Original Research Article

Green Synthesis of Zinc Oxide Nanoparticles Using Clove Oil Extract (*Syzygium aromaticum*) and evaluate their antibacterial activity against MRSA

Abstract

There is a crucial necessity for the formulation of efficient antimicrobial agents owing to the increasing prevalence of hospital-acquired bacterial infections triggered by multidrug-resistant microbes that result in significant deaths and illnesses around the world. Hence, The current study aimed to synthesis zinc oxide nanoparticles using clove extract (SaZnONPs) by an eco-friendly technique and investigated their antibacterial activity against *S. aureu (MRSA).*Clove extract serves as a capping and reducing agent for making ZnO-NPs. Formation of SaZnO NPs were then confirmed by using a variety of analytical techniques. Clove extract was initially incubated with zinc nitrate solution, and the color shift of the solution that followed is proof that the zinc nitrate ion was reduced to ZnO nanoparticles. UV(Ultraviolet and visible spectroscopy) revealed SaZnO nanoparticles surface plasmon resonance band at 329.26nm. Furthermore, SEM (Scanning electron microscopy) image showed that atoms' average size is 41 nm, and it reveals that they are spherical in shape. The antibacterial activity of the SaZnO-NPs was tested against Staphylococcus aureus(MRSA) using agar well diffusion, minimum inhibitory concentration. The clove extract phytochemicals facilitate the synthesis of stable ZnO-NPs and showed good antibacterial activity.

Keywords: SaZnONPs, plasmon resonance, clove extract, MRSA.

Introduction

Staphylococcus aureus (*MRSA*) is a significant human pathogen that causes a number of clinical infections [1]. The rise of multi-drug resistant strains like Methicillin- Resistant Staphylococcus aureus (MRSA) in community and hospital settings has made treating infections caused by S. aureus very difficult in recent decades[2,3]. The mecA or mecC gene, which encodes a penicillin-binding protein (PBP2a) or (PBP2ALGA) with a poor affinity for -lactams, is the cause of methicillin resistance[4]. The demand for the creation of novel and efficient antibiotic alternatives has increased due to the emergence, dissemination, and durability of resistance to various antimicrobials. The development of nanotechnology have considerable potential of nanomaterials as antimicrobial agents. Compared to conventional antibiotics, nanomaterials offer a wider range of microbicidal activity. [5]. Among the most promising metallic nanomaterials, ZnO nanoparticles have been identified. Because ZnO-NPs are safe and stable for human cells, there has been an increase in interest in them as effective antibacterial agents in recent years[6, 7]. Biosynthesis of metal and metal oxide NPs with plant extraction is an innovative branch of nano biotechnology, which has been recognized depend on green chemistry [8]. Because plants contain carbohydrates, proteins, enzymes, and phytochemicals like

phenols, terpenoids, ketones, aldehydes, and amides that are responsible for the spontaneous reduction of ions, it is actually possible to create inorganic NPs using plant extracts and their derivatives[9]. The most popular processes for creating ZnO NPs are hydrothermal synthesis and vapor-liquid-solid synthesis. Due to their non-hazardous, affordable, biodegradable, and environmentally benign properties, microorganisms, phyto extracts, and natural biomolecules have recently been used in the production of nanoparticles [10,11]. Syzygium aromaticum (clove) is a member of the Myrtaceae plant family and has fragrant dried flower buds. In addition to glycosides and flavonols, clove also includes 10% fixed oil, 15-20% essential oil, 6-7% non-essential ether extract, and 13% tannin. [12]. It possesses antibacterial and antioxidant properties. In the commercial world, they are employed in the production of the fragrance and pharmaceuticals industry [13]. Clove essential oil's main ingredient, eugol (4-allyl-2-methoxyphenol), has strong antibacterial, antioxidant, and insecticidal properties [14]. The current study illustrated an environmentally acceptable method for making zinc oxide nanoparticles (ZnO NPs) utilizing clove (Syzygium aromaticum) extract. This work was aimed to Biosynthesis of SaZno nanoparticles using plants, and Iinvestigate the antibacterial activity of the ZnO nanoparticles which are obtained by clove extract in-vitro.

Materials and methods

Plant extract preparation

Collection of plant

The origin of *S. aromaticum* that was purchased from a resident market.. All amount of clove buds cleaned three times with distilled water, disinfected with 5% sodium hypochlorite solution (NaOCl), and then allowed to dry. Using a mechanical mortar, the dried plant was ground into powder. The hydro-distillation extraction was performed by clevenger equipment. The amount of *Syzygium aromaticum* L. used for hydro-distillation was 200 g.

