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Research Article

EVALUATION OF ANTIMICROBIAL ACTIVITY AND MUTAGENICITY OF COPPER NANOPARTICLE SYNTHESIZED USING GREEN CHEMISTRY

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ABSTRACT

Copper nanoparticles (CuNPs) have antimicrobial properties, which have contributed to their widespread usesuch as in water treatment, foodprocessing etc. Existing genotoxicityassays are useful for evaluating the risks associated with the use of Nanoparticles. In this study, the mutagenicity of Copper Nanoparticle was assessed using standard assay, the Salmonella reverse mutation assay (Ames test). Seven different concentrations (0.005, 0.015, 0.05, 0.15, 0.5, 1.5 and 5 µL/mL) of this Nanoparticle were tested using five histidine deficient mutant tester strains of Salmonella typhimurium (i.e., TA1537, TA1535, TA98, TA100, and TA102) in the presence and absence of the S9 mixture. At all the concentrations tested, copper nanoparticles did not significantly increase the number of revertant colonies compared with the negative and positive control with or without S9 mixture.

INTRODUCTION

Nanoparticles are particles with a size not greater than 100 nm, with spherical, cubic and needle-shaped forms (5-100 nanometers to micrometers) (Allakeret *et al*, 2014). Copper is an essential element for the metabolism of animal and plant cells. It is a trace element present in most organisms, and there are more than 30 types of proteins that contain it. Today copper consumption worldwide is about 18 million tons per year (Sanhueza *et al*, 2016**)**. Its antimicrobial activity is recognized worldwide and has recently been registered by the US Environmental Protection Agency as the first solid antimicrobial material. As early as 2200 B.C. it was used to sterilize wounds and drinking water (Sanhueza *et al*, 2016**)**.
Laboratory and clinical studies have been conducted to demonstrate its effectiveness. In recent years, copper has been used as a building material for hospitals and medical centers as its antimicrobial properties proved useful in the fight against infections. These altered features can impart very desirable properties, including being less expensive, harder, and generally more efficient in particular industrial applications. The synthesis of compounds such as nanoparticles with antimicrobial properties is important and has potentially promising applications in the fight against the increasing number of pathogens resistant to currently available antimicrobials. These pathogens pose a continuous threat to human and animal health (Usman *et al*, 2013 & Kruk *et al*, 2015). Nanomaterials can have drastically different properties than their corresponding bulk (normal/not nanoscale) materials because of their size. Due to increased use of nanoparticles, exposure to nanomaterials is also expected to increase which exhibits an adverse biological effects, such as mutagenicity and

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genotoxicity (Li *et al*, 2012).There is only a limited amount of data relating to the toxicity and genotoxicity of nanomaterials. From a regulatory standpoint, it is important to determine if the current genotoxicity assays used to evaluate the safety of new products are adequate for detecting the potential toxicity and/or genotoxicity of nanomaterials. For assessing genotoxic hazard, the Salmonella reverse mutation assay (the Ames test) is widely used genotoxicity assay (M.C. Cimino, 2006).The Ames test has been propagated by Bruce N. Ames and his associates has gained world-wide acceptance as an initial test for identification of chemicals that are able to cause mutagenic activity.

The purpose of this study was to determine whether or not biosynthesized CuNPs can be tested successfully for genotoxicity hazard using the Ames test.

MATERIALS AND METHODS

The present work was carried out at Department of Microbiology, B. P. Baria Science Institute, Navsari and the Ames test was carried out at Jai Research Foundation, Vapi,Gujarat, India.

Materials

The plants: *Mallotus phillipensis, Kalanchoe integra, Eujenia jambula, Citrus sinensis, Aegle marmelos and Flacourtia indica* were collected from the Botanical Garden of Navsari Agriculture University (N.A.U.), Navsari. Their botanical identification and authentication was done by the authorities of the Herbarium of N.A.U.

Organisms:The bacterial cultures for the present study were procured from NCIM, Pune, India. They included Gram positive, Gram negative bacterial strains and fungal strains.

