iJRASET
International Journal For Research in Applied Science and Engineering Technology

INTERNATIONAL JOURNAL FOR RESEARCH

IN APPLIED SCIENCE & ENGINEERING TECHNOLOGY

Volume: 6 **Issue:** XI **Month of publication:** November 2018

DOI:

www.ijraset.com

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International Journal for Research in Applied Science & Engineering Technology (IJRASET)
ISSN: 2321-9653; IC Value: 45.98; SJ Impact Factor: 6.887
Volume 6 Issue XI, Nov 2018- Available at www.ijraset.com

Bacterial Toxicological Assay of Calcium Oxide Nanoparticles against some Plant Growth Promoting Rhizobacteria

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Abstract: ***Nowadays, Nanoparticles are widely used in all major fields such as Pharmaceuticals, Agricultural, Phyto-remediation, Biochemical Sciences and Nutraceuticals etc. In the field of agronomic practices, these nanoparticles play a major role in using advancement. The present investigation was worked on the inhibitory concentration of chemically synthesised calcium oxide nanoparticles against some Plant growth promoting rhizobacteria viz., Azotobacter chroococcum, Pseudomonas putida, P. fluorescens, Bacillus subtilis, B. licheniformis, Paenibacillus polymyxa and Rhizobium species isolated Melilotus indica (L.) All. and Cassia tora L. Calcium Oxide powder was also used as comparative analysis and Streptomycin was used as a Standard drug. Antibacterial assay done by using Broth Micro-dilution (BMD) prescribed by CLSI and data were analysed on Spectramax 384plus, Microplate data analysis, Molecular device. The results came very prominently with Minimum Inhibitory Concentration (MIC) was shown on P. putida, P. fluorescens, Bacillus subtilis and B. licheniformis. Low MIC value was observed (0.001 mg/ml) on A. chroococcum. No MIC value was recorded against Rhizobium species and P. polymyxa. The present investigation showed that Calcium Oxide Nanoparticles can be used with low concentration (i.e. less than 1500 ug/ml or 1500 ppm) as "Nano-Biofertilizers" mixing with PGPRs as they were showed no inhibitory effects on rhizobacteria like A. chroococcum and Rhizobium species and showed inhibitory effects on other at very higher concentration i.e. more than 3000 ug/ml.***

Keywords: CaO Nanoparticles, Antibacterial assay, MIC, Nano-Biofertilizers, etc.

I. INTRODUCTION

Nanoparticles (NPs) have become the most widely used materials for modern scientific research, especially in electronic and medical sciences because of a wide range of properties (1). Many methods include microwave, hydrothermal, sol-gel, sonochemical and solvothermal have been developed to prepare various types of NPs (1, 2). Among the huge variety of nanoparticles synthesized till date, the metal oxide nanoparticles, such as Al2O3, ZnO, MgO, CuO, TiO2, and CaO are well known for their inherent antimicrobial activity (3). Also, due to their high stability and unique physicochemical properties, these nanoparticles are highly significant nano-materials in material science. Nanoparticles of calcium oxide, calcium hydroxide and calcium acetate are accepted as food additives. Calcium oxide used food grade as preservative, acid regulator, dough conditioner and prevents food elements from aggregation and increases its bioavailability (4). In addition, metal oxides nanoparticles demonstrate antimicrobial in previous studies (5, 6), which may be produced from its alkalinity and presence of active oxygen particles. Metal oxides generally considered safe for human and animals compared to other organic materials. Calcium oxide nanoparticles (CaO NPs) are low cost, easily accessed and biocompatible. So, metal oxides could be good substitutes in medical and food processing purposes (7). Consider Nanotechnology can be applied in food production and processing with applying of nanoparticles which may be organic or inorganic. Organic nano-material as protein, fat, carbohydrates and etc. inorganic nano-material may be metals like calcium and magnesium or non-metals as selenium and silicate which may be applied in food, food additives or food packaging. The term nano-food is used by applying nanoparticles or nanotechnology in any step of food production (8). Nanoparticles can be used as nano-sized food additives which either be naturally or artificially produced in food applications. However, manufactured nano-sized particles may cause harm to human or environment especially if penetrate the cell membrane (9). There is a little knowledge about nanofood, although nanoparticle can be produced naturally during the manufacture of food products. Also, Nanofabrication helps in

