Comparative study of antibiofilm activity of Lime juice and Lithium dioxide nanoparticles against *E.coli* isolated from local made cheese

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Abstract

Thirty specimens of fresh white cheese, in different markets at different cities of Iraq were analyzed microbiologically. Isolates of *E.coli* that have been collected from the samples of cheese, were investigated. Capacity for biofilm producing was demonstrated by two method, Tissue culture plate method (TCP) and agar (CRA). After that, 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 *E.coli* isolates produce biofilm but in different degrees. The results also displayed that Lime extract and LiO_2NPs had antibiofilm effect against *E.coli* 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 elimination of this contamination can be done by using lime extract and LiO_2NPs.

Key words: Biofilm, LiO2NPs, Lim juice, *E. coli*, Cheese.

Introduction

Cheeses are mainly classified based on the moisture content into very hard, hard, semi so and so cheeses. The fresh white cheese has been considered as soft, and based upon the nutrients and the moisture content it can be subject to growth of a variety of the pathogens and micro-organisms (1).

Production of cheese is a very important activity of the dairy plants all over the world. White cheese is the main type of the cheese that is produced in the local market. It has been characterized as un-ripened cheese, which is ready to eat directly post processing, it has been identified by the white color and soft texture product that is obtained by the enzymatic

coagulations of the milk and complemented or not by action of the specific lactic acid bacteria; Those properties provide the optimal conditions for pathogenic and spoilage micro-organism multiplications (2, 3).

Mainly it is produced from cows and sheep milk, furthermore it is often seasonally produced from the ewes or goat milk or from combination of the two, with no addition of the starter culture or salt under the artisanal or un mechanized conditions and it's handled at a variety of the manufacturing. Its acidity shouldn't be higher than 1.90% as the lactic acid and has to be kept under the refrigeration despite the fact that it has quite a limited keeping capability even under the refrigeration. The production approach might be summarized in milk coagulating for 40min-60min by rennet (which is a natural complex of the enzymes that are produced in any mammal stomach for the digestion of mother's milk, and is typically utilized for producing cheese) post the heating to approximately 35^{o}C and after that, pressing in the cheese cloth. Thee cheese is typically consumed directly after the production or utilized for producing some Arabian confectioneries and food. A variety of the micro-organism types can enter cheese throughout the manufacturing and consequent handling (3); Milk is contaminated by organisms that are found on the animal's exterior surfaces as well as surfaces of the milk handling tools, like the pipeline, milking machines, and containers, which results in infections and threating to the health of the consumer's by diseases like brucellosis, tuberculosis, listeriosis and typhoid fever (**4).** The Coliform bacteria represent the fundamental contaminants of the dairy products and raw milk, which includes fresh cheese. Coliforms are destroyed easily by the heat treatments that is typically utilized for milk, being one of the indicators of the process failures or post-processing contaminations in the pasteurized foods hey are responsible as well for milk deterioration, yield losses, unwanted fermentation, and defects in the cheese. *E. coli* represents the main fecal contamination indicator in the foods. *E. coli* alone could be representing one of the health risks, due to the fact that particular strains are pathogenic to the human beings (2,3). Contaminations typically occur at a farm where the milk is produced. *E. coli* and coliform bacteria could enter the milk and milk products quite easily; the existence of the *E. coli* in fresh white cheese indicates low quality milk, poor production practices or contamination after processing due to fecal contaminations; in addition to that, it is an indication of the presence of the enteropathogenic or toxigenic bacteria that are the main hazard to the public health. The entero-pathogenic *E. coli* could result in cause severe diarrhea and vomiting in the young children and

infants. Which is why, identifying and enumerating *E. coli* is commonly utilized as indication for evaluating the fresh white cheese safety (1, 4).

A bio-film can be characterized as aggregate of the microorganisms living together as a community and are usually found to be attached to the solid surfaces in the moist environments. The microbes in the bio-film secrete various protective substances that are referred to as EPS, enhancing the efficiency of their survival. *E. coli* bio-films have been found to be the main causative factor of a wide range of the intestinal infections. The dense bacterial cells in the bio-film communicate with one another through chemical signaling path-way, which is referred to as quorum sensing (QS). Throughout the QS, the bacterial cells secrete the auto-inducer (AI) substances to extra-cellular milieu and as soon as required high density has been attained, they are responsible for the upregulation of the formation and maturation of the bio-film. The auto-inducer helps bacterial cells in bio-film in the secretion of the virulence factors, modulation of host immune responses and accruing the genetic changes. The bio-film renders penetration of the traditional antibiotics hard and make cells be of a lower susceptibility to antibiotics (5).

