



Green Synthesis of Chromium Nanoparticles by Aqueous Extract of *Melia azedarach*, *Artemisia herba-alba* and Bacteria Fragments against *Erwinia amylovora*

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Authors' contributions

This work was carried out in collaboration among all authors. Authors FMAE, ARA, SSS, SFE and OMK designed this work, wrote the manuscript and revised it. Author OMK conducted the experiments, performed the measurements of the samples and analysis of data. Authors SFE and SSS coordinated the data collection. All authors read and approved the final manuscript.

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ABSTRACT

This study was carried out at the Tissue Culture and Germplasm Conservation Research Laboratory, Horticulture Research Institute, Agricultural Research Center, Giza, Egypt. in cooperation with Department of Horticulture, Faculty of Agriculture, Benha University, Egypt. from 2016 to 2020.

Medicinal plants contain many reduction substances e.g. terpenoids, flavonoids, tannins, and glycosides. *Melia azedarach* and *Artemisia herba-alba* are rich in these compounds that are able to reduce chromium metal (VI) to chromium nanoparticles (Cr₂O₃). Chromium oxide nanoparticles were green synthesized by the reduction of potassium dichromate solution with *Melia azedarach*

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and *Artemisia herba-alba* plant extract. In biological methods, Cr₂O₃ nanoparticles were synthesized by two biological agents of *Erwinia amylovora* dry matter and extraction. The resulting Cr₂O₃ nanoparticles were characterized by transmission electron microscopy (TEM), UV-VIS absorption spectroscopy. The antibacterial effect of Cr₂O₃ nanoparticles against *E. amylovora* gave the highest inhibition zone for Cr₂O₃ nanoparticles reduction by *Artemisia* followed by *Melia* (31.0 and 25.0 mm respectively). These particles were shown to have an effective bactericide on contaminated callus pear cells by *Erwinia* bacteria which gave survival 75% and 50% and 0.0% contamination.

Keywords: Chromium nanoparticles; *Erwinia amylovora*; *Melia azedarach*; *Artemisia herba-alba*; fire blight; *Pyrus communis*.

1. INTRODUCTION

Melia azedarach L., commonly known as mahanimbin belongs to family Meliaceae. It is a large evergreen tree found throughout India and similar to Neem. Traditionally it is used as an anthelmintic, antilithic diuretic, antioxidant, astringent and stomachic. Vario's scientific studies reported the analgesic, anticancer, antiviral, antimalarial, and antibacterial, antifeedant and antifertility activity of *M. azedarach* [1].

The effectiveness of extracts from fruits and leaves of *M. azedarach* L. has been previously demonstrated against insects [2,3,4,5]. The antifeedant effects of *M. azedarach* extracts are known for many insects [3,4,6,7] *M. azedarach* fruits are believed to be toxic, but toxicity assays of the fruit extract carried out on mammals have not shown any adverse effects when orally administered to rats [8].

The genus *Artemisia*, small herbs and shrubs, is one of the largest and most widely distributed genera of the Compositae (or Asteraceae) [9,10,11]. Members of this genus have botanical and pharmaceutical interest due to their characteristic scent and taste and are used in the liqueur-making industry [9,12].

Artemisia herba-alba Asso, known also as desert wormwood (known in Arabic as Shih, and in French as Armoise herba-blanche. Fr. [13] has been used in folk medicine by many cultures and used in Moroccan folk medicine to treat arterial hypertension and/or diabetes [14,15]. Herbal tea from this species has been used as an analgesic, antibacterial, antispasmodic, and hemostatic agents [16]. During an ethnopharmacological survey carried out among the Bedouins of the Negev desert, it was found that *Artemisia herba-alba* relieved stomach disorders [9]. This plant is

also suggested to be important as a fodder for livestock in the plateau regions of Algeria where it grows abundantly [17,18]. Ascaridae from hogs and ground worms were killed by the oil of the Libyan *A. herba-alba* in a short time [19,20].

The *Artemisia* genus was employed in traditional medicine by many cultures since the ancient periods. The genus was used as a natural pesticide and also in the treatment of human diseases [21]. *Artemisia* is used as analgesic agents, antibacterial, anti-parasite and hemostats, anthelmintic, anti-diarrheal and diuretic whereas several extracts and EOs showed a certain performance of biological activities such as anti-hyperglycemic, antioxidant and anti-inflammatory. Moreover, some species of the genus are frequently used as anti-rabic and for the treatment of certain diseases such as malaria, hepatitis, cancer, and infections by fungi, bacteria, and viruses [22,23,24].

