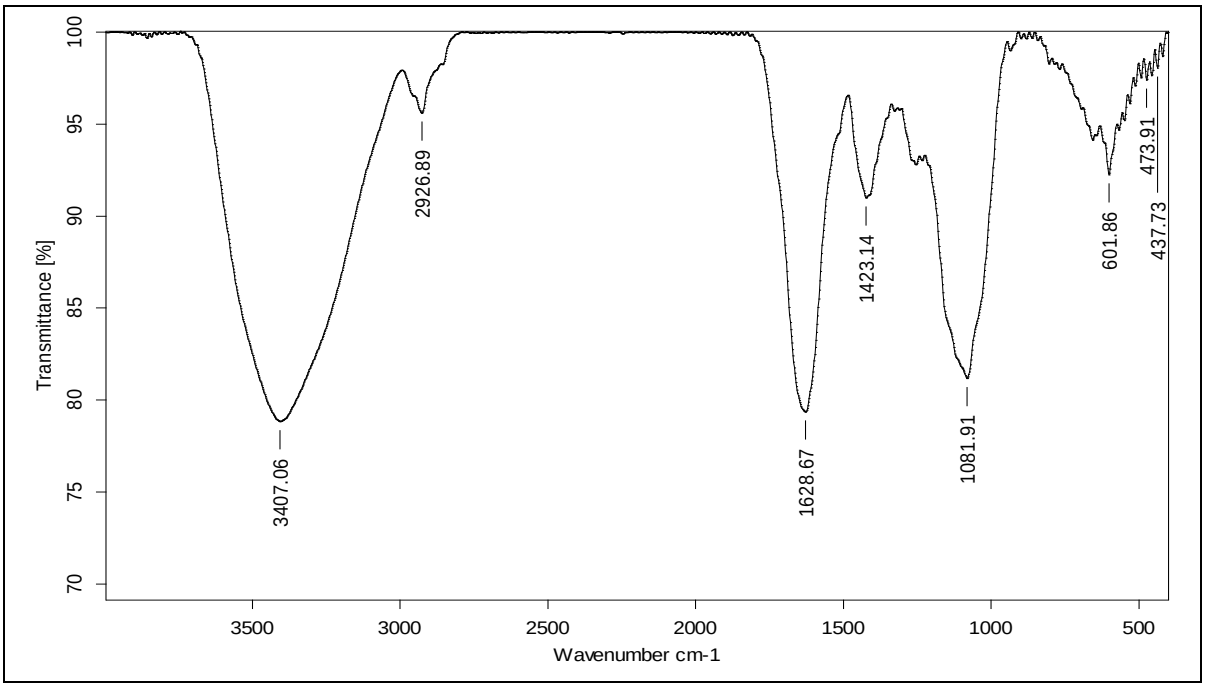
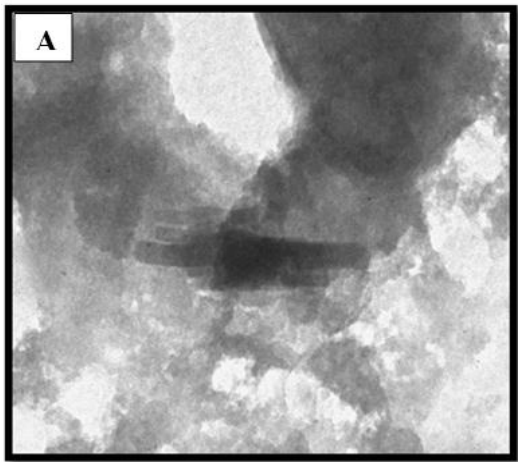
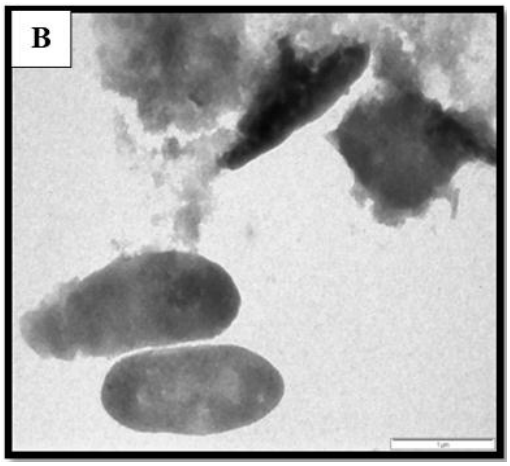


Fig 3: FTIR spectra of biosynthesized copper nanoparticles by *Ocimum sanctum*.

