SYNERGISTIC EFFECT OF COPPER OXIDE NANOPARTICLES FOR ENHANCING ANTIMICROBIAL ACTIVITY AGAINST *K. PNEUMONIAE* AND *S. AUREUS*

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ABSTRACT

This study was aimed to assess the antimicrobial activity of copper oxide nanoparticles (CuO NPs) created by method of thermal green way using basically a maize starch. Mucoid were appeared of *Klebsiella pneumoniae* bacterial colonies and the positive results with some biochemical tests. On the other hand, *Staphylococcus aureus* appeared pigmented colonies surrounded by a yellow halo because of mannitol fermentation. According to the 24 time incubation period, the CuO NPs antimicrobial activity showed of bacterial growth pathogenic *K. pneumonia* was 0.52 ± 0.04 cell/ml than control 1.60 ± 0.01 cell/ml .Aven as *S. aureus* appeared the number of bacterial growth as follow 0.79 ± 0.07 cell/ml compared with control 1.90 ± 0.01 cell/ml. The biologically effect for enhancing antimicrobial activity the percentage of resistant was decreasing from 66.6% to 22.2% when used copper oxide nanoparticles. Also, *S. aureus* sensitivity test showed resistant percentage was decreased from 55.5% to 33.3% at 24 hours.

Keywords: antimicrobial activity; bacterial growth; cuo nanoparticles; antibiotic resistance.

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تأثير التآزري لجسيمات اوكسيد النحاس النانويه لزيادة فعالية المضادات المايكروبيه ضد بكتريا *K. pneumonia* و *S. aureus*

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مدرس

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المستخلص

هدفت هذه الدراسة الى تقيم الفعالية الضد مايكروبية لجسيمات النانوية أوكسيد النحاس(CuO NPs) مخلق بواسطة طريقة التوليف الأخضر الحراري باستعمال النشا الذرة. ظهرت المستعمرات مخاطيه للبكتيريا *Klebsiella pneumonia* و نتائج إيجابية مع بعض الاختبارات الكيميائيه.من ناحية أخرى، *Staphylococcus aureus* ظهرت مستعمرات مصطبغة محاطة بهالة صفراء بسبب تخمير مانيتول. ووفقا لفترة الحضانة 24 ساعة, أظهر CuO NPs نشاطه كمضادات المايكروبيه للنموالبكتيري *K. pneumonia* المسببة للأمراض 0.04 ± 0.52 خلية /مل مقارنة مع السيطرة 0.01 ± 1.60 خلية /مل .ايظا *S. aureus* أظهرت عدد النمو البكتيري0.07 ± 0.79 خلية / مل مقارنة مع السيطرة 0.01 ± 1.90 خلية / مل.اما التأثير البيولوجي لتعزيز نشاط مضادات الميكروبات كانت النسبة المئوية للمقاومة تناقصت من 66.6٪ إلى 22.2٪ عند استخدام أوكسيد النحاس النانوية. أيضا، اختبار حساسية *S.aureus* أظهرت نسبه المقاومة انخفاض من 55.5٪ إلى 33.3٪ في 24 ساعة.

الكلمات المفتاحية: نشاط مضاد للمايكروبات، النموالبكتري، جزيئات اوكسيد النحاس النانويه، مقاومه للمضادات