Historically, wormwood was a productive kind in the search for new biologically active compounds. The Phytochemical investigated showed that this genus is rich in secondary metabolites such as essential oils (sesquiterpenes and monoterpenes), flavonoids, coumarins, caffeoylquinic acids, sterols and acetylenes [25].

Furthermore, the determination of the potential antimicrobial activity of *Artemisia Afarensis* EO could be more informative for the future use in controlling phytopathogens and also in clinical treatment as natural antimicrobial agents.

The organisms like *E. amylovora* and *Agrobacterium vitis* were reported to be phytopathogens, causing damage in culture. These phytopathogens cause diseases in any plant tissue it invades, mainly by wilts and rots and a tumor known as crown gall disease (*A. vitis*) [26].

Pyrus communis (Pear) is one of the most important deciduous fruit trees all over the world. It is second rank after apple in production. Moreover, *Pyrus communis* is considered as the most valuable and compatible rootstock of pear under Egyptian conditions. It can grow in different kinds of soils. The traditional propagation technique of pear trees by grafting on quince, seedlings or clonal selection of *Pyrus communis* is not completely satisfactory because of the lack of compatibility with some cultivars, heterogenesis of the pear seedlings and excess growth and also due to the sensitivity of the grafted plants to pear decline [27].

In the present study, a phytochemical screening and the antimicrobial potency of aqueous extracts of *A. herba-alba* and *Melia azedarach* were investigated. *Artemisia herba-alba* to our knowledge has not previously been investigated as an antimicrobial agent study apart from some rare works on phytochemistry.

2. MATERIALS AND METHODS

This study was carried out at Tissue Culture and Germplasm Conservation Research Laboratory, Horticulture Research Institute, Agricultural Research Center, Giza, Egypt. in cooperation with Department of Horticulture, Faculty of Agriculture, Benha University, Egypt from 2016 to 2020.

2.1 Bacteria Source and Plant Material

The drying leaves of *Artemisia herba-alba* were collected from the Experimental Garden of Medicinal and Aromatic Dept. Horticulture Research Institute A. R. C.

The fruits of *Melia azedarach* were collected from the Experimental Greenhouse of Temper Dept. Horticulture Research Institute A. R. C. The seeds and embryos were removed from the fruits. and removed the cover of embryos until usage.

The purified and identified cultures of *Erwinia amylovora* were obtained from the culture collection Borg Al-Arab Academy- Microbiological Lab. By Dr. Sayed Hafez. For preparation of stock suspension for tested bacterial isolate *E. amylovora*, one ml from old culture was aseptically plated onto potato dextrose agar (PDA) and incubated at 37°C for 24 hours. The bacterial growth was washed off with sterilized distilled water to produce suspension and stored in a refrigerator at 4°C.

2.2 Aqueous Extraction

Leaves of *A. herba-alba* were washed with distilled water and dried at room temperature for three days. Powdered leaves (20 g) were mixed with 100 ml of distilled water in an Erlenmeyer flask. The extraction was done on a magnetic heater-stirrer at 80°C for 30 min. The solution was filtered and stored at 4°C until required for use.

The fruits of *M. azedarach* were dried at room temperature for one week and ground in a mortar (10 g) and mixed with 100 ml distilled water in a 250 ml flask. The extraction was done on a magnetic heater-stirrer at 80°C for 30 min. The solution was filtered and stored at 4°C until the time of use.

One gram of *E. amylovora* colonies were separated under aseptic conditions and added in sterile aluminum foil in the oven at 60°C for three hours. The bacteria dry matter was dissolved in 100 ml distilled water and stirred for 30 min. metal solution of the desired concentration of 100 mg/l. After incubation for 24 h, 100 ml of bacterial suspension was centrifuged at 4000 rpm for 10 min. The supernatant was filtered and stored at 4°C until the time of use.

2.3 In vitro Culture of *Pyrus communis* cv. Le-Conte

Explants were thoroughly washed in tap water with a 5% detergent solution (Teepol) for 20 min, followed by 2–3 washes in sterile distilled water. The explants were cut into sizes (2-3.5 cm in length) after removing the leaf sheaths. The cut pieces (micro nodes) were surface sterilized with colorex 20% for 15 min and rinsed 4–5 times with sterilized double-distilled water and then trimmed to 1.0–1.5 cm in length. In each media, free plant growth regulators were supplemented with 30 mg/l sucrose and 0.7% agar. After explant growth and differentiated to plantlets callus was induction with cut leaves to small pieces and cultured on MS media containing 2,4-D at 3 mg/l. The culture was incubated at 25°C ±2 under fluorescent lamps with light intensity of 3000 lux at 16 h photoperiods. The development of shoots was monitored every week.

