Determination of Synergistic Inhibitory Activity of some Nanoparticles and Antibiotic Against Pathogenic Bacteria Caused Acne

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Abstract

Background: Acne is a common adolescent disease with social impact on affected individual and his family. Many drug formulations available for acne treatment but with varied treatment response and associated with patient none compliance.

Aim: Determine the inhibitory effect of antibiotics and nanoparticles combination against bacteria that cause acne.

Materials and methods: This study was conducted at Laboratories of the Department of Biology/ College of Education for Pure Sciences/ University of Tikrit. 124 samples were collected by swap survey of hospitalized patients with acne in Salahuldean General Hospital during the period from 1/8/2018 to 1/4/2019. After the diagnosis using biochemical tests as well as Vitek2 Compact System, the ability of Gram positive and negative bacterial species that produce virulence

factors such as Hemolysin, Urease, Biofilm, Lecithinase, Lipase and β -Lactamase were investigated.

Results: Twelve types of antibiotics were used and the isolates showed different degrees of sensitivity to antibiotics. For the inhibition of titanium dioxide nanoparticles, the results showed that TiO_2 nanoparticles had the highest inhibition capacity for Gram-positive bacteria, while the inhibition of Gram-negative species was relatively low. The combination of TiO_2 nanoparticles and antibiotic shows inhibitory diameter ranged between 25 to 50 mm against Gram positive bacteria for nano concentration of 50 and 100 mg respectively. While Gram negative bacteria show inhibitory diameter ranged between 20-30 mm for TiO_2 nanoparticles concentrations were 50 and 100 mg respectively.

Conclusion: Isolated organisms demonstrated high resistance rate to antibiotics; however, antibiotic combination with TiO_2 induced synergistic inhibitory activity against Gram positive and Gram negative bacteria. And the effect was higher in gram positive as compared to gram negative bacteria. This finding suggests new drug formulation for acne treatment.

Keywords: Acne, TiO_2 , *Staphylococcus aureus*, *Citrobacter freundii*, *Strep. Pyogenes*, *Ps. aeruginosa*, *Ps. stutzeri*, *Enterobacter cloacae*, *Staph. epidermidis*, *Staph. saprophyticus*, *Klebsiella oxytoca*, *Raoultella ornithinolytica*

Introduction

Acne is one of the most common skin problems and defined as a disorder of the sebaceous glands of the skin resulting in increased secretions causing obstruction and inflammation of the ducts [1], attributed to chronic inflammation that occurs in the subcutaneous fat layer. It characterized by the formation of comedones, papules and pustules and often scars on the face, neck and upper area of the back and arms. Symptoms may appear in the twenties or thirties and may last for many years that are not associated with a specific age group. Although more common among the age group of adolescents, however it was found that 15% of the cases were in adults [2]. However, in some cases the acne lesions continue to appear with age progression. Acne lesions distributed mainly on the face and can spread to nearby areas such as the neck and shoulders. The most important factors in the formation of acne is the frequency of taking

some medicinal drugs or the result of hormonal changes, as well as affected by diet composition, especially the unbalanced diet which may be attributed to stress [3].

The most common microbes that can cause inflammation in cysts or acne pimples are *Staphylococcus* bacteria [4], especially *Staph. epidermidis*, and *Staph. aureus*, which are naturally coexisting microbes in the nose and on human skin [5]. Recent studies and research had indicated that the delay or lack of treatment for acne cases in a timely manner has caused unpleasant side effects such as burning and redness in the face as well as the appearance of dark marks and spots in the skin that are difficult to get rid of such lesions [6]. Additionally, the acne response to available treatment approaches varies and influence by multiple factors and there was no absolute effective treatment. Thus this study was conducted to find an alternative treatment approach.