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INTRODUCTION

Staphylococcus aureus and *Klebsiella pneumoniae* common pathogens colonization in human can cause the advance of disease. *S. aureus* lead to toxic shock syndrome soft tissue and skin infections (28,29) , bacteremia (34). *S. aureus* can causes in children severe infections, like pneumonia, sepsis and otitis. Whereas generally asymptomatic when nasopharyngeal carriage with its(31). likewise, the healthy individuals nasopharynx is a probable reservoir for transmission of *S. aureus* to other individuals (16).The *Enterobacteriaceae* family, *K. pneumonia* is a bacteria of gram-negative, rod-shaped, aerobic and non-motile. Their raise on agar media appeared mucoid colonies and are able fermentation of lactose (22). In human, this bacteria is found in intestinal tracts and nasopharyngeal, reason for hospital-aquired infections involve bloodstream, urinary tract infections(UTI) and respiratory tract infections (26). As well as, after skin surgery *K. pneumoniae* is also identified on wound (12).The emergence of methicillin-resistant *S. aureus* (MRSA) and World Health Organization reported only a few antibiotics are effective against of *K. pneumoniae* (16, 36). This state causes develop into not easy treatment of diseases and generate for the life of human further serious problem(24), can cause increased health care costs, wide hospitalization and possibly will in the end reason to increased morbidity and mortality (35).Thus, needed to develop and recognize new strategies next-generation antibiotics. A latest group as an substitute to antibiotics and antimicrobials show promise is metal nanoparticles, depend on highly prepared metal nanoparticles have been developed that make biocidal agents and non-specifically aim most bacteria and fungi(9). Found different microorganisms, including gram-positive *S. aureus* (33), gram-negative(11) and fungi effect by these nanoparticles (Ag, Au and CuO). CuO nanoparticles used extensively not as an industrial material, in addition as an antimicrobial agent. Cu and Cu complexes have been utilized as algaecides, fungicides and bactericides (10). Furthermore, evaluated CuO antimicrobial properties in several type, including antibiotic-resistant microbes as *S. aureus* (2). Hence, performed this study to observe the enhancing antimicrobial activity of CuO nanoparticles against opportunistic pathogens gram-negative *K. pneumonia* isolated from pus specimen and gram-positive *S. aureus* strains. Potential projection rotate about rising inventive methods and products to avoid, control, and care infections of microbial in the current pandemic disease.

MATERIALS AND METHODS

In this study, using a maize starch and ($CuSo_4.5H_2o$)**.** The solvents ethanol 96% and ammonia solution agents were procured from sigma. The flasks in addition to dishes were gained from Assistant (Assipette)/Germany. Deionize water (DDW) was used in all methods.

Preparation of CuO nanoparticles

Preparation CuO nanoparticals by exactly weighed 2.476 g (0.1 M) copper sulphate (CuSo4. 5H2O) powder/CDH-India and dissolving into beaker containing 100ml of DDW under strong stirring. At the same time, dissolved 0.1 g maize starch (Baghdad-Iraq/ local market) in deionize water (3ml) with moved at 10 min toward arrive at white color starch suspension. Then the suspension color was altered to a bold blue when added starch solution to copper sulphate solution with continues stirring. Later added 0.5M (15 drop wise) an abundance amount of ammonia solution until pH solution reached 10, later than 20 min. Then for 2min put the mixture in microwave (LG/Korea), the mixture set was formatted is CuO nanoparticles that changed to a suspension black color. Then centrifugation at ten min (8000 rpm) was used. Finally, wash by DDW and ethanol many times the precipitant to make it free impurities organic and from sulphate, ammonia. By oven for 2 hours precipitant was dried at 150ºC. Finally, obtained black expected CuO NPs powder(18). In previous study, the properties nanoparticles showed were very pure, globular shape, the ranging diameter size of particles was from (47.41 to 109.49)nm and constant, in addition average crystallite measure is 9.8nm. However, the average distribution (d50) is71.17nm (1).To prepare a stock CuO suspension using0.001g prepared black powdered dissolving with 5ml DDW (stock solution). Than was used Sonicatar instrument

(Heraeus/Germany) to homogenizing and fine dissolving for more analysis.

Bacterial isolation

K. pneumoniae isolation was done by streaking loopful from brain heart infusion broth culture previously isolated from pus specimen on MacConky agar and on EMB agar for primary selection of pathogenic *K. pneumoniae* (3, 4, 5, 17). According to MacFaddin(21), the biochemical tests were employed Catalase, Oxidase, Motility, Urease, Indol, Methyl Red(MR), Voges-Proskauer and Citrate utilization. Beside these tests, Api 20E system kit (Bio-Meriaux, France) and VITEK 2 system kit (Bio-Meriaux, France) were also checked for identification of *K. pneumoniae*. On the other hand, *S. aureus* also previously identified from purulent wound and on manitol salt agar. The bacterial isolated were activated on brain heart infusion broth(himedia) and incubated over night at 37 ^{o}C.

Antibacterial activity tests

The activity antimicrobial of 40µg/ml concentration CuONPs was investigated against two strains; *K. pneumonia* is represented gram-negative organisms and *S. aureus* is represented gram positive bacteria. Activated bacterial strains on nutrient broth sterile (Himedia/India). Prepared bacteria suspension by alone colony was inoculated for 24h in nutrient broth with turbidity adjust using 0.5 McFarland standards. in brief, (100 µl /40 µg/ml) of CuO NPs were made was added to NB media sterile (5ml). Then inoculated with activated bacterial strains(0.1 ml). Later at37°C incubated these tubes for 24 hours. Control was involved; 0.1 ml of nanoparticles inoculated with nutrient broth media only at 37ºC. Finally, measured the bacterial growth after incubation using uv-vis spectrophotometer (DRG/ USA) at 625nm(18). The triplicate reading values of mean for each bacterial strain was recorded.

