

Potential Activity of Zinc Oxide Nanoparticles and Ethanolic Olive Leaf Extract Against Oxacillin Resistant *Staphylococcus aureus* *in vitro*

Dhafar Al-Ugaili, Fadhil A.M.A. and Saleh A. Wohaiieb

Department of Biotechnology, College of Science, Al-Nahrain University, Baghdad-Iraq.

E-mail: ayad_university@yahoo.com.

Abstract

This study was carried out to evaluate the enhanced effect of ZnO nanoparticles and a crude ethanolic extract of olive leaves upon the antibacterial activity of oxacillin against oxacillin resistant *Staphylococcus aureus* (ORSA) isolate from hospital environment. A positive and induced effect for ZnO nanoparticle was detected upon the antibacterial activity of the antibiotic through the increase in the diameter of the inhibition zone for the antibiotic as it recorded (15mm) for the mixed activity to the antibiotic with nanoparticles compared with that of (10mm) for the zinc oxide nanoparticle alone and the disappearance of the inhibition zone for the antibiotic alone. The antibacterial efficacy of the crude ethanolic olive leaf extract was evaluated alone or in combination with either oxacillin or zinc oxide nanoparticles against oxacillin resistant *Staphylococcus aureus* using disc diffusion method. The results revealed that olive leaf extracts (2.25-9 mg/disc) neither exhibited antibacterial activity when used alone, nor potentiated the antibacterial activity of zinc oxide nanoparticles (100µg/disc) when used in combination. However, in the presence of the highest concentration of olive leaf extract (9mg/disc), the antibacterial activity of oxacillin (1µg/disc) (as a diameter of inhibition zone) increased from zero mm to a maximum of 14 mm, suggesting a potentiation of the antimicrobial activity of oxacillin against the already existing oxacillin-resistant strain of *Staphylococcus aureus*.

Keywords: *S.aureus* MRSA/ORSA, synergism, ZnO nanoparticles, ethanolic olive leaf extract, antibacterial activity

Introduction

Increasing resistance to antibiotics with the wide-spread use of immunosuppressing drugs and a rise in many infections all emphasize the necessity to find and develop new antimicrobial agents [1]. The use of nanoparticles is gaining impetus in the present century as they possess defined physico-chemical properties including ultra-small size (nano is a Greek word synonymous to "dwarf" meaning extremely small), large surface to mass ratio, high reactivity and unique interactions with biological systems [2]. Antibacterial properties of nano metal oxides have been discovered as new generation of antimicrobial agent. Nano-ZnO particles are effective in inhibiting Gram-positive, Gram-negative bacteria and even spores that are high-temperature resistant and high-pressure resistant [3], [4] and researchers have offered the use of zinc ion as superior disinfectant from hospitals infectious microorganisms [5]. Furthermore, ZnO nanoparticles, have selective toxicity to

bacteria and only exhibit minimal effect on human cells, which recommend their prospective uses in agricultural and food industries especially with the growing need to find alternative methods for formulating new type of safe and cost-effective antibiotics in controlling the spread of resisted pathogens in food processing environment [6],[7], [8]. As well as, ZnO is listed as "generally recognized as safe" (GRAS) by the U.S. Food and Drug Administration. The small size and the high surface to volume ratio i.e., large surface area of the nanoparticles enhances their interaction with the microbes to carry out a broad range of probable antimicrobial activities [9]. Therefore, nano-sized particles of ZnO have more pronounced antimicrobial activities than large particles, since the small size (less than 100 nm) and high surface-to-volume ratio of nanoparticles. Among the factors that may influence the antibacterial activity of ZnO, are the concentrations of the metal oxides particles [10], the particle size of the metal oxide

powder [11] and the specific surface area of the powder [12]. Thus, the antimicrobial activity of the nanoparticles is known to be a function of the surface area in contact with the microorganisms.