2.4 Nanoparticles System

2.4.1 Chromium phosphate preparation

14.5 g of potassium dichromate was dissolved in 50 mL deionized water and stirred for 15 min. An

orange solution of potassium dichromate solution was obtained.

2.4.2 Preparation nanoparticles

In a typical experiment, 50 ml of potassium dichromate solution was mixed with 50 ml of either dry bacteria extraction or bacterial solution in a beaker. After mixing and serried at 60°C for 1 hour. The color of the solution changed from orange to green indicating the formation of chromium oxide nanoparticles.

2.5 Antibacterial Assay

2.5.1 Potato dextrose agar method

The nanoparticles synthesized using *Arachis-hypogaea* were tested for antibacterial activity by agar well diffusion method against *E. amylofora*. The pure bacterial culture was subcultured on nutrient agar and potato dextrose agar (PDA) respectively. Wells of 10 mm diameter were made on nutrient agar and PDA plates using gel puncture. The strain was swabbed uniformly onto the individual plates using sterile cotton swabs. Using a micropipette, different concentrations of the sample of nanoparticles solution (10 μ l, 20 μ l, 30 μ l and 40 μ l) was poured onto each well on the plates. After incubation at 37°C for 24 h, the different level of zone of inhibition of bacteria was measured.

2.5.2 Nanoparticles characterization

Characterization of the prepared Cr_2O_3 nanoparticles was carried out by different techniques. UV-VIS spectra were measured using a TU-1901 model UV- VIS double beam spectrophotometer (Beijing Purkinje General).

Samples for transmission electron microscopy (TEM) analysis were prepared by dropping 50 μ l

of the reaction mixtures onto carbon-coated copper TEM grids and were allowed to dry. Excess solution was removed and the grids were further dried before viewing with a TEM microscope (JEOL, Japan; Model JEM2010F) operating at an accelerating voltage at 120 kV.

3. RESULTS AND DISCUSSION

3.1 Nano-chromium Characterization

Fig. 1. presents the UV-vis absorption spectra from 225 nm to 545 nm of the solution containing the nano chromium particles. The solutions containing nano chromium particles that were reduced by various extractions (Fig. 1). The UV-vis absorption spectra of the nanoparticles solution showed a dominant absorption peak at about 360 ~ 580 nm [28]. (Fig. 1) presents the UV-vis absorption spectra (from 225 nm to 545 nm) of the solution containing the nano chromium particles. A dominant absorption peak was observed at 525 nm and 562 nm for nanoparticles formed by *A. herba-alba* and *M. azedarach* respectively indicating the presence of the nano chromium particles. The Fig. 1 also shows that the position of the absorption peak is still stable in the wavelength area with nanoparticles containing *A. herba-alba* concentration from 1 to 3 times (data not shows). Moreover, the size of the nano chromium particles increased with a redshift of the UV-vis absorption spectrum with applying *A. herba-alba* following by *M. azedarach*, dry matter of bacteria.

On the other hand, according to Fig. 1 the highest absorption peaks frequency was observed for *A. herba-alba* indicating to reducing agents in *A. herba-alba* extraction that reduced chromium to Cr_2O_3 following by *M. azedarach* extraction and dry matter bacteria.

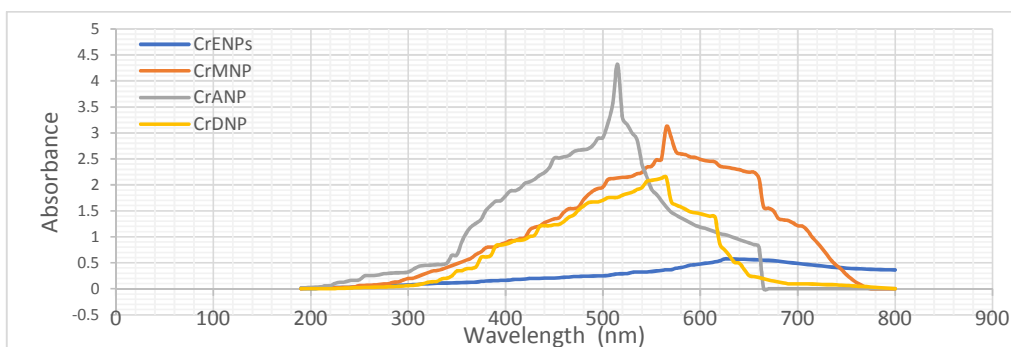


Fig. 1. UV-vis absorption spectrum of Cr_2O_3 nanoparticles in aqueous solution of *M. azedarach*, *A. herba-alba*, dry matter and bacterial extraction