1. *Staphylococcus aureus* (NCIM 2079)
2. *Escherichia coli* (NCIM 2065)
3. *Pseudomonas aeruginosa* (NCIM 2863)
4. *Salmonella typhi* (NCIM 2501)
5. *Klebsiella pneumonia* (NCIM 2883)
6. *Aspergillus niger* (*NCIM 1004*)
7. *Candida albicans* (*NCIM 3102*)

The tester strains used for the bacterial reverse mutation test were *Salmonella typhimurium* TA1537, TA1535, TA98, TA100, and TA102. The *Salmonella typhimurium* strains used in this study were mutants derived from *Salmonella typhimurium* LT2. The strains used in the study were obtained from Molecular Toxicology Inc., 157 Industrial Park Dr. Boone, NC 28607, U.S.A.

Chemicals: Copper (II) sulfate pentahydrate salt-CuSO4.5H2O (LobaChemie), DMSO (Finar), Methanol (Finar), Bacto agar (Becton, Dickinson and Company), S9 mixture from rat liver (M/s G. P. Meshram, Nagpur, India), Nutrient broth no. 2 (Oxoid), Positive controls (9-Aminoacridine hydrochloride monohydrate, Sodium azide, 2- Nitrofluorene, Mitomycin-C, 2-Aminoanthracene) procured from Sigma Aldrich. B-NADP, Ampicillin, glucose-6-phosphate and histidine were purchased from Sigma Aldrich. The other chemicals used in the study were obtained from Merck (Merck & Co., Inc., Kenilworth, USA).

Methods

Preparation of Copper Nanoparticles

10 gm. leaves powder of *Mallotus phillipensis, Kalanchoe integra, Eujenia jambula, Citrus sinensis, Aegle marmelos and Flacourtia indica* were each mixed with 100 ml organic solvent (methanol) separately in each flask. Each mixture was incubated for 48-72 hours on rotary shaker. Mixture was filtered using whatmann filter paper no.1 and evaporated under hot air oven to yield the powder form (crude extracts) of pure extract. Stock solutions of crude extracts were prepared by mixing organic solvent with appropriate amount of dried extracts to obtain a final concentration of 100 mg/ml. The solvent used was 20% DMSO. The filtrates were collected and stored at 4 ˚C for further experiment to biosynthesize nanoparticles. For the copper nanoparticles synthesis, 1 ml of eachleaf extract was added to 100 ml of 1mM aqueous copper sulphate solution in a 250 ml Erlenmeyer flask. The flask was then kept overnight at room temperature. The copper nanoparticles solution thus obtained was purified by repeated centrifugation at 12,000 RPM for 15 min followed by re-dispersion of the pellet in ethanol. Then the copper nanoparticles were dried in oven at 80º C. After completion of biosynthesis process resulting copper nanoparticles were subjected to characterization using Scanning Electron Microscope. (Antimicrobial activity and Ames test).

Evaluation of Antibacterial Activity

Mallotus phillipensis, Kalanchoe integra, Eujenia jambula, Citrus sinensis, Aegle marmelos and Flacourtia indica were used for biosynthesis of Copper nanoparticles. These biosynthesized nanoparticles were tested individually against test organisms for antimicrobial activity by agar well diffusion method (Perez*et al,* 1990). For this study Gram positive organism (*Staphylococcus aureus*), Gram negative organisms (*Pseudomonas aeruginosa*,*Escherichia coli*, *Salmonella typhimurium* and *Klebsiella pneumonia*) and fungal strains (*Aspergillus niger* and *Candida albicans*) were used. On the basis of zone of inhibition, the susceptibility of a particular test organism to antimicrobial agent was determined. Pure cultures were sub cultured into nutrient broth and incubated at 37˚ C for 24- 48 hours. Wells of 8 mm diameter were made on pre-incubated nutrient agar plates using gel puncture. Each test organism was spread uniformly onto the individual plates using spread plate technique. Using sterile micropipette tips, 100 µLof each Copper nanoparticlewas pipetted into the mentioned well in all the plates. After incubation, the diameters of zone of inhibition were measured in triplicate.

