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MOLECULAR INVESTIGATION AND COMPARATIVE STUDIES OF THE BACTERICIDAL ACTION BETWEEN SILICA AND ZINC OXIDE NANOPARTICLES AGAINST *KLEBSIELLA PNEUMONIAE*

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ABSTRACT

Klebsiella pneumoniae has been linked to numerous infections that is responsible for high mortality and morbidity rates. In our study, we evaluated and contrasted the antibacterial properties of zinc oxide and silica nanoparticles against strains of *Klebsiella penumoniae* that were obtained from mastitic cow milk. The bacterial strains were identified by biochemical and molecular characterization based on PCR to detect certain genes including rmpA, K2 and fimH genes. According to the PCR results, the size of the genes under investigation was 535, 641 and 508 for rmpA, K2, fimH, respectively. Using *Aspergillus niger* for silica nanoparticle synthesis and Chlorella for ZnO nanoparticle synthesis, the nanoparticles were synthesized using a green synthesis process. The physicochemical properties of the nanoparticles were displayed through execution of several characterization techniques, such as scanning electron microscope (SEM) and transmission electron microscope (TEM), in order to provide additional information regarding the size, shape, and nanoparticles morphology. Minimum inhibitory concentration (MIC) and inhibition zone values were determined by using the agar well diffusion method to test the antibacterial activity of both types of nanoparticles. Only zinc oxide type of nanoparticles had shown efficacy against isolated *Klebsiella pneumoniae* strains, as MIC and inhibition zone diameter for it were 625 µg/ml and 13 mm, respectively. Therefore, the research concluded that zinc oxide is a safe alternative to antibiotics for combating Klebsiella pneumoniae strains.

Keywords: Zinc Oxide, Silica, Klebsiella pneumoniae, Nanoparticles, Bactericidal

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INTRODUCTION

Klebsiella pneumoniae has become a prominent pathogen that causes pneumonia and other major infections, including blood stream and urinary tract infections. The increasing prevalence of the strains of *Klebsiella pneumoniae* to multi-drug resistant poses a critical challenge to the community, particularly in hospital settings where nosocomial infections are common (Garza-Ramos *et al.,* 2015). The resistance of those strains to conventional antibiotics has led to heightened morbidity and mortality rates, necessitating the exploration of alternative therapeutic strategies (Hladicz *et al.,* 2017). Like other novel medical approaches implemented for diseases treatment, such as biological molecules and enzymes, nanoparticles have attracted attention for their potential as antimicrobial agents (Haneen& Neihaya 2019, Jawad *et al.,* 2023and Seror *et al.,* 2023). Nanoparticles of silica and zinc oxide (ZnO) have demonstrated potential in having bactericidal effects against a wide range of pathogens (Akbar, 2021 & Alhazmi, 2022) The application of green synthesis techniques for formulation of the nanoparticles not only minimizes environmental impact, but also enhances their biocompatibility, making them suitable candidates for medical applications (Ijaz *et al.,* 2020). The unique physicochemical properties of those nanoparticles are characterized by crystalline state, particle size, morphology, and porosity. ZnO NPs possess a diverse range of antimicrobial properties that can be used to fight microorganisms, such as *E. coli, Staph. aureus, Pseudomonas aeruginosa* and *Bacillus subtilis* (Jin & Jin. 2021). The antimicrobial effect against microorganisms can be related to many aspect ratios and the different shapes of zinc oxide nanoparticles, including spheres, platelets, rods, and needles (Raghupathi *et al.,* 2011). The development potential of Silica nanoparticles increases the prevalence of research hotspot. Inorganic materials, such as silica nanoparticles (sNPs) are produced in a variety of particle ranges from 1 to 100 nm. Their distinctive features include a specific surface area that is easily synthesized and amplified, easy surface modification, and robust delivery systems (Li *et al.,* 2020).

The aim of the research is to provide a novel antimicrobial strategy that can deal with the major issue of antibiotic resistance. The findings may give instructive details about the application prospective of those nanoparticles in clinical settings, ultimately improving treatment outcomes for infections caused by multi drug resistance (MDR) strains of *Klebsiella pneumoniae.*

MATERIALS AND METHODS

1. Process of sampling

Forty milk samples were collected randomly from 40 cows in 7 dairy farms that were diagnosed with clinical mastitis. Each sample was collected in 30 ml volumes into sterile centrifuge tubes, which were then transported to lab. Without delay on ice for further examination.

