

Comparative study of antibiofilm activity of lime juice and lithium dioxide nanoparticles against *Salmonella* isolated from local cheese

دراسة مقارنة للنشاط المضاد للغشاء الحيائي لعصير الليمون وجسيمات ثاني أكسيد الليثيوم النانوية ضد بكتيريا السالمونيلا المعزولة من الجبن المحلي

Zainab Zamel Khalaf

College of Science Department of Biology / Baghdad university

زينب زامل خلف

قسم علوم الحياة/كلية العلوم/جامعة بغداد

E-mail : zainabalmusawi@yahoo.com

Abstract

Thirty specimens of fresh white cheeses, presented for sale in different markets at different cities of Iraq were analyzed microbiologically in this study. Isolates of *Salmonella*, included in the specimens collected from November 2017 to January 2018, were investigated. Capacity for biofilm producing was demonstrated by two method, Tissue culture plate method (TCP) and Congo red agar (CRA). After that the antibiofilm activity of lime extract and LiO_2NPs was studied as each one of them alone and then the synergistic effect was done by TCP method. The results showed that all *Salmonella* isolates produce biofilm but in different degrees. The results also displayed that Lime extract and LiO_2NPs had antibiofilm effect against *Salmonella* when used alone and when the combination done between each one of these materials. In conclusion, it was observed that the specimens of fresh white cheese included in this study contained microbial contamination at a health-threatening level but we can eliminate this contamination by using lime extract and LiO_2NPs .

Key words: Biofilm, LiO_2NPs , Lim juice, Congo red, *Salmonella*, Cheese.

المخلص

أخذت ثلاثين عينة من الاجبان الطازجة البيضاء المعروضة للبيع في اسواق مختلفة لمدن عراقية متنوعة وتم تحليل البكتيريا الموجودة فيها، تم فحص عزلات السالمونيلا ، المدرجة في العينات التي تم جمعها من تشرين الثاني 2017 الى كانون الثاني 2018. تم توضيح القدرة على إنتاج الأغشية الحيائية بطريقتين ، طريقة صفيحة الأنسجة (TCP) وطريقة الكونغو ريد (CRA)، وبعد ذلك تمت دراسة النشاط المضاد للغشاء الحيائي لمستخلص الليمون وجزيئات اوكسيد الليثيوم متناهية الصغر كل على حده ومن ثم دراسة التأثير التآزري لكليهما معا بطريقة صفائح النسيج (TCP). أظهرت النتائج أن جميع عزلات السالمونيلا تنتج غشاء حيائي ولكن بدرجات مختلفة وكذلك بينت أن مستخلص الليمون و جزيئات اوكسيد الليثيوم النانوية كان لهما تأثير مضاد للغشاء الحيائي عند استخدام كل منهما على حده وعند الجمع بينهما في النتيجة، لوحظ أن عينات الجبن الأبيض الطازج المدرجة في هذه الدراسة تحتوي على تلوث جرثومي بمستوى يهدد الصحة ولكن يمكننا القضاء على هذا التلوث باستخدام مستخلص الليمون وجزيئات اوكسيد الليثيوم النانوية.

Introduction

Cheese is an excellent source of protein, fat and minerals such as calcium, iron and phosphorus, vitamins and essential amino acids and therefore is an important food in the diet of both young and old people [1, 2]. It's produced throughout the world in wide-ranging flavors, textures, and forms [3]. The characteristic flavor of cheese is achieved by the addition of starter culture such as lactic acid bacteria which are produced lactic

acid from lactose, so the cheese was fermented [4]. Cheese is a nutrient that is quite rich in protein and calcium, so it is exhibit a very rapid growth of microorganisms which infect through various sources [5]. Biofilm production in milk tanks and milk development position resulting from the capacity of bacteria to stick and acumulative on stainless steel surfaces. Occasionly for microbial pollution of the processed dairy food caused by biofilms maturation in milk treating places[6]. *Salmonella enterica*, *Escherichia coli* O157:H7 and *Shigella* spp. are among the major food borne pathogens affecting people worldwide [7].

