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Microwave oriented sonochemical method for synthesis of copper sulfide nanoparticles as antifungal agents

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Abstract
Copper sulphide nanoparticles in aqua-dispersed form were prepared by reaction of copper ions with different sulfide sources by sonochemical irradiation method. The products were characterized by Transmission Electron Microscopy and UV-Visible spectroscopy. The antifungal potential of nanoparticles was screened against *Drechslera oryzae, Alternaria alternata, Ustilago tritici* and *Ustilagonoidea virens.*

Keywords: Nanoparticles, copper sulfide, antifungal and fungi

Introduction
Among the class of nanomaterials, nanoparticles is rapidly growing field in the nanotechnology era [1]. These nano-sized materials have interesting properties that change with change in their size to produce better, longer lasting, cleaner, safer and smarter products for the home, agriculture, medicine, industry, communications and transportation [2]. The variety of bioactive materials feel limitation in their bio-applications owing to their low/zero solvent dispersibility or solubility. Nano transformations of materials into formulation form provide solution to this problem. These aqua-dispersed nanoparticles inflicted diverse physiochemical and topological properties to the materials, thus results in their augmented biological potentials.

Copper salts are known form centuries for their antifungal action. There are various commercial copper fungicides are in use which includes Bordeaux mixture, cupric chloride, copper oxychloride, cuprous chloride, copper soap and copper naphthenate [3]. Therefore, direct use of copper as copper salts causes copper toxicity due to high solubility and tissue bioavailability of Cu^{2+} ions [4]. These ions get ultimately detoxified in nature by sulfidation [5]. Also, bound form copper, is bio-available low toxic form, which still carries the inherent antifungal properties of copper, intact. Copper sulfide (CuS) can be considered equivalent to bound form of copper which is thermally stable, non-toxic [5, 6, 7, 8, 9] and insoluble in most of the solvents.

Prompted by the bioactivites of copper, stability and non-toxicity of copper sulfide (CuS) and power of nanotechnology, we planned to transform copper sulfide (CuS) into aqua dispersed form for evaluation of their antifungal potential against various phytopathogenic fungi.

Material and Methods
Instrumentation Used
Absorption spectra of nanoparticles were recorded in aqueous solution, using UV-Vis1800 Shimadzu Double-beam Spectrophotometer. The measurements were carried out using quartz cell in the wavelength range of 350-800nm. The morphology and size of nanoparticles were recorded in Hitachi Transmission Electron Microscope Hi-7650 at an accelerated voltage of 200kV by castin..g a drop of particle solution onto a 200-mesh carbon coated copper grid from EMN laboratory, Punjab Agricultural University, Ludhiana.

Synthesis of copper sulfide nanoparticles (CuS NPs) – 1 mmol of copper nitrate trihydrate ($Cu(NO_3)_2.3H_2O$) was dissolved in ethylene glycol. 1mmol of different sulfide sources *viz.* thiourea $SC(NH_2)_2$, sodium sulfide Na_2S, thioacteamide CH_3CSNH_2, sodium thiosulfate pentahydrate $Na_2S_2O_3$. $5H_2O$and sodium diethyl-dithiocarbamate (DTC) $C_5H_{10}NNaS_2.3H_2O$ in

distilled water were prepared. During sonication, 30 ml of copper solution was added dropwise to 30 ml of different sulfide source solution along with a pinch of cetyltrimethylammonium bromide (CTAB) as a surfactant, with the observation of appearance of light green or brown colour (in case of DTC). The solution was irradiated with microwave radiations for 30 seconds during which it turned to dark green. The solution was allowed to cool down at the room temperature. 4-Aminobutyric acid (GABA), 0.3g dissolved in 20 ml distilled water was added to above prepared solution during sonication and the process was continued for another 15 minutes to get stabilized copper sulfide nanoparticles.

Stock Solution: The prepared copper sulfide nanoparticles (CuS-NPs) of 35.85 µg/ml was obtained, were kept as stock solution, which was further diluted by adding distilled water. Series of dilutions were done to 30, 20 and 10 µg/ml respectively.