CuO NPs synergistic effect with diverse antibacterial agents

K. pneumonia and *S. aureus* incubated with the CuO NPs (40µg/ml) was subjected to evaluate the synergistic effect with diverse antimicrobials agents usually was used in present test. The Kirby-Bauer disk diffusion method was used on Mueller-Hinton agar(MHA) plates for determined synergistic effect (7). Antimicrobial disks (Bioanalyse/Turkey) involve; Methicillin (ME) (10µg), Trimethoprim/ sulphamethoxazole (SXT) (25µg), Amoxillin/ clavulanic acid (AMC) (30µg), Gentamicin (CN) (10µg), Levofloxacin (LEV) (5µg), Ciprofloxacin (CIP) (10µg), Cefixime (CFM) (5µg), Cefotaxime (CTX) (5µg) and Amikacin (AK) (30 µg) were used (Table 1). CuO NPs (40 µg/ml) with activated bacterial strain suspension (1.5 X 10^{8}) CFU/ml at McFarland 0.5 were mixed. 0.1 ml of a mixture were inoculated by dispersed regularly in Mueller Hinton agar (Himedia /India) plates via swab, then antimicrobial disks were dispensed. Afterward incubated these plates for 24 h. at 37°C. After that calculated the zone of inhibition(ZOI) in millimeters (mm) around each antimicrobial disk, and compared to the activated bacterial (0.1 ml) inoculated directly on plate of Mueller-Hinton agar (Control). In addition, the degree sensitivity was determined relation to rules of National Committee for Clinical and Laboratory Standards Institute (NCCLs) (37)

Table 1. Antibiotic agents used for susceptibility testing

Antimicrobial disks	Disc Content
Methicillin (ME)	(10µg)
Trimethoprim/sulphamethoxazole (SXT)	(25µg)
Amoxillin/clavulanic acid (AMC)	(30µg)
Gentamicin (CN)	(10µg)
Levofloxacin(LEV)	(5µg)
Ciprofloxacin (CIP)	(10µg)
Cefixime (CFM)	(5µg)
Cefotaxime (CTX)	(5µg)
Amikacin (AK)	(30 µg)

Statistical analysis

IBM SPSS computer program version 25.0 was used to calculate the median, standard error (SE), probability (two tailed) by using ANOVA table.

RESULTS AND DISCUSSION

Characteristics of bacterial isolates

The colonies of *K. pneumoniae* were appeared mucoid on MacConky agar (Figure 1) and the positive results with sugar catalase, Simmons' citrate, urease and Voges-Proskauer except negative results in MR, Indol, oxidase and motility tests were identified in biochemical

testing (Table 2). Result of Api-20E system was agreed with previous biochemical tests (Figure 2). The consequences similar to Patel, *et al.* (25). On the other hand, the results mannitol fermentation of *S. aureus* on mannitol salt agar appearance pigmented colonies surrounded by a yellow halo and lush (Figure 3).

Figure 1. A result of *K. pneumoniae* on MacConky agar

Table 2. The biochemical tests of *K. pneumoniae* bacterial isolates

Test	Catalase	Oxidase	Indole producti	Methyl -	Voges-	Citrate utilizatio	Motility	Urease
K.pneumoniae	+	_	_	_	+	+	_	+