Preparation of clove oil (*Syzigyum aromaticum)*

Clove oil was extracted from dry buds using hydro-distillation (water distillation) by Clevenger apparatus. Two hundred gram of dried buds were taken and completely cleaned with double-distilled (DD) water to remove dust particles, then dried outside in the sun and placed in 1000ml glass flask, 500 ml of distilled water was placed. The flask was joined to the machine and the operation continue for 3 hours. The flask was placed in a heating mantle and electrically heated. The temperature of the flask was first raised progressively to 100°C from a starting point of roughly 80°C.The extraction procedure continuous at this temperature until no further droplets of oil was coming out of the condenser. The oil was collected after isolating the water , and to increase the water disposal a little of anhydrous sodium sulfate was used. The extract oil was kept in the refrigerator at a temperature of 4c̊. [15].

Preparation of SaZnO nanoparticles:

SaZnO nanoparticles were created using green synthesis method according to [16] with slight alteration. In a beaker with 300 ml of deionized water, 20 mg of Zn(NO3)2 was

dissolved with a stirring for ten minute using magnetic stirrer. Then, 50mL of clove extract was added slowly into the beaker containing the Zn(NO3)2 solution under stirring condition and controlled pH above 9, until yellow color solution was obtained. A prepared organic phase of ethanol was made and added to the mixed solution in the glass beaker. The resulting suspension was centrifuged at 14,000 rpm after being filtered (using Whatman filter paper 1) to remove any precipitated materials. The solution was placed in a 20 ml beaker, transported straight to an ultrasonicator, and the emulsion was sonicated for five minutes at a power of 100 W and a frequency of 42 kHz.

Separation and Identification of Sa ZnO nanoparticles

UV–Vis spectra analysis

Using UV-Vis spectrophotometry, the zinc oxide nanoparticles were evaluated for their maximum absorbance. UV-vis spectrum: sample of 0.2 gm of ZnONPs was taken, and the absorption was measured using a scanning spectrophotometer at intervals of 0.5 in the wave length range from 200 to 1100 nm. The peak increase positively with increase reaction time of ZnONPs synthesis [17].

Scanning electron microscopy (SEM)

The structural morphology of zinc oxide nanoparticles was examined and measured by Scanning Electron Microscopic (SEM). The shape and size of synthesized SaZnONPs. were examined at advanced nano search center by using Scanning Electron Microscopy (SEM)[18].

Particle size distribution

A partical size analyzer was used to determine the particle size distribution (polydispersity index and mean diameter)(Malvern Instruments, Worcestershire, United Kingdom). It exploited the Mie theory of light, which says that the size of any particles present is related to the scattering pattern created when light passes through a sample. [19]. SaZnO nanoparticles Before being examined in a size analyzer, the sample was treated in a sonicator water bath at 35C for 30 min. to prevent particle aggregation.The outcomes were monitored on a computer screen.

Antibacterial activity of SaZnO-NPs

Isolation and characterization of bacterial isolates

Collection of specimens and bacterial identification were done by selecting five samples of pathogenic bacteria *Staphylococcus aureu* (MRSA strain). These samples were obtained from Microbiology lab at College of VET medicine / University of Baghdad. Samples were inoculated on nutrient agar and blood agar and then incubated at 37°C overnight below aerobic-circumstances. Many of these isolates were established for microscopic morphological, cultural and biochemical studies.

Preparation of Standard Bacterial Suspension

By comparing to the Standard McFarland solution (0.5), the quantity of MRSA bacteria in each milliliter of the stock suspension was standardized [20]. Briefly, bacterial suspension equivalent to 0.5 McFarland (1.5 x 10^8 CFU /ml) was arranged from overnight bacterial culture. The absorbance of this index was 0.136 as noted by spectrophotometer at a wave length of 450 nanometer. 0.1 ml of the prepared bacterial suspension was diluted in 14.9 ml of Mueller-Hinton broth and incubated at 37 ºC for 1 hr. to obtain 10^6 CFU/ml bacterial suspensions [21,22].

