Synergistic Activity of Antibiotics and Bio Active Plant Extract: Astudy Against Mdr Gram Negative Bacteria in Kirkuk City

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ABSTRACT

Increasing multidrug resistance (MDR) is becoming a problematic issue worldwide. Finding alternatives to cope with this rising problem is becoming crucial for community healthcare. The present study aimed to investigate the antibacterial activityCopper oxide nanoparticles (CuONPs) synthesised using hawthorn fruit extract against different MDR gram-negative bacteria isolates. CuoNPs were synthesized from Fresh hawthorn fruits (Crataegus). The biosynthesized copper oxide nanoparticles were Characterization of the synthesised nanoparticles was performed using UV-visible spectroscopy, Scanning Electron Microscopy (SEM), Fourier-transform infrared spectroscopy (FT-IR), and X-ray diffraction (XRD) to confirm CuONP production and assess their properties. Antibacterial activity was determined by disc diffusion and the healthy diffusion method. The considerable inhibitory action of hawthorn fruit extract was observed in gram-negative bacteria isolates. Also, the green synthesised CuoNPs demonstrated remarkable antibacterial activity against two human pathogenic bacteria when combined with commercially available antibiotics. The current study suggested that CuoNPs have become an essential approach for nanobiotechnology applications in the development of antibacterial treatments for different bacterial infections, particularly for non-treatable antibiotic resistance pathogen.

1. Introduction

Antibiotic resistance is an emerging global health problem resulting from the constant use (and misuse) of antibiotics in healthcare, the agricultural industry, and elsewhere (1, 2). This resistance is a particular concern of predominant pathogen-causing urinary tract infections (UTIs) such as Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, and Enterobacter spp.. these pathogens are frequently nosocomial. They can cause severe topical and systemic infections (3) and spread in hospital intensive care units (ICU) (4). Strikingly, most clinical isolates are multi-drug resistant (MDR), extensively drug-resistant (XDR), and pan-drug resistant (PDR) bacteria (5). To prevent re-entering the “post antibiotics” era, there is an urgent need for new therapeutic agents against the MDR, XDR, and PDR pathogens. To fight these bacteria, scientists and researchers suggest several new therapeutics alternatives or complements to antibiotics against the pathogens. (6,7).

Recent advancements in nanotechnology may provide solutions to overcome limitations in excessive copper fungicide use, mainly by reducing application doses. Among the various metal nanoparticles that were developed and evaluated for their antimicrobial activity, copper nanoparticles (CuNPs) became the promising treatments because of their lower production cost and ubiquitous availability (8). Furthermore, the Hawthorn (Crataegus spp.) is commonly used as a medicinal food and medicinal material in China and other European countries to prevent cardiovascular and hepatic diseases. It was proved that administering hawthorn extracts to patients contributes to reducing total cholesterol and LDL and increasing HDL cholesterol. The inner membrane integrity and protein engineering equip the lysin with the tools to overcome the outer membrane (9).The biological effects of hawthorn phenolics have been mainly tested by employing extracts obtained from leaves, flowers, and fruits using ethanol, methanol, water, or mixtures of these solvents. Hawthorn-based herbal products are nowadays marketed as alternative treatments for several diseases, such as hypertension, angina, arrhythmia, and the early stages of congestive heart failure (10).Copper nanoparticles are a viable option for application in antibacterial coatings, wound dressings, and water purification systems because studies have

demonstrated their antimicrobial solid characteristics against various bacteria, fungi, and viruses.

In medicine, copper nanoparticles have shown potential applications in anticancer, antibacterial, antifungal, antiviral, disinfection, antibiotic adsorption, contrast agent, MRI, PET, biomedical, bioimaging, theragnostic, personalised medicine, cytotoxicity, biocompatibility, tissue regeneration, wound healing, and dental materials (11,12).

2. Methodology

Preparation Extraction of Hawthorn Fruits

Fresh hawthorn fruits (Crataegus) were obtained from local suppliers and thoroughly washed with water and distilled water to remove impurities. The cleaned, dried, seedless hawthorn fruits were crushed into powder fruit and sieved to a particle size of fewer than 220 micrometres.100 grams of the hawthorn fruit powder were macerated in 500 mL of 95% ethanol for 24 hours, with the extraction process repeated thrice. Using a rotary evaporator, the resulting extracts were filtered, combined, and concentrated to 100 mL. The maceration method was chosen to efficiently extract bioactive compounds while preserving the hawthorn fruit composition. The ethanolic extraction was then utilised for various applications, including synthesising copper oxide nanoparticles.