A number of food safety agencies around the world have notified of the dangers of raw-milk cheeses. The U.S. Food and Drug Administration states that soft raw-milk cheeses can cause "severe infectious diseases like salmonellosis [8]. *Salmonella* that caused this infection characterize as a Gram-negative, facultative anaerobe and rod-shaped bacterium belonging to the family Enterobacteriaceae. Some *Salmonella* serovars frequently foodborne, are capable to become invasive [9]. The alarming incidence of antibiotic resistance in bacteria of medical importance makes there is a constant need for novel and efficient healing agents [10], so we require to apply other antimicrobial agents such as plant extract for example is *Citrus aurantium L*. *Citrus aurantium L* has long been inspired in food industry as a part of liqueurs and marmalades, and its extracts have also been using in traditional Chinese medicine to encourage essential energy and exchange, eliminate phlegm, and divide stagnation [11]. Various citrus fruits have been learned for its antimicrobial action comprehensive of both gram positive and gram negative bacteria [12]. Furthermore citrus fruits consumption has been associated with minor risk of numerous chronic diseases [13]. Many researchers study the antimicrobial effect of Citrus fruits alone and as a mixture with other herbs [14, 15] and lime juice has been revealed to have elevated antimicrobial action. Other materials be able to used as antimicrobial agent such as nanoparticles and for many years, it was recognized for its important broad - spectrum antimicrobial action against Gram-negative and Gram-positive bacteria, fungi, protozoa and certain viruses [16]. The word "nano" itself belongs to the length scale that is one thousand times smaller than the micro scale. The large surface area of nanoparticles came from its size and make it in contact with the bacterial cells and hence, it will have a higher proportion of interaction than bigger particles, the size of nanoparticles smaller than 10 nm make them in interact with bacteria and produce electronic effects, which enhance the reactivity of nanoparticles. Currently, Nano therapeutics have been used as antibiofilm through the incorporation of nanoparticles into several substances[17, 18, 19]. From all the information that remembered above this study was aimed to screening bacterial contamination by *Salmonella* in local cheese, determine biofilm producing isolates by different methods and study the antibiofilm effect of lime extract and LiO₂NPs against *Salmonella* isolates.

Materials and Methods

Isolation and Identification:

Local fresh- white cheese were collected randomly from local markets in different cities of Iraq during the period from November 2017 to January 2018. Thirty Samples were transferred directly by refrigerator containers to the laboratory and examined immediately without further storage. Five grams of cheese were suspended in 10 ml of buffered peptone water and incubated for 24 h at $37 \pm 1^\circ \text{C}$. Loop-full from this broth was streaked on to MacConkey agar (Himedia/ India), *Salmonella Shigella* agar (S.S agar) (Himedia/ India) and Xylose lysine desoxycholate agar (XLD) (Himedia/ India), incubated at $37 \pm 1^\circ \text{C}$ for 24 h. After that, the

suspected colonies were transferred directly to Triple Sugar iron agar (TSI) and Urea agar base (Himedia/India) tubes by stabbing the bottom and streaking the slope, and incubated for 24 h at 37 °C (10), for further identification Epi 20 tests were done.

Biofilm assay:

Tissue culture plate method (TCP)

The ability of bacterial isolates to form biofilm was achieved by using tissue culture plate method (TCP), all bacterial isolates were cultured in brain heart infusion broth (BHI) containing 1% glucose in 96-well polystyrene tissue culture plates and incubated at 37±°C for 24 hrs under aerobic conditions. After that, the bacterial cells were washed three times with distilled water, and then the adhering cells in each well were fixed with 200 µl of absolute methanol for 20 minutes. The plates were emptied and left to dry overnight. The adhering cells were stained with 0.1% crystal violet in volume 200 µl for 15min, and excess stain was rinsed off. The plates were washed again with D.W and air-dried overnight. The crystal violet dye fixed to the adherent cells by using 200 µl of 96% ethanol per well, and the plates were read at 490 nm using a spectrophotometer. The experiment was performed in triplicates, and the absorbance of wells containing sterile BHI broth was used as the control, the result calculate in compare with control as in Table (1).

Table (1): Classification of bacterial adherence by tissue culture plate method (20)

OD values	Biofilm formation
< OD _c	Not producer
OD < OD ≤ 2*OD _c	Weak
2*OD _c < OD ≤ 2*OD _c	Moderate
4OD _c < OD _t	strong

OD= optical density, c= control, t = test

Congo red methods:

This method was done following the method illustrated by Freeman *et al* [21], who styled an alternative method of screening biofilm formation by *Staphylococcus* isolates; which needs the use of especially prepared solid medium that represent mixture from BHI broth (37 gms/L), sucrose (50 gms/L), agar no.1 (10 gms/L) and Congo red stain(0.8 gms/L). Congo red stain was prepared formerly alone as aqueous solution and autoclaved at 121°C for 15 minutes, discretely from additional medium constituents and was then added when the medium had cooled to 45°C. Culture medium plates were cultured with bacterial isolates and incubated for 24 to 48 hours at 37°C. Positive result was known by dry black colonies, moderately producer makes gray color and weak slime producers usually pink [22].

