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SUSTAINABLE BIOSYNTHESIS OF COPPER OXIDE NANOPARTICLES USING *GALIUM APARINE* EXTRACT FOR ANTIBACTERIAL APPLICATIONS

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ABSTRACT

The present study was designed to examine the antibacterial activity of the green synthesized copper oxide nanoparticles (CuONPs) utilizing *Galium aparine.* CuONPs were fabricated using fresh vegetative parts of *Galium aparine.* UV–visible absorption spectra showed that absorbance peak at 260 nm. AFM exhibited spherical and semi-spherical NPs.size range between 10-90nm with an average diameter 32.1nm. roughness with average 1.22nm. Image of FE-SEM was demonstrated CuONPs with dense, relatively uniform distribution, spherical and semi spherical morphology. Also, they tend to agglumerate or cluster together. FTIR illustrated shifting in functional group peaks with changes in peak intensity indicate the interaction between functional groups of plant extract components and metal ions leading to NPs synthesis. Biosynthesized CuONPs were used to evaluate their antibacterial efficacy against four bacterial species: *S. aureus, Strept. mutans, K. pneumoniae and E. coli* using well diffusion and disc methods. NPs displayed significantly lower MIC values than gentamycin as standard refrence for all tested bacteria, showing greater inhibitory effects against bacteria, higher concentrations produced a significantly larger inhibition zones, in contrast lower concentrations or control displayed insignificant or no inhibition. They exhibted broad spectrum antibacterial activity against both gram- positive and gram-negative bacteria.

Keywords: Copper Oxide Nanoparticles; *Galium Aparine* L.; *Staphylococcus aureus, Streptococcus mutans, Klebsiella pneumoniae, Esherichia coli,* Antibacterial Activity.

1 INTRODUCTION

1.1 Nanotechnology

Has gained considerable attention as aversatile technology with extensive applications in various fields. where they utilized in fields particulary medicine, energy and industry, agriculture, cosmetics and many other implementations. It offers numerous advantages due to its high surface area to volume ratio, resulting in distictive physicochemical properities that support its application in diverse industrial and scientific sectors (Alishah *et al*., 2017). Nanotechnology

appeared as advancing field in the prevent and manage infections diseases, providing novel approaches via the development of nanomaterials with antimicrobial activities (Arif, 2023; Khudhair, 2023).

1.2 Copper Oxide Nanoparticles (CuONPs)

Plants with an antioxidant properities utilized in NPs synthesis. These plants parts possess reducing properities that faciliate the reduction of precursor salts. Wide range of medicinal plants exhibt antioxidant owing to their diverse phytochemical constiutents, such as carboxylic acids, proteins, phenols, tannins, flavonoids, terpenoids...etc. The biomolecules present in plant extract play a crucial role in reducing metal precursors and stabilizing the synthesized NPs (Bhavyasree and Xavier, 2021; Mali *et al*., 2020; Rajeshkumar and Rinitha, 2018). Analytical grade copper compounds, including copper acetate, copper sulfate, copper nitrate, and copper chloride are used as precursors, while plant parts such as leaves provide natural bioactive compounds (Bhavyasree and Xavier, 2021; Mali *et al*., 2020), seed (Rajeshkumar and Rinitha, 2018; Venkatramanan, *et al*., 2020) flowers (Sackey, *et al*., 2020; Karunakaran, *et al*., 2020), roots (Chandrasekaran, *et al*., 2020), fruit (Amer and Awwad, 2021).

CuONPs one of metal oxide nanoparticles gained substantial attention due to their remarkable physicochemical properities, such as high ratio of surface to volume, extraordinary reactivity and wide spectrum antimicrobial activity (Khudhair, 2023). I's relatively low-cost metal in comparision with gold and silver (Jia *et al*., 2012). CuONPs derived from inorganic metals have attracted considerable attention in recent years for several important reasons. Their ready availability, low production costs and antimicrobial properities have contributed significantly to their growing recognition. CuONPs have shown remarkable antimicrobial activity against abroad spectrum of harmful microorganisms, including fungi and bacteria. The synergy between cost-effectiveness and antimicrobial performance emphasizes their growing relevance in various applications, such as water purification, materials engineering and medical care, where effective and economical antimicrobial solutions are increasingly required (Sankar *et al*., 2014).

The antimicrobial potential of CuONPs result from multiple mechanisms including the formation of reactive oxygen species (ROS), copper ions release (Cu^{+} and Cu^{2+}), damage the bacterial cells membrane, interference with important enzymatic functions and DNA degradation. CuONPs exhibted bactericidal activity against Gram negative and Gram positive bacteria, including clinically significant pathogens for example methicillin resistant *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Eschericia coli* (Khairy *et al*., 2024; Abed *et al*., 2021). Overall, CuONPs represent promising materials for healthcare innovation, with potential applications in infection managment, diagnostic technologies, drug delivery systems and therapeutic development. Their potent antimicrobial activity, copuled with their distinctive nanoscale characteristics, offers opprtuities to adress pathogenic organisms and medical interventions (Jain *et al*., 2021).

Conventional methods for CuONPs fabrication including chemical methods like microwave irradiation, sol-gel and precipitation, as well as physical msuch as electrochemical reduction, thermal decomposition and vapor deposition, which involve generate toxic by-products, high energy utilization and hazardous chemicals, Threaten human health and environmental safety(Khudhair, 2023; Arif, 2023).These limitations have led researchers to explore for eco-friendly, inexpensive and sustainable substitutes. Biosynthesis, which, uses natural resources such as fungi, algae, bacteria and extracts of plant as reduction and stabilizing agents emerged as aviable an effective strategy for overcoming these limitations (Khudhair, 2023; Arif, 2023; Okoye *et al*., 2023).