Antifungal Evaluation of Aqua-dispersed CuS-NPs: All the test compounds were screened against four phytopathogenic fungi, *viz. Drechslera oryzae* (Rice), *Alternaria alternata* (Wheat), *Ustilago tritici* (Wheat) and *Ustilagnoidea virens* (Rice) by applying spore germination inhibition technique [10].

Drechslera oryzae and *Alternaria alternata* were cultured on potato dextrose agar (PDA). The isolates of phytopathogenic fungi were provided from experimental area of the plant pathology department of the PAU (Punjab Agricultural University 2016) and standard Tilt and Vitavax which served as the positive control were obtained from their respective manufacturers. The spores of *Ustilago tritici* and *Ustilagonoidea virens* was obtained from loose smut infected samples obtained from susceptible varities of wheat and rice respectively experimental area of PAU (Punjab Agricultural University 2016). Spores suspension of the test fungus were prepared by adding autoclaved distilled water at a concentration of 1 x 10^9 spores/ml and were used for bio-efficacy testing of copper sulfide nanoparticles in dispersed form *in vitro*.

Spore suspension was made by adding autoclaved distilled water to bit of respective fungi. Suspension was filtered through three layers of sterilized cheese cloth in order to remove mycelial particles under aseptic conditions. Haemocytometer was used to form standardized spore suspension (1 x 10^9 spores/ml). Small droplets (0.02 ml) of test solution and spore suspension in equal amount were seeded in the cavity of the cavity slides. These slides were placed in Petri plates lined with moist filter paper and were incubated for 24 hrs at 25±1ºC in case of *D. oryzae*, *A. alternata*, *U. tritici* and for 72 hrs at 25±1°C in case of *U. virens.* The numbers of spores germinated were counted and per cent spore germination inhibition was calculated by the following formula:

$$\text{Per cent spore germination inhibition} = \frac{\text{Spore germination in control} - \text{Spore germination in treatment}}{\text{Spore germination in control}} X100$$

All the tests are performed in triplicate and the reported data is the mean of three replicate tests performed with each antifungal compound. The SPSS statistical software was used for calculation of mean and analysis of the results recorded for antifungal evaluation. The results recorded in triplicates were subjected to descriptive analysis and univariate analysis of variance followed by post hoc Tukey's test to confirm its effective demarcation from other treatments as well as control set. $P < 0.05$, i.e., statistical significance at 5% level of significance was chosen as criterion for compilation of all the results.

Spore suspension prepared in autoclaved distilled water was used as control and number of spore germinated of test fungus were recorded for comparison of the results.

Results and Discussion

Chemistry

Series of surface protected copper sulfide nanoparticles (CuS-NPs) in aqua-dispersed form were synthesized by sonochemical irradiation method. Copper ions in ethylene glycol were allowed to interact with different sulfide sources, while sonication. Thiourea $SC(NH_2)_2$, sodium sulfide Na_2S, thioacteamide CH_3CSNH_2, sodium thiosulfate pentahydrate $Na_2S_2O_3.5H_2O$ and sodium diethyl-dithiocarbamate (DTC) $C_5H_{10}NNaS_2.3H_2O$ were different sulfide sources used. The change in colour from transparent to green indicated the formation of copper sulfide nanoparticles in aqua-dispersed form. These dispersed nanoparticles were stabilized with capping agent 4-aminobutyric acid (GABA). The prepared samples were characterized by Transmission Electron Microscopy (TEM) and UV-Visible spectroscopy.

The information about the size and structure of copper sulfide nanoparticles (CuS-NPs) was obtained by Transmission electron microscopy (TEM). The images of copper sulfide nanoparticles were showed that the particles were individual spherical as well as the aggregated particles in the nanometer range which formed kernels of CuS nanoparticles (Table 1). The results indicated that the nucleation rate of CuS is faster than the growth rate of CuS nanoparticles [11]. No correlation has been found between the source of sulfur and shape or size of nanoparticles.