Preparation of LiO₂ NPs suspension

The preparation of nanoparticles was done following to [23], one hundred milligrams of LiO₂NPs was added to 10 ml of sterile D.W. and shaken vigorously. The suspending solution was treated by ultrasound (40 kHz) for 30 min, autoclaved at 121 °C for 20 min and then cooled down to room temperature.

Preparation of Lime juice:

The preparation of lime Juice extract was done according to the methods reported by [12]. Lemon fruits were obtained from the market, samples were cut into halves using sterile knife and the liquid was squeezed out from it individually in sterile container. Care was taken to avoid contamination of the lemon juice during

squeezing; this extract was marked as 100% concentrated juice extract. Concentration of 50% was prepared by diluting the 100% extract with right volume of sterilized distilled water.

Antibiofilm study of lime juice:

The antibiofilm activity of lime juice was studied by the same steps that followed for detecting biofilm producing isolates as mentioned earlier, but the different concentrations of lime juice were added in volume 100 with the same volume for bacterial suspension in the wells as triplicate for each concentration, incubated at $37 \pm ^\circ\text{C}$ for 24 hr, after that all wells were washed three times with D.W, stained with 0.1% crystal violet, and read O.D by ELIZA reader at 490 nm.

Detection the antibiofilm activity of LiO_2 against bacterial isolates:

The same procedure mentioned was followed but different concentrations of LiO_2 NPs were added with bacterial suspension in the wells as triplicate for each concentration, incubated for 24 hr at $37 \pm ^\circ\text{C}$, after that all wells were washed, stained, and read O.D at 490 nm.

Detection the synergetic antibiofilm activity of LiO_2 and Lime juice against bacterial isolates:

The anti-biofilm activity of LiO_2 NPs in combination with Lime juice was studied by using TCP assay also. Individual wells were filled with 100 μl of the bacterial suspension, after that LiO_2 NPs as mixture with lime extract was added (50 μl from each one of them). The plate was incubated for 24 hours at $37 \pm ^\circ\text{C}$, and the wells then washed 3 times with D.W, stained with crystal violet for 15 minutes. The stain was rinsed off, resolubilized with ethanol and optical density was measured at 490 nm. The control was considered to represent 100% of biofilm formation isolates and achieved from the first result of biofilm formation.

Results and Discussion:

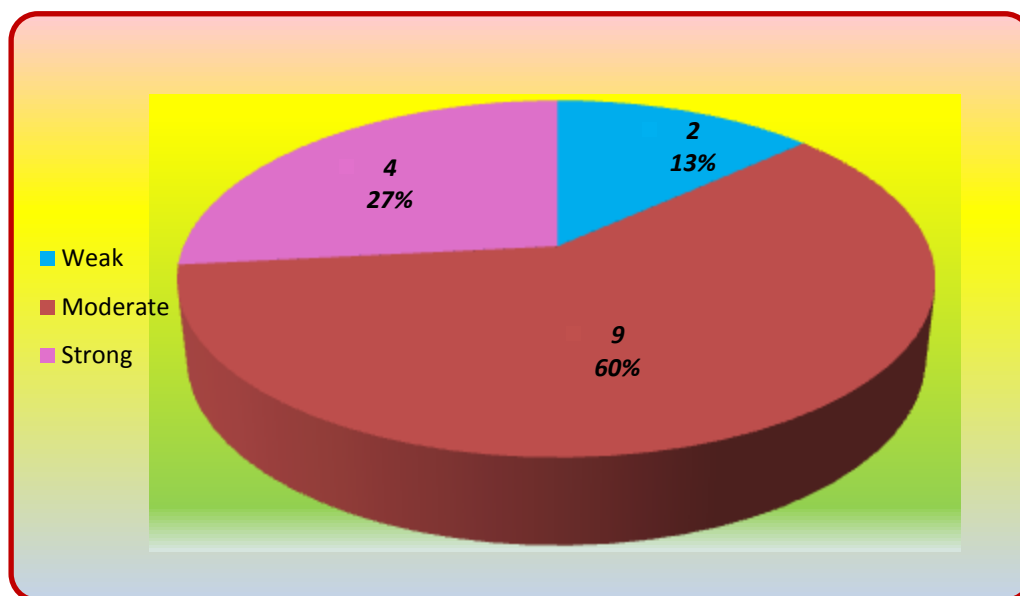
Isolation and identification:

The results of present study showed the presence of 84 isolates belong to different bacterial species in cheese samples, Data revealed that (19.04%) of the cheese samples were contaminated with *Salmonella enterica* serovar typhi. Study by Nazal,[24] in Iraq reported that (5%) of the cheese samples were infected with *Salmonella spp.* This may belong to the low quality of raw milk utilized in cheese producing or due to unsanitary conditions during processing and handling of cheese [25]. Three major food borne pathogens affecting people worldwide are *S. enterica*, *E. coli* O157:H7 and *Shigella spp.* [7]. In the United States, analysis of epidemiological data on food borne disease outbreaks indicated that *Salmonella* is the most common bacterial etiologic agent, accounting for 52% of outbreaks attributed to bacteria [26].