Ames Test

Genotype Confirmation Test

The genotype of the tester strain was confirmed for all the strains. The tester strains of *Salmonella typhimurium* were tested for histidine dependence, biotin dependence, histidine and biotin dependence, rfa mutation, uvrB mutation through sensitivity to ultraviolet light and the R-factor resistance for ampicillin and tetracycline (Mortelmans K. and Zeiger E., 2000, Maron D.M. and Ames B.N., 1983).

Sterility Check for the Operating System

A sterility check for the operating system was performed along with mutagenicity test for the following:

Top Agar

Top agar prepared and used for the study (2 mL) was poured aseptically onto MGA plate.

S9 Mix

A volume of 0.1 mL of S9 mix (5% v/v S9 mix for initial toxicity-mutation test and 10% v/v S9 mix for confirmatory mutation test) prepared for treatment was added aseptically to 2 mL of top agar, mixed thoroughly and this mixture was poured onto MGA plate.

Solvent

A volume of 0.1 mL of dimethyl sulfoxide,used as vehicle was added aseptically to 2 mL of top agar, mixed well and this mixture was poured onto MGA plate.

Test Item

A volume of 0.1 mL of the test item stock solution of the highest concentration was added aseptically to 2 mL of top agar, mixed well and this mixture was poured onto MGA plate.

ONB Solution

A volume of 0.1 mL of ONB solution was added aseptically to 2 mL of top agar, mixed well and the mixture was poured on to MGA plate.

0.2 M Sodium Phosphate Buffer

A volume of 0.1 mL of 0.2 M of sodium phosphate buffer was added aseptically to 2 mL of top agar, mixed well and the mixture was poured on to MGA plate.

MGA Plate

Blank MGA plate was maintained for the sterility check.

Mutagenicity test

Copper nanoparticle was tested for mutagenicity test using all five tester strains of *Salmonella typhimurium* (TA1537, TA1535, TA98, TA100, and TA102). The experiment was conducted both in the absence and presence of metabolic activation system (5% v/v S9 mix).

The first stock solution (stock A) of the test item was prepared by dissolving 500 µL of test item in dimethyl sulfoxide (DMSO) and volume was made up to 10 mL (50 µL/mL). Further stock solutions, stock B to H, were prepared by further dilutions.

Tubes containing 2 mL of molten top agar with 0.5 mM histidine/biotin were maintained at 45 ± 2 °C. A volume of 500 µL of 0.2 M phosphate buffer was added in the absence of metabolic activation system and 500 µL of 5% v/v S9 mix was added in the presence of metabolic activation system. Volume of 100 µL of the relevant stock solution of test item, dimethyl sulfoxide and relevant positive control were used for treatment, as a negative control and as a positive control, respectively. Finally, 100 µL of bacterial culture was added to the tubes and mixed. Cultures used were checked for cell viability prior to testing. This treatment mixture was poured on MGA plates and allowed to solidify. Triplicate sets were maintained for each concentration of copper nanoparticle, positive control and negative control. The petriplates were incubated at 37 ± 1 °C for 48 hours and then examined to assess the bacterial lawn inhibition and reduction in number of colonies.Plates were maintained in triplicates for each test concentration of copper nanoparticle as well as negative and positive controls. The numbers of revertant colonies were recorded after 48 h incubation period at 37± 1 °C.

Treatment with 2-aminoanthracene in the absence of metabolic activation system was also performed for tester strain TA100 in both initial toxicity-mutation test to verify the efficiency of the S9 fraction used in the study.

RESULTS AND DISCUSSION

Characterization of Nanoparticles

SEM images of copper nanoparticles biosynthesized from different herbal extracts using copper sulphate are shown in Fig.1 (A-F). The average particle size of the coppernanoparticles is around 52-89 nm.Copper Nanoparticles synthesized from *Mallotus phillipensis* have particle size between 52-56 nm, which is smallest size nanoparticle amongst nanoparticles synthesized from other five herbal plant extracts. So, based on small size, only copper nanoparticles synthesized from *Mallotus phillipensis* were evaluated for mutagenicity test.