Finding healing powers in plants is an ancient thought that used as traditional medicine all over the world and predate the introduction of antibiotics and other modern drugs [13],[14], [15]. The antimicrobial efficacy attributed to some plants in treating diseases has been beyond belief. Owing to their popular use as remedies for many infectious diseases, searches for plants containing antimicrobial substances are frequent [16], [17], [18], and [19]. Throughout the history of civilization, the olive plant has been an important source of nutrition and medicine. Olive leaf from olive tree (*Olea europaea*) that is native to the Mediterranean countries, is a plant which can survive for hundreds of years and is known to naturally possess strong resistance to microbial attack [20],[21],[1]. Phenolic compounds including oleuropein, tyrosol, hydroxytyrosol, caffeic acid, gallic acid, syringic acid, p-coumaric acid and luteolin, isolated from olive fruit and leaves, have shown antimicrobial activities against viruses, retroviruses, bacteria, yeasts, fungi and other parasites [20], [1]. *in vitro* research demonstrated the effectiveness of olive leaf extract against a wide range of pathogens, including *Escherichia coli*, *Streptococcus pyogenes*, *Pseudomonas fluorescens*, *Helicobacter pylori*, *Compylobacter jejuni*, *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus*, *Salmonella typhimurium*, *Erwinia carotovora*, *Candida albicans* and *Plasmodium falciparum* [22], [18],[1].

Although the *in vitro* antibacterial activity and efficacy of metal zinc oxides, which used for pharmaceutical applications, have been known for a long time, little is known about the antibacterial activity of ZnO nanoparticles against oxacillin-resistant *Staphylococcus aureus* and no published data had been documented at local academic level. In addition, drug synergism between known antimicrobial agents and bioactive plant extract has been reported [23]. The search for bioactive constituent of plant extracts with

significant antibacterial potentials and of interaction with the currently used antibiotics may represent a promising approach toward combating the rise in bacterial resistance to these antibiotics in medical practice.

Material and Methods

Staphylococcus aureus isolates were supplied from previous study from swab samples collected from nose (both anterior nares), hand-swab (especially hands of the hospital personnel) and ear-swab were collected from different patients attending the four hospitals, health care workers such as staff-nurses, ward boys and indoor environment of these hospitals. Preliminary identification of the isolates was performed as described by [24] on the basis of colonial morphology, cultural characteristics on agar media, grams staining, catalase activity, coagulase test, and growth on mannitol salt agar and thermonuclease activity of clinical isolates. In addition; a confirmatory examination was carried out using the (VITEK 2 System) at Al-Kaddimia Teaching Hospital.

The antimicrobial activity of OLE and nano-ZnO suspension was tested against selected *S. aureus* isolate that give positive result for the presence of (*mecA*⁺) gene and (*hla*⁺) gene which represents important virulence factors of *S. aureus* clinical isolates in addition to their wide range of resistance to the majority of the tested antibiotics.

Preparation of crud ethanolic olive leaf extracts (OLE)

Olive leaves were collected from Al-Jadrhia garden and identified as *olea europaea* by prof. Dr. Ali Al-Mossawy, Biology department, College of Science, Baghdad University. The dried olive leaves were powdered using a coffee grinder, and then extracted with 70% ethanol at a 20% w/v concentration. Fifty grams of the processed plant were extracted in 250 ml of the ethanol (70%) using the soxhlet apparatus. The obtained extract was then evaporated to remove the solvent under reduced pressure by rotary evaporator at 40°C and the yield of extraction was approximately 3g, i.e 6% (w/w) and was frozen at -20°C. to be used in subsequent analyses, the extract was dissolved

in dimethyl sulfoxide and then sterilized by filtration to give an approximate concentration of 300 mg/ml. the whole leaves ethanolic extract was used in the present study.

Determination of plant antimicrobial activity

Antimicrobial activity of the olive leaves extract was measured by the paper disc diffusion method as described by [1] with a slight modification. Sterile 6 mm filter papers discs were impregnated with 30 μ l sterilized olive leaf extracts of various concentrations (75, 150, 225 and 300 mg/ml) to give a concentrations of (2.25, 4.5, 6.75 and 9.0 mg/disc, respectively). The agar plates inoculated with ORSA were incubated for 1 h before placing the extract. Following this, the sterile discs impregnated with the extract was placed onto the surface of the agar plate. Oxacillin discs (1 μ g/disc) alone and in combination with the paper disc impregnated with the 300mg/ml OLE were also used as a positive standard references as to test the potential synergistic effect of the oxacillin-OLE combination, respectively. Plates were incubated at $35 \pm 0.1^\circ\text{C}$ for 48 hr. After incubation, all plates were observed for zones of growth inhibition, and the diameters of these zones were measured in mm.