1.2.1 Galium aparine

(*G. aparine* L.), commonly known as catchweed bedstraw or cleavers, belongs to the Rubiaceae family. It' s an annual herbaceous plant, widely distributed in North America, Europe and parts of Asia. It was considered as source of different biological active phytoconstituents, such as flavonoids, phenolic acids, alkaloids, saponins, tannins, coumarins, anthroquinones, fatty acids, lipophilic compounds and iridoid glycosides (Al-Sanafi, 2018; Shynkovenko *et al*., 2026). Polyphenolic contenents of *G. aparine* extract were identified, they are gallic acid, ferulic acid and quercetin (Valse *et al*., 2014). The plant has long history for using in traditional medicine, it used as choleretic, blood purifier and diuretic. In addition to its use for infection of urinary tract, treatment of skin disorders for example: psoriasis, eczema, chronic wounds and ulcers (Canli *et al*., 2026).

Extracts of plants contain different phytochemicals including alkaloids, terpenoids, flavonoids, saponins, polysaccharides and phenolic compounds, which act as both reducing agents for ions of metals, capping and stabilizing agents in NPs synthesis (Khudair, 2023; Okoye *et al*., 2023). This method provides several benefits compaired with convential techniques, including simplicity, expandability, economic efficiency and environmental sustainability (Khairy *et al*., 2024; Arif, 2023). Green synthesis of CuONPs successfully carried out using different plant sources. Khudhair (2023) utilized gum Arab extract in bio- fabrication of CuONPs, producing spherical and semispherical NPs with diameter average 36.47nm, as confirmed by FE-SEM analysis. UV-visible absorption spectrum exhibted peak at 224nm (Kudhair,2023). CuONPs synthesized using *Aloe vera* leaf exteract appered with spherical shape, size of particles range 40-100nm with significant antibacterial activity with inhibition zone: *S. aureus*(17.1mm), *P. aeruginosa*(17mm)

and *E. coli*(16.8mm) (Abed *et al*., 2021). Using ethanolic extracts of *Azadirachta indica* (Neem) and *Simmondsia chinensis* (Jojoba)for fabrication of CuONPs, characterized as semi spherical NPs with sizes range from 10.7-30.9nm and showing MIC values of 62.5-125µg/mL against pathogens: *E. coli*, *P. aeruginosa, K. pneumoniae, Acinobacter spp*. and *Sterptomonas maltophilia* (Khairy *et al*., 2024). Leaf extract of *Vernoonia amygdalina* utilized for CuONPs biosynthesis, showing in FTIR ananlysis the involvement of alkene, hydroxyl, the alkene functional groups from plant phytochemicals in NPs formation and stabilization (Okoye *et al*.,2023). *Aspergillus niger* extract was utilized to biosynthesis CuONPs, producing spherical shape NPs with average size of 15nm. FTIR analysis, which used to identify the functional groups including C=C, N-H and Cu-O bonds, Antibacterial activity of CuONPs, was depended on concentration. The highest inhibitions zones noted aganist *Salmonella typhimurium*(12.2mm) and *E. coli*(11.1mm) at 200 µg/mL (Arif, 2023).

Antibacterial activity of bio-synthesized CuONPs widely reported against abroad range of pathogenic bacteria. The mechanism action of them is multifactorial and includes electrostatic copper ions and negative charge of interactions between the positive charge of bacterial cell surface, lead to membrane disruption, permeability increasing and cytoplasmic contenents leakage (Arif, 2023; Abed *et al*., 2021). Also, the CuONPs can penterate bacterial cells and interact with DNA, inhibiting replication and interfering with enzyme activity (Abed *et al*., 2021). Generation of ROS, for example hydroxyl radicals and superoxide anions, activate oxidative stress, which damage the cellular components for example lipids, nucleic acids and proteins (Khairy *et al*., 2024). Antimicrobial activity of CuONPs influenced by various factors such as, particle shape, size, surface charge and concentration, with the smaller particles exhibted enhancing the activity as a result to their larger surface area and improve cellular penetration (Khudair,2023; Abed *et al*., 2021). Green synthesized CuONPs have demonstrated significant antibiofilm and ant-quorum sensing activites, which are especially important in chronic infections and biofilm related diseases (Khairy *et al*., 2024). The appearance of multidrug-resistant (MDR) of bacterial pathogens has become one of critical challenges facing the modern medicine, which threatening the activity of traditional antibiotic therapies, consequently increasing pressures on healthcare systems around the world (Mahdi and Mustafa,2026) The excessive and inappropriate use of antibiotics has accelerated the development of resistant bacterial strains, for example: Carbapenem resistant *P. aeruginosa*, Methicillin resistant *S. aureus* (MRSA) and Enterobacteriaceae producing extended -spectrum β-lactamase (ESBLs), reducing the effectiveness of numerous first line therapies (Khairy *et al*., 2024).

The efficacy of antimicrobial of CuONPs is associated with several synergistic mechanisms, including the production of reactive oxygen species (ROS)and the release of copper ions (Cu and Cu^{+2}), DNA fragmentation, damage of bacterial cell membrane and implairment (Khairy *et al*., 2024; Abed *et al*., 2021). Significantly, CuONPs have exhibted bactericidal effects against both Gram negative and positive bacterial species, including Methicillin resistant *S. aureus* (MRSA), *P. aeruginosa*, *E. coli* and *K. pneumoniae* (Mahdi and Mustafa, 2025; Khairy *et al*., 2024; Abed *et al*., 2021)

Gentamicin is an aminoglycoside antibiotic primarily effective against aerobic Gram-negative bacteria, including *E. coli*, *Pseudomonas*, and *Klebsiella*. It also exhibits synergistic activity against certain Gram-positive bacteria when combined with other antibiotics. However, it is ineffective against anaerobes due to its oxygen-dependent cellular uptake mechanism (Mohan *et al*., 2015). Gentamicin is a broad-spectrum aminoglycoside antibiotic extracted from *Micromonospora purpurea*. It ’s most widely used due to the relatively broad spectrum of its antibacterial properties and resistance to heat. Gentamicin has the ability to kill *E. coli* bacteria It exhibits powerful bactericidal activity by irreversibly binding to the bacterial 30S ribosomal subunit, which disrupts protein synthesis and causes the misreading of genetic code, work by inhibiting protein synthesis mechanism (Sulistiyaningsih *et al*., 2017; Mullins *et al*., 2016; Dong *et al*., 2011). Gentamicin widely used as chemotherapy drugs to prevent and treat bacterial. Infections. Gentamicin is commonly used to treat severe infections caused by *S. aureus*, *E. coli*, *P. aeruginosa*, *Klebsiella spp*. (Dongmei *et al*.,2016).