Study by Arif [27], investigated that *salmonella* species cause contamination for three samples from total 120 cheese samples. Other Study by Ashraf and Tadashi, [28] reported that the occurrence of *S. enterica* was 6.6% in meat products and 2.0% in dairy products. Pasteurization destroys pathogenic bacteria which frequently may be present in milk, including those causing of tuberculosis (*Mycobacterium bovis*), listeriosis (*Listeria monocytogenes*), campylobacteriosis (*Campylobacter*), Salmonellosis (*Salmonella*) and several other foodborne illnesses (e.g., *Escherichia coli* O157:H7). Since this kind of cheese formed from unpasteurized milk thus, *Salmonella* contamination was estimated, furthermore the bad aseptic condition through the industrial, handling and distributing of this product in our local markets increase this capacity for contamination [29].

Biofilm assay

The gold-standard method for this study was TCP method, in compare with data from TM and CRA methods [30]. The findings of recent study reported that all bacterial isolates has the ability for producing biofilm but in different degrees, *Salmonella* isolates formed biofilm in the following percentage, 4 isolates gave strong biofilm, 9 isolates produced moderate biofilm, while only two isolates showed weak biofilm (see figure (2)). *Salmonella* spp. has confirmed the ability to biofilms producing on several surfaces including different kinds of stainless steel (AISI 304 or 316), polyethylene, polystyrene, acrylic, glass, and metal inert gas (MIG) and tungsten inert gas (TIG) melts [31].



Figure(1): Biofilm producing by *S. enterica* serovar typhi

Biofilm formation capacity may be an important factor for the persistence of *Salmonella* on food get in touch with surfaces in the factory surroundings [32]. Biofilms have been commonly associated in chronic infections and environmental persistence of *Salmonella enterica*, as a result simplifying enhanced colonization of surfaces and expanding the ability of the bacteria to be diffused to new hosts [33]. In conclusion, it was found that there was relationship between the time to clearance of *S. typhi* from typhoid patients after recovery (as gauged by PCR on stool samples) and biofilm production capability. All salmonellae tested in this study are able to form strong biofilms and this result was in agreement with present study [34].

Salmonellosis that caused by *Salmonella* spp. are represent the most important zoonotic diseases that commonly worldwide, and the most common type of salmonellae infection is enterocolitis. It was found that *Salmonella enterica* serovars Enteritidis and Typhimurium were the most frequent serotypes secluded from food borne infections, which are capable to of forming biofilm on several surfaces including biotic and abiotic for example: glass, stainless steel, , plastic and living tissues [35,36]. The contact of *Salmonella* serotypes on biofilm formation capability has not yet been completely explained, and there are few experiments that compare the differences among the serotypes. Descriptions inherent to a specific serotype can effect on its ability for biofilm production [37, 38, 39].

Congo red method:

This is the other procedure by which the biofilm was detected but represent less accurate from microtiter plate method. The present work showed that *Salmonella* species produce Lipopolysaccharides but in variable values. There are only 4 isolates (26.66%) gave positive results that appeared as black crystalline color, 2 isolates(13.33%) gave gray color that's mean moderate result while all other isolates in percentage (60%) gave negative results appeared as orange color. see figure (2). This method was not reliable because of less precision as see by other researchers such as Hassan *et al.*[30] who explained that CRA method suggested very little association with the other approaches and parameters such as sensitivity, specificity and accuracy were very low, by this method, some isolates were found to be false positive and others were false negative. Knobloch *et al* [40] did not advised for using CRA procedure for biofilm formation in their study, from 128 isolates of *S. aureus* in this method detected only 3.8% as biofilm producers as compared to TCP which detected 57.1% as biofilm producing bacteria. The CRA plate method is not recommended method for detecting biofilm. The same results have been reported by other researchers [41].