Green Synthesis of Copper Oxide Nanoparticles (CuONPs) Using

Hawthorn Extract

Copper oxide nanoparticles (CuONPs) were synthesised using hawthorn fruit extract as a reducing and stabilising agent. A one millimolar aqueous solution of 2 grammes of copper oxide (CuONPs.H2O) was produced for the synthesis. After that, varying volumes of hawthorn extract (3, 5 and 10 mL) were added into the copper solution. The change in colour which suggested reduction was observed within 30 minutes after agitation at room temperature. The mixture was further allowed to incubate at ambient temperature for over night and thereafter subjected to centrifugation for 15 minutes at 8000 xg to sediment the copper nanoparticles. Afterwards, the nanoparticles were washed several times with deionized water through centrifugation and were then dried using an air heat exchanger. (13).

UV-visible spectroscopy, SEM, FT-IR and XRD were used to confirm the synthesis of CuONP and to determine their properties. The synthesis of copper oxide nanoparticles using hawthorn extract in green synthesis process is an eco-friendly method for synthesizing copper oxide nanoparticles with potential uses in various fields.

Characterisation of Copper Oxide Nanoparticles

UV-visible spectroscopy is used frequently for characterization of copper oxide nanoparticles (CuONPs). Copper oxide (CuO) has typically a UV absorption edge in the range of 280 nm and 360 nm. By employing UV-visible absorption spectroscopy it is possible to investigate the optical characteristic of CuONPs which gives information about the band gap energy and the size of the nanoparticles. The absorption spectra analysis shows that the band gap energy of CuONPs is higher than that of bulk CuO due to quantum confinement effect. In addition, UV-visible absorption spectroscopy enables the study of the effect of doping on the optical properties of CuONPs. (14).

X-ray diffraction (XRD) analysis is one of the most widely used techniques for studying the packing of material like copper oxide nanoparticles (CuONPs). This method involves exposing the nanoparticles to X-ray radiation so as to produce diffraction patterns. The observed patterns are very useful in understanding the crystallographic arrangement and phase homogeneity of the synthesized nanoparticles. It is crucial to gain such understanding to determine the atomic-level properties of CuONPs and optimize their properties for specific applications. (15). Polychromatography is a stable quantitative technique used to identify, quantify and separate the various components in complex samples. In this approach, the elements are separated based on their physical and chemical properties using a gas chromatograph and the separated components are detected and quantified using a mass

spectrometer.

An FTIR analysis of copper nanoparticles (CuONPs) is performed by first washing the nanoparticles and the filtrate and dried pellet with deionized water and then drying the pellet to obtain a powder.

The pellet powder is then determined with the help of KBr disk in the laser wave number of 400-4000 cm. In the case of CuONPs FTIR spectroscopy may be used to confirm specific information about the state of the surface of the nanoparticles and the chemical covalent bonds that have been formed in the atom groups that are discernible on the surface of the CuONPs. (16).

Agar Diffusion Method to Evaluate the Antibacterial Activity of Hawthorn

Extract, CuONPS and their Combination with Antibiotics

The agar well diffusion assay is a widely employed technique for determining the antibacterial properties of plant or microbial samples and other natural substance like honey. The technique involves preparing agar plates that will be inoculated by smearing the broth evenly over the surface of the agar plate, creating a lawn culture, and creating wells in the agar where the antimicrobial agent is introduced. The antimicrobial agent diffuses into the agar medium, inhibiting the growth of the microorganism and creating a zone of inhibition around the well. The diameter of the zone of inhibition is then measured and used as an indicator of the agent's antimicrobial activity.

The agar well diffusion method evaluated the antibacterial activity of hawthorn extract, CuONPs, and their combination with each antibiotic (ciprofloxacin, trimethoprim, and ceftriaxone). Bacterial suspensions of Escherichia coli and P. aeruginosa were prepared in sterile saline solution, and their turbidity was standardized to the 0.5 McFarland standard. Wells measuring five millimetres in diameter were created using a sterile cork borer in Mueller-Hinton agar plates.