Measurement of Antimicrobial Activity of SaZnO-NPs was done by three method:

1-Agar well diffusion method:

The agar well diffusion method was take on according to [23], for evaluating the antibacterial activity of SaZnO-NPs against *MRSA*. Standardized bacterial suspension (1.5×10^8cfu/ml) of *S. aureus* was carefully mixed with sterile Mueller Hinton agar. 25 ml of the sterile agar was dispersed into sterile Petri dishes and left for 10 minutes in room temperature to allow to dry, and six mm. diameter wells were bored in the agar. The SaZnO-NPs was reconstituted in distilled water to a concentration from 5µg/ml to 0.312µg/ml and then 100µl was added to wells. The plates were at 37 C^o for 24 hours after allowing the extract to diffuse into the agar at room temperature. Thereafter, the diameter of the inhibitory zone was measured to the closest millimeter (mm).

2- Microtiter plate Dilution

SaZnO-NPs (10 mg/ml) was made in Mueller-Hinton broth, from this broth, two folds dilution was downgraded from 40 µg for clove oil in U- shape (200 µl well capacity) 96 well micro-titer plate, Each well was inoculated with 100 µl of 10^6 CFU/ml *S. aureus* and incubated on 37ºC for 24hrs. For colorimetric identification of bacterial growth, 15µl of 0.125% triphenyltetrazolium chloride dye was added to each well of the test and re-incubated for two hrs. [24] in order to determined MIC and MBC which was determined as the lowest concentration of extracts kill the bacteria [25].

***3-In-vitro* Time Kill Curve Kinetic**

The time-kill curve assay of SaZnO-NPs against *MRSA* were based on the National Committee for Clinical Laboratory Standards [26]. Briefly, bacterial suspension prepared as mention beforea and the Clove extract had been dissolved in Mueller-Hinton broth to prepare 10 mg/ml stock solution, after that, clove extract concentrations from 4x MIC to 0.25x MIC were prepared. Bacterial colonies were calculated at 0, 1, 2, 4, 6, and 24 h through the incubation time by making serial dilutions and spreading of 20 µl of each dilution on Mueller-Hinton agar plate (triplicate); colonies range 30-300 CFU/plate was accepted [27].

The trapezoidal method was used to estimate the area under the time-kill curve of concentration of SaZnO-NPs as below [28]:

$$\boldsymbol{AUC_{kill}} = \sum \left(\frac{\mathbf{log}(\boldsymbol{Cn}) + \mathbf{log}(\boldsymbol{Cn+1})}{\mathbf{2}} . \Delta \boldsymbol{t} \right)$$

Where the AUC_{kill} is the area under the killing curve of SaZnO-NPs, log (Cn) is the logarithm of a specific concentration at a specific time, log (Cn+1) is the logarithm of the next concentration while (Δ t) resemble the difference between their times.

Statistical Analysis

The Statistical Analysis System- SAS [29] program was used to detect the effect of difference factors in study parameters. Least significant difference –LSD test (Analysis of Variation-ANOVA) was used to significant compare between means in this study.

RESULT AND DISCUSSION

Nanoparticles characterization

Visual observation

The color shift from dark yellow to light yellow after combining with clove extract is the first sign of the creation of SaZnO nanoparticles as show in (Figure 2).

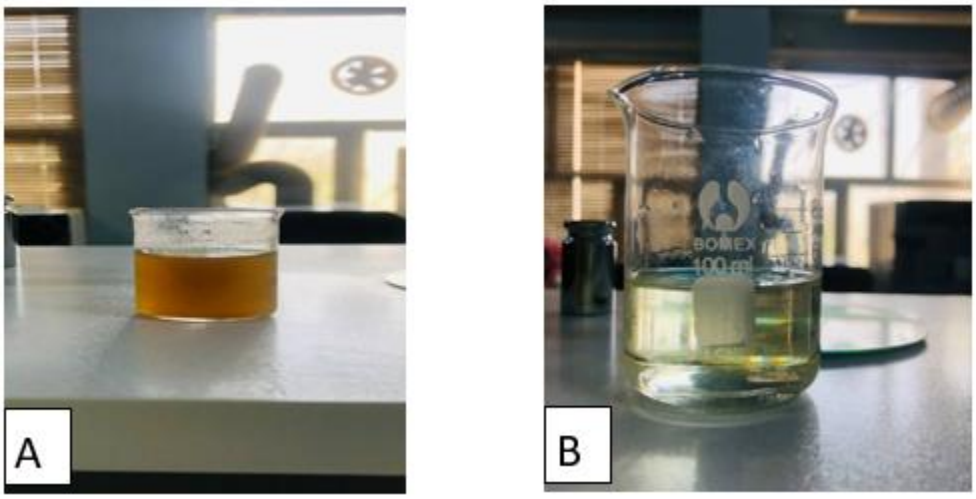

Figure 1. The changing in color of *clove* extracts from dark yellow to light yellow. (a) Clove extract solution. (B) Extract after reaction with Zn nitrate solution to form ZnO nanoparticles

UV-visible Spectroscopy

UV-Vis spectroscopy was used to assess the absorbance of a solution of SaZnO nanoparticles; the results showed that the SaZnO nanoparticles' highest absorbance occurred at the absorbance peak of 329.26 nm. as show in (Figure 2).