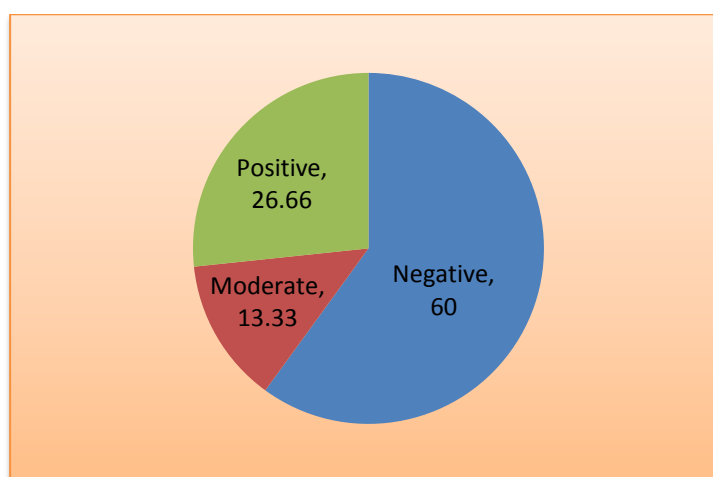


Figure (2): Congo red results for *S. enterica* serovar typhi

Zuberi* and Nadeem [42] reported in their study when compared this method in compare with other results the CRA plate method for detecting biofilm production, reported that only four isolates produced black crystalline morphology, while 111 showed orange colonies

Antibiofilm study of lime juice:

There are few studies about the effect of lime juice against bacterial biofilm and most of them discussed the antibacterial effect of bacteria as planktonic cells. The results of present study revealed that all concentration of lime juice affect on biofilm in *Salmonella*, the higher antibiofilm effect against *Salmonella* 26 and 27 was achieved by concentration 12.5% see figure(3) and (4). Previous studies have shown lemon extract have significant antimicrobial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella pneumoniae* [43]. Other research showed that Lemon juice is not only an astringent but it is also a good antimicrobial agent [44]. Bansode and Chavan,[45] displayed that the fresh crude lemon juice produced the highest antimicrobial activity against *Salmonella para typhi* B and *Shigella sonnei* followed by *E.coli*.

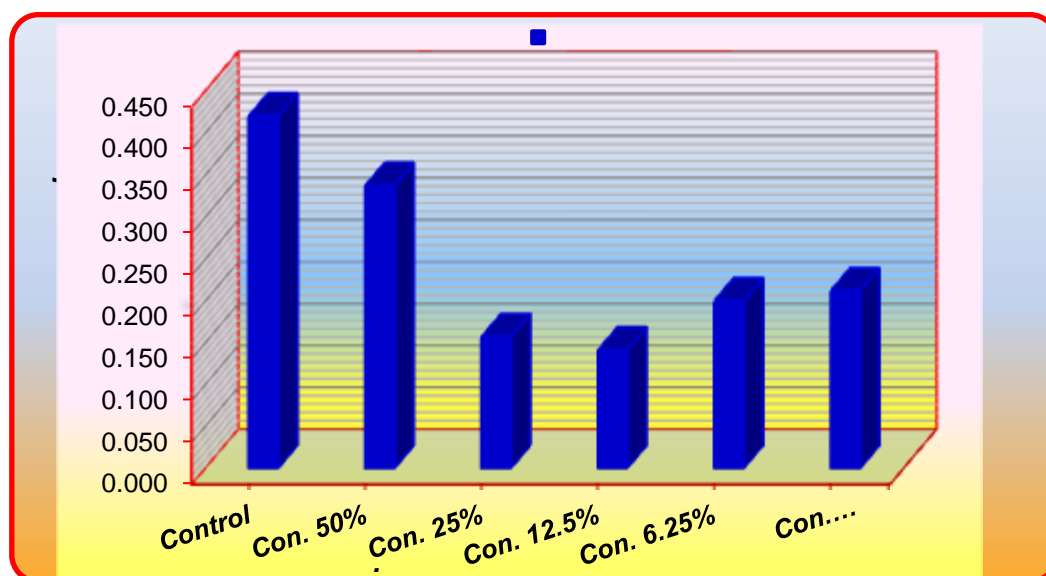


Figure (3): Antibiofilm study of lime juice against *Salmonella 26*

The figure above showed the effect of different concentration of lime juice against bacterial biofilm, the results demonstrated that lime juice was a good antibiofilm agent as appeared from the percentage of each concentration when compared it with the control (biofilm of bacteria without lemon).