the absorption and transportation of fat in our body (10). Calcium hydroxide was a material with numerous chemical, industrial, environmental, and architectural applications (11-18). The produced nanolimes were successfully employed in form of hydro-alcoholic suspension on mural paintings, stuccoes and frescoes (19) as well paper and wood deacidification (20); refurbishments of architectonical surfaces were considered too (21-24). CaO and $Ca(OH)_2$ nanoparticles possesses compatibility and sustainable action towards the main historical substrates with following advantages: having the capability to penetrate deeper into the cell, less limitation due to the particle size, high reactivity and fast reactions, such as carbonization, in the treated zones, high purity and defined composition (25, 26). Chemical synthesis of CaO NP by precipitation process in supersaturated aqueous solutions of reactants of aqueous sodium hydroxide solution (NaOH) added drop by drop in an aqueous calcium chloride ($CaCl_2$).$2H_2O$ solution maintained at high temperature (60°C). The super-saturation degree, the high temperature and the slow mixing time tended to favour $Ca(OH)_2$ nucleation rate with respect to particle growth, promoting the precipitation of nanosized calcium hydroxide particles. After synthesis, the produced white precipitate phase was washed, in order to remove the sodium chloride that was produced during their action, and water was partially substituted with an alcoholic medium to improve particles disagglomeration and stability (27). Disagglomeration allowed to have a higher specific surface, leading to more reactive and more penetrating particles; besides, the higher stability of Ca(OH)2 alcoholic suspensions in comparison to aqueous media, reduced the tendency to form a white film onto the surfaces to be consolidated (28). The nanoparticles were synthesized by an original method that allows obtaining them easily and drastically reducing the time of synthesis with respect to other procedures reported in the literature (29-31). The straightforwardness and quickness of our synthesis method could allow to scale-up the nanolime production opening different possibilities in engineering fields, where the use of lime play a fundamental role (32,33).

II. MATERIALS AND METHOD

The six rhizobacteria were procured from MTCC Chandigarh, India. Two species of Rhizobium were isolated from different plant viz., nodules of *Melilotus indica* and rhizospheric soil of *Cassia tora*. All 8 bacterial inocula were made compared with 0.5 McFarland standard solutions. Calcium oxide nanoparticle was synthesised with reacting the CaCl2.2H2O with NaOH solution and 0.5 ml Poly-ethyl Glycol (PEG) was added for stability. Characterization of chemically synthesised CaO NPs was done with SEM, XRD and UV-vis.

The toxicity of CaO NP on PGPRs was done by using antimicrobial assay recommended by CLSI with some modifications. Broth Micro Dilution was done and data were analysed at 625 nm on Spectramax 384plus, Microplate data analysis. CaO powder (collected from SRL Pvt. Ltd) was used to compare with synthesised nanoparticles. The Broth Micro-dilution protocol was used as recommended by CLSI- Antibacterial assay. Column-1 contains formaldehyde, Column-2 contains MHB as broth control, Column-3 and Column-4 contains the drug in each row. Row A and B of streptomycin. Row C, D, E and F contain CaO NP. Row G and H contain CaO powder. Column-3 is known as a column of drug control. Now dilute the drugs horizontally from column-4 to column-11 by using multichannel micropipette. Column-12 was filled with bacterial inoculum as the positive control. The extract solutions over horizontally diluted 1:1 in MHB in 96 well plates were incubated at 37 °C for 24 hours (34, 35). Inhibitory concentration and it was determined as the lowest concentration without turbidity. Streptomycin used as Drug (Standard) control. Formaldehyde was used as a negative control.

III. RESULTS AND DISCUSSION

The chemically synthesised CaO nanoparticles was cubical crystals with grain size 100 nm (Figure1) and are found more stable with the shelf life of 6-8 month (in dry condition). UV-visual spectroscopy showed the peak at 370 nm and XRD peaks (2 Θ=1207, 1877) confirms the nanometre-sized crystals.