Table A. Concentration of *A. herba-alba* and *M. azedarach* as a stabilizer and nanoparticles materials

Stabilizers	Cr ₂ O ₃
<i>Melia azedarach</i>	50:50 ml
<i>Artemisia herba-alba</i>	50:50 ml
Bacteria dry matter sol	50:50 ml
Bacteria extraction	50:50 ml

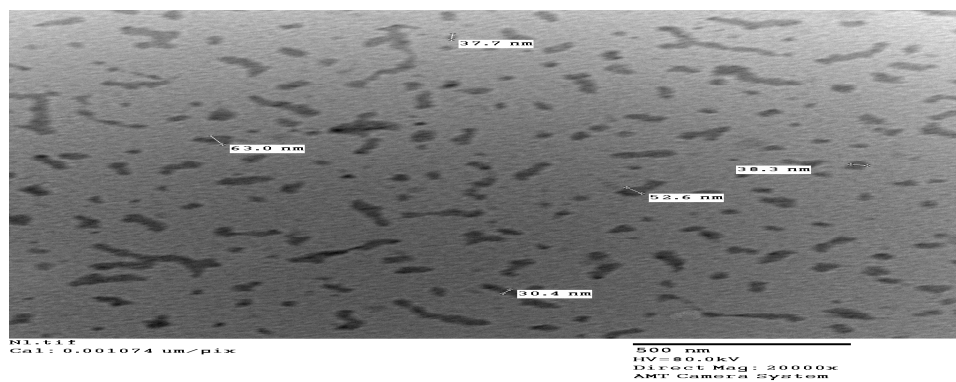


Fig. 2. TEM micrograph of Cr₂O₃ nanoparticles in aqueous solution of *A. herba-alba* after dilution with water three times

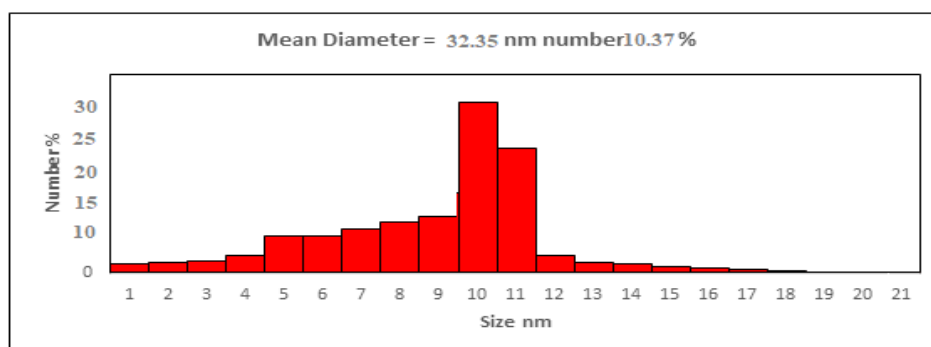


Fig. 3 Chromium nanoparticles size laded on *Artemisia* extraction

TEM has been employed to characterize the size, shape, and morphologies of formed chromium nanoparticles. TEM image is the evidence that the morphology of chromium nanoparticles is nearly spherical and few nanoparticles were agglomerated. The nanoparticles were not in direct contact even within the aggregates and were surrounded by a thin layer of organic material, indicating stabilization of the nanoparticles by *M. azedarach* agent. Figs. 2 and 3. presents the TEM images of the chromium particles prepared in aqueous solution of *Artemisia* and size of chromium particles was 32.35 nm as average of 10.37 number of particles counted.