SEM images of Copper Nanoparticles Prepared using Different Herbal Extracts

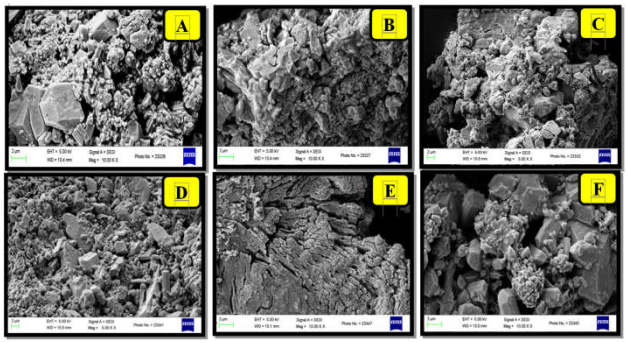
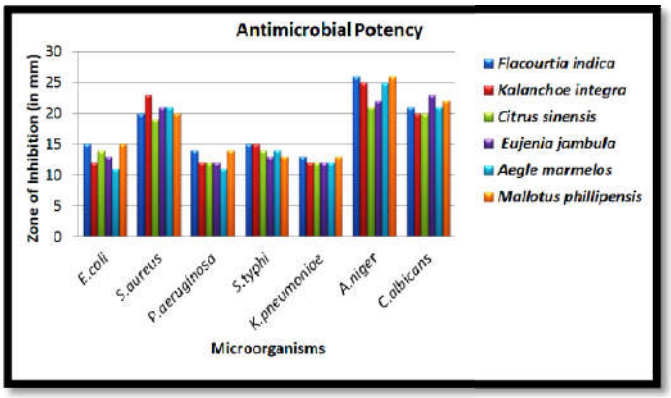


Figure 1 SEM images of Copper Nanoparticles prepared using different herbal extracts**,** (A) *Aegle marmelos*, (B) *Citrus sinensis*, (C) *Eujenia jambula*, (D) *Flacourtia indica*, (E) *Mallotus phillipensis*, (F) *Kalanchoe integra*

Antimicrobial Potency of Copper Nanoparticles

After 24 hours of incubation, the antimicrobial potency of CuNPs biosynthesized using *Mallotus phillipensis, Kalanchoe integra, Eujenia jambula, Citrus sinensis, Aegle marmelos and Flacourtia indica* were graphically represented in the Graph-1.



Graph 1 Antimicrobial potency of Copper nanoparticles against tester strains

From the graph it was clearly seen that copper nanoparticles synthesized from all the six herbal plants extracts have highest antifungal activity against *Aspergillus niger* and *Candida albicans* as compared to antibacterial activity. Kanhed *et al,* (2014) reported the in vitro antifungal activity of chemically synthesized CuNPs with a commercially available antifungal agent against four different plant pathogenic fungi, viz., *Fusarium oxysporum, Curvularialunata, Alternaria alternata* and *Phoma destructiva*. CuNPs showed activity against all the plant pathogenic fungi used in the experiment. The results demonstrated that *Curvularialunata* and *Alternaria alternata*were comparatively resistant to the commercial antifungal agent (bavistin) but showed sensitivity towards CuNPs. The present study also confirmed the antifungal nature of CuNPs against test strains.

In case of antibacterial activity, *Staphylococcus aureus*was more susceptible to all biosynthesized copper nanoparticles than other bacterial tester strains. Usman *et al,* (2013) reported the synthesis of pure CuNPs in the presence of a chitosan stabilizer through chemical means with the evaluation of antimicrobial activity of the nanoparticles by using several test microorganisms like methicillin-resistant *Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa, Salmonella choleraesuis* and *Candida albicans.*

AMES test

Sterility test for the operating system

The results of sterility test revealed that the operating system used for the Ames test was sterile. The results are tabulated in below Table 1.