2. Isolation of *Klebsiella pneumoniae* Strains

A MacConkey plate and 100 milliliters of milk were plated on macConky plate and then incubated aerobically at 37°C for 24 to 48 hours. The typical colony was subcultured on MacConkey plate and blood agar. A nutrient broth was used to cultivate single colonies for 24 to 48 hours at 37°C. After that, they were stored using 15% glycerol at -80°C (Yang *et al.,* 2021).

3. Bacterial strains identification

3.1. Biochemical methods

To verify the presence of *Klebsiella pneumoniae*, a variety of biochemical assays were performed on the isolated colonies. Tests included catalase, oxidase, urease, methyl red, and citrate utilization tests. The results were interpreted according to standard microbiological protocols (McFadden 2000).

3.2.PCR

The procedure of polymerase chain reaction (PCR) was used to confirm the identity of the bacterial strains. Primers specifically designed to target the 16S rRNA gene of bacteria were utilized. The detection of a specific band corresponding to the anticipated size validated the identities of the strains when the PCR results underwent electrophoresis on agarose gel. Process of DNA extraction from samples was achieved using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH). 200 µl of the sample suspension was incubated for 10 minutes at 56 °C with 200 µl of lysis solution and 10 µl of proteinase K. Subsequent to incubation, the lysate was combined with 200 µl of 100% ethanol. The sample was subsequently purified and centrifuged, following the manufacturer's guidelines. 100 µl of elution buffer was employed to elute the nucleic acid. Metabion-Germany supplied the primers utilized (Table 1). In a 25 µl reaction, 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan) was employed, accompanied by 1 µl of each primer at a concentration of 20 pmol, 5.5 µl of water and 5 µl of DNA template. The operation was executed using an Applied Biosystem 2720 heat cycler (Yeh *et al.,* 2007; Siu *et al.,* 2011).

Table 1: Cycle conditions, amplicon sizes, target genes and primer sequences.

Gene	Sequences of Primers	Amplified segment (bp)	1ry Denat	Amplification (35 cycles)			Final extension	Reference
				2ry denat	Annealing	Extension		
rmpA	ACTGGGCTACCTCTGCTTCA CTTGCATGAGCCATCTTTCA	535	94˚C, 5 min.	94˚C, 30 sec.	50˚C, 40 sec.	72˚C, 45 sec.	72˚C, 10 min.	(Yeh *et al*.,2007)
K2	GACCCGATATTCATACTTGACAGAG CCTGAAGTAAAATCGTAAATAGATGGC	641	94˚C, 5 min.	94˚C, 30 sec.	50˚C, 40 sec.	72˚C, 45 sec.	72˚C, 10 min.	(Siu *et al*.,2011)
fimH	TGCAGAACGGATAAGCCGTGG GCAGTCACCTGCCCTCCGGTA TGCCGCAGGACTCGGAGGTC	508	94˚C, 5 min.	94˚C, 30 sec.	50˚C, 40 sec.	72˚C, 45 sec.	72˚C, 10 min.	(Garza *et al*.,2015)

4. Biosynthesis of Nanoparticles

4.1. Preparation of Chlorella Aqueous Extract and Zinc Oxide Nanoparticles Synthesis

Chlorella (a green microalga) was harvested and washed with distilled water. Aqueous extract was produced through boiling 10 grams of chlorella for 15 minutes in 100 milliliters of distilled water, followed by filtration to obtain the clear extract. Zinc oxide nanoparticles were produced by adding 10 milliliters of the Chlorella extract to 90 mL of zinc acetate solution (0.1 M) and heating the mixture for 2 hours at 60°C. The produced precipitate was collected, washed using distilled water, then kept at room temperature to be dried (Morowvat *et al.,* 2023).

4.2. Preparation of Aspergillus niger Culture and Silica Nanoparticles Synthesis

Potato dextrose agar (PDA) was used to cultivate *Aspergillus niger* at 28°C for 5 days. Sterile saline was added to the culture to create a spore suspension and filtered through sterile gauze. For silica nanoparticles synthesis, 10 mL of the *Aspergillus niger* spore suspension was mixed with 90 mL of tetraethyl orthosilicate (TEOS) solution (0.1 M) then incubated at room temperature for 24 hours. The yield of silica nanoparticles was collected, washed with ethanol, and air dried (Morowvat *et al.,* 2023).