The purpose of this research was to green-synthesize CuONPs by utilizing *G. aparine* L. extract of fresh vegetative parts and investigate the antibacterial activity of these NPs in different concentrations on four pathogenic species of Gram-positive and Gram-negative bacteria: *Staphylococcus aureus, Streptococcus mutans, Kiebsiella pneumoniae* and *Eschericia coli*. The aim of this study to know the antibacterial activity of biosynthesized CuONPs and their potential as a sustainable antimicrobial nanomaterials suitable for biomedical and environmental applications. The goal is to develop an eco-friendly and cost-effective alternative to conventional chemical synthesis methods that often involve toxic reagents and high energy inputs.

2 MATERIALS AND METHODS

2.1 Green synthesis of Copper Oxide Nanoparticles

The method of El-Abeid *et al*., (2024) was followed with minor modifications for green synthesis of CuONPs from the extract of *G. aparine* plant.

2.2 Preparation of *G. aparine* extract

G. aparine were collected from AL Nahrain University gardens. Fresh vegetative parts were washed thoroughly under running water to remove any contaminated and pollutants then rinsed with deionized distilled water (D.D.W.). To prepeare aqueous extract, in flask 250ml size, 100 ml of D.D.W were combined with 25g of vegetative parts of *G. aparine* and heating at 65°C for 60 min on heating stirrer. Allowing the extract to cool to room temperature and left for overnight, then filtered by Whatman No.1 until pure aqueous extract obtained, which stored at 4 ◦ C until use.

2.3 Synthesis of copper oxide nanoparticles

10 ml of pure aqueous extract added gradually to 90 ml of 10 nM of Copper sulphate pentahydrate (CuSO4.5H2O) in flask with size 250ml heats on magnetic stirrer hot plate at 80°C for 60-70 min, CuONPs finally produced, color change of mixture was an evidence of CuO NPs synthesis. Solution of CuONPs centrifuged for 30 min at 6000 rpm, supernatant was discarded, to purify CuONPs, the pellets were washed with deionized distilled water with repeated centrifugation three times. Dried in vacuum oven at 60 °C for 24 hours and then underwent to characterization of CuONPs.

2.4 Characterization of CuONPs

Using Ultraviolet-visible spectroscopy (UV-Vis), Atomic Force Microscopy (AFM) Scanning Electron Microscope (SEM) and Fourier-Transform Infrared Spectroscopy (FTIR),

3 ANTIBACTERIAL ACTIVITY OF CUONPS

3.1 Determination of Minimum Inhibitory Concentration (MIC)

Two-fold serial dilutions (1-1024µg/mL) of Gentamicin and exopolysaccharide were prepared from stock (10mg/ml) in microtiter plate using Muller-Hinton broth as dilluent. An inoculation of all wells with 20µL with suspension of bacteria comarable to McFarland standrad no.0.5(1.5× 108CFU/ML) was except for the negative control wells. The microtiter plates were incubated for 18-20 hrs. at 37°C, then addition of 20µL of resazurin dye to all wells and incubated for 2hrs. to observe any changes of color. Visually determination of sub-MIC concentrations in broth micro dilutions as the lowest concentrations at which color change from blue to pink in the resazurin broth assay. an antibiotic(Gentamicin) replaced the CuONPs (positive control). In column 2, only the culture medium, the microbial suspension and the resazurin were present (negative control) (Ohikhena *et al*., 2017).

3.2 Well Diffusion Assay

Single colonies of *S. aureus, Strept. mutans, E. coli,* and *K. pneumoniae* were taken and cultured on muller hinton agar, then incubated at 37°C for 18 hrs. The colonies were transffered into 5ml of seterile normal saline and mixed by using vortex mixer. The suspension turbidity of was adjusted to 0.5McFarland standard, corresponding to 1.5 $\times 10^8$ CFU/ml. Asterile of cotton swab was immersed in standardized bacterial suspension. Plates of Muller Hinton agar were streaked with cotton swab in two to three directions. In order to allow the absorption of excess moisture. Inoculated plates left at room temperature for 10 mins. Four concentrations of CuONPs were prepered, including µg/mL. Wells on agar were created with attention to leave an equal distance between them to prevent overlapping inhibition zones. Each well filled with 80µL of each CuONPs concentration by using sterile Pasteur pipette. Agar plates were incubated at 37°C under aerobic conditions, then antibacterial activity was evaluated by measuring the inhibition zone diameter in millimeters(mm) around each well by using ruler. The experiment is repeated, but using filter paper discs saturated with the different concentrations of CuONPs (Hlila,2017).

4 RESULT AND DISCUSSION

As shown in **Figure1**: The fabrication of biosynthesized CuONPs, a solution of 90 mL copper sulfate was mixed with 10 mL of fresh vegetative of *G. aparine* extract at ratio 9:1(v/v) and stirred continuously at 65°C for 60 min. As shown in **Figure1**, color of the solution changed from light blue to dark green during the reaction. This evidence of the formation of CuONPs,This was confirmed using UV– vis spectroscopy, which showed the electronic spectrum caused by the surface plasmon resonance (SPR) effect (Chandraker *et al*., 2019; Ansilin *et al*., 2016). It's agree with El-Abeid *et al*., (2024) and Okoye *et al*., (2023).