Figure 2. A positive result of Api20E for *K. pneumoniae*

Figure 3. A result of *S. aureus* on mannitol salt agar

Antibactericidal tests

The results bactericidal impact of biosynthesis 40µg/ml (200µl from stock solution added to 5ml DW)concentrations of copper oxide nanoparticles against pathogenic *K. pneumonia* and *S.aureus* strains. According to the 24 time incubation period, showed of pathogenic *K. pneumonia* was recorded 0.52 ± 0.04 cell/ml compared with control 1.60 ± 0.01 cell/ml (Table3) (Figure4) .While *S.aureus* showed the number of bacterial growth absorption as 0.79 ± 0.07 compared with control 1.90 ± 0.01 cell/ml (Table3) (Figure5). From consequences exposed previous, the 40mg/ml concentration of copper oxide nanoparticles has impact antimicrobial against G-ve and G+ve bacteria, and all were recorded significant alteration than control. Structure of cell wall bacteria, particle size, in addition to the degree of bacterial cell suitability test was achieved that impact these consequences represent and to settle on the impact of bactericidal assorted attachment the organisms with NPs (20). The great quantity of amines and carboxyl groups on cell surface of bacteria, that raise the Cu ions attraction towards both bacteria and ascribed to the template CuO NPs that lead to the greater sensitivity of these groups to the CuO NPs(19). Mechanisms diverse have been planned to translate the antibacterial conduct of metal oxides. (6) agreement with our current study, all have behavior antimicrobial higher, which is represented that nanoparticle smaller sizes that helped the nanoparticles access during membrane of bacteria and react with component of its.

Table 3: Antibacterial effect of 40µg/ml concentration of green synthesized CuO NPs against two pathogenic bacteria strain

Control	24 hours
1.60 ± 0.01 [a A]	0.52 ± 0.04 [d E]
Staphylococcus aureus (Mean ± SE)	
Control	24houres
1.90 ± 0.01 [a A]	0.79 ± 0.07 [d D]
Duncan test: the similar letters stated to non significant differences	

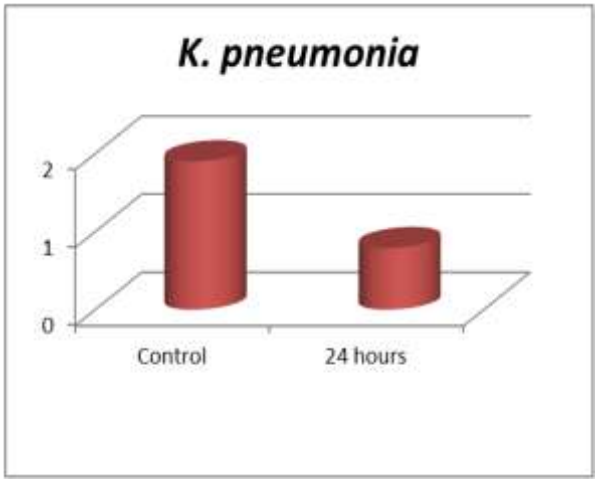

Fig. 4. Antibacterial impact of green produced CuO NPs 40µg/ml concentrations against *K. pneumonia*

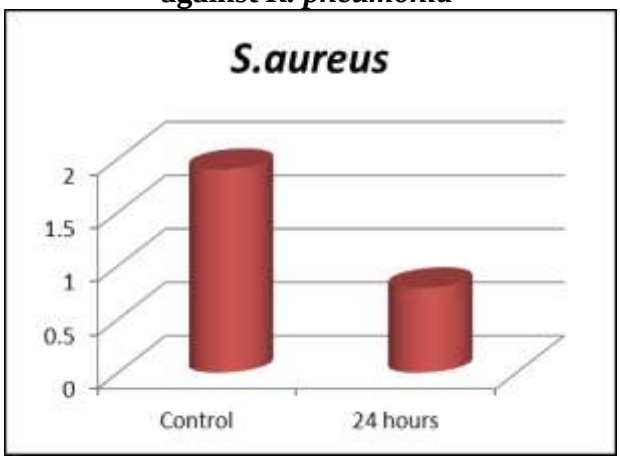

Fig. 5. Impact antibacterial of CuO NPs green produced(40µg/ml)against *S.aureus*