5. Characterization of Nanoparticles

Several methods were used to characterize the produced nanoparticles. Transmission electron microscopy (TEM) was employed to determine the size and morphology of the nanoparticles. Scanning electron microscopy (SEM) was used to analyze the surface features (Khaing *et al.,* 2018).

6. Nanoparticles Bactericidal Activity Evaluation

The diffusion method of agar wells was applied to assess the produced silica and zinc oxide nanoparticles' antibacterial efficacy. Wells made on Mueller-Hinton agar (plates inoculated with *Klebsiella pneumoniae)* were filled with varying concentrations of nanoparticle. Bactericidal efficacy was assessed by measuring the inhibition zones following the incubation period of 24 hours at 37°C for the plates. Additionally, by creating successive dilutions of the nanoparticles and measuring bacterial growth following incubation, minimum inhibitory concentrations (MIC) were ascertained (Hindler *et al.,* 1994).

RESULTS

1. *Klebsiella pneumoniae* strain isolation

Serial dilution method was applied to the collected milk samples. The inoculated plates were incubated at 37°C 24-48 hours on MacConkey agar plates growing typical colonies for *Klebsiella pneumoniae* (red, mucoid and with a diffuse red pigment color) were picked up for further identification **(Table 2)**.

Table 2: The number of isolated strains of Klebsiella pneumoniae

Number of samples (Total)	The numbers of isolated strains of *K. pneumoniae*
40	16

2. *Klebsiella pneumoniae* strains identification

***2.1* Biochemicals**

Isolated colonies were investigated additionally, by applying a number of biochemical tests (**Table 3)**.

Table 3: *Klebsiella pneumoniae* has been identified by the following biochemical tests

Biochemical tests	Result
Catalase test	+ve
Voges- Proskauer test (VP)	+ve
Citrate test	+ve
Urease test	+ve
Indole test	-ve
Oxidase test	-ve
Methyl red test	-ve

2.2. PCR

Electrophoresis on agarose gel (1.5%) was used to separate the PCR products. Each slot of the gel was loaded with twenty microliters. To determine the band position, a generuler 100 bp ladder was employed **(Figure 1)**.

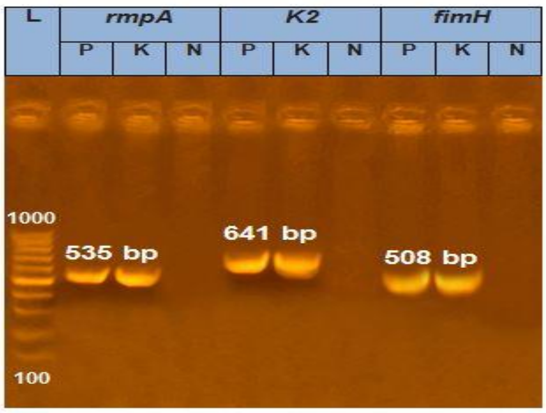

Figure 1. Band position corresponded to each amplified gene. The band positions for rmpA, K2, fimH are 535, 641 and 508 respectively.

3. Nanoparticle characterization

3.1. Scanning electron microscope (SEM)

Scanning electron microscope results of silica nanoparticles and zinc oxide nanoparticles displayed the spherical shaped silica nanoparticles and mixture of rod and zinc nanoparticles' spherical shapes