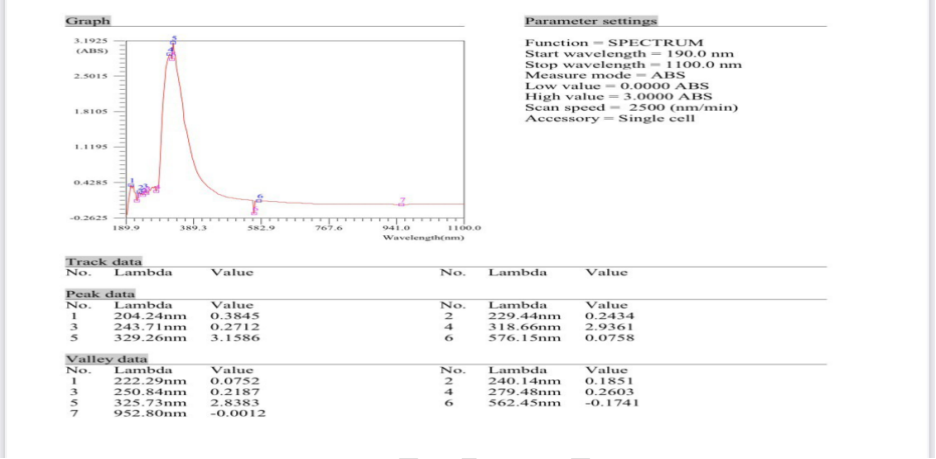

Figure 2. UV-VIS absorbance spectroscopy for SaZnO nanoparticles from clove extract

Scanning Electron Microscopy (SEM)

Figure 4 illustrates a typical SEM picture of the produced SaZnO NPs, which appear different magnifications of ZnO nanoparticles. This images makes it obvious that there are spherical to hexagonal-shaped particles with a grain size of 41.4 nm in aggregated states.

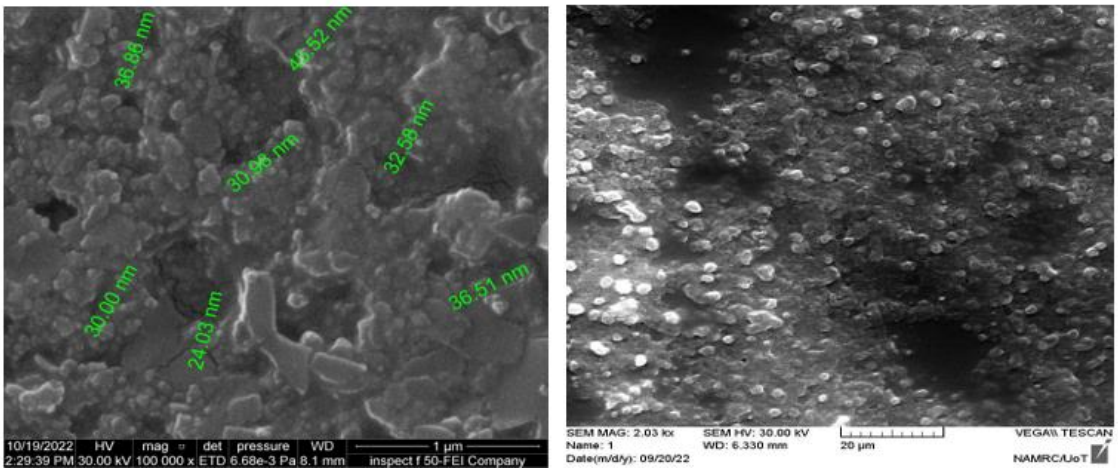

Figure 3. Scanning Electron Microscopy image of particle size and morphology of SaZnONPs.

Particle size analyzer

The size of the nanoparticles was measured using a size analyzer that relied on dynamic light scattering. The distribution of size analysis showed that the synthetic SaZnONPs had an effective diameter of about 41 nm. (39.7 ± 4.1) as show in **(Figure 4**)

Figure 4. Particle size of SaZnO NPs.