Materials and Methods

Collection of samples

One hundred and twenty four patients were enrolled in the study and samples were collected from people who attend to the hospital with age range of 11-24 years. The study was conducted in Salahuldean General Hospital for the period between August 2018 to April 2019. The samples were taken from pimples or comedones. The study protocol was approved by the Ethical Committee of Tikrit University College of Education for Pure Sciences. Verbal informed consent was taken from each individual before his/her enrollment in the study.

Detection of the production of virulence factors

● Hemolysin production test

The bacterial isolates to be selected for the ability to produce the hematopoietic enzyme were transplanted to the center of the prepared hematopoiesis and incubated at 37 ° C for 24 hours. The positive result is when the blood decomposes and the appearance of a colorless transparent area in the case of complete decomposition or on the appearance of a green belt in the case of partial decomposition around the colonies [7].

● Urease test

This test was used to detect the ability of bacterial isolates to produce urease enzyme which catalyzes breakdown of urea. Urea agar

was inoculated with isolates and incubated at 37°C for 24 hours. The change of the medium color from yellow to pink indicates the positive reaction hence means urea catalyzed to ammonia by urease enzyme [8].

● **Lecithinase and Lipase production test**

Bacterial isolates were cultured on dishes containing medium egg yolk (Egg yolk agar) and incubated at 37 ° C for 48-24 hours. The emergence of clear areas around the developing bacterial colony indicates the production of the enzyme Lecithinase. While Lipase production performed by submerging the dish with 20% Cuso4-7H2O blue aqueous sulphate solution for 20 minutes, then dried by incubator for 30 minutes after removing the excess solution, as the appearance of greenish blue color around the growth zones indicates the production of lipase enzyme [9].

● **Beta-lactamase production test**

An Iodometric spot method was used where a circular piece of filter paper was placed in sterile Petri dishes, then 0.02 g of penicillin G in powder form was transferred to the center of the filter paper and then drops of distilled water were added. Then part of the colony was transferred and placed in the center of the paper and mixed with penicillin, and the dishes were left at room temperature for 10 minutes. Then drops of Iodine were added and positive test shows a white spot that appears in the center of the paper after 5 minutes of incubation [10].

● **Detection of Bacterial biofilm production methods**

Tube method was performed by placing a loopful of bacterial inoculum from agar plate into a glass test tube containing 5ml of trypticase soy broth (TSB) and incubated overnight at 37°C. Each tube was decanted, washed with phosphate buffer saline (pH 7.2), stained with 0.25% Saffranin, and then gently rotated to ensure uniform staining and the contents were gently decanted. The tube were then placed upside- down to drain. The color of the inner surfaces of the tubes was observed. Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube. The absence of a film or the presence of a ring at the liquid-air interface was interrupted as a negative result (-). Based on biofilm production, the positive results were recorded as strong (+++), moderate (++) and weak (+) [11].

Antibiotic Sensitivity Test

Antibiotics susceptibility was carried out on all isolate using Kirby Bauer disc diffusion method. Results were interpreted by measuring the zone of inhibition in mm [12]. Twelve types of antibiotics were used in this study: Levofloxacin (LEV), Metronidazole (MET), Norfloxacin (NOR), Piperacillin (PRL), Imipenem (IPM), Azetreonam (ATM), Ofloxacin (OFX), Carbenicillin (PY), Netilmicin (NET), Neomycin (N), Azithromycin (AZM), Meropenem (MEM).

Titanium dioxide TiO₂ nanoparticles

TiO₂ nanoparticles were obtained from the postgraduate laboratory, Department of Biology, College of Education for Pure Sciences, University of Tikrit which was prepared from *Aspergillus flavus* [13].

Manufacture of Titanium TiO₂Nanoparticles

Cell-free filtration was obtained after incubation of 10 g of fungus strands of *Aspergillus flavus* and 100 ml of 1 mM TiO₂ mineral for nanoparticle synthesis [14]. The entire mixture was transferred to a shaking incubator (150 rpm) at 28 ° C and allowed to interact for 48 hours, another vial containing 10 g of wet biomass and 100 ml of sterile distilled water (this vial was used as a control). While the third bottle was used as a control also because it contains only 100 ml mineral solution. All vials were incubated at 28 ° C in the dark for 96 hours with agitation (150 rpm). The biologically transformed particles were collected periodically and monitored for their characterization [14].