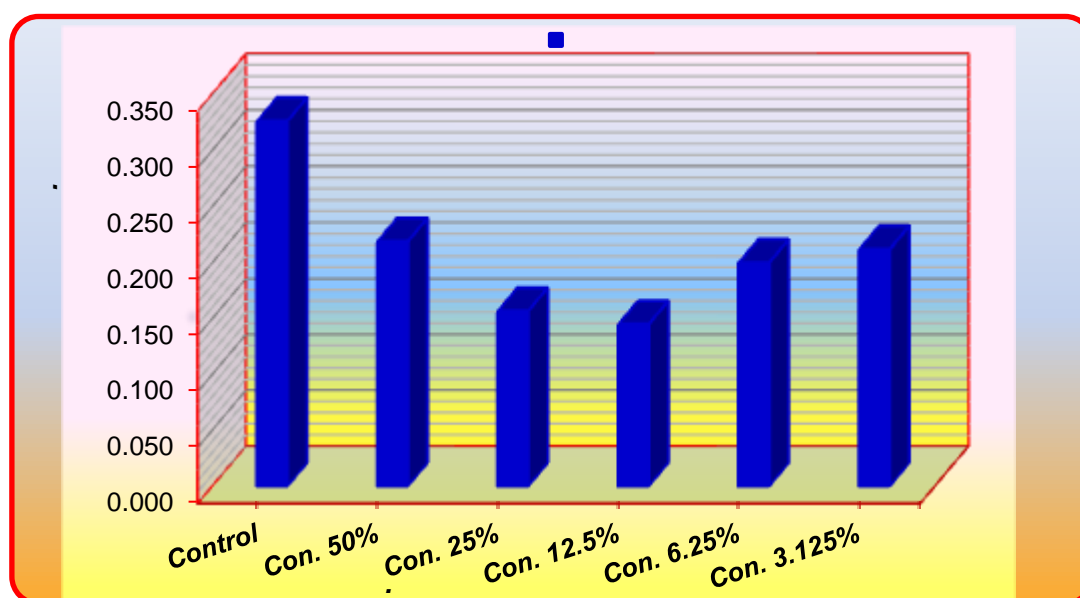


Figure (4): Antibiofilm study of lime juice against *Salmonella 27*

Detection the antibiofilm activity of LiO₂ NPs against bacterial isolates:

The antibiofilm activity of LiO₂ was carried by microtiter plate also; LiO₂ NPs were prepared in different concentrations and used as antimicrobial materials on biofilm of *Salmonella*. The current study revealed that all prepared concentration of LiO₂NPs had the antibiofilm activity against *Salmonella* 26 and 27 but higher activity was achieved by 7.8 µg / ml concentration. figure (5) and (6). Studies about the LiO₂ activity on biofilm are limited but local study by [46] reported that Nanoparticles(Ag and Li O₂) had effects against adherent cells and mature biofilm that formed by *C. albicans* .The mechanisms causal the antimicrobial activities of NPs are not totally understood and differ from the formations of oxidative and/or free radical formation stressors to DNA damage [47].