Antimicrobial activity of ZnO Nanoparticles

The potential synergistic antimicrobial effect of ZnO nanoparticles (35 nm) was measured using disc diffusion method as described by [25]. Briefly, 6 mm diameter filter paper discs were impregnated with zinc oxide nanoparticles (100 μ g/disc). To determine combined effects, zinc oxide impregnated discs (100 μ g/disc) were further impregnated with 30 μ l of various concentrations of crude OLE (75, 150 and 225 mg/ml). Also, saturated oxacillin discs (1 μ g/disc) were further impregnated with zinc oxide nanoparticles (100 μ g/disc).

The muller-Hinton agar plates inoculated with ORSA about 1 hr before placing the zinc oxide impregnated discs on the plates. After incubation at 37°C for 24 hr, the zones of inhibition (mm) were measured.

Results

Depending on the conventional cultural procedures; a primary test for species identification was done by Gram staining and standard biochemical tests and results revealed that 61 isolates were detected as *S. aureus* as they appear Gram positive. The clinical isolates ferment mannitol and produced yellow color due to the acid production; they were catalase positive due to the production of catalase enzyme which distinguishes them from *Streptococcus* spp. Isolates were positive for coagulase which distinguished them from other *Staphylococcus* spp. characterizing them as *S. aureus*. In addition; a confirmatory examination was carried out using the (VITEK 2 System) at Al-Kaddimia Teaching Hospital; this documented system enable rapid identification for the suspected specimens to the species level. The tested pathogenic isolates were found to exhibit obvious level of resistance against the antibiotics used and the susceptibility pattern for these clinical *S. aureus* isolates are shown in Fig.(1).

The percentages of resistance for *S. aureus* isolates to ticarcillin-clavulanic acid, penicillin, oxacillin, cefepime, cefoxitin, cephalothin were (93.4, 78.7, 77.1, 68.9, 65.6 and 55.7 %), respectively, followed by a moderate resistance against erythromycin (47.5%) and tetracyclin (27.9%) while, the percentage of resistance of gentamycin and amikacin was (18%), ciprofloxacin (14.8%) and (13%) for chloramphenicol. On the other hand, the tested isolates exhibited a high degree of susceptibility towards vancomycin (6.6%), tobramycin (9.8%) and imipenem with only (3.3%).