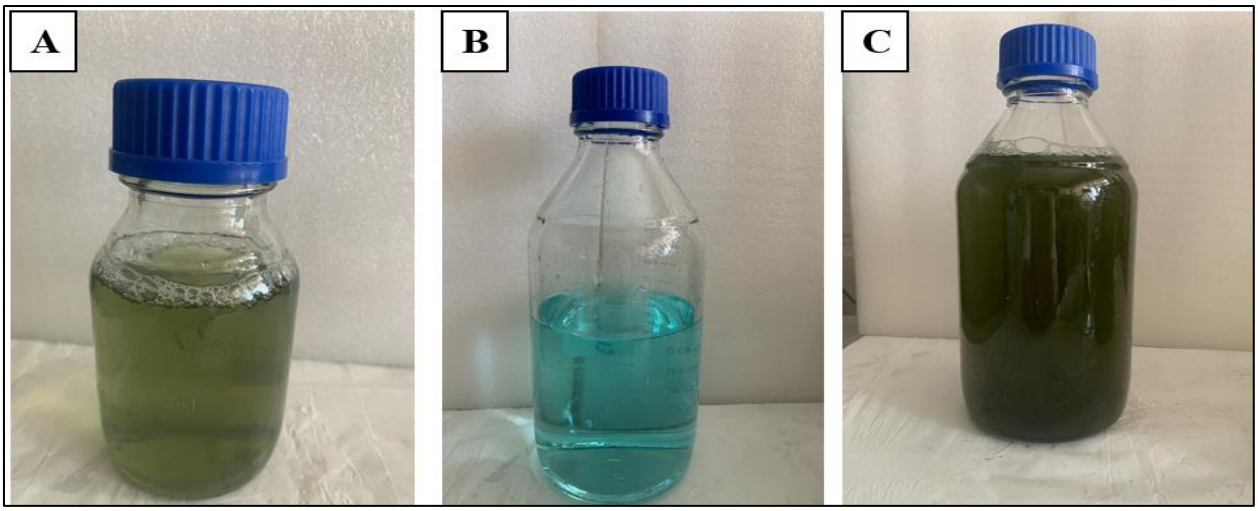


Figure 1 (A): Aqueous extract of *Galium aparine* B- Mixture of extract with 10mM of CuSO4.5H2O (C)green synthesized CuONPs solution

UV-visible analysis Absorption spectrum of the green synthesized CuONPs using fresh vegetative parts of *G. aparine* was noted over wavelength range between 200-800nm to analyze their optical properities. The spectrum distinct maximum at approximately 260nm, then the sbsorbance decreases sharply between approximately 270 and 350nm, followed by more gradual decline, as illustrated in **Figure 2**. The crrent study closely agree with Abed *et al*. (2021). They used an aqueous extract of *Aloe vera* for CuONPs green synthesis, the color of mixed solution from the aqueous extract with copper sulphate pentahydrate was turned from light green to dark green. Also, UV-visible spectroscopy confirmed the absorption peak at 260 nm. El-Abeid *et al*., (2024) observed the absorption peak at approximately 290nm for biosynthesized CuONPs using *Ziziphus spina-christi* leaf extract, this difference may represent differences in conditions of fabrication, sample composition, particle characteristics.

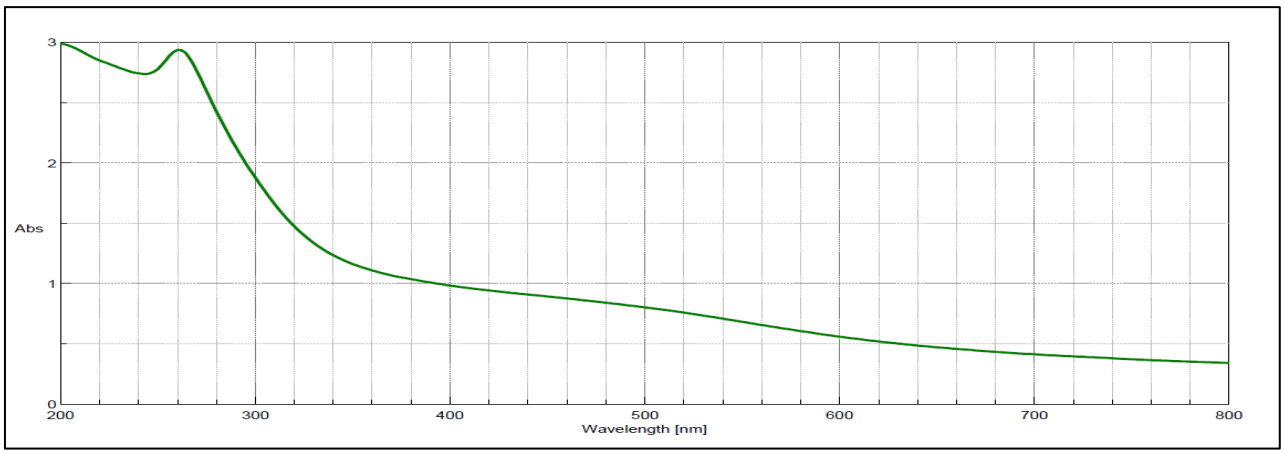


Figure 2: UV-vis spectroscopy of CuONPs

High resolution topography data by AFM demonstrated the size, shape and surface features of CuONPs biosynthesized. 2D and 3D AFM images with distribution histogram revealed that predominantly spherical and semi-spherical NPs. The particle size distribution Histogram confirms narrow and uniform size range between 10-90nm with an average diameter 32.1nm. Green synthesized CuONPs exhibted well-defined, separated NPs with smoother surfaces, improved homogeneity and reduced roughness significantly (average 1.22nm). the height distribution and granularity analyses showed the reduction in the particle size a following the formation of NPs, indicating an active reduction of Cu^{+2} and stabilization of CuONPs by plant molecules, as shown in **Figure3**