Concentrations of 128, 256, 512, and 1024 µg/mL of hawthorn extract, CuONPs, and their combination with each antibiotic (separately) were prepared for testing, following the clinical breakpoints recommended by the Clinical and Laboratory Standards Institute (CLSI). The wells were loaded with 100 µL of each test solution, and the plates were then incubated at 37°C for 24 hours. The antibacterial activity was determined by measuring the diameter of the inhibition zone surrounding the wells using a metric ruler.

3. Result and Discussion

Characterisations of copper oxide nanoparticles

UV-Visible Spectroscopy of Copper Oxide Cuo Nanoparticles

UV-Vis spectroscopic analysis refers to the measurement of ultraviolet-visible light absorption by samples. It is used in this case to analyses the green synthesis of CuO nanorods. This research provides information on the optical and structural properties of the nanorods which would help in the determination of size, shape and purity of the nanorods and in evaluating the efficiency of the synthesis process. (24). Copper oxide nanoparticles (CuONPs) were synthesised by incorporating a copper precursor into hawthorn berry extract. The change of color of the solution from yellow to dark brown was as a result of the addition of the copper precursor, and the formation of CuONPs as shown in (Figure 1).

In UV-visible spectrum, CuONPs have absorption peaks at approximately 200 nm and 600 nm as illustrated in the figure 2 below. The first one at 265 nm is attributed to the inter-band transition of core electrons while the second one at 670 nm is due to the Surface Plasmon Resonance (SPR) shown by the CuONPs. The SPR peak has the diagnostic potential for the assessment of CuONPs synthesis and provides data on their dimensions and crystal structure. (25). An identifiable and well-defined surface plasmon resonance (SPR) peak indicates the creation of spherical and evenly distributed copper oxide nanoparticles (CuONPs). The height and shape of the peak depends on the concentration of CuONNs and presence of capping or stabilizing agents. Recent studies also focus on the applicability of UV-

visible spectroscopy for the real-time tracking of CuONPs formation. (26).

With the progress of the chemical reaction, the intensity of the SPR peak gradually increases, which shows the formation of CuONPs. Positional changes of the peak can give useful information concerning the changes in the dimensions and morphology of the nanoparticles. (27).

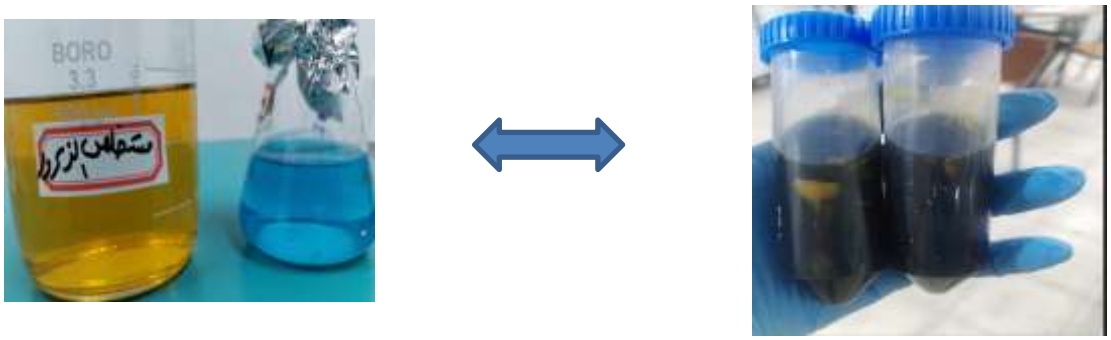

Figure (1): Copper oxide nanoparticles (CuONPs) were synthesized using hawthorn berry extract

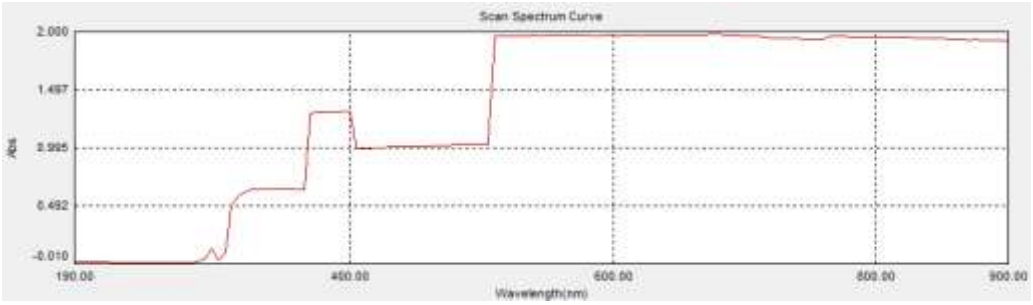

Figure (2): UV-Visible spectroscopy of copper oxide nanoparticles

X-ray diffraction of copper oxide nanoparticles

The XRD analysis of copper oxide nanoparticles prepared with hawthorn (*Crataegus monogyna*) extract is depicted in (Figure 3). The peaks observed at 2θ angles of 32.50, 35.40, 38.90, 48.70, 58.40, 61.40, 65.80, 66.30, 72.50, and 74.90 correspond to the crystal planes of copper oxide at orientations (110), (002), (111), (202), (020), (202), (113), (311), (113), (220), and (311) respectively.