Table 1 Sterility Test for the Operating System

Sterility Check For	Mutagenicity Test
Top agar	Sterile
S9 mix	Sterile #
Solvent (DMSO)	Sterile
T.I. Stock Solution *	Sterile
MGA Plate (Blank)	Sterile
ONB Solution	Sterile
0.2M Sodium Phosphate Buffer	Sterile

Key:* = Highest Concentration of Test Item Stock Solution, # = 5% v/v S9 mix T.I. = Test Item, DMSO = Dimethyl Sulfoxide, MGA = Minimal Glucose Agar, ONB = Oxoid Nutrient Broth

Genotype confirmation test for tester strains

All the tester strains retained their genetic characteristics while performing the genotype confirmation test. The results are tabulated in below Table2 and Table 3.

Table 2 Genotype Confirmation Test for Tester Strains

Name of the Test		*Salmonella typhimurium* Tester Strains				
		TA1537	TA1535	TA98	TA100	*TA102*
Histidine Dependence		NG	NG	NG	NG	NG
Biotin Dependence		NG	NG	NG	NG	G
Histidine and Biotin Dependence		G	G	G	G	G
rfa Mutation		ZI	ZI	ZI	ZI	ZI
DNA repair (*uvrB*)		NG*	NG*	NG*	NG*	G*
R - factor Resistance	Ampicillin Resistance	NG	NG	G	G	G
	Tetracycline Resistance	NG	NG	NG	NG	G

Key: NG = No Growth, G = Growth, ZI = Zone Of Inhibition, * = On Irradiated Side

Table 3 Cell Viability Test for Tester Strains

Tester Strains Used	Mutagenicity Test	
	OD	Viable cell Number (CFU/mL)
TA1537	0.565	2.53×10^9
TA1535	0.419	1.98×10^9
TA98	0.402	1.91×10^9
TA100	0.439	2.05×10^9
TA102	0.524	2.37×10^9
Control Blank	0.000	0.00

Key: OD = Optical density, CFU = Colony forming unit

Mutagenicity Test

The results of the study indicate that the values of negative control in all strains were within historical range of respective strains. Positive controls exhibited a clear increase in the number of revertants when compared with the concurrent negative controls. Increase in revertants were not observed in tester strain TA100 treated with 2-aminoanthracene in the absence of metabolic activation but clear increase was observed in the presence of metabolic activation. This demonstrated the efficiency of the S9 fraction used in this assay.

Cytotoxicity was characterized by inhibition of the background bacterial lawn and/or reduction in the number of revertant colonies. Normal growth was observed up to the concentration of 5 µL/plate and no increase in the revertant colonies was observed both in the absence and presence of the metabolic activation system (5% v/v S9 mix) in all the tester stains.

Results revealed that there was no positive mutagenic effect in tester strains TA1537, TA1535, TA98, TA100 and TA102 up to the tested concentration of 5 µL/plate of copper nanoparticle in the absence and presence of metabolic activation, when compared to negative control. Although statistical significance was observed in strain TA1535 (absence of S9), mean revertant colonies at different concentration level as between 14.330 to 16.670 (against 13.00 in negative control). There for revertant colonies were within the range of historical control data and hence this was considered as biologically non-significant.

Table 4 Mean Count of His^+ Revertant Colonies in Negative Control, Positive Controls and Treatment Plates in the Absence of Metabolic Activation (Mutagenicity Test)

Concentration of copper nanoparticle (µL/plate)	His^+ Revertant Colonies/Plate [Absence of Metabolic Activation]									
	TA1537		TA1535		TA98		TA100		TA102	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD