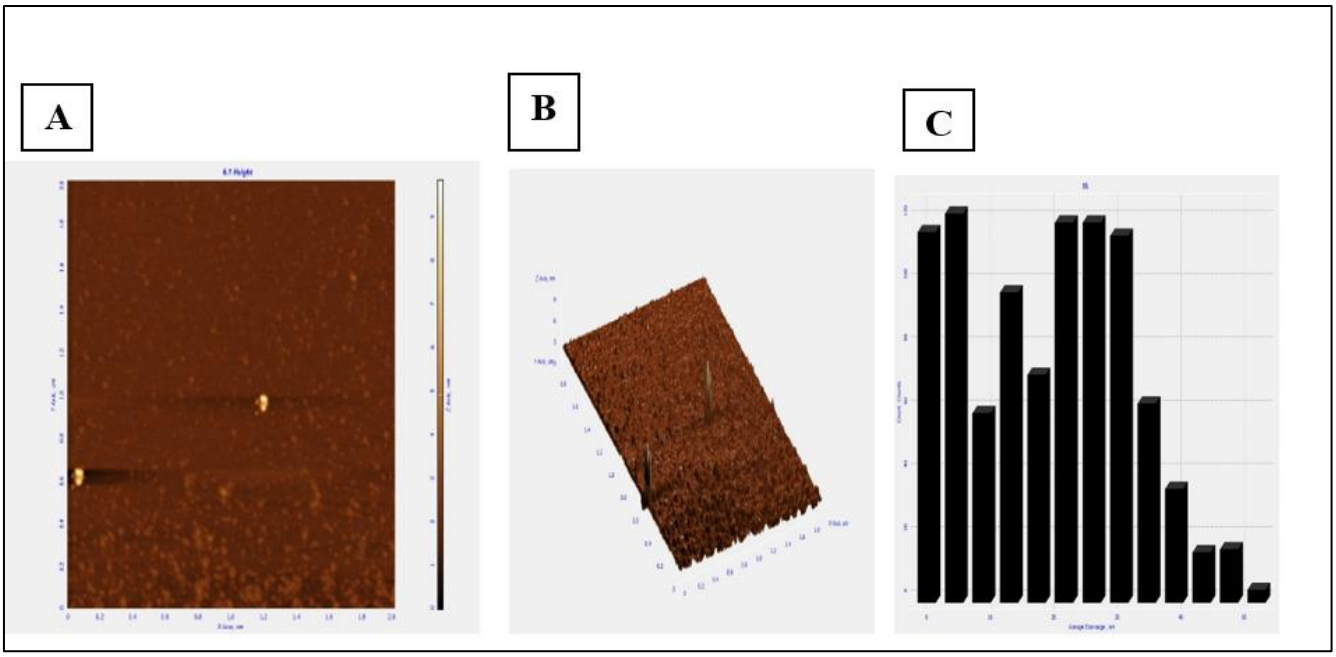


Figure 3: AFM of CuONPs: A- 2D B-3D C- Granularity accumulation distribution of CuONPs

Figure 4 highlights that CuONPs were appeared densely packed rather than sparsly scattered. Field Emission Scaning Microscopy (FE-SEM) used to examine the surface morphology. The image exhibts adense, relatively uniform distribution of CuONPs. The nanoparticles reveal a predominantly spherical to semi spherical morphology, densely packed rather than sparsly scattered. Although some particles agglomeration was observed, this is a common occurance in nanopowders due to their high surface energy, the individual primary particles remain distinguishable. Images with high magnification confirms the successful formation of nanostructures with narrow size distribution, which is essential for acheieving high surface area to volume ratio, which improve antibacterial activity. Image provides higher resolution, this high magnification enables for clearer observation of the idividual semi- spherical primary particles, indicating the nanostructural integrity. These results are agree with what has been concluded by Arif, (2023) and Khudhair, (2023), that green synthesized CuONPs exhibt aspherical and semi spherical morphology. Also the NPs tend to agglumerate or cluster together.

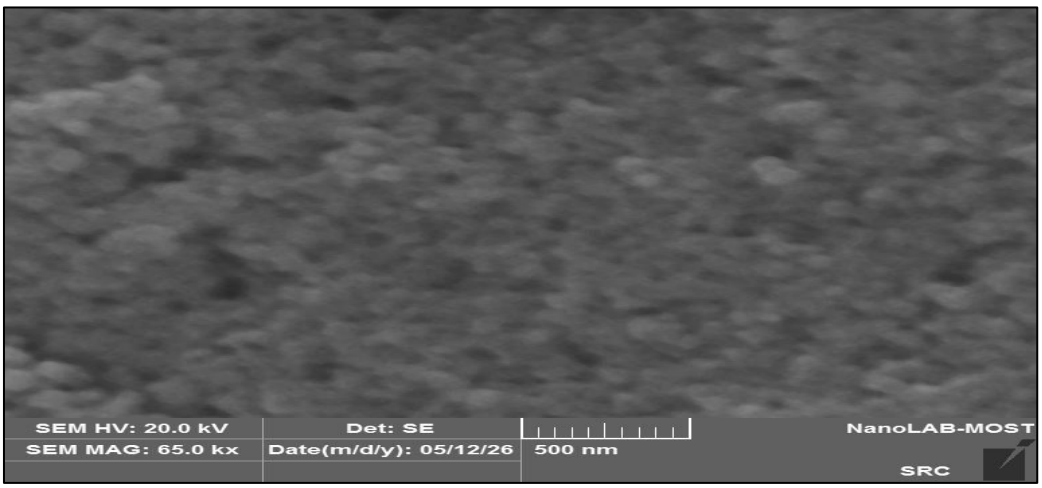


Figure 4: FE-SEM of CuONPs

Figure 5 illustrated that FTIR was utilized to analyze the aqueous extract of fresh vegetative *G. aparine* and the green synthesized CuONPs, to identify the biomolecules responsible for the reduction, capping and stabilization of CuONPs. The aqueous extract of fresh vegetative parts of *G. aparine* exhibts significant peak at [~3400 cm^{-1}] (O-H stretching of alcohols and phenols), [~1640 cm^{-1}] (C=O stretching of amides or C=C stretching of aromatic rings), [~1380 cm^{-1}] (C-H bending), [~1060 cm^{-1}] (C-O stretching ether, ester or alcohols) and [~610 cm^{-1}] (C-S stretching of specific ring vibrations), which were attributed to the functional groups of bioactive constituents (Vlase *et al*., 2014; Shynkovenko *et al*., 2018). The peaks at [~615], [~587], and [~548 cm^{-1}] in CuONPs spectra were reflect the vibrational characterstics

of metal-oxide bonds (52). The broad peak at [~3352 cm^{-1}] for NPs is assigned to hydroxyl(O-H) functional groups, demonstrating the contribution of biomoecules or water in NPs. The shifting in functional group peaks with changes in peak intensity indicate the interaction between the components of plant extract and metal ions, leading to NPs synthesis.