Results and Discussion

Bacterial isolates virulence

The result showed that there is a variation in the virulence factors of the isolated bacterial species, Table 1. All tested isolates of *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, and *Pseudomonas stutzeri* isolates completely produced hemolysin. While only 33.3% of tested *Staphylococcus aureus* and 50% of *Enterobacter cloacae complex* induced hemolysin. However, *Staph. epidermidis*, *Staph. saprophyticus*, *Citrobacter freundii*, *Klebsiella oxytoca*, and *Raoultella ornithinolytica* did not produce hemolysin.

The current study shows that all *Staph. aureus*, all *Klebsiella Oxytoca*, 71.42% of *Staph. epidermidis*, 40% of *Staph. saprophyticus*,

and 50% of *Enterobacter cloacae* complex produced urease. The bacterial species *Raoultella ornithinolytica*, *Citrobacter freundii*, *Pseudomonas.spp*, and *Strep.pyogenes* did not produce urease. Seven isolates (46.66%) of *Staph.aureus*, two isolates (40%) of *Staph. Saprophyticus*, 3 isolates (100%) of *Pseudomonas species*, 4 isolates (100%) of *Enterobacter cloacae* complex, 3 isolates (60%) of *Citrobacter freundii*, one isolate (100%) of *Klebsiella oxytoca* and single (100%) isolate of *Raoultella ornithinolytica* produced biofilms. Whereas bacterial species that did not producing biofilms included *Staph.epidermidis* and *Strep.pyogenes*.

The results of the current study showed that 8 isolates (53.33%) of *Staph.aureus*, 5 isolates (71.42%) of *Staph.epidermidis*, 3 isolates (60%) of *Staph.saprophyticus*, 3 isolates (100%) of *Pseudomonas species*, two isolates (50%) of *Enterobacter cloacae* complex, 5 isolates (50%) of the type *Citrobacter freundii*, one isolate (100%) of *Raoultella ornithinolytica*, produce the lecithinase enzyme. While the results showed that *Klebsiella oxytoca* and *Strep.pyogenes* did not produce lecithinase. The results also showed that 8 isolates (53.33%) of *Staph.aureus*, 5 isolates (71.42%) of *Staph.epidermidis*, 3 isolates (60%) of *Staph.saprophyticus*, 3 isolates (100%) of *Pseudomonas.spp*, 5 isolates (50%) of *Citrobacter freundii*, and 2 isolates (50%) of *Enterobacter cloacae* complex produced lipase enzyme. Whereas, *Strep.pyogenes*, *Raoultella ornithinolytica* and *Klebsiella oxytoca* did not produce lipase enzyme.

The results of the current study showed that 15 isolates (100%) of *Staph. aureus* isolates, 5 isolates (71.42%) of *Staph. epidermidis* isolates, 3 isolates (60%) of *Staph.saprophyticus* produced beta-lactamase. The results also showed that *Raoultella ornithinolytica*, *Klebsiella oxytoca*, *Pseudomonas species*, *Enterobacter cloacae* complex, and *Citrobacter freundii* did not produce beta-lactamase.

Antibiogram

The sensitivity test for Gram-positive bacteria isolates against tested antibiotics indicated a varied susceptibility. All *Staph. aureus* were resistant to 4 antibiotics, (Piperacillin, Carbenicillin, Neomycin, Azetreonam), and 80% resistance to Metronidazole. While showed a weak resistance to Netilmicin. In contrast, absolute sensitivity (100%) to Levofloxacin, Imipenem, Ofloxacin, Azithromycin and Meropenem). *Staph. saprophyticus* isolates were 100% absolute resistance to Metronidazole, Piperacillin, Carbenicillin and

Aztreonam. While demonstrated a weak resistance of 20% to Azithromycin, and 60% and 80% to Neomycin and Netilmicin respectively. In contrast, absolute sensitivity to Levofloxacin, Meropenem. Imipenem and Ofloxacin was demonstrated.