investigation CuO NPs synergistically impact with diverse antibacterial agents

The 40µg/ml of CuONPs incubation with bacterial strains (*K. pneumonia*, and *S. aureus*) during period 24h.Than the bacterial strains sensitivity test against diverse antimicrobial was done suggested by (13), using method of disc diffusion. Depending on guideline of the (NCCLs). The consequences sensitivity of *K. pneumoniae* to copper oxide nanoparticles and biologically effect for enhancing antimicrobial activity were converted from resistance to sensitive for Methicillin, Ciprofloxacin, Cefataxime and Cefixime at 24hours compared with the resistance control. Furthermore, *K. Pneumoniae* isolates showed a high sensitive rate to Gentamicin, Levofloxacin and Amikacin (100%), and the percentage of resistant was decreasing from 66.6% to 22.2% when used Copper oxide nanoparticles at 24 hours(Table4) (Figure6). Also, sensitivity test of *S. aureus* appeared change against antimicrobial agents in the level of resistant involve: Trimethoprim sulphamethox and Cefataxime which was converted from resistant to sensitive at24 hours that copper oxide nanoparticles enhancing antimicrobial activity compared with the resistance control. Thus the resistant percentage was decreased from 55.5% to 33.3%. Whereas *S. aureus* (MRSA) results showed high sensitivity rate to Gentamicin, Levofloxacin, Ciprofloxacin and Amikacin at a percentage (100%). As well as, *S. aureus* was showed high degree of resistant to Methicillin, Amoxillin/ clavulanic acid and Cefixime at a percentage (100%) (Table5)(Figure 6). In addition to, sensitivity of *K. pneumoniae* to copper oxide nanoparticles and biologically effect for enhancing antimicrobial activity higher than *S. aureus*. The structural of the cell membrane in addition to the compositional contrasts could be ascribed to variation in the antimicrobial agents impact as well as CuO nanoparticles against *K. pneumonia* and *S. aureus* (15).Thicker peptidoglycan cell membranes for Gram- positive bacteria contrasted with thin peptidoglycan cell membranes for Gram-ve bacteria and a low antibacterial effect of CuO of Gram- positive bacteria for the reason that CuO NPs are difficult to enter thicker peptidoglycan cell (32). Multidrug resistance between bacteria or/and some genetic mutations were occurred and randomly used of antibacterial agents may be that due to rise of resistance to most recently antibacterial agents as results in the present study elicited that, and mentioned by Stock and Wiedemann (30). In addition to mutation, The highly resistant organisms was producing from that present genes of resistance in plasmid , and creation

of biofilm that assist resistance which is because of antimicrobial slow diffusion during wall of cell bacteria (23), also multidrug efflux systems (27) and alter their permeability to the drug (8). The NPs-mediated removal of the microbes may be microbiostatic, or the impact may be microbicidal, wherever in the growth of bacteria is detained and the killing host's immune cells is potentiated and stop metabolic activities of bacteria. As alternative antimicrobial agents, utilize of polymer-based nanomaterials, functionalized with ligands, antibodies or antibiotics or alone for treating cruel bacterial infections. Assist to control the increasing risk of bacterial resistance can use combinatorial treatment with metallic NPs as adjunct to the existing antibiotics (14).

Table 4. The synergistic effect of CuO NPs on *K. pneumoniae* with different results

Isolate	Hours	Methicillin (ME)	Trimethoprim /sulphamethoxazole (SXT)	Amoxillin/ clavulanic acid (AMC)	Gentamicin (CN) (10µg)	Levofloxacin (LEV) (5µg)	Ciprofloxacin (CIP) (10µg)	Cefixime (CFM)	Cefataxime(CTX)	Amikacin (AK) (30 µg)	Percentage of resistance
Klebsiella pneumoniae (control)		R	R	R	S	S	R	R	R	S	66.6%
	24 hours	S	R	R	S	S	S	S	S	S	22.2%
		50% R	100%R	100%R	100%S	100%S	50%R	50% R	50 %R	100%S	

Table 5. The synergistic effect of CuO NPs on *S. aureus* with different results

Isolate	Hours	Methicillin (ME) (10µg)	Trimethoprim /sulphamethoxazole (SXT)	Amoxillin/ clavulanic acid (AMC) (30µg)	Gentamicin (CN) (10µg)	Levofloxacin (LEV) (5µg)	Ciprofloxacin (CIP) (10µg)	Cefixime (CFM) (5µg)	Cefataxime(CTX) (5µg)	Amikacin (AK) (30 µg)	Percentage of resistance
S. aureus (control)		R	R	R	S	S	S	R	R	S	55.5%
	24 hours	R	S	R	S	S	S	R	S	S	33.3%
		100% R	50%R	100%R	100%S	100%S	100%S	100%R	50%R	100%S	

Figure. 6. Bacteria cultures representing the zones of inhibition around disk were exposed to CuO NPs(40 µg/ml) (A)pathogenic *K. pneumonia* demonstrated inhibition zone created with sensitive to ME, CIP , CTX and CFM at 24hours ,(B) *S. aurous* representing inhibition zone formed sensitivity to CTX and SXT at24 hours

CONCLUSON

According to the findings of the experiments, copper nanoparticles is beneficial as antibacterial agent and resistance rates to antibiotics were decreased after 24 hours incubating in casing of mixture with CuO NPs. Hence, antimicrobial agents and CuONPs, is effect synergistically.

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