Transmission Electron Microscopy (TEM) analysis

The size and shape of green synthesized copper nanoparticles were analyzed using TEM particles were rod, cylindrical and elliptical with particle size of 25nm.Particles were well dispersed which was confirmed by TEM studies. Selected area electron diffraction (SAED) patterns of the copper nanoparticles synthesized using tulsi leaf extract were investigated using HRTEM. The corresponding SAED of copper nanoparticle patterns sample showed spotty ring patterns without any additional diffraction spots shown in (fig 4).





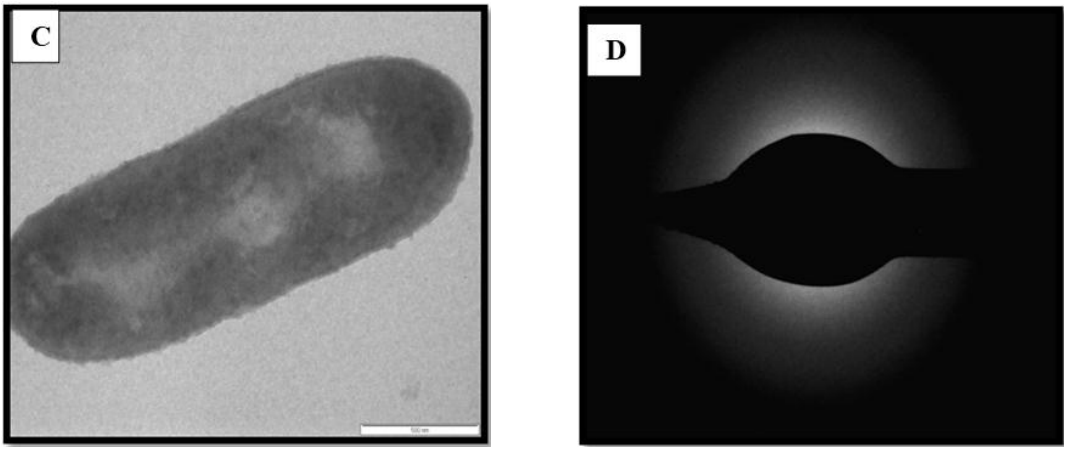


Fig 4: TEM image of Biosynthesized copper nanoparticle (A), higher magnified TEM image of biosynthesized copper nanoparticles (B, C), with selected area electron diffraction (D).

Particle size analyzer (LM-20)

The nanoparticles tracking analyzer measurements showed average particle size of copper nanoparticles was 25 nm with total concentration 300.29 particles/ frame and 13.53 particles/ ml (fig.4).

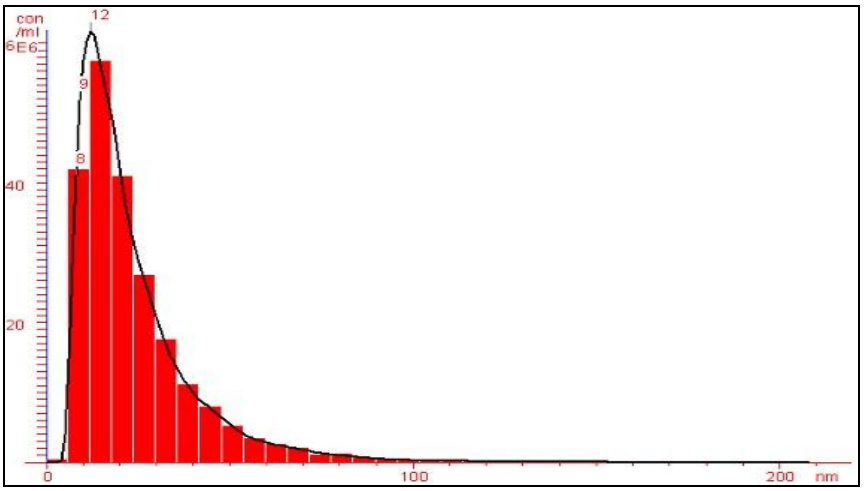


Fig 5: Particle size spectra of biosynthesized copper nanoparticles.

Malvern zeta sizer

The zeta potential is related to the surface charge density and high magnitudes of zeta potential denote stability of the nanoparticles in suspension. The biosynthesized copper nanoparticles had -14.9 mV zeta potential value (Fig. no. 5).