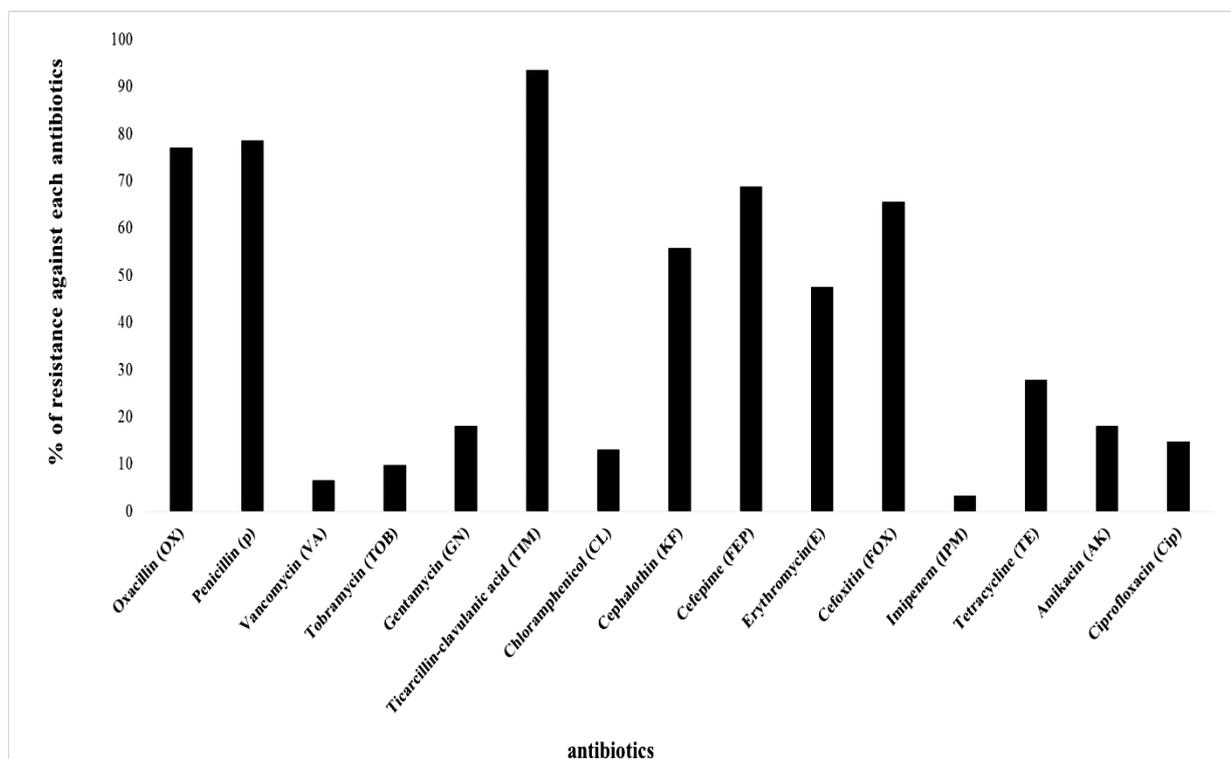


Fig. (1) Antibiotic susceptibility profile of the tested *S. aureus* isolates to different groups of antibiotics.

Results showed that (77%) of the total number of isolates exhibited multiple resistances as determined by their ability to resist more than three types of antibiotics as described in Table (1). These isolates were oxacillin resistant and thus they were designated as multidrug-resistant oxacillin-resistant *S. aureus* (MDR-ORSA).

From the total number of the *S. aureus* isolates; 23 were chosen for further identification using a set of *nuc* primer. All the selected isolates (n=23) expressed *S. aureus* specific sequence gene in their PCR products which confirmed the obtained results that all the isolates were *S. aureus*. In addition; *mecA* gene was detected in 19 out of 23 oxacillin-resistant *S. aureus* (journal of Al-Nahrain University-Science in press).

**Table (1)
Multiple antibiotic resistance of ORS
Aisolates.**

No. of antibiotic	No. of the isolates	Percentage (%)
3	2	(3.3)
4	3	(4.9)
5	7	(11.5)
6	7	(11.5)
7	8	(13.1)
8	6	(9.8)
9	6	(9.8)
10	4	(6.6)
11	2	(3.3)
13	1	(1.6)
14	1	(1.6)

For OLE; the *in vitro* antibacterial activity was expressed against oxacillin-resistant *S. aureus* as the mean diameter of inhibition zones (mm) produced by the extract alone and in combination with the oxacillin. The results are presented in Fig.(2) and Table (2). Neither oxacillin alone (1µg/disc) nor increasing concentrations of OLE (2.25-9 mg/disc) produced any inhibition zone

when used alone with ORSA. However, when oxacillin was combined with highest concentration of OLE (9 mg/disc), a pronounced inhibition zone (14 mm) was noticed.

Table (2)

Antibacterial profile of olive leaf extracts (OLE) alone or in combination with oxacillin and/ or Zinc oxide nanoparticles against oxacillin-resistant *S. aureus*.

Drug concentration	Inhibition zone (mm)*
Oxacillin (1µg/disc)	NA
Zinc oxide (100 µg/disc)	10
Oxacillin (1µg/disc) + Zinc oxide (100 µg/disc)	15
Olive leaf extract (OLE) (2.25 mg/disc)	NA
OLE (4.5 mg/disc)	NA
OLE (6.75 mg/disc)	NA
OLE (9 mg/disc)	NA
Oxacillin (1µg/disc) + OLE (9 mg/disc)	14
Zinc oxide (100 µg/disc)	11
OLE (6.75mg/disc) + Zinc oxide (100 µg/disc)	10

* Zone of inhibition, including the diameter of the filter paper disc (6mm): mean values of two readings.

-NA= no activity.

The antimicrobial activity of nano ZnO suspension was tested and the results were shown in Fig.(3) and Table (2), the zinc oxide nanoparticles are found to enhance the antibacterial activity of oxacillin against the selected bacterial isolate of *S. aureus* in such a way that when the standard oxacillin antibiotic disc was used; no inhibition zone was obtained around the disc. Furthermore, ZnO nanoparticles alone were recorded to produce an inhibition zone of (10 mm), while the combination of nano- ZnO with oxacillin gave an inhibition zone of (15 mm) around the (oxacillin-ZnO) disc. Our results were found to be in accordance with that obtained by [25] published report on the antibacterial properties of ZnO nanoparticles as a promising new unconventional antibacterial agent that could

be helpful to confront methicillin-resistant *S. aureus* and other drug-resistant bacteria.