The X-ray diffraction (XRD) study reveals that the copper oxide nanoparticles (CuONPs) display distinct crystallinity. The peak locations and relative intensities closely correspond to the standard phase CuONPs diffraction pattern found in the International Center of Diffraction Data card (JCPDS-80-1916), as shown in Figure 3. The Debye-Scherrer formula was used to calculate the average particle size of the synthesised CuO nanoparticles. This method takes into account the whole width at the half-maximum value of XRD diffraction lines, the X-ray radiation wavelength, the half diffraction angle, and the Scherrer constant.

Utilising this formula, the mean particle size of the CuO nanoparticles produced from hawthorn extract was calculated to be around 20 nm. (28,29). In general, the XRD analysis verifies the effective production of copper oxide nanoparticles with necessary crystalline characteristics by the use of hawthorn extract as an environmentally benign and sustainable approach.

XRD Laboratory-University of Kashan

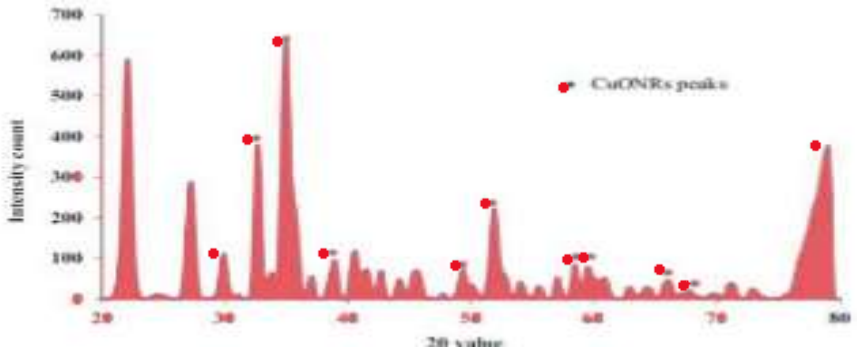

Figure (3): XRD of copper oxide nanoparticles

Fourier transform-Infrared (FTIR) Spectroscopy

Fourier Transform Infrared (FTIR) spectroscopy is widely employed to analyse infrared spectra arising from the chemical bonds within a molecule. This study acquired IR spectra for the biosynthesised Copper Oxide Nanoparticles (CuONPs). The FTIR spectral analysis of CuO Nanorods (NRs) revealed distinct peaks, as depicted in (Figure 4). Notably, a semi-broad band around 3408 cm−1 indicated the stretching frequency of hydroxyl groups, providing insights into the surface morphology of the synthesised nanoparticles. A peak observed at 1056 cm−1 corresponded to the presence of ester bonds between copper species and hydroxyl groups. Comparative analysis of the obtained spectrum with relevant literature substantiated the successful biosynthesis of the CuONPs. (30,31).

FT-IR Laboratory-University of Kashan

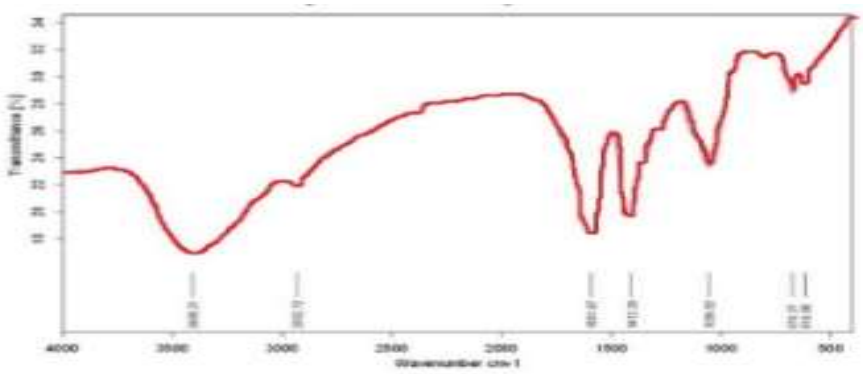

Figure (4): The FT-IR spectra of synthesized CuO-NPs

The results demonstrate that hawthorn extract exhibits a concentration-dependent antimicrobial effect. At higher concentrations of 50 µg/ml and 100 µg/ml, more antimicrobial activity was observed with p-values < 0.05. This suggests a significant difference in inhibition zone diameters at these higher concentrations.