3.2 Antimicrobial Effect of Greening Nano-chromium

3.2.1 Direct antibacterial effect

In this trail the effect of nanomaterial on bacteria without a biological system was examined. The antibacterial activity of chromium nanoparticles was investigated against pathogenic fire blight bacteria of gram-negative strains (*E. amylofora*) using a disc diffusion technique (Fig. 4). The diameter of the inhibition zones around each disc with chromium nanoparticles is represented in Table 1. The highest antibacterial activity was observed against *E. amylofora* for chromium / *A.*

herba-alba (31 mm) followed by chromium / *M. azedarach* (25 mm) and chromium solution Cr (19.0 mm) while the least activity noticed against *M. azedarach* (2 mm) and *A. herba-alba* (4 mm). The number of bacterial colonies grown on agar plates as a function of the different concentrations of chromium nanoparticles gradually declined when the concentrations of nanoparticles were increased and loaded in *A. herba-alba*.

Disc with chromium nanoparticles is represented in Table 1. The highest antibacterial activity was observed against *E. amylovora* for chromium / *A. herba-alba* (31 mm), chromium / *M. azedarach* (25 mm), Cr (19.0 mm), followed by *M. azedarach* (2 mm) and *A. herba-alba* (4 mm). The number of bacterial colonies grown on agar plates as a function of the different concentrations of chromium nanoparticles gradually declined when the concentrations of nanoparticles increased and loaded in *A. herba-*

alba. Results demonstrated that newly synthesized chromium nanoparticles are promising antibacterial agents against the pathogens employed.

The formation of complexes with sulfhydryl groups can inactivate vital cell surface enzymes and hinder the respiration at the cell membrane [29]. Inducing a proton leakage through the membrane, discharging K^+ ions, inhibition of energy-dependent sodium transport in membrane, inhibition of cell division and damaging bacterial cell envelopes, interaction with hydrogen bonding processes are also recorded as antimicrobial actions of NS [30,31,32]. Silver nanoparticles can also disturb the integrity of the plasma membrane of fungal cells [33]. The association of silver nanoparticles with the envelope of certain viruses has been suggested to prevent them from being infected [34].

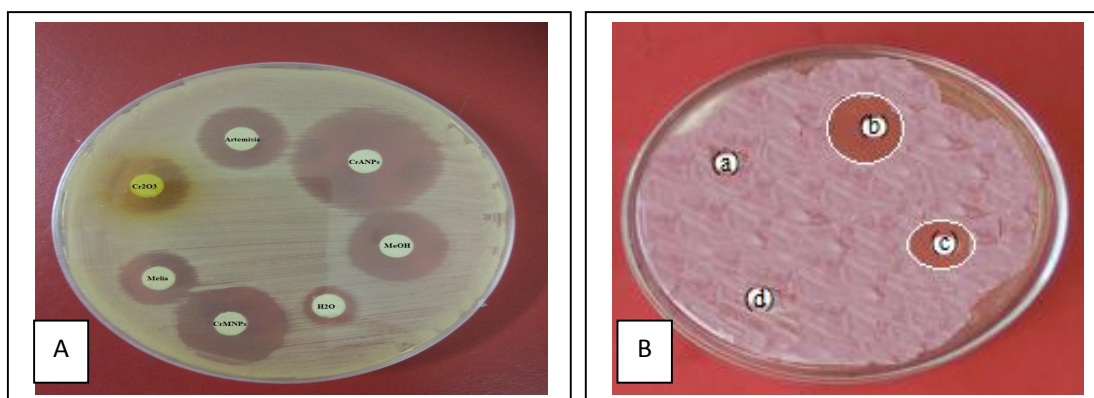


Fig. 4. Antibacterial activity of synthesized chromium nanoparticles by (A) natural extracts (B) by bacteria fragment and extractions against *E. amylovora* bacteria. (a) bacteria extraction (b) dry matter

Table 1. Effect of chromium nanoparticles and plant extractions on *E. amylovora* inhibition zone growth of *in vitro* cultures

Treatments	Inhibition zone in mm
Water	0.00
Methanol	10.0
<i>Melia azedarach</i>	2.00
<i>Artemisia herba-alba</i>	4.00
Chromium solution	19.0
Chromium/ <i>Melia</i> NPs	25.0
Chromium/ <i>Artemisia</i> NPs	31.0
Chromium/ Dry matter NPs	10.0
Chromium/ extraction NPs	13.0
LSD 5 %	2.871