The FTIR for this study align with its known phytochemical profile. which confirms that *G. aparine* contain tannins, phenols, alkaloids, flavonoids (such as rutin, luteolin and quercetin), and phenolic acids (such as caffeic and chlorogenic acids) (Al-Sanafi, 2018; Valse *et al*., 2014). These compounds possess carbonyl (C=CO) and hydroxyl(-OH) groups that actively contribute in the reduction of metal ions and act as capping agents (Shynkovenko *et al.,* 2018).

The recent study agree with Baral *et al*., (2025), who observed that FTIR of *Mentha* leaf extract shift are highly consistent. Both studies observe abroad O-H stretching band shifting slightly upon formation of NP (from 3285 cm^{-1} in *Mentha* extract to 3352cm^{-1} in NPs). Additionally, the appearance new, sharp peaks in the 500-650cm^{-1} region affirms the successful formation of Cu-O bond, a phenomenon noted cross different biosynthesized CuONPs (Naz *et al*., 2023).

In addition, the AQS and antimicrobial study by Canli *et al*. (2026) emphased that ethanolic extract of *G. aparine* was rich in long chain of fatty acids (palmitic and linolenic acids) and terpenoids (phytol and neophytadiene). these lipophilic compounds, also contribute to the C-H stretching and C=O bands identified in the FTIR spectrum of aqueous extract. Further indicating that a complex matrix of phytochemicals was responsible for bioreduction, capping and stabilization of CuONPs.

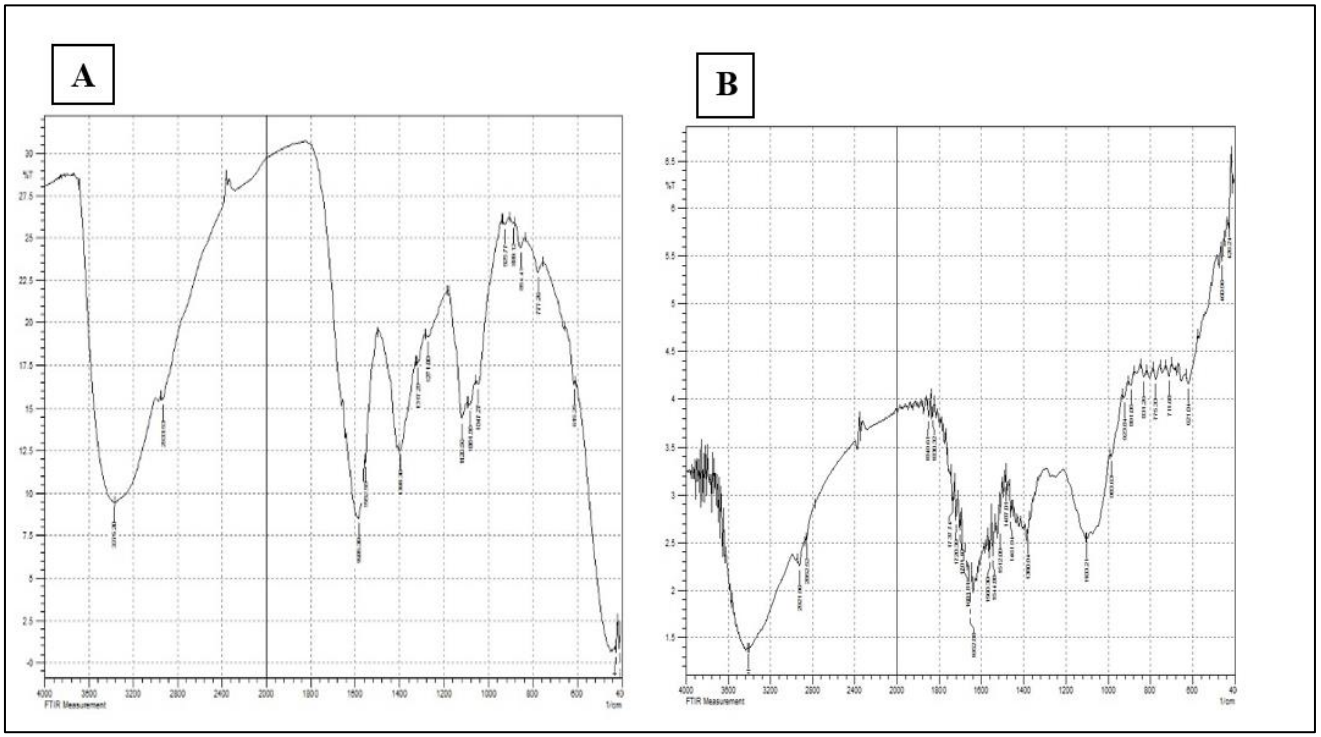


Figure 5: FTIR OF : A- Aqueous extract of *G. aparine* B- CuONPs

4.1 Minmum Inhibitory Concentration(MIC) and Sub -MIC Evaluation

The **Table 1 and figure6** shows the evaluation of MIC and sub-MIC values of greensynthesized CuONPs compared with Gentamicin antibiotics as standard refrence using the broth microdilution method across two Gram positive and two Gram negative bacterial species: *S. aureus, Strept. mutans, K. pneumoniae and E. coli*, as shown in **figure 6**. The MIC represents the lowest concentration of an antimicrobial agent that prevents visible microbial growth under the specified experimental conditions. Lower MIC values generally indicate greater inhibitory activity against the tested bacteria(Ali *et a*l., 2023).

4.1.1 The comparative of efficacy:

The biosynthesized CuONPs displayed significantly lower MIC values than the standard refrence(Gentamicin)across all tested bacteria, showing greater inhibitory effects against bacteria . For example, against *S. aureus* and *E.coli*, the MIC values of NPs was recorded at 128µg/mL(with sub-MIC at 64µg/mL),while Gentamicin required higher concentrations of 512 and 256µg/mL respectively (Abomughaid, 2025; Ali *et al*., 2023).