Biosynthesis of zinc oxide nanoparticles from clove extract in this study was identified by clearly changing in color as a step was showing in figure1. The SPR (surface plasmon resonance) in the metal nanoparticles is indicated by the color change[30]; After the clove extract was incubated with the zinc nitrate solution, a color change was noticed, which is proof that the zinc nitrate ion was reduced to ZnO nanoparticles. Bio-reduction is the process of reducing metal ions to metal nanoparticles with the help of phytochemicals such polyphenols, polysaccharides, alkaloids, vitamins, and amino acids. Flavonoids act as reducing elements for metal ions. However, the functional groups of flavonoids are what cause the formation of nanoparticles[17].

Figure (2) clarified the UV-visible spectra of prepared ZnONPs from clove extract. SaZnO nanoparticles' absorbance peak at 329.26 nm revealed that this wavelength's significant absorption peak is caused by zinc oxide's band-gap absorption, which is the result of electrons being removed from its valence band and transferred to its conduction band[31]. Zinc oxide nanoparticles exhibit good UV absorption in the range of 200 to 400 nm, making them useful for medical applications like sunscreen or antiseptic ointments [32].

To describe the size, form, morphology, and distribution of created nanoparticles, the SEM analysis is used. Many sizes and shapes were produced through the green synthesis of SaZnO NPs from clove extract, but the most common shape was spherical with a diameter of about 41 nm. These nanoparticle forms were consistent with the research of Vahidi et al. [33], who used Pelargonium leaf extract to create ZnO NPs with the same morphological characteristics. This test suggests that the SaZnO -NPs employed in this

investigation are high-quality materials. Our results are in coordination with earlier studies[34].

The SaZnONPs size was calculated via dynamic light scattering. The average particle size, according to the size of the nanoparticles dispersion analysis, was 41.4 nm. (Figure4). Small particles are known to be more effective than large ones in terms of NP activity, which is influenced by their size[35]. Numerous earlier studies revealed that NPs' activity varied depending on their particle size [36].

Antibacterial results of SaZnO-NPs

The multidrug resistant strains of *S. aureus* are increasing, making the treatment more challenging. The prevalence of MRSA among *S. aureus* isolates is the highest over the world.The results showed that the concentration 0.312 µg/ml SaZnO nanoparticles were effective to prevent *MRSA* from growing, these concentrations were shown to have a positive value that inhibit the growth 0.625 µg/ml of SaZnO nanoparticles killed *MRSA* so it considered as the (MBC) that tested in micro-dilution assay.

The plant extracts may temporarily exhibit an action against infections, but they are susceptible to destruction after a while due to the presence of many chemical components in their structure [37], however after usage in the biological synthesis of nanoparticles, the active substance including proteins,vitamins, enzymes, alcohol and amino acids compounds that present in the plant extract and are responsible for the development of the nanoparticles act as reducing and covering substance for many months [38].

According to the study by [39] ZnO-NPs have broad-spectrum antibacterial properties and can even eliminate MRSA and other drug-resistant pathogenic bacteria. The possible mechanisms of action of ZnO-NPs against bacteria include the following:(a) excessive production of reactive oxygen species by bacteria, (b) disruption of key enzymes in the respiratory chain due to damage to microbial plasma membranes; (c) metal ion accumulation in microbial membranes; (d) inhibition of metabolic processes by electrostatic attraction between nanoparticles of metal and microbial cells; and (e) suppression of microbial proteins/enzymes through increased production of H2O2. [40].

Strong anti-microbial properties are possessed by metal nanoparticles, particularly ZnO NPs; the mechanism for this is that ZnO-NPs will produce hydrogen peroxides (H2O2), and these peroxides will disturb the lipid and protein bilayers, which will destroy bacterial cells [41]. In addition, another antimicrobial action of ZnO-NPs involves the production of reactive oxygen species (ROS) and accumulation of the nanoparticles in the cytoplasm which causes cell death. Bacterial DNA proteins and lipids will be damaged by the generated ROS, leading to cell death [42, 43].

On other hand, the concentration and size of ZnO nanoparticles significantly influenced bacterial growth. Therefore, ZnO-NPs may have a size-dependent antibacterial effect. Nanoparticles with smaller size can penetrate through the microbial cell membrane more easily and enter into the mitochondria leading to mitochondrial oxidative stress and apoptosis,thus inhibits growth of the cell and promotes its death [43].