Toxicity assay of CaO NP was found to be most effective against Paenibacillus polymyxa with 0.153 mg/ml (153 ppm), CaO powder 0.301 mg/ml (301 ppm) and streptomycin with 0.280 mg/ml (280 ppm), whereas found least effective against Rhizobium sp. (Melilotus indica) with CaO NP 2.540 mg/ml (2540 ppm), CaO powder 3.900 mg/ml (3900 ppm) and Streptomycin with 0.912 mg/ml (912 ppm) followed with B. subtilis with CaO NP 1.392 mg/ml (1392 ppm), CaO powder 1.89 mg/ml and streptomycin 0.290 mg/ml (Figure2). No toxicity was reported in A. chroococcum (CaO powder 0.14 mg/ml, streptomycin 0.290 mg/ml); B. licheniformis (CaO powder 0.23 mg/ml, streptomycin 0.450 mg/ml); P. fluorescens (CaO powder 0.187 mg/ml; streptomycin 0.018 mg/ml),, P. putida (CaO powder 0.156 mg/ml; streptomycin 0.008 mg/ml) and Rhizobium sp. (C. tora) (CaO powder 0.56 mg/ml; streptomycin 0.003 mg/ml) (Figure4).

Inhibitory concentration (IC_{50}) was found more toxicity of CaO NP against P. polymyxa with CaO NP 0.153 mg/ml (153ppm), CaO powder 0.302 mg/ml, streptomycin 0.460 mg/ml. Less toxicity was found on B. licheniformis with CaO NP 2.713 mg/ml (2713

ppm), CaO powder 2.560 mg/ml, streptomycin 0.210 mg/ml. No inhibitory effectiveness was recorded on A. chroococcum, P. putida and Rhizobium sp. (C. tora) (Figure 3).

The present investigation was found that CaO NP was toxic only to some PGPRs viz., P. polymyxa. Comparative analysis found that CaO powder (Merck) was more toxic than synthesised CaO NP. Also, the toxicity of CaO NP against some PGPRs (B. subtilis, B. licheniformis and Rhizobium sp.. (Melilotus indica) were observed with higher concentration i.e. above 1000 ppm. This clearly showed that CaO NP was least toxic to the rhizobacterial inoculants (found above 1000 ppm) and can be used with rhizobacteria for the formulation of Nano-Biofertilizers.