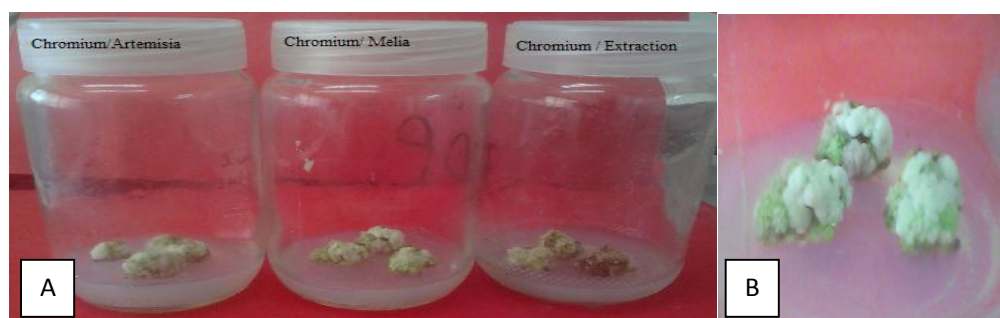


Fig. 5. Effect of chromium nanoparticles against fire blight bacteria on pear cell growth

Table 2. Effect of nanoparticles materials and plant extractions on *E. amylovora* contaminated callus by *E. amylovora* growth of *in vivo* culture

Treatments	Contamination %	Survival %	Color
Water	100.0	0.0	Black
Methanol	60.0	0.0	Brown
Chromium/ <i>Melia azedarach</i> NPs	0.0	50.0	Green
Chromium/ <i>Artemisia</i> NPs	0.0	75.0	Green
Chromium/ Dry	50.0	25.0	b/g
Chromium/ extraction NPs	50.0	25.0	b/g
LSD 5%	22.908	12.33	

3.2.2 Indirect antimicrobial effect

According to the data presented in Table (2) the callus material was exposed to the chromium nanoparticles by using *M. azedarach* and *A. herba-alba* as a stabilizing agent of particles compared to 70% methanol after immersion of 1 hour in *E. amylofora* bacterial suspension. The contamination percentage was 0.0% for chromium nanoparticle loaded on *M. azedarach* and *A. herba-alba* followed by those treated with bacterial dry matter and their extractions (50% for each). On the other hand, the highest survival percentage was observed in the treatment of chromium with *A. herba-alba* was the highest value (75%) recorded in this parameter.

3.2.3 Antimicrobial Effect of Biological Nano-chromium

Bacterial fragments of cell walls containing its polysaccharides binding with chromium atoms formed nano-chromium Cr_2O_3 . The direct effect of chromium nanoparticles on fire blight bacteria was weak which scored 10 mm for dry material and 13 mm for extraction. This inhibition returns to some fragment of dry matter of bacteria containing some polysaccharides receptors that reduces chromium to Cr_2O_3 nanoparticles in this concern, [35] reported that high chromate concentration prevents multiplication of bacteria.

They observed There was a significant difference ($p \leq 0.05$) between the growth of the isolates at different concentrations and time.

On the other hand, the indirect effect of dry matter and extracts of bacteria on bacterial growth were observed on callus; the growth of callus turned to brown in color (Fig. 5) and slower growth rate of callus was observed as compared to with that treated with *Artemisia* and *Melia*. This reaction returned to some free polysaccharides attach the callus cell membrane surface and linked with bacteria receptors that increase signal transduction in the cells to increase oxidized enzymes activity (peroxidase, polyphenoloxidase and catalase) and increasing polyphenols compound in the cells.

In this case, [36] found that the values indicated that nano particles of Cr_2O_3 synthesized from all the three methods (biological, electrochemical in presence $\text{K}_2\text{Cr}_2\text{O}_7$ and electrochemical with platinum) showed inhibiting effect towards different bacteria. However, the nano Cr_2O_3 (41 nm) synthesized with electrochemical with platinum method showed inhibition effect close comparable to that of the standard. The figure shows the zone of inhibition for bacterial growth on agar plates. Hence, the results clearly demonstrated that the newly synthesized Cr_2O_3 nanoparticles are promising antimicrobial

potential as an antimicrobial agents against target bacterium.

4. CONCLUSION

It can be concluded that a colloidal solution of NC at lower concentrations (e.g. 100 ppm) can be used as a disinfectant for plant cells, especially for *in vitro* cultures; however, at high concentrations, it inhibits cell growth, which may be due to its toxicity against eukaryotic cells. In pear plants, the use of NC at more than 100 ppm against *E. amylovora* fire blight bacterium could decontaminate *in vitro* infected callus cell.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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