Sensitivity assay

Different concentrations of SaZnONPs was using in agar well diffusion methods, resulting in different sizes of inhibition zone against methicillin resistance *staphylococcus aureus*. The sizes of inhibition zones were different according to concentration of the SaZnONPs. Results indicated that MRSA strain were sensitive significantly ($P<0.05$) to SaZnONPs in a dose dependent concentration 5, 2.5 ,1.25 ,0.625 and 0.312 µg/ml. Increasing in the diameter measurement of zone of inhibition in *MRSA* growth was proportionally related to SaZnONPs concentrations, (Table 1), (Figure 5).

Table 1: *in-vitro* antibacterial efficacy of SaZnO-NPs in different concentrations against *MRSA* (measured as the diameter of the inhibitory zone in millimeters).

Groups / Conc.	Con.(µg/ml)				
	5µg/ml	2.5µg/ml	1.25µg/ml	0.625µg/ml	0.312µg/ml
Sa ZnO-NPs	25.0±0.97 A a	19.0±0.77 A b	17.0 ±0.82 A ab	14.0 ±0.57 A bc	12.0 ±0.61 A c
LSD value	3.337				

- **Values represent mean ± S.E**
- **Different letters denoted a significant difference ($p\leq0.05$) among the groups.**

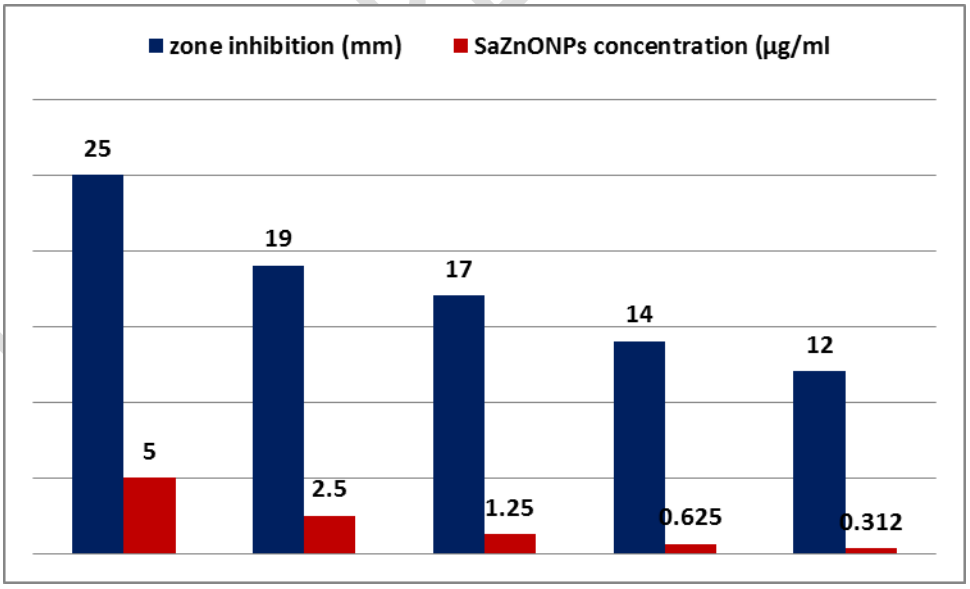

Figure 5: Proportional relationship between different concentration of SaZnO-NPs, Clove oil and the mean of inhibition zone (mm) against MRSA.