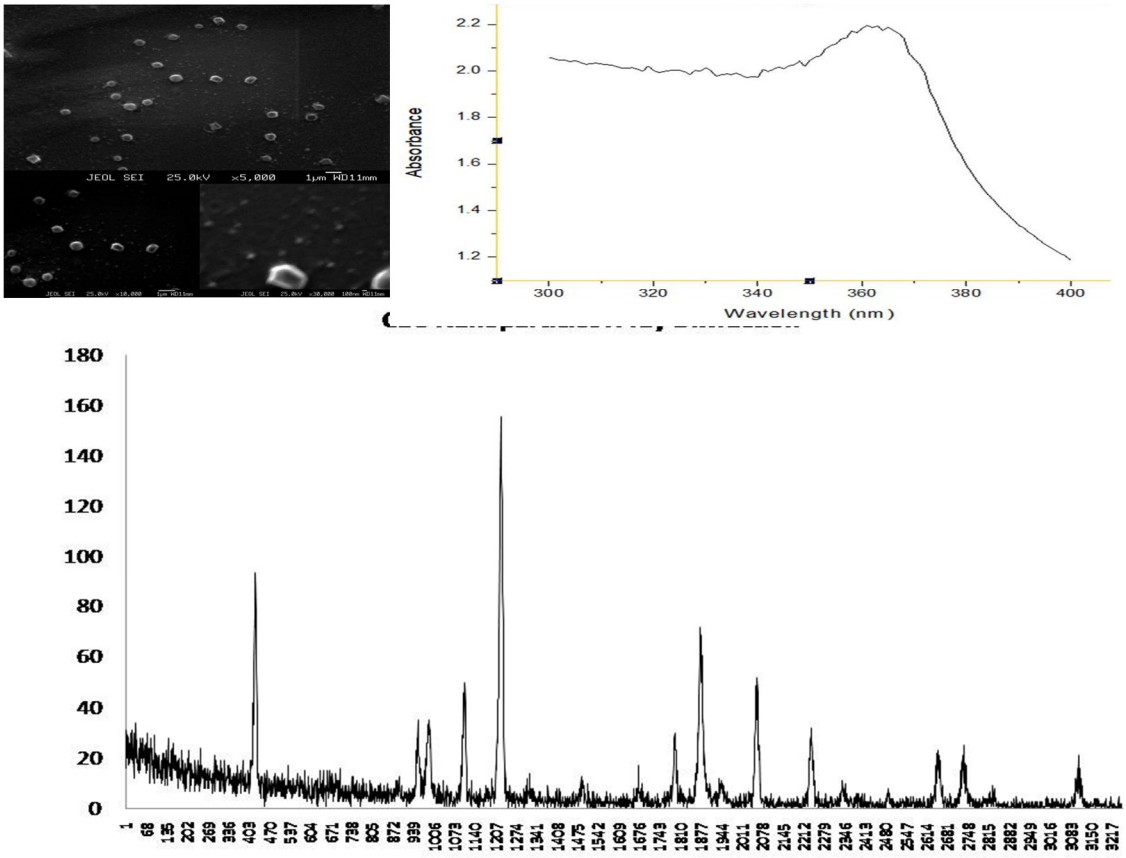
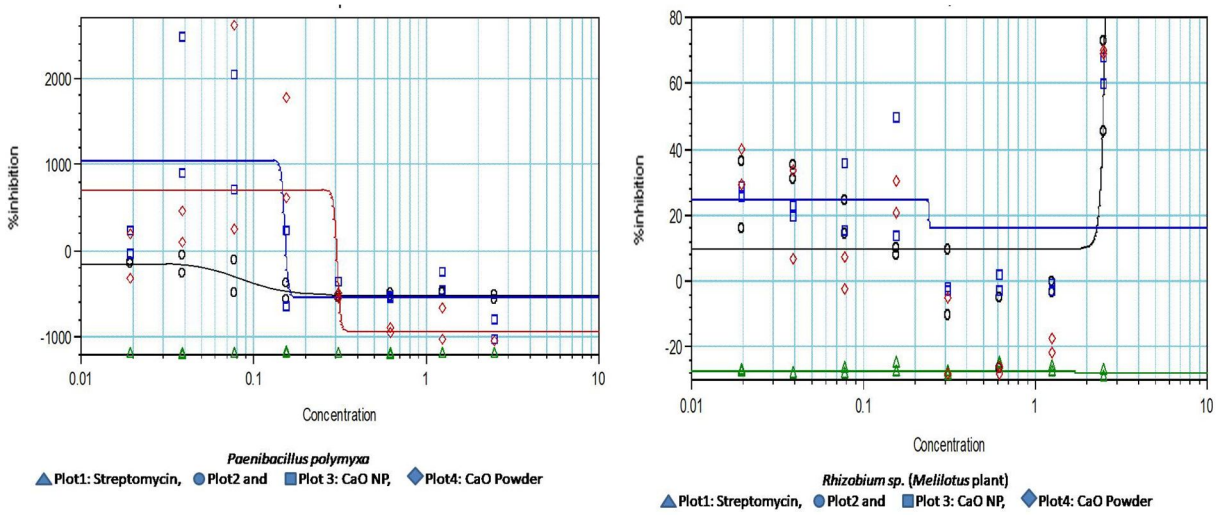


Figure1: Characterization of CaO nanoparticles i.e. SEM, UV-vis and XRD.