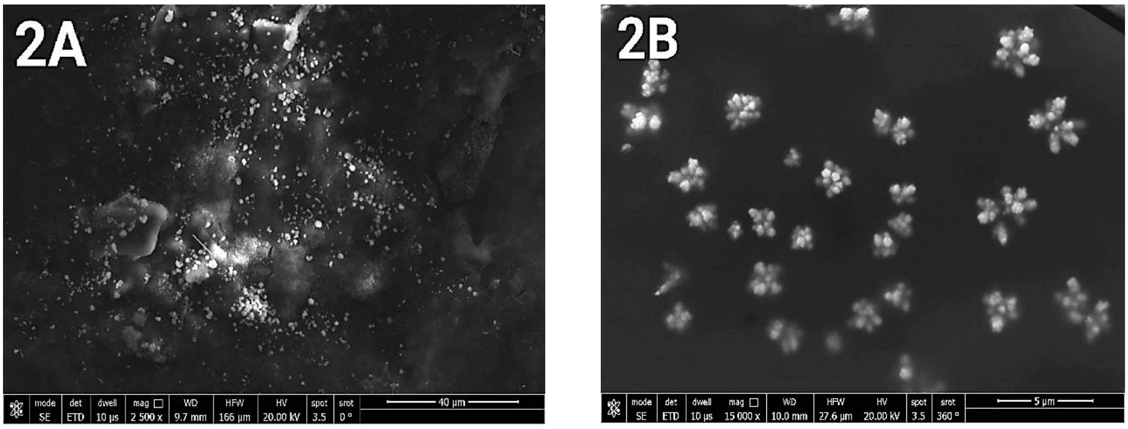

Figure 2: Scanning electron microscope image of both, silica nanoparticles and zinc oxide nanoparticles. (A). Silica nanoparticles. (B) Zinc oxide nanoparticles.

3.2. Transmission electron microscope (TEM)

SEM results of silica nanoparticles and zinc oxide nanoparticles revealed that they have an average silica particle size of 20 nm and a zinc oxide particle size of 30 nm.

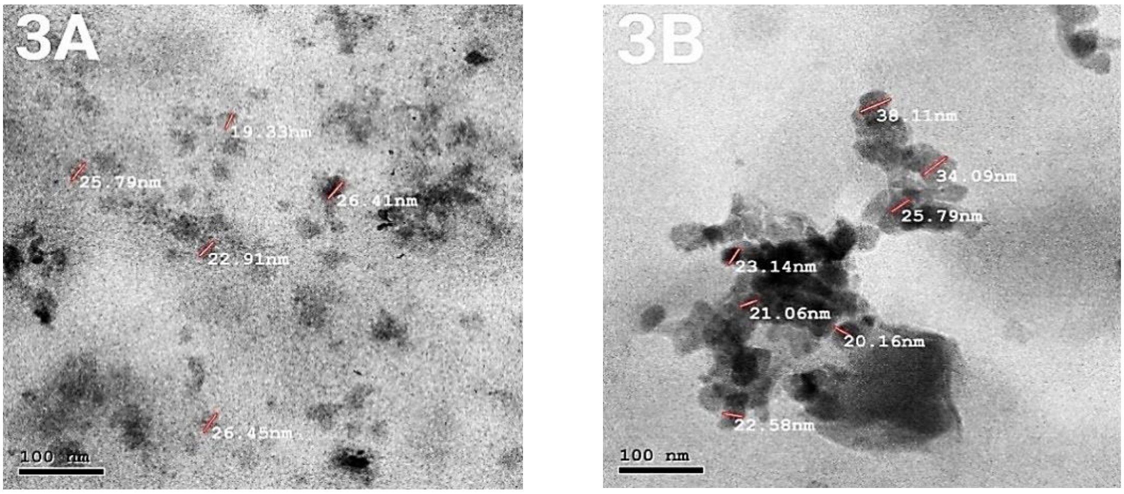

Figure 3: Transmission electron microscope of silica nanoparticles and zinc oxide nanoparticles. (A). Silica nanoparticles. (B) Zinc oxide nanoparticles.

4. Assessment of bactericidal activity

Minimum inhibitory concentration (MIC) value and inhibition zone diameter were measured in order to reveal the efficacy of the two types of nanoparticles against *Klebsiella pneumoniae* **(Table 4 & Figure 4)**.

Table 4: The inhibition zone and minimum inhibitory concentration (MIC) of zinc oxide and silica nanoparticles against *Klebsiella pneumoniae*

Tested nanoparticle agents	Inhibition zone (mm)	Minimum inhibitory concentration (μg/ml)
Zinc oxide	13	625
Silica	____	____

Figure 4: Inhibition zone diameter of both zinc oxide nanoparticles (4 A) and silica nanoparticles (4 B) against *Klebsiella pneumoniae*