NC (DMSO)	10.67	1.53	13.00	2.65	20.67	9.29	126.67	9.29	240.67	32.72
0.005	9.00	3.61	14.33	1.15	18.67	4.51	124.33	15.31	254.00	28.83
0.015	9.33	1.53	13.00	1.73	21.33	3.06	126.00	4.58	228.67	19.66
0.05	8.67	2.52	13.67	3.79	21.67	3.21	139.33	11.06	238.33	11.02
0.15	10.33	2.89	14.33	8.39	20.33	3.79	133.67	9.02	249.00	18.33
0.5	10.33	4.04	12.67	3.79	22.00	7.81	123.00	13.86	226.00	14.00
1.5	9.67	0.58	14.33	2.52	22.00	2.00	124.33	6.51	233.67	18.01
5	9.33	3.79	16.67	4.62	17.00	5.29	125.00	21.17	254.00	16.64
PC	204.67	32.02	350.00	50.86	536.33	8.33	727.67	31.34	983.00	7.81
2Aa	-	-	-	-	-	-	127.33	9.24	-	-

Key: SD = Standard Deviation, NC = Negative Control, DMSO = Dimethyl Sulfoxide, PC = Positive Control {TA1537 = 9-Aminoacridine Hydrochloride Monohydrate (75 µg/plate), TA1535 = Sodium Azide (0.5 µg/plate), TA98 = 2-Nitrofluorene (7.5 µg/plate), TA100 = Sodium Azide (5 µg/plate), TA102 = Mitomycin-C (0.5µg/plate)}, 2-Aa = 2-Aminoanthracene (5 µg/plate for TA100), - = Not Applicable

Table 5 Mean Count of His+ Revertant Colonies in Negative Control, Positive Control and Treatment Plates in the Presence of Metabolic Activation (Mutagenicity Test)

Concentration of copper nanoparticle (µL/plate)	His+ Revertant Colonies/Plate [Presence of Metabolic Activation (5% v/v S9 mix)]									
	TA1537		TA1535		TA98		TA100		TA102	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
NC (DMSO)	12.33	2.52	17.00	3.00	23.67	2.89	126.00	18.73	240.00	33.78
0.005	12.00	2.65	16.33	3.06	22.00	1.73	138.33	20.31	233.00	19.52
0.015	10.00	1.73	18.33	4.51	18.67	2.52	120.67	20.43	232.67	28.15
0.05	12.00	2.00	17.67	6.51	24.00	11.53	127.33	11.59	262.67	13.50
0.15	10.67	3.06	17.33	6.66	22.33	4.16	134.33	18.93	242.33	29.96
0.5	11.67	1.15	18.00	6.93	20.00	3.61	137.67	4.16	254.67	16.07
1.5	10.00	3.46	15.00	2.65	24.67	3.51	122.00	17.78	247.00	33.87
5	11.67	1.53	15.00	5.57	18.67	5.86	140.67	22.55	236.33	9.29
PC-2Aa	220.67	31.21	393.33	11.72	559.33	28.18	811.00	19.00	993.67	54.08

Key: SD = Standard Deviation, NC = Negative Control, DMSO = Dimethyl Sulfoxide, PC = Positive control, 2Aa = 2-Aminoanthracene (10 µg/plate for TA1537, TA1535, TA102 and 5 µg/plate for TA98 and TA100)

CONCLUSION

Use of different herbal plant extracts such as *Mallotus phillipensis, Kalanchoe integra, Eujenia jambula, Citrus sinensis, Aegle marmelos and Flacourtia indica* for the synthesis of CuNPs is a novel step towards the biogenic synthesis of CuNPs. It is an ecofriendly, non-toxic and rapid approach. Further, the use of biogenic CuNPs against different pathogens confirmed its effectiveness against wide range of microorganisms. Due to antimicrobial potential of these copper nanoparticles, it can be used in different formulations like nanofungicides, nano antimicrobials and nanofertilizers, which could serve dual purpose, by protecting the crop plants from its pathogens and also providing nutrients to the plants. Finally, and most important is that it is a cost effective approach as raw materials involved in the synthesis are very cheap.

From the results of this study, it is concluded that copper nanoparticle is non-mutagenic to all of the five strains of *Salmonella typhimurium* viz., TA1537, TA1535, TA98, TA100, and TA102 when tested under the specified conditions.

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