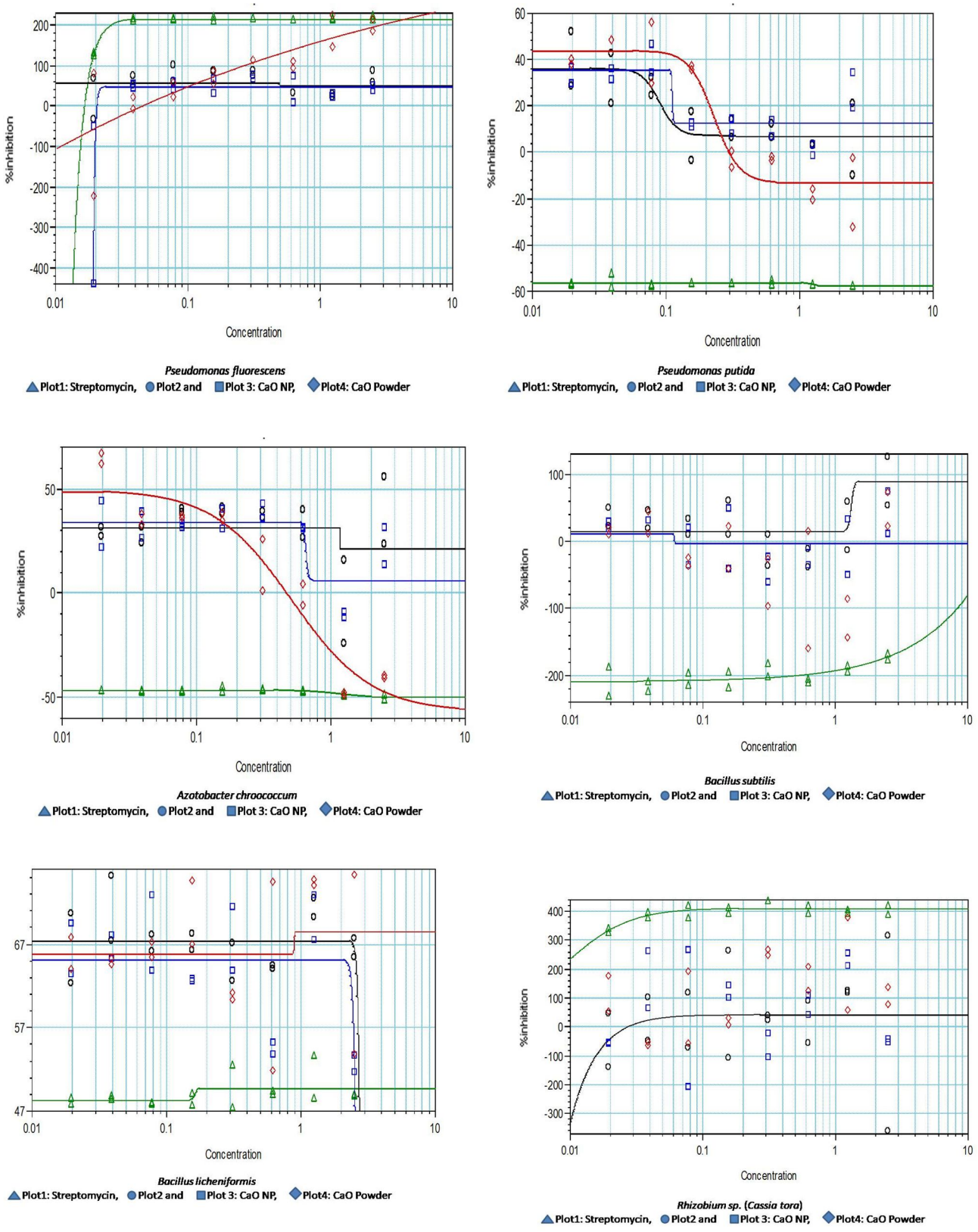


Figure2: Toxicity assay of CaO NP and CaO powder against some PGPRs.

Table1: Inhibitory concentration (IC_{50}) value in mg/ml of CaO NP and CaO powder value against some PGPRs

PGPRs	IC_{50} (mg/ml)		
	Streptomycin	CaO Nanoparticles	CaO Powder
A. chroococcum	0.190	0.00	1.56
B. subtilis	1.968	1.319	1.88
B. licheniformis	0.210	2.713	2.56
P. fluorescens	0.017	0.496	0.108
P. putida	0.002	0.00	0.12
P. polymyxa	0.460	0.153	0.302
Rhizobium sp. (M. indica)	0.800	2.478	3.89
Rhizobium sp. (C. tora)	0.002	0.00	1.24