4.1.2 Sensitivity of bacterial species:

Strept. mutans showed to be the most susceptible bacteria, displaying an MIC of 64µg/mL for CuONPs and 256µg/mL for Gentamicin. Incomparison, Gram negative bacillus *K. pneumoniae* exhibted MIC of 256µg/mL for NPs and 512µg/mL for Gentamicin. Potentially related to the characteristics outer membrane barrier of Gram negative bacteria (Arif, 2023; Okoye *et al*.,2023).

4.1.3 Antibacterial Activity via Well Diffusion Assay:

The Antibacterial performance of CuONPs was Further evaluated by measuring the zone of inhibition in millimeters at different concentrations (Well and Disc Diffusion methods), as illustrated in **Table 2**. And **Figure 7**

4.1.4 Response Dependent on Dose:

Both liquid and paper diffusion assays highlighted aclear dose-dependent increase in antibacterial activity. The higher concentrations produced a significantly larger inhibition zones, in contrst lower concentrations or control displayed insignificant or no inhibition (Abed *et al*., 2021)

4.1.5 Comparision of Disc Diffusion Methods:

Disc Diffusion Method resulted larger inhibition zones compared contrast to the Well Diffusion Method(e,g, reaching up to 32mm for *S. aureus* and 30mm for *Strept. mutans* and *E. coli* at concentration256 µg/mL)indicating active release and diffusion of active copper species from the solid matrix into the agar medium(Khudhair, 2023).

4.1.6 Wide-Spectrum Activity:

The observation of large inhibition zones against both gram-positive(*S. aureus, Strept. mutans*) and gram-negative(*E. coli, K. pneumoniae*) bacteria confirm the broad spectrum biocidal capability of green synthesized NPs (Abomughaid, 2025; Ali *et al*., 2023

4.2 Mechanisms of Antibacterial Activity

The strong inhibitory activity can be showed by CuONPs to several well-documented nanotoxity mechanisms.

4.2.1 Interaction of Electrostatic:

The positive charge of copper(Cu^{+2})released by the NPs are attracted electrostatically to the funstional groups on bacterial cell walls with negative charge, leading to disrupte the membrane then increased permeability. (Arif, 2023)

4.2.2 The Oxidative stress:

The generation of intracellular Reactive Oxygen Species(ROS), for example hydroxyl radicles(OH^{-1})and superoxide anions, stimulates sever oxidative damage for proteins, lipids and nucleic acids(DNA/RNA). (Abomughaid, 2025; Ali *et al*., 2023)

4.2.3 Cellular uptake:

NPs and Cu^{+2} ions pentrate the bacterial cell, binding to essential cellular enzyme and disrupting important respiratory and metabolic processes, finally resulting the cell lysis and death. (Abed *et al*., 2021; Okoye *et al*.,2023)

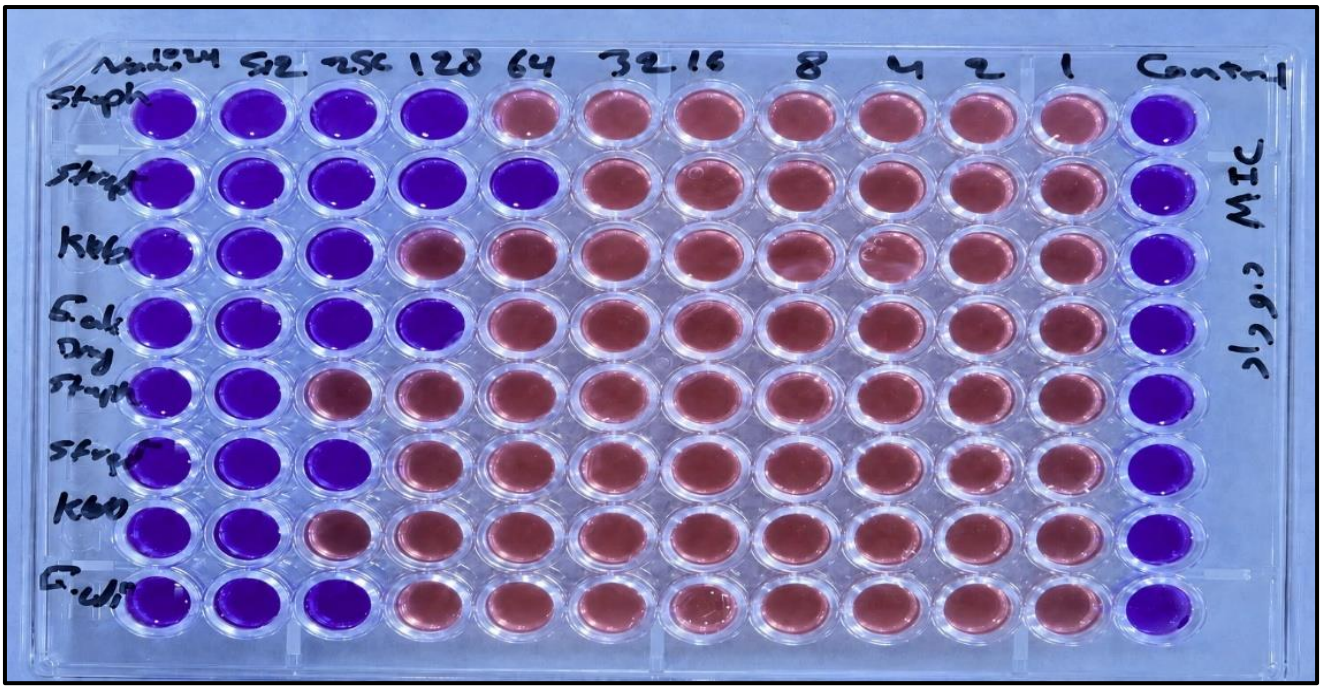


Figure 6: The experiment of evalution of MIC and Sub-MIC of Green-synthesized CuONPs compaired to Gentamycin antibiotic as standard refrence. Using 96 well microplate, the blue color indicates inhibition of growth, while pink indicates that bacteria are active.