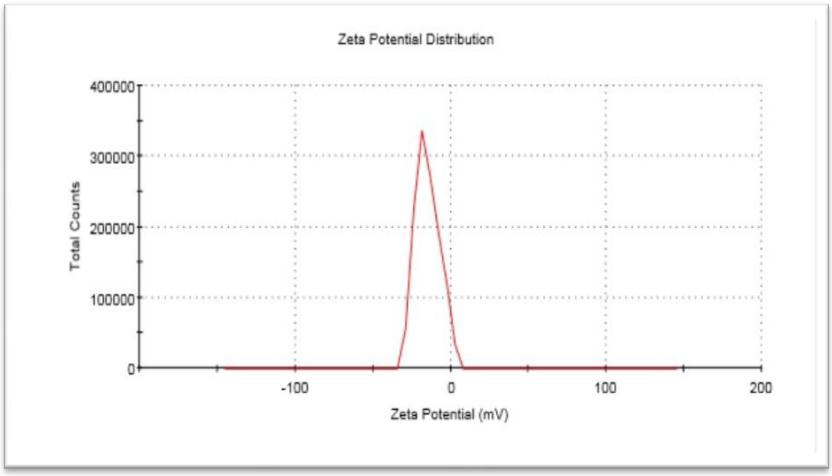
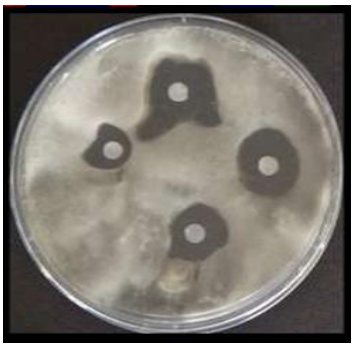


Fig 6: Zeta potential of copper nanoparticles by zeta size analyzer.

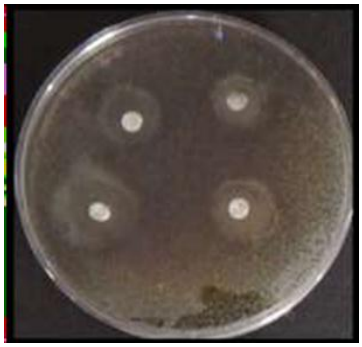
Antimicrobial activity of copper nanoparticle

The antimicrobial effect of biologically synthesized copper nanoparticles was analyzed on the basis of the zone of inhibition. Copper nanoparticles exhibited strong antimicrobial activity against plant bacterial pathogen such as *Rhizoctonia solani*, *Xanthomonas axonopodis* pv. *citri*, *Xanthomonas axonopodis* pv. *punicae* and plant fungal pathogens, such as *Alternaria carthami*, *Aspergillus niger*, *Colletotrichum gloeosporioides*, *Colletotrichum lindemuthianum*, *Drechslera sorghicola*, *Fusarium oxysporum* f.sp. *carthami*, *Fusarium oxysporum* f.sp. *ciceri*, *Fusarium oxysporum* f.sp. *udum*, *Macrophomina phaseolina*, *Rhizoctonia bataticola*, *Rhizopus stolonifer* (Fig 7). The maximum activity of copper nanoparticles was found against bacterial pathogen *Xanthomonas axonopodis* pv. *punicae* (17.25±0.95) while the minimum activity was found against *Rhizoctonia solani* (10±0.81). The MIC of the copper nanoparticles against bacteria was found to be 0.01 mg/ml for *Rhizoctonia solani and* 0.03mg/ml for *Xanthomonas axonopodis* pv. *punicae* and *Xanthomonas axonopodis* pv. *citri.*

The maximum activity of copper nanoparticles was found against fungal pathogen *Alternaria carthami* (18.5±1.7) while the minimum activity was found against *Rhizopus stolonifer* (10.5±0.5). The MIC of the copper nanoparticles against fungus *Alternaria carthami*, *Aspergillus niger*, *Fusarium oxysporum* f.sp *udum* was found to be 0.06 mg/ml and 0.03 mg/ml for *Colletotrichum gloeosporioides*, *Colletotrichum lindemuthianum*, and *Drechslera sorghicola*, *Fusarium oxysporum* f.sp. *carthami*, *Fusarium oxysporum* f.sp. *ciceri*, *Macrophomina phaseolina*, *Rhizoctonia bataticola and Rhizopus stolonifer.* Control activity of copper sulphate and tulsi plant extract was also tested. Tulsi plant extract did not shown any antimicrobial activity whereas copper sulphate shown relatively less activity as compared to copper nanoparticles.