Staph. epidermidis with absolute resistance to Piperacillin, Carbenicillin, Neomycin and Aztreonam and 80% resistance to Metronidazole, 40% resistance to Netilmicin. Meropenem, Norfloxacin, and 100% absolute sensitivity to Ofloxacin and Azithromycin. *Strep. pyogenes* isolates showed 100% absolute resistance to Neomycin, Piperacillin, Carbenicillin and Metronidazole; and 92.3%, 69.2% and 77% to Aztreonam, Netilmicin and Azithromycin respectively. It also showed low resistance of 15.3% and 23% to Imipenem and Meropenem. However, 100% sensitivity was demonstrated to Levofloxacin, Norfloxacin and Ofloxacin.

The Gram-negative isolates tested in this study were absolutely resistant to Netilmicin, Azithromycin Neomycin, Piperacillin, and Metronidazole. In contrast, 100% sensitive to Ofloxacin, Norfloxacin and Levofloxacin was demonstrated. In addition, it showed variation in sensitivity to other antibiotics. All *Citrobacter freundii* isolates were resistance to Neomycin, Netilmicin, Carbenicillin, Piperacillin and Metronidazole. While 60%, 60%, 90% and 80% of the isolates were resistant to Aztreonam. Imipenem, Meropenem, Azithromycin, respectively. However, low sensitivity of 30%, 20% and 10% against Ofloxacin, Norfloxacin, Levofloxacin were shown.

Enterobacter cloacae complex tested isolates showed 100% resistance to Neomycin Netilmicin, Carbenicillin, Piperacillin, Metronidazole. While, 50%, 50%, 75% and 25% of the isolates were resistant to Aztreonam, Azithromycin, Meropenem and Imipenem respectively. In contrast, 100% sensitivity to Ofloxacin, Norfloxacin Levofloxacin was demonstrated. One isolate of *Raoultella ornithinolytica* was 100% sensitive to Norfloxacin Levofloxacin, Imipenem, Ofloxacin, Netilmicin and Meropenem, and showed 100% absolute resistance to Metronidazole, Piperacillin. Aztreonam and Carbenicillin.

Ps. aeruginosa isolates showed absolute resistance to Metronidazole, Piperacillin, Aztreonam, Carbenicillin, Neomycin, Azithromycin. While shows a resistance rate of 44%, 44.3% and 22.2% to Meropenem, Netilmicin and Imipenem, respectively. Absolute sensitivity to Levofloxacin, Norfloxacin and Ofloxacin was

demonstrated.. While single isolation of *Ps. stutzeri* showed absolute resistance to Metronidazole, Piperacillin, Azetreonam, Carbenicillin, Neomycin and Azithromycin, and resistance rate of 22%, 22.3% and 11.1% to Meropenem, Netilmicin and Imipenem, respectively. While, shows 100% sensitivity to Levofloxacin, Norfloxacin and Ofloxacin.

The single bacterial isolate of *Klebsiella oxytoca* showed 100% resistance to Metronidazole, Netilmicin, Piperacillin, Carbenicillin, Neomycin, Azetreonam and Azithromycin.