Using UV-Vis spectroscopy technique, the absorption properties of nanoparticles was studied. In all the prepared capped samples, slight blue shift was observed with values ranging from 214-218 nm which is due to the combined effect of size reduction [12] and effect of capping agents. Absorption peaks of copper sulphide samples were close to each other due to size range similarity in NPs. The range of CuS-NPs along with the corresponding absorption peaks are give in table 1.

Table 1: Size Range and absorption data of prepared samples of aqua-dispersed CuS-NPs

S. No	Sample name	Source of Sulfide	Absorption peak (in nm)	Range of Particles size (in nm)
1.	1G	Thiourea	216	4-18
2.	2G	Thioacteamide	214	7.5-12.5
3.	3G	Sodium Thiosulfate	216	2.5-17.5
4.	4G	Sodium Sulfide	218	6.6-29.7
5.	5G	DTC	216	16-40

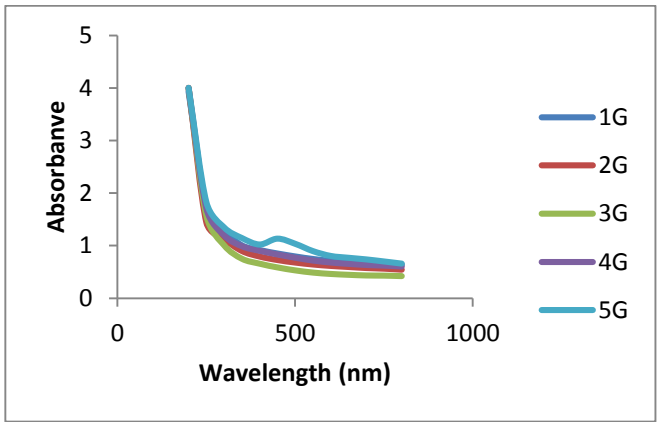

Fig 1: UV-Vis spectra of sample 1G, 2G, 3G, 4G and 5G

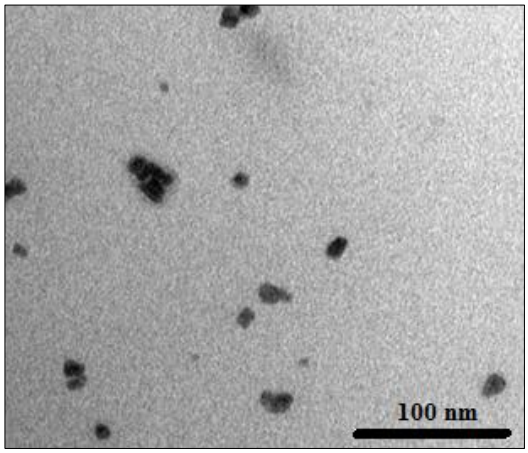

Fig 2: TEM analysis of sample 1G

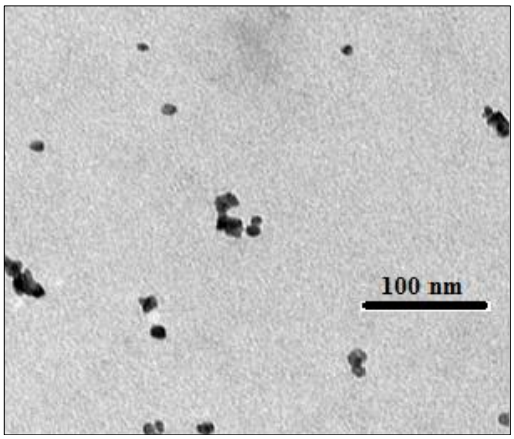

Fig 3: TEM analysis of sample 2G

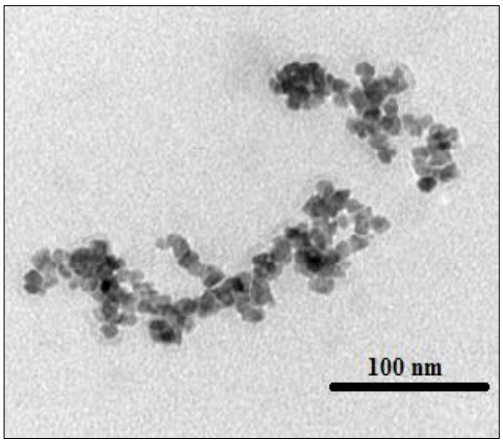

Fig 4: TEM analysis of sample 3G

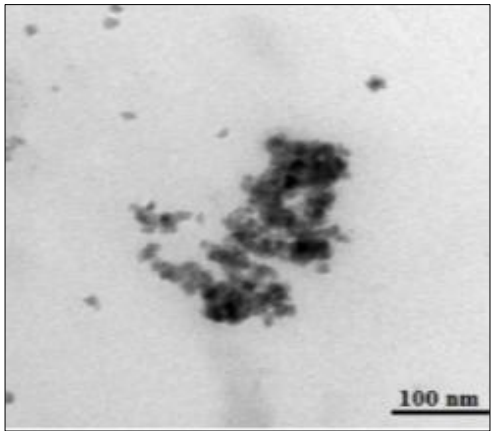

Fig 5: TEM analysis of sample 4G

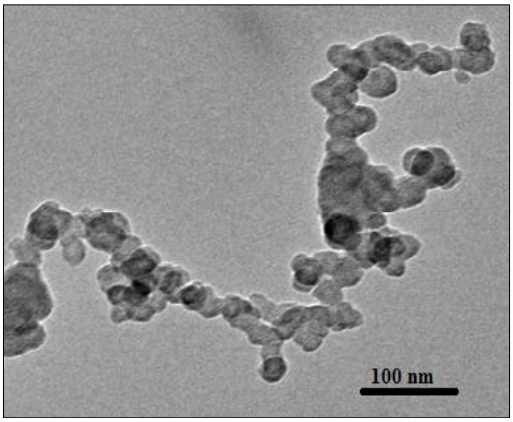

Fig 6: TEM analysis of sample 5G

Antifungal activity of Aqua-dispersed CuS-NPs