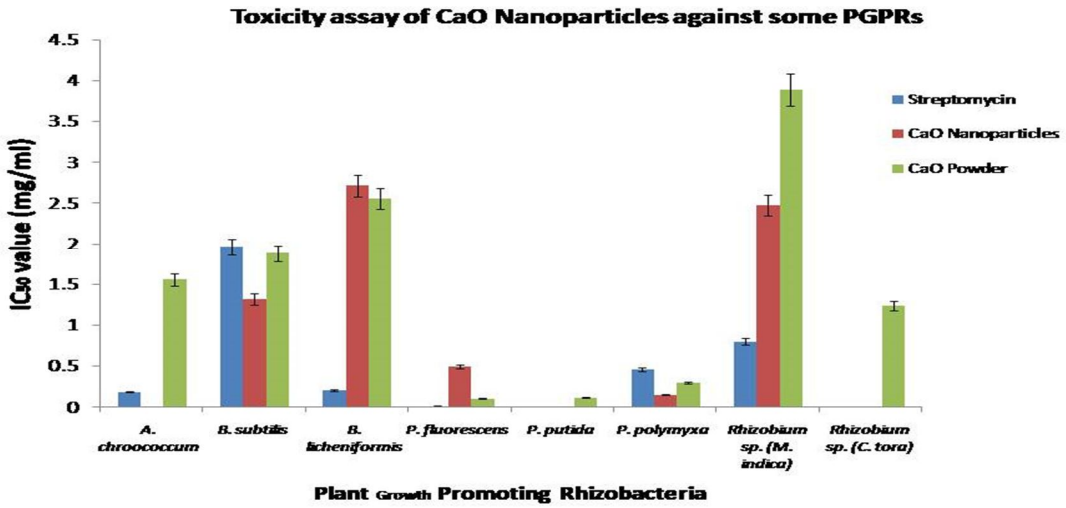


Figure3: Inhibitory concentration (IC_{50}) value in mg/ml of CaO NP and CaO powder value against some PGPRs

Table2: Minimum Inhibitory concentration (MIC) value in mg/ml of CaO NP and CaO powder value against some PGPRs

PGPRs	MIC (mg/ml)		
	Streptomycin	CaO Nanoparticles	CaO Powder
A. chroococcum	0.290	0.00	0.14
B. subtilis	1.800	1.392	1.89
B. licheniformis	0.450	0.00	0.23
P. fluorescens	0.018	0.00	0.187
P. putida	0.008	0.00	0.156
P. polymyxa	0.280	0.153	0.301
Rhizobium sp. (M. indica)	0.912	2.540	3.90
Rhizobium sp. (C. tora)	0.003	0.00	0.56

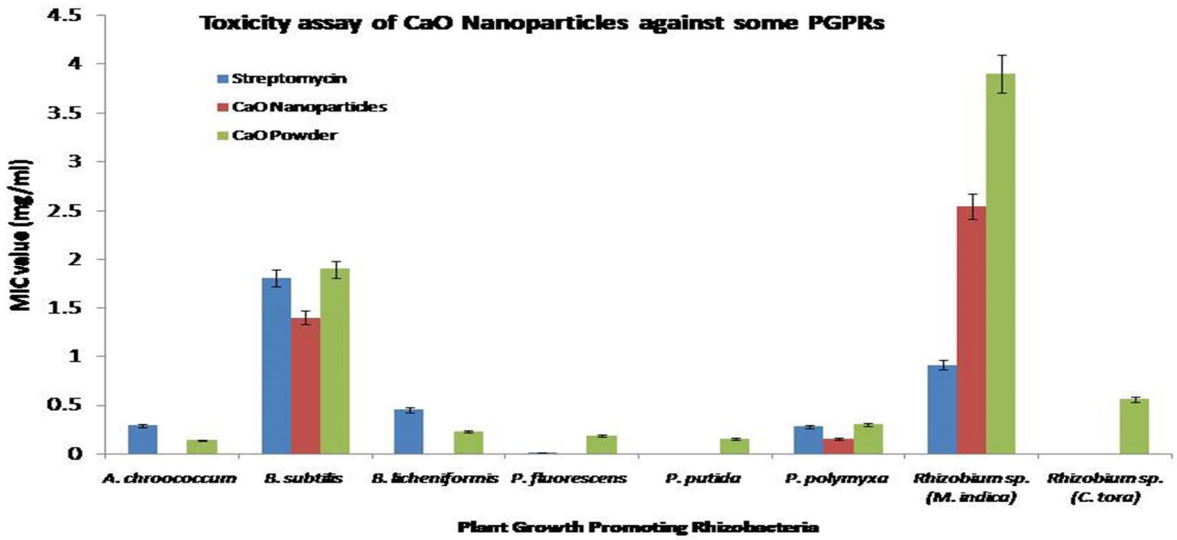


Figure4: Minimum Inhibitory concentration (MIC) value in mg/ml of CaO NP and CaO powder value against some PGPRs

IV. ACKNOWLEDGEMENTS

Authors were thankful to Head, Department of Botany for providing the lab facilities; Dr. M.M. Dwivedi, NCEMP, University of Allahabad for CaO NP characterization; Dr. Ashutosh Pathak for valuable suggestions and UGC for the funds.