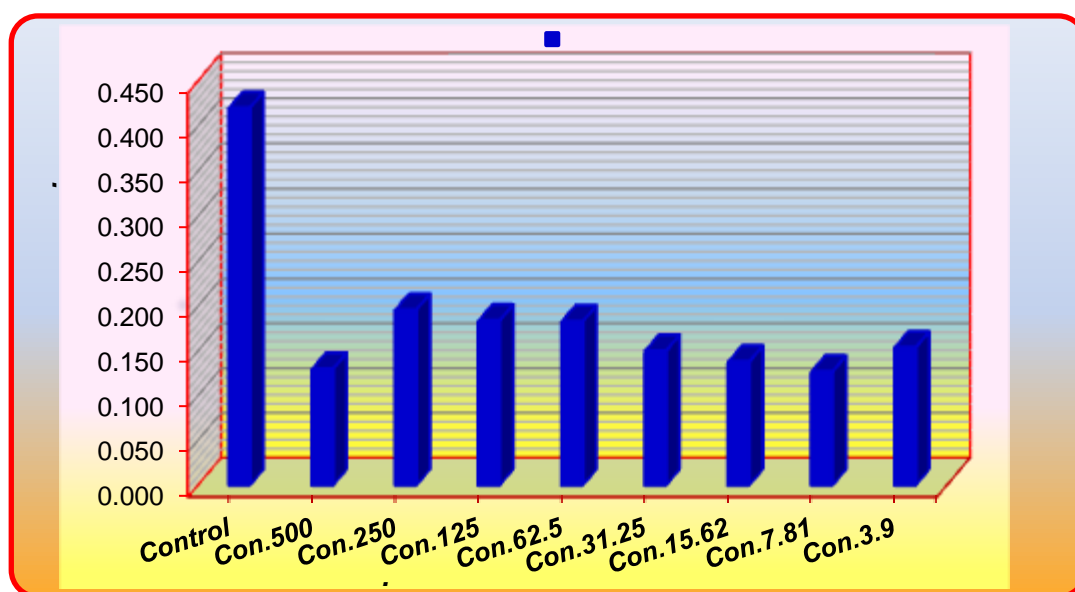


Figure (5): Antibiofilm activity of LiO₂ against *Salmonella* 26

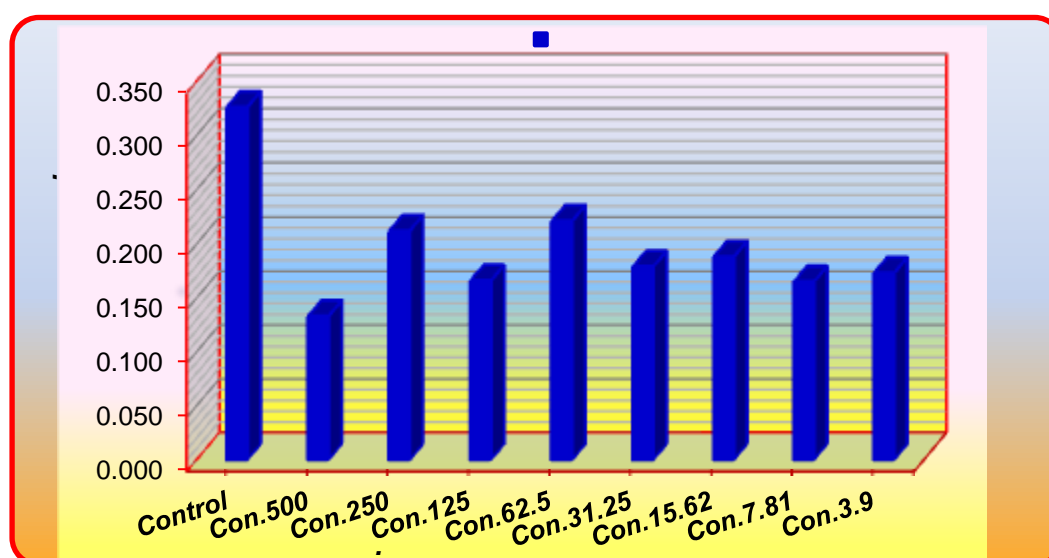


Figure (6):Antibiofilm activity of LiO₂ against *Salmonella* 27

Detection the synergistic antibiofilm activity of LiO_2 NPs and Lime juice against bacterial isolates:

The synergistic effect of LiO_2 NPs and Lime juice was also studied, it was found that when the combination between them was done the same antibiofilm activity was achieved but the higher effect against *Salmonella* 26 observed by the concentration 125 $\mu\text{g/ml}$ from Lime juice and 12.5 $\mu\text{g/ml}$ from LiO_2 NPs. The biofilm of *Salmonella* 27 gave highly effect by concentration 62.5 $\mu\text{g/ml}$ from lime juice and 6.25 $\mu\text{g/ml}$ from LiO_2 NPs. Many studies mentioned that the using of antimicrobial agents combination was more effective than the using of any agents alone like the study by [48] who reported the antifungal effect of silver nanoparticles in combination with chlorhexidine digluconate and nystatin against *Candida albicans* and *C. glabrata* biofilms. Local result achieved by [46] showed that the nanoparticles (Ag and LiO_2) combination with antifungal (Itraconazole) obtained significant inhibition in mature biofilm as compared with the adherent cells, Significant inhibition happened at most combinations of nanoparticles (Ag and LiO_2) with antifungal (Itraconazole) that used. So these findings were in agreement with the present result on the effect of lime extract and LiO_2 NPs against biofilm.