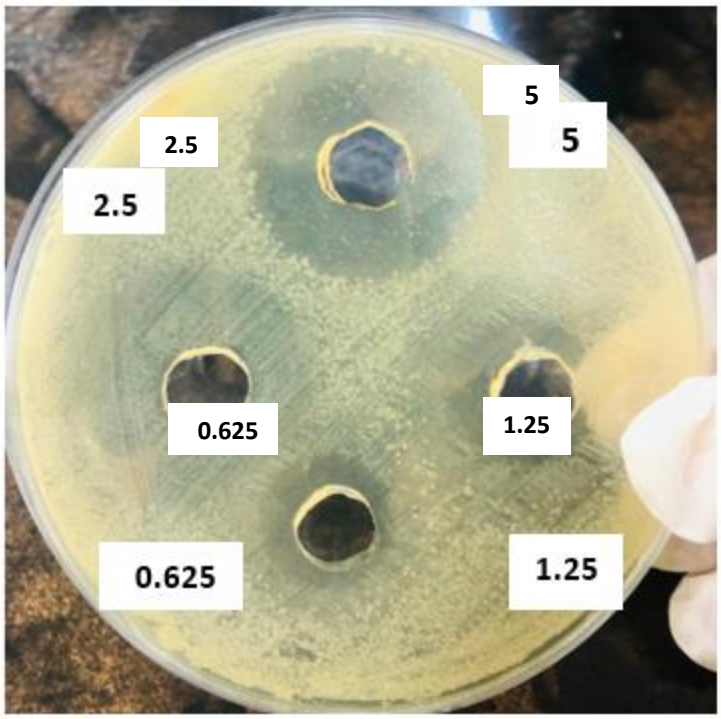

Figure 6: Sensitivity of *MRSA* to different concentrations of SaZnO -NPs

The results of SaZnONPs showed that the inhibitory effect increased as the concentration increased and reached its peak inhibition zone of 25.0mm at 5 µg/ml. this is in line with [44] who observed that 1% ZnO-NP solution exhibited maximum inhibition of the zone against *Staphylococcus aureus* (21 mm). while Irfan *et al.*, [45] who used Moringa oleifera for synthesis ZnO-NPs and detected good activity against *methicillin-resistant Staphylococcus aureus (MRSA)* at concentrations higher than as 30 µg, 20 µg, and 10µg) and produced inhibition zone in the range of 17, 12 and 6 mm. respectively.

In general Pati *et al.* (46) have demonstrated that zinc oxide nanoparticles damage the integrity of bacterial cell membranes, decrease the hydrophobicity of cell surfaces, and suppress the transcription of oxidative stress-resistance genes in bacteria. By promoting the formation of ROS, they improve intracellular killing of bacteria. These nanoparticles prevent the development of biofilms and inhibit pathogens' hemolysin toxin from inducing hemolysis.

In order to demonstrate the size-dependent antibacterial activity of ZnO-NPs, it has been stated that the smaller size of ZnO-NpS has stronger antibacterial activity than microscale particles [47] and these in accordance with our results as we obtained nanoparticles in size 41nm. The small size of ZnO-NPs has the ability to inter the microbial cells because of their high surface area to volume ratio [48]. Therefore, smaller ZnO-NPs are more potent antibacterial agents than the larger one *[49]*. *S. aureus* growth is inhibited when the size is 12 nm, but as the size above 100 nm, the inhibitory effect is decreased [50].

According to this study's finding we can say that ZnO-NPs reinforced with clove extract and can be utilised as an alternative to commercially available antibacterial agents. Since applying plant extract to produce metal oxide, significant benefits have been obtained because of the production of the functional molecules which reduce metal ions during synthesis of nanoparticles [51].

Time kill curve kinetics

The time-kill curve kinetics of *Sa*ZnO nanoparticles are based on the highest MIC recorded from the micro-dilution assay which were 0.312µg/ml for the *MRSA* strain. The concentrations used *in vitro* study were 0.25x MIC, 0.5x MIC, 1x MIC, 2x MICs, 4x MICs.

*Sa*ZnONPs showed bactericidal activity at concentration of 4xMICs, 2xMICs by reducing ≥ 3 log10 of the total number of CFU/ml of *MRSA* at 4 and 6 hr respectively as show in (Figure 6) in comparison to control, 0.25x MIC and 0.5x MIC; on the other hand, 1x MIC for two agents showed a drop in growth curve at 1st six hours. Then, there was continued inhibition of growth as reported at the 24th hour (Figures 7).

The area under the time of killing curve of *Sa*ZnO nanoparticles calculated and compared to control inoculum growth rate, and the difference in the area under the curve values among different treatments were set as an endpoint whereas the lowest area under the curve refers to the highest bactericidal effect as reported in the (Table 2).

The results showed that all of 2xMICs and 4xMICs achieved the highest significant bactericidal effect ($P \geq 0.05$) in comparison to other treatments. The 1xMIC concentration achieved purely bacteriostatic effect ($P \geq 0.05$) in comparison to all concentrations and control groups; both of 0.5xMIC and 0.25xMIC failed to achieves a significant bacteriostatic or bactericidal effect ($P \leq 0.05$) in comparison to 1xMIC, 2xMIC and 4xMIC.