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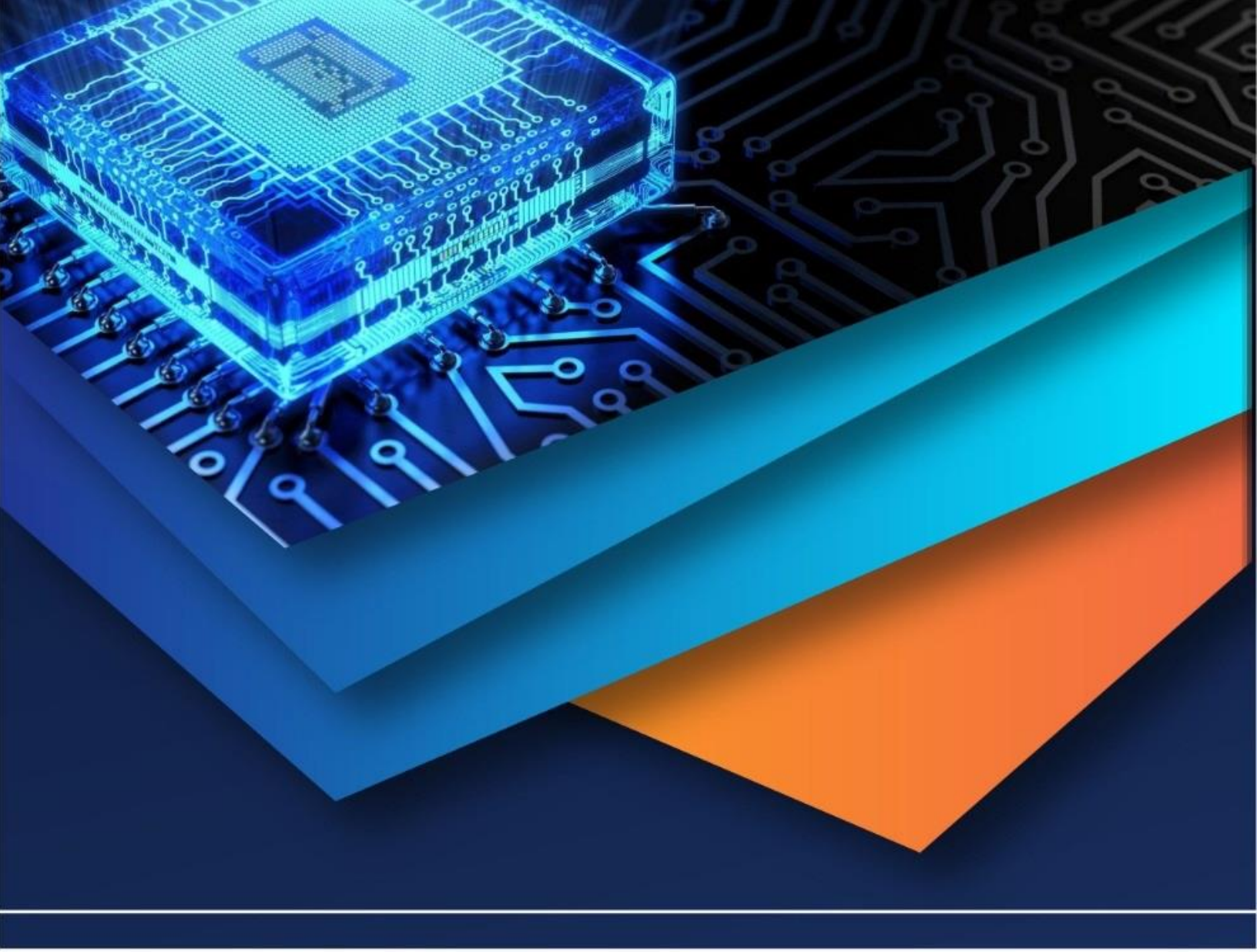

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