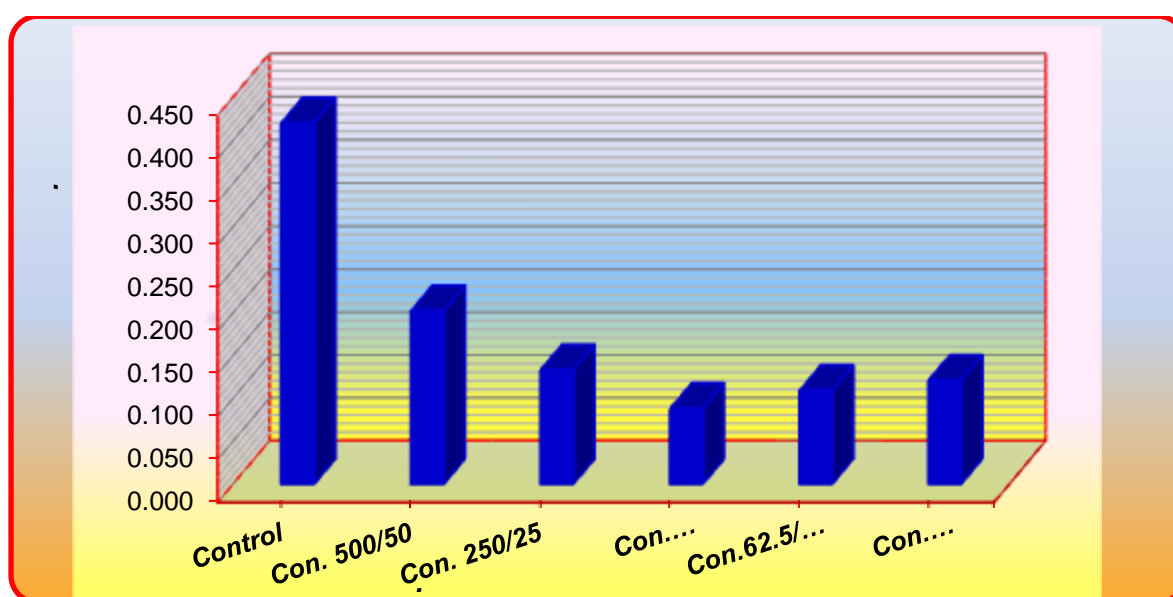


Figure (7): The synergistic antibiofilm activity of LiO_2 and Lime juice against *Salmonella*26

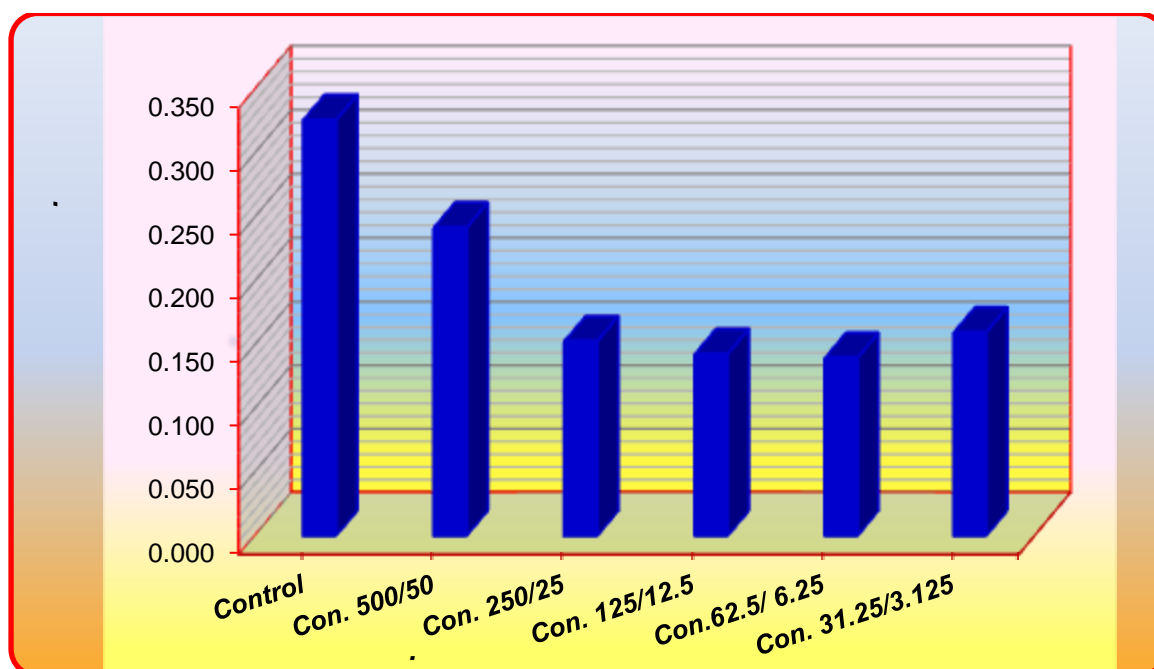


Figure (8): The synergetic antibiofilm activity of LiO_2 and Lime juice against *Salmonella*27

Conclusions:

The results of current study revealed that lime extract and LiO_2 NPs had the antibiofilm effect against *Salmonella* isolates when each one of them was used alone or when the combination between them was accur.

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