DISCUSSION

The scientific community is now more concerned about *Klebsiella pneumoniae* strains that have undergone mutations, which generate the bacteria's hypervirulent or resistant forms. (Merino *et al.,* 1992). To put it concisely, *Klebsiella pneumoniae* is a bacteria that is gram-negative with a capsule, a unique characteristic that allows it to thrive in soil, surface waters, and medical equipment (Bagley 1985 & Rock *et al.,* 2014) As evidenced by previous studies on the use of nanoparticles as antibacterial agents, nanotechnology has revealed a number of advantageous properties that can be applied to both scientific and industrial operations. The unique characteristics of nanoparticles, such as their chemical, physical, and biological characteristics make them appealing for a variety of applications. The use of nanoparticles, which have antibacterial activity against bacteria, fungus, viruses, and protozoa, has significantly enhanced microbiological applications (Renata & Gerson 2020). In the present study, the antibacterial qualities of biosynthesized nanoparticles were evaluated against strains of *Klebsiella pneumoniae,* that were isolated from mastitic cow milk (Frey *et al.,* 2013). Both PCR and biochemical assays had been used to identify the bacterium, and all identification techniques had been validated like earlier publications that were published in the same context (Yogesh *et al.,* 2011). Since green synthesis is believed to be more ecologically friendly than chemical synthesis, it was used to biosynthesize the nanoparticles for manufacture and characterization. The physicochemical report of silica and zinc oxide nanoparticles was also obtained by means of a variety of techniques, such as TEM and SEM for the purpose of visualizing and imaging the nanoparticles. Previous research had confirmed the characterization and production procedures (Nuha, 2023). The agar well diffusion technique has been used to assess the use of nanoparticles as an antibacterial agent; inhibition zone and MIC values have been obtained, and DMSO was employed as a control. The antibacterial activity of silica and zinc oxide nanoparticles could be evaluated using the agar well diffusion methodology, which is agreeable with numerous results of earlier research (Seetharam *et al.,* 2018). The results revealed that only zinc oxide possessed an efficacy against *Klebsiella pneumoniae*.

CONCLUSION

From the obtained results, we can conclude that zinc oxide nanoparticles can be used to suppress the *Klebsiella pneumoniae* as a good antibacterial agent instead of the antibiotics especially for bacteria which may resist the conventional treatment by antibiotics.

DATA AVAILABILITY STATEMENT

The article Material lists the original contributions of the study; if you have any more queries, you can get in touch with the corresponding author.

DECLARATION OF CONFLICT OF INTEREST

According to the authors, there were no financial or commercial ties that might have been perceived as a conflict of interest in conducting the study.

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التوصيف الجزيئي والدراسات المقارنة للتأثير القاتل للبكتيريا بين جسيمات النانو من السيليكا وأكسيد الزنك ضد بكتيريا الكلبسيلا الرئوية

اسراء خلف عنيد ، أمنه حسين موسى ، غفران قاسم عبد الخالق ، نور على زايد ، مها فوزى لطفى ، ايمان محمود شرف

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ارتبطت بكتيريا الكلبسيلا الرئوية بالعديد من الأمراض المعدية المسؤولة عن ارتفاع معدلات الوفيات. في دراستنا قمنا بتقييم ومقارنة الخصائص المضادة للبكتيريا لأكسيد الزنك وجسيمات السيليكا النانوية مع سلالات كلبسيلا الرئوية المُستخلصة من حليب الأبقارالمصابة بالتهاب الضرع، وذلك لاستبدال أنظمة العلاج بالمضادات الحيوية القياسية. تم تعريف البكتيريا من خلال الفحوصات البيوكيميائية الحيوية وتفاعل البوليميراز المتسلسل ثم تم اختيار جينات لتشغيل عملية تفاعل البوليميراز المتسلسل، وكانت مواقع النطاقات للجينات الثلاثة هى rmpA و K2و fimH ، ٦٤١،٥٣٥ على التوالى تم استخدام فطرالاسبرجيلس نيجر لتخليق جسيمات السيليكا النانوية 508 والكلوريلا لتخليق جسيمات أكسيد الزنك النانوية باستخدام عملية تخليق صديقة للبيئة لتجنب الآثار البيئية السلبية. تم توصيف الخصائص الفيزيائية والكيميائية للجسيمات النانوية باستخدام المجهر الإلكتروني الماسح وذلك لتوفير معلومات إضافية حول حجم وشكل ومورفولوجيا الجسيمات(SEM)والمجهر الإلكتروني النافذ(TEM) النانوية، حُددت قيم التركيز المثبط الأدنى للبكتيريا لكلا النوعين من الجسيمات النانوية. أظهرت الجسيمات النانوية من نوع أكسيد الزنك فقط فعالية ضد سلالات الكلبسيلا الرئوية المعزولة، حيث بلغ الحد الأدنى للتركيز المثبط الأدنى (MIC) وقطر منطقة التثبيط ٦٢٥ ميكروغرام/مل و١٣ مم, على التوالي. وعليه استخلص البحث ان أكسيد الزنك هو البديل الآمن للمضادات الحيوية ضد سلالات الكلبسيلا الرئوية.