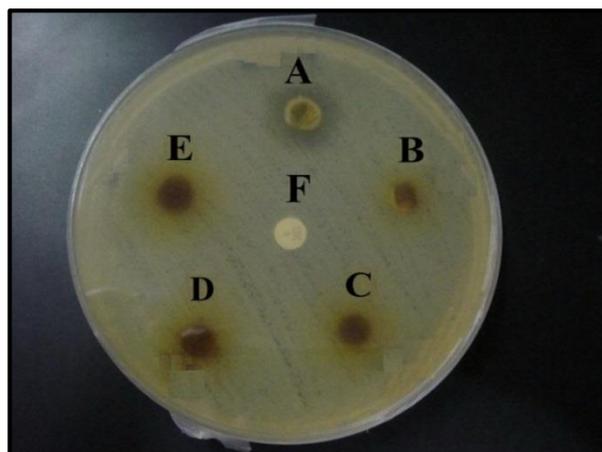


Fig.(2) Antibacterial activity of olive leaves ethanolic extract with oxacillin against oxacillin resistant *S. aureus* isolate.

A: olive leaves extract (300 mg/ml) + standard oxacillin antibiotic disc.

B: olive leaves extract (75 mg/ml)

C: olive leaves extract (150 mg/ml)

D: olive leaves extract (225 mg/ml)

E: olive leaves extract (300 mg/ml)

F: oxacillin antibiotic disc.

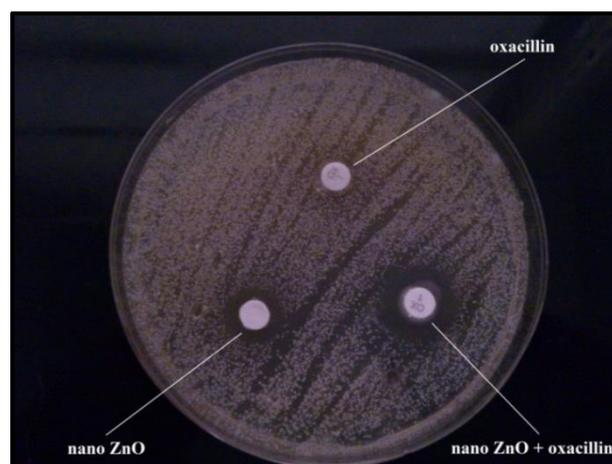


Fig. (3) Combined antibacterial activity of ZnO nanoparticles (35 nm) with oxacillin against oxacillin resistant *S. aureus* isolate.

The combined antibacterial activity of olive leaves ethanolic extract and ZnO nanoparticles were tested against our selected *S.aureus* strain and as shown in Fig.(4) and Table (2) the inhibitory effect of ZnO alone was (11mm) and there was no synergistic effect for olive leaves extract in the presence of Zn.

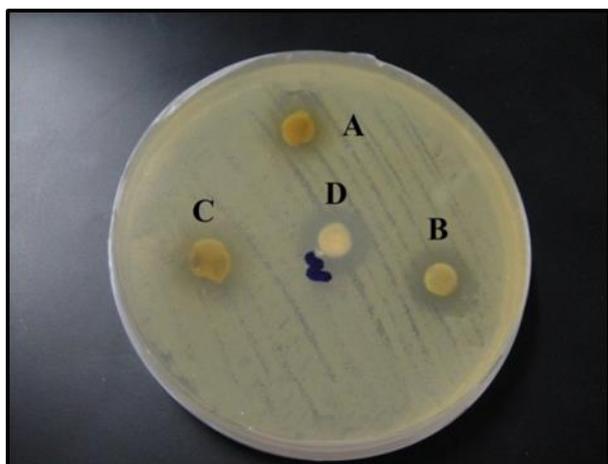


Fig. (4) Combined antibacterial activity of olive leaves ethanolic extract with nano-ZnO (100µg/disc) against oxacillin resistant *S. aureus* isolate.