All prepared surface protected copper sulfide nanoparticles in aqua-dispersed form were found to be effective with ED_{50} values less than 7 µg/ml (Table 2) in most of the cases. The most of the samples had effective values in the range 5-6 µg/ml. Sample 3G and 5G were found to be toxic to *D. oryzae* fungi with ED_{50} 5 µg/ml which were multifold lower than the standard fungicides tilt 25 EC exhibiting value 22 µg/ml. Samples 2G and 3G inflicted best result with ED_{50} 6 µg/ml against *A. alternata* which was much lower than the standard fungicides tilt 25 EC exhibiting value 17 µg/ml respectively. Against *U. tritici*, most effective samples 2G and 5G were found with ED_{50} 5 µg/ml. Standard fungicide Vitavax exhibited 20 times lower value ED_{50} value 120 µg/ml against this test fungus. The prepared samples were found to be moderately effective against *U. virens*. Sample 3G was found to be toxic to the test fungi with ED_{50} values 6 µg/ml. It was observed that there was no significant correlation in terms of activity and size of NPs was observed.

Table 2: Antifungal potential of aqua-dispersed CuS-NPs

	ED_{50} values (µg/ml)			
Sample No.	***D. oryzae***	***A. alternata***	***U. tritici***	***U. virens***
1G	9	9	7	11
2G	6	6	5	12
3G	5	6	6	6
4G	6	7	6	8
5G	5	7	5	9
Tilt *	22	17	-	20
Vitavax**	-	-	120	-

*Standard fungicide against, *D. oryzae*, *A. alternata* and *U. virens*.
** Standard fungicide against *U. tricti*

Conclusion

The extraordinary results for bio-efficacy of the formulated copper sulphide nanoparticles suggested its further tailoring and field trials and the work for the same is under process.

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