Table 1. Virulence factors of the tested bacterial isolates

Virulence Factor						No	Bacterial Species
Betalactamase	Lipase	Lecithinase	Biofilm	Urease	Hemolysen		
% +	%+	%+	%+	%+	%+		
100	53.3	53.3	64.7	100	33.3	15	<i>Staphylococcus aureus</i>
71.4	71.4	71.4	0	71.4	0	7	<i>Staphylococcus epidermidis</i>
60	60	60	40	40	0	5	<i>Staphylococcus saprophyticus</i>
0	0	0	0	0	100	13	<i>Streptococcus pyogenes</i>
0	50	50	60	0	0	5	<i>Citrobacter freundii</i>
0	50	50	100	50	50	4	<i>Enterobacter cloacae complex</i>
0	0	100	100	0	0	1	<i>Raoultella ornithinolytica</i>
0	100	100	100	0	100	2	<i>Pseudomonas aeruginosa</i>
0	100	100	100	0	100	1	<i>Pseudomonas stutzeri</i>
0	0	0	100	100	0	1	<i>Klebsiella oxytoca</i>

Figure.1. Detection of Virulence factors

Table 2. Antibiotic sensitivity test

Antibiotics in mg	<i>Staph. aureus</i> NO[44]	<i>Staph. epidermidis</i> NO [21]	<i>Staph. saprophyticus</i> NO [17]	<i>Strep. pyogenes</i> NO [13]	<i>Citrobac. freundii</i> NO [10]
	%	%	%	%	%
ATM 30	100	100	100	92.3	60
MEM 10	0	40	0	23	90
AZM 30	0	0	20	77	80
N 10	100	100	60	100	100
NET 30	40	40	80	69.2	100
PY 25	100	100	100	100	100
OFX 10	0	0	0	0	30
IMP 10	0	0	0	15.3	60
PRL 30	100	100	100	100	100
NOR 10	20	40	0	0	20
MET 30	80	80	100	100	100
LEV 5	0	0	0	0	10

Table. 3. The effects of TiO₂ nanoparticles on the gram positive and negative bacterial isolates

TiO ₂ nanoparticles		Bacterial isolate	
		<i>Staphylococcus aureus</i>	<i>Ps. Aeruginosa</i>
First Run	Concentration	50%	50%
	Inhibition diameter (mm)	20	5
Second Run	Concentration	100%	100%
	Inhibition diameter (mm)	25	10

The titanium oxide nanoparticles (TiO₂) showed inhibitory efficacy at concentrations of (50% - 100%) against isolated and antibiotics multi-resistant bacteria. It was noted that the titanium dioxide nanoparticles have an effective antibacterial effect against both Gram negative and positive bacteria. In addition, when using 50% TiO₂ concentration, the inhibition diameter was 20 mm for *Staph.aureus*, while it was 5 mm for *Ps.aeruginosa*, Table 3. Fortunately, TiO₂ concentration of 100% was more effective inhibitor for both *Staphylococcus* and *Pseudomonas aeruginosa*. Thus the inhibition of bacterial growth was dose dependent, which may interfere with host cell function. The present study finding was agreed with the previously reported studies [15-21]

Determination of synergistic inhibitory activity of titanium dioxide nanoparticles (TiO₂) and antibiotic resistance

Table 4 shows that the combination of TiO₂ nanoparticles and antibiotics (AZM, MET and N) is with synergistic inhibitory effect against the tested bacterial isolates. The inhibitory diameters for *Strep. pyogenes* isolates were 50mm, 40mm and 20mm at 50% concentration when combined with different antibiotics. While they are 50mm, 50mm and 30mm at 100% concentration which indicate that the doubling of concentration not add any synergistic effect. Additionally, the inhibition diameter of *Staph. saprophyticus* isolates at 50% concentration were 30mm, 25mm and 20mm, which is not increased at 100% concentration. The same pattern was demonstrated by *Staph. epidermidis*. Thus this results indicated that combination of

nanoparticles with antibiotic at 50% concentration is with synergistic effect which may be suggested for treatment of infections induced by the tested bacteria. Additionally, the combination of nanoparticles and antibiotics (AZM, MET and N) may be suggested as alternative therapeutic approach for the treatment of infection induced by multiple resistant bacteria [22-26].