- A: olive leaf extract (6.75 mg/disc) + nano- ZnO (100 µg/disc)
 B: olive leaf extract (4.5 mg/disc) + nano- ZnO (100 µg/disc)
 C: olive leaf extract (2.25 mg/disc) + nano- ZnO (100 µg/disc)
 D: nano- ZnO (100 µg/disc)

Discussion

Several studies have demonstrated that OLE possesses potent antibacterial activities against oxacillin-resistant *S. aureus* [20]; [26] [27] and [1]. This was not the picture revealed in our study, which suggest that OLE alone lacks any antibacterial effect. It is not clear at present, the reasons for this discrepancy between our finding and the above-mentioned studies; yet it may be due to the high degree of resistance of the selected *S. aureus* isolate to a wide range of tested antibiotics which render it among the most virulence hospital acquired bacterial infections. Our results suggest that, under the present experimental conditions, OLE exhibited controversial effects against ORSA *in vitro*. It failed to show antibacterial activity when applied alone to the agar plate, yet it potentiated the antibacterial activity of oxacillin when used in combination against ORSA. No comparable report in the available literature reported that OLE can potentiate the antibacterial activity of oxacillin against ORSA *in vitro*, which is already resistant to this antibiotic. Therefore, this sporadic positive finding should not be over interpreted in the midst of the present study negative

findings concerning the antibacterial potential of OLE

The presence of an inhibition zone clearly indicates that the mechanism of the biocidal action of ZnO involves disrupting the membrane. The antimicrobial ability of nano-ZnO might be referred to their small size which is 250 times smaller than a bacterium. This makes them easier to adhere with the cell wall of the microorganisms causing its destruction and leads to the death of the cell. Also, the high rate of generation of surface oxygen species from ZnO leads to the death of the bacteria. So the enhanced effect of nano-ZnO upon the antibacterial activity of oxacillin is observed by the notable increase in the diameter of the inhibition zone around the disc. This is considered to be due to this synergistic effect of antibiotic-zinc nanoparticles combination. At the concentration tested, zinc nano particle significantly improved antibiotic efficacy against *S. aureus*. Our results are found to be in accordance with that obtained by [28] published report on the antibacterial properties of ZnO nanoparticles as a promising new unconventional antibacterial agent that could be helpful to confront methicillin-resistant *S. aureus* and other drug-resistant bacteria

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المضاد الحيوي بدون اضافة لم يعطي اي نتيجة. قدرت الفعالية المضادة للبكتريا للمستخلص الخام لأوراق الزيتون بدون اي اضافة و التأثير التآزري للمستخلص مع مضاد الأوكساسيلين أوجزيئات أوكسيد الزنك النانوية بأستخدام نفس العينة قيد الدراسة والتي تمثل عزلة بكتريا المكورات العنقودية المضادة للأوكساسيلين وبأستخدام طريقة التنشيط بالأقراص. أظهرت النتائج ان مستخلص اوراق الزيتون وبالتراكم المستخدمة (٢,٢٥-٩ ملغم/ قرص) لم يعطي اي تأثير مثبت على البكتريا عندما استخدم بدون اي اضافة ولم يكن له أي تأثير محفز للتأثير المضاد البكتيري لجزيئات الزنك النانوية (١٠٠ مايكروغرام/ قرص) عندما استخدم كمزيج. بالرغم من ذلك فإن الفعالية ضد المايكروبية لمضاد الأوكساسيلين قد أظهرت زيادة وبالأعتماد على قطر منطقة التنشيط من (٠ ملم) الى (١٤ ملم) عندما قيست بوجود التركيز العالي (٩ مايكروغرام/ قرص) لمستخلص أوراق الزيتون مما يسجل تأثير محفز لفعالية المضاد ضد بكتريا المكورات العنقودية المقاومة للأوكساسيلين.

الخلاصة

أجريت هذه الدراسة لتقييم التأثير المحفز لجزيئات اوكسيد الزنك النانوية والمستخلص الخام لأوراق الزيتون على الفعالية المضادة للبكتريا لمضاد الأوكساسيلين ضد بكتريا المكورات العنقودية الذهبية المقاومة لعقار الأوكساسيلين والمعزولة من بيئة المستشفى. التأثير الأيجابي لجزيئات اوكسيد الزنك النانوية حدد اعتماداً على الفعالية المضادة للبكتريا مقارنة بمضاد الأوكساسيلين بدون اي اضافة من خلال زيادة قطر هالة التنشيط والتي سجلت (١٥ ملم) للفعالية المشتركة ما بين خليط المضاد الحيوي والجزيئات النانوية بالمقارنة مع فعالية الجزيئات النانوية بدون اي اضافة حيث سجلت (١٠ ملم) مع ملاحظة ان استخدام