Similar to hawthorn extract, the data for CuONPs also shows a concentration-dependent antimicrobial effect. Significant differences in inhibition zone diameters were observed at 25 µg/ml, 50 µg/ml, and 100 µg/ml (p-value < 0.05).

Ciprofloxacin (CIP) exhibited limited antimicrobial activity against the MDR *E. coli* isolates. A statistically significant difference in the diameter of the inhibition zone was observed only at the highest concentration of 100 µg /ml (p-value < 0.05).

CIP had no antibacterial action at lower doses which means that higher concentrations may be required to prevent the growth of such resistant bacteria. TMP-SMX and CRO presented concentration

dependent antibacterial activity against MDR E. coli strains. Significant variations in the magnitude of the zones of inhibition were noted at doses of 50 µg/ml and 100 µg/ml (p-value < 0.05). These results also suggest that higher concentrations of TMP-SMX and CRO produce enhanced antibacterial effect, which may imply that the antibiotics are more effective against resistant bacteria.

A p-value below 0. 05 revealed that the synergistic interaction of CIP-CuONPs-hawthorn extract, TMP-SMX-CuONPs-hawthorn extract and CRO-CuONPs-hawthorn extract exhibited significant antibacterial effect against MDR E. coli isolates. Further, there was a decrease in width of the inhibitory zones as the concentrations were raised and the findings indicated a directly proportional relationship between the concentration levels and the efficiency against bacteria.

Table 1: Average Inhibition Zone Diameters for Different Concentrations of Hawthorn Extract, CuONPs, and a Combination of Antibiotic + CuONPs + Hawthorn against *E. coli*

Materials and/ or antibiotics	**Diameter of Inhibition Zone in Millimeters (Mean ± SD)**			
	Concentration			
	12.5 µg/ml	**25 µg/ml**	**50 µg/ml**	**100 µg/ml**
Hawthorn Extract	0.12 ± 0.6^{N}	3.24 ± 3.918^{N}	8.48 ± 3.45^{S}	12.52 ± 1.228^{S}
CuONPs	5.44 ± 3.53^{N}	11.52 ± 2.91^{S}	15.92 ± 1.67^{S}	17.64 ± 1.32^{S}
CIP	4.19 ± 5.91^{N}	9.48 ± 7.73^{N}	14.44 ± 9.43^{N}	20 ± 8.94^{S}
CIP + CuONPs + Hawthorn	9.76 ± 8.11^{N}	22.56 ± 4.17^{S}	31 ± 4.52^{S}	37.2 ± 3.13^{S}
TMP-SMX	0^{UD}	1.96 ± 2.52^{N}	5.8 ± 2.51^{S}	10.72 ± 2.83^{S}
TMP-SMX + CuONPs + Hawthorn	20.44 ± 7.4^{S}	27.92 ± 2.77^{S}	32.16 ± 2.82^{S}	38 ± 2.4^{S}
CRO	0.12 ± 0.58^{N}	2.68 ± 2.44^{N}	6.2 ± 1.74^{S}	10.16 ± 3.84^{S}
CRO + CuONPs + Hawthorn	24.72 ± 2.21^{S}	27.68 ± 2.27^{S}	28.84 ± 2.1^{S}	30.04 ± 2^{S}

***N: Not significant** ***S: Significant** ***UD: Undefined**

The synergistic interaction between antibiotics and copper oxide nanoparticles enables the bypassing of resistance mechanisms exhibited by multidrug-resistant E. coli strains, making this combination a therapeutic strategy. This finding is supported by studies 1 and 2 in this context. Furthermore, the present study is consistent with prior research conducted in 2024, which reported the strong antimicrobial activity of CuONPs against E. coli, K. pneumoniae, and S. aureus, resulting in a substantial reduction in antibiotic resistance among these bacteria. (17). And the synergistic effect of copper nanoparticles and antibiotics enhanced antibacterial potential (18).