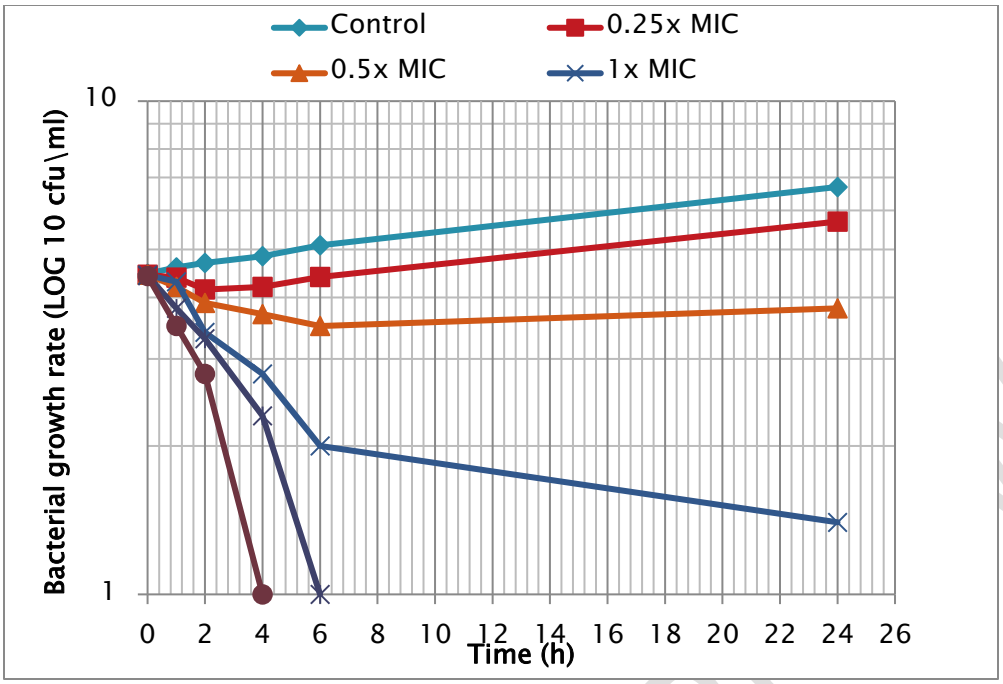

Figure 7: Time kill curve kinetics of SaZnO -NPs against MRSA

Table 2: Area under the time-kill curve of *Sa* ZnO NPs against *MRSA (h*log CFU/ml).*

Antibacterial	Control	0.25xMIC	0.5xMIC	1xMIC	2xMIC	4xMIC
Sa ZnONPs MIC=0.312µg \ml	121.12 ± 0.25 A	115.71 ±0.31 A	76.15 ±0.41 B	49.12 ±0.44 C	20.37 ± 0.52 D	11.77 ±0.14 D
LSD	9.1*					

- **Values represent mean ± S.E**
- **Different letters denoted a significant difference (p≤0.05) among the groups.**

Time killing curve kinetic is a combined and extensive tool to assess both bacteriostatic and bactericidal effects of the antibiotics; it depends on the change in the logarithmic number of bacterial colonies through the defined chronological pattern [52]. More accurate descriptions of antimicrobial activity are provided by a measure of bacterial killing (kill kinetics) than by the MIC, and it has also shown better sensitivity developments to physicians than disc diffusion methods [53; 54]. When the concentration was increased more than two fold in MICs, each of 2xMICs, and 4xMICs concentrations of *Sa*ZnO nanoparticles showed a distinguished bactericidal effect at the 6 hr and 4 hrs respectively. These is nearly as same as what reported by [55], where they found that

ZnO nanoparticles at 2xMICs killed more than 99.91% of *S. aureus ATCC* at the same time that we report considering the difference in the isolates that were used in studies. At higher ZnO-NPs concentrations(2 and 4x MIC), noticeable bactericidal activity is seen. After 6 hours of being subjected to ZnO-NPs, the bacteria are completely destroyed.

In the present study, it was also found that the greater concentration of SaZnO NPs, the shorter the time are required for the bactericidal action on the tested bacteria. Likewise, Hoseinzadeh *et al.,* [56] reported that the time killing for *S. aureus* and *E. coli* at 2xMIC was 3 and 6 h respectively.

Time killing kinetic of SaZnO nanoparticles against *MRSA* was concentrations and time dependent[57; 54]. This in turn suggests a more rational basis for establishing the ideal dose for antimicrobial treatment regimens., in order to prevent the development and spread of antimicrobial resistance[54] **.**

Conclusions

The ability of S. aromaticum's aqueous extract to create ZnONPs has been established. Compared to the traditional physical and chemical metal oxide NP fabrication methods, the green synthetic approach for the synthesis of ZnO NPs uses clove extract as a natural reducing and stabilizing agent, making this synthesis method simple, energy-efficient, and cost-effective. The reduction of zinc nitrate to ZnONPs was approved by UV–visible spectrophotometer, SEM, partial size analyzer and zeta potential analyzer techniques. In addition, SaZnO-NPs showed strong antibacterial effectiveness. As a result, they could be employed as an antibacterial agent and a viable alternative to traditional antibiotics.

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