Table.4. Inhibitory activity of interference between TiO₂ nanoparticles and antibiotics used against gram negative and gram positive bacterial isolates

Bacterial isolates	Inhibition diameter in mm					
	TiO ₂ + N (50)	TiO ₂ + N (100)	TiO ₂ + AZM (50)	TiO ₂ + AZM (100)	TiO ₂ + MET (50)	TiO ₂ + MET (100)
<i>Streptococcus Pyogenes</i>	50	50	40	50	20	30
<i>Staphylococcus saprophyticus</i>	30	30	25	25	20	20
<i>Staphylococcus epidermidis</i>	35	35	25	25	20	20
<i>Staphylococcus Aureus</i>	35	35	35	35	30	30
<i>Klebsiella Oxytoca</i>	30	30	30	30	30	30
<i>Raoultella ornithinolytica</i>	20	20	30	30	20	20

Figure. 2. Synergistic inhibitory activity of TiO_2 nanoparticles and some multi-resistant antibiotics toward *Staph. Aureus* bacteria

The gram positive bacterial isolates showed that the synergy inhibition diameters of TiO_2 molecules and the antibiotics (MET, N, AZM) against *Strep. pyogenes* isolates were (50, 40 and 20) mm at a concentration of 50%; and reached (50, 50 and 30) mm at the concentration of 100%. While the inhibition diameter of *Staph. saprophyticus* isolates at the concentration of 50% were (30, 25 and 20) mm; and reached (30, 25 and 20) mm at 100% concentration. For *Staph. epidermidis* isolates, inhibition diameters were (35, 25 and 20) at both concentrations of 50% and 100%. Whereas, the inhibitory diameters of *Staph. aureus* isolates at the 50 and 100% concentrations were (35, 35 and 30) mm.

The gram negative bacterial isolates showed that *Klebsiella oxytoca* inhibition diameter was 30 mm at both concentrations 50% and 100%. The inhibiting diameters of the *Raoultella ornithinolytica*

isolates reached (20, 30 and 20) mm at the two concentrations of 50% and 100%, Table 4.

TiO₂ nanoparticles combination with antibiotics (MET, AZM, and N) as treatment approach for bacterial infections is a promising alternative as this study indicated and the conclusions of previously reported studies [22-26]

Figure. 3. Synergistic inhibitory activity of TiO₂ nanoparticles and some multi-resistant antibiotics toward *Raoultella ornithinolytica* bacteria

Conclusions

1. *Staph.aureus* was the common bacterial isolate (35.48%) of the gram-positive bacteria from acne lesions. While, *Citrobacter freundii* was the predominant of the gram negative species (8.06%) in acne lesion. This may attributed to the modulation of the treatment approach of acne.

2. *Staph. aureus* isolates from acne lesion show ability to produce all tested virulence factors (Hemolysin, Urease, Biofilm, Lecithinase, Lipase and β -Lactamase). While this character not demonstrated by other gram positive and gram negative bacterial isolates. This phenomenon may hurdle the acne treatment approach and may be responsible for high rate treatment failure.
3. The gram positive isolates were 100% resistant to Piperacillin, Aztreonam, Carbenicillin, Neomycin, while some gram negative isolates such as *Pseudomonas species*, *Klebsiella oxytoca* and *Raoultella ornithinolytica* were 100% resistant to Metronidazole, Piperacillin, Aztreonam, Carbenicillin, Neomycin, Azithromycin
4. The concentration of 100% nanoparticles of titanium dioxide showed very high inhibitory efficacy of 25 mm against *Staph.aureus* while with less inhibitory effect against *Pseudomonas aeruginosa* (10 mm).
5. The highest inhibition diameter of 50 mm was when the neomycin combined with TiO₂ nanoparticles at 50% and 100% concentrations against *Strep. pyogenes* and the lowest inhibitory activity was 20 mm in diameter when metronidazole combined with TiO₂ nanoparticles at 50% and 100% concentrations against *Staph. saprophyticus* and Neomycin with *Raoultella ornithinolytica*.

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