Furthermore, the synergistic antibacterial mechanism of silver-copper bimetallic nanoparticles emphasises the enhanced antibacterial efficacy and reduced toxicity of Ag-Cu NPs compared to individual nanoparticles (19).

It has been indicated that the CuONPs also exhibited potent antibacterial activity against *E. coli*, with inhibition zones ranging from 14 ± 0.31 mm to 16 ± 0.53 mm at different concentrations; the antimicrobial activity of CuONPs could be attributed to their small size, large surface area, and ability to interact with the bacterial cell membrane. In contrast to the presented results of hawthorn fruit extract, it has been suggested that the hawthorn fruit extract has significant antibacterial and antioxidant effects (19).

Table 2: Average Inhibition Zone Diameters for Different Concentrations of Hawthorn Extract, CuONPs, and a Combination of Antibiotic + CuONPs + Hawthorn against *P. aeruginosa*.

Materials and/ or antibiotics	**Diameter of Inhibition Zone in Millimeters (Mean ± SD)**			
	Concentration			
	12.5 µg/ml	**25 µg/ml**	**50 µg/ml**	**100 µg/ml**

Hawthorn Extract	0^{UD}	2.33 ± 3.5^{N}	8.8 ± 2.13^{S}	11.7 ± 1.18^{S}
CuONPs	5.3 ± 4.43^{N}	12.8 ± 2.84^{S}	15.6 ± 2.7^{S}	18 ± 2.81^{S}
CIP	0.33 ± 1.24^{N}	1.53 ± 3.26^{N}	6.86 ± 5.35^{N}	12.8 ± 7.63^{S}
CIP + CuONPs + Hawthorn	12.06 ± 3.172^{S}	22.8 ± 3.95^{S}	29.86 ± 4.64^{S}	33.6 ± 8.55^{S}
TMP-SMX	0^{UD}	1.4 ± 2.21^{N}	6.73 ± 1.52^{S}	11.13 ± 4.31^{S}
TMP-SMX + CuONPs + Hawthorn	18.13 ± 6.469^{S}	25.93 ± 2.43^{S}	31.4 ± 2.18^{S}	35.86 ± 2.7^{S}
CRO	0^{UD}	0.93 ± 1.87^{N}	5.4 ± 2.09^{S}	9.06 ± 1.48^{S}
CRO + CuONPs + Hawthorn	25.4 ± 1.818^{S}	27.13 ± 1.74^{S}	29.6 ± 2.47^{S}	29.73 ± 1.43^{S}

***N: Not significant** ***S: Significant** ***UD: Unndefined**

The synergistic increase in the antimicrobial activity against the resistant strains when the natural extracts were used in conjunction with antibiotics was also determined. This finding strengthens the findings of the present study, which demonstrated that hawthorn extract, CuONPs, and antibiotics exhibited additive activity against MDR *P. aeruginosa.* (20).

Moreover, enhanced efficiency of metal nanoparticles against bacterial infections in the presence of antibiotics. CuONPs have been shown to possess synergistic effects with antibiotics against P. aeruginosa. CuONPs when combined with ciprofloxacin, gentamicin and amikacin were found to provide larger inhibitory zones than the use of antibiotics alone. (21). Furthermore, the enhancement of antibacterial activity against P. aeruginosa isolates was observed when CuONPs were combined with amoxicillin-clavulanic acid, ceftazidime and imipenem. (22). Enhanced antibacterial activities of the CuONP-antibiotic formulations may be ascribed to the intrinsic antibacterial properties of CuONPs, the ability of CuONPs to enhance antibiotic diffusion, and potential inhibition of resistance factors in P. aeruginosa. (23).

4. Conclusion and future scope

Of course, CuONPs nanoparticles have a high possibility in reducing the MDR bacterial activity and increasing the antibacterial effect. The use of nanoparticles has been widely observed in antimicrobial and therapeutic uses. The present work also established that the antibacterial activity of CuoNps could be realized in a one-step, green synthesis at a low cost using extract from hawthorn fruits (Crataegus). The CuONPs that were synthesized in the present study had appreciable antibacterial activity against the gram-negative bacteria. The biosynthesis clean, non-toxic, and eco-friendly approach can be used as a better replacement to the conventional CuONPs synthesis process for biomedical applications. Therefore, the application of our nanoparticles may assist in preventing the growth of the studied bacteria

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