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 (6). A variety of the citrus fruits have been learned for its anti-microbial action comprehensive of the gram positive as well as gram negative bacteria (7). Furthermore citrus fruits consumption has been related to minor risk of numerous chronic diseases (8). Many researchers study the antimicrobial effect of Citrus fruits alone and as a mixture with other herbs (9,10) and lime juice has been revealed to have elevated antimicrobial action. Other materials be able to use as antimicrobial agent such as nanoparticles and for many years, it was recognized for its important broad-spectrum anti-microbial action against the Gram-negative and -positive bacteria, protozoa, fungi, and certain viruses (11).

The word “nano” itself belongs to the length scale that is 1,000 times smaller compared to the micro scale. The large surface area of nanoparticles came from its size and make it in contact with bacterial cells and as a result, it will have a higher rate of the interactions compared to larger particles, the nanoparticle size which is smaller than 10nm makes them in interact with the bacteria and produce electronic effects that improve the nanoparticles’ reactivity. Currently,

Nano therapeutics have been used as antibiofilm through the incorporation of nanoparticles into several substances (12, 13, 14).

From all the information that remembered above this study was aimed to screening bacterial contamination by *E.coli* in local cheese, determine biofilm producing isolates by different methods and study the anti-biofilm effect of lime extract and LiO_2NPs towards the *E. coli* tested isolates.

Materials and Methods

Isolation and Identification:

From the period November 2017 to January 2018; Thirty Samples of the local fresh-white cheese have been randomly obtained from local markets in different cities of Iraq. Those samples have been directly transferred in refrigerated containers to the lab for immediate examination. Five grams of each sample was suspended in 10ml of the buffered peptone water and then incubated for 24h at 37C. after incubation; each sample`s suspension was streaked on several differential and selective media, such as: MacConkey agar (Himedia/ India), *Salmonella Shigella* agar (S.S agar) (Himedia/ India) and Xylose lysine desoxycholate agar (XLD) (Himedia/ India), incubated for 24 hours at 37C. then the suspected colonies were transferred to culture on Triple Sugar iron agar (TSI) and Urea agar base (Himedia/ India) tubes through stabbing the bottom and streaking the slope. After a 24h incubated at 37^{o}C; Epi 20 tests were preformed for further identification.

Biofilm assay:

Tissue culture plate method (TCP)

The capability of bacterial isolates in producing bio-film has been preformed by using tissue culture plate method (TCP), all bacterial isolates were cultured in brain heart infusion broth (BHI) containing 1% glucose, poured in 96-well polystyrene tissue culture plates and then incubated at a temperature of 37^{o}C for 24hrs under the aerobic conditions.

After that, bacterial cells have been washed 3 times by the distilled water, and after that, adhering cells in every one of the wells have been fixed with 200µl of the absolute methanol for 20min; the plates have been emptied and then left overnight to dry. Adhering cells have been stained of with 0.10% crystal violet in a 200µl volume for 15 min, and the excess stain has been

rinsed off. Plates have been washed once more by DW and air-dried overnight. Crystal violate dye fixed to adherent cells with the use of 200µl of 96% of the ethanol in each one of the wells; ultimately, plates have been read at 490nm with the use of a spectrophotometer. The experimentation has been carried out in triplicates and results have been compared with absorbance of the wells that contain sterile BHI broth as the control, as it is showed in table1.

Table1: Classification of the bacterial adherence with the tissue culture plate approach (20)

OD value	Bio-film formations
< OD c	Not producer
OD < OD ≤ 2ODc	Weak
2ODc < ODt≤ 2ODc	Moderate
4 ODc<OD t	strong

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

Preparation of LiO_2 NPs suspension

Nanoparticles preparation was preformed according to (15), 100 ml of the LiO_2NPs has been added to 10ml of the sterile DW and vigorously shaken. The