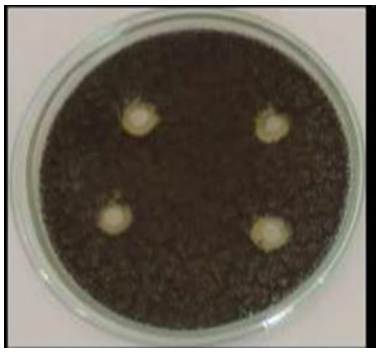


Alternaria carthami



Aspergillus niger

Colletotrichum gloeosporioides



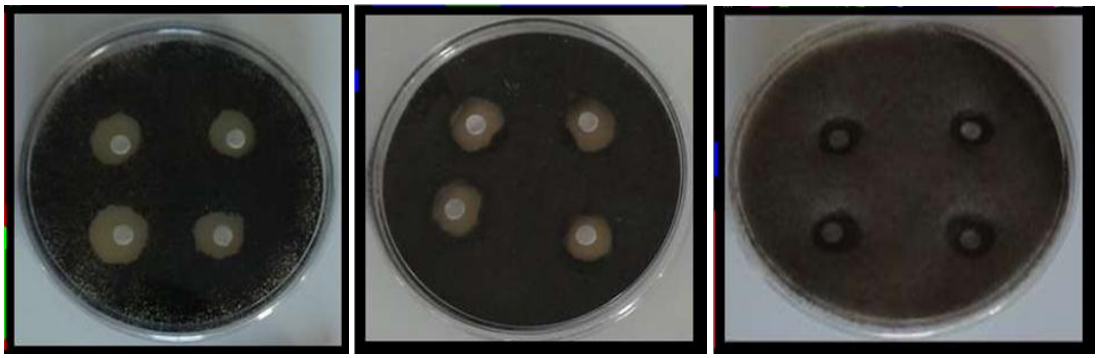
Colletotrichum lindemuthianum

Drechslera sorghicola

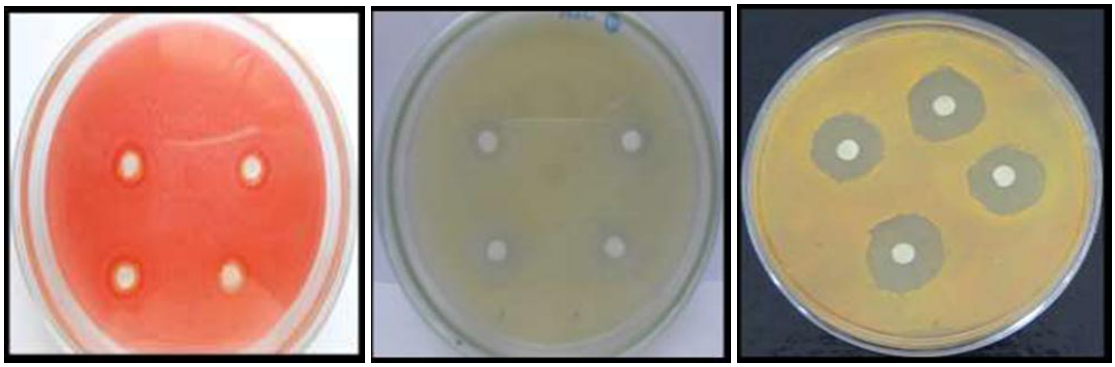
Fusarium oxysporum f.sp. carthami

Fusarium oxysporum f.sp. *cicero*

Fusarium oxysporum f.sp. udum



Macrophomina phaseolina *Rhizoctonia bataticola* *Rhizopus stolonifer*



Rhizoctonia solani *Xanthomonas axonopodis pv. Citri* *Xanthomonas axonopodis pv. Punicae*

Fig 7: Antimicrobial activity of copper nanoparticle against plant pathogenic bacteria and fungi.

Table 1: Antimicrobial activity of copper nanoparticles.

S. No.	Name of the Microorganism	Diameter of zone of inhibition (mm)	MIC (mg/ml)
Bacteria			
1.	*Rhizoctonia solani*	10±0.81	0.01
2.	*Xanthomonas axonopodis* pv. *Citri*	13.5±1.29	0.03
3.	*Xanthomonas axonopodis* pv. *Punicae*	17.25±0.95	0.03
Fungi			
1.	*Alternaria carthami*	18.5±1.7	0.06
2.	*Aspergillus niger*	12.75±1.7	0.06
3.	*Colletotrichum gloeosporioides*	11.5±1.0	0.03
4.	*Colletotrichum lindemuthianum*	15.25±0.5	0.03
5.	*Drechslera sorghicola*	12±0.8	0.03
6.	*Fusarium oxysporum* f.sp. *carthami*	14.75±1.25	0.06
7.	*Fusarium oxysporum* f.sp.*ciceri*	13.5±1.25	0.03
8.	*Fusarium oxysporum* f.sp. *udum*	18±0.8	0.06
9.	*Macrophomina phaseolina*	12.5±0.5	0.03
10.	*Rhizoctonia bataticola*	10.5±0.5	0.03
11.	*Rhizopus stolonifer*	11.75±1.5	0.03

Conclusions

In the present study, the successful synthesis of copper nanoparticles was achieved by tulsi leaf extract. The potential antimicrobial activity of copper nanoparticles can be exploited in field of agriculture to check the activity of different phytopathogens. The synthesized copper nanoparticles in this study will presumably useful in formulation of various biopesticides, insecticides and ecologically feasible effective management strategy against harmful pathogenic microorganisms.

Acknowledgement

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