Table 1: The evaluation of MIC and Sub-MIC of Green-synthesized CuONPs compaired to Gentamycin antibiotic as standard refrence

Bacterial species	CuONPs(µg/mL)		Gentamicin(µg/mL)	
	MIC	Sub-MIC	MIC	Sub-MIC
Staph. aureus	128	64	512	256
Strept. mutans	64	32	256	128
K. pneumoniae	256	128	512	256
E. coli	128	64	256	128

Table 2: The Antibacterial Activity of Green-synthesized CuONPs using Well and Disc diffusion Methods

Bacterial species		Size of Inhibition zone(mm)				
		256 µg/mL	128 µg/mL	64 µg/mL	32 µg/mL	C(D.D.W.) 0.0 µg/mL
Well Diffusion	Staph. aureus	24	16	-	-	-
	Strept. mutans	24	19	15	-	-
	K. pneumoniae	22	-	-	-	-
	E. coli	26	19	-	-	-
Disc Diffusion	Staph. aureus	32	24	15	-	-
	Strept. mutans	30	25	18	14	-
	K. pneumoniae	26	20	-	-	-
	E. coli	30	22	18	-	-

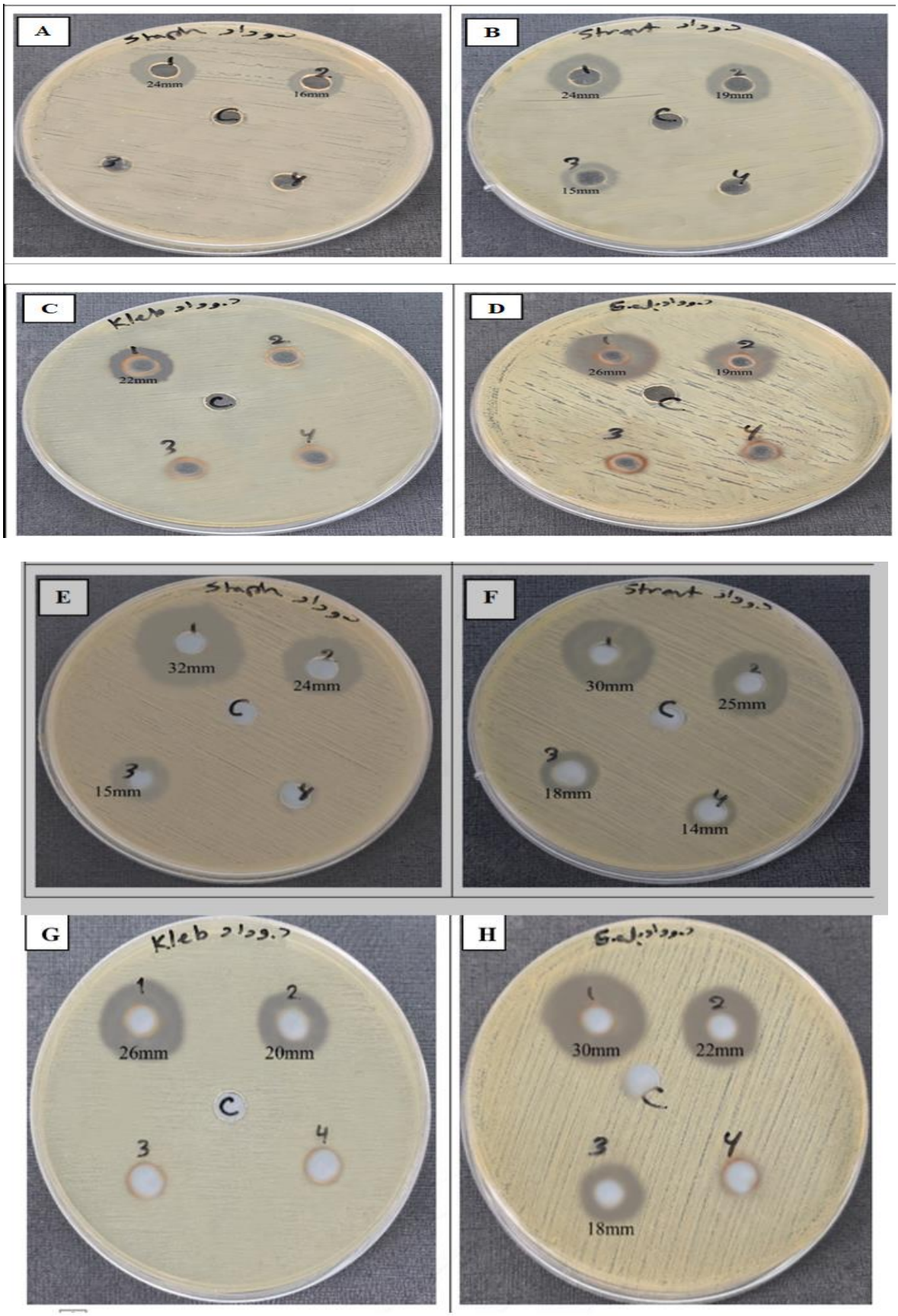


Figure 7: Inhibition Zone of *S. aureus, Strept. mutans, K. pnemoniae* and *E. coli* in response to treatment with the green synthesized CuO NPs, using Well Diffusion: (A, B, C. D) and Disc Diffusion methods (E, F, G, H) respectively

5 CONCLUSION

The current study successfully achieved the green synthesized CuONPs using aqueous extract of fresh vegetative parts of *G. aparine*, the NPs characterized by UV- visible, AFM, FE-SEM and FTIR techniques, these CuONPs exhibited efficiently antibacterial activity against four pathogenic bacteria, two of them Gram positive: *S. aureus* and *Strept. mutans*, the other were Gram negative bacteria: *K. pneumoniae* and *E. coli*. According to the findings, biosynthesized CuONPs showed higher significant antibacterial activity than Gentamycin antibiotic. Therefore, the green synthesized CuONPs have opened up new possibilities in medicine, particulary for the treatment of various infection diseeases. Overall, this method offers an ecofriendly and cost-effective alternative to conventional chemical and physical synthesis with promoting sustainable development and reducing potential environmental impacts

STATEMENTS ON COMPLIANCE WITH ETHICAL STANDARDS

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Disclosure of conflict of interest

The author declare no conflicts of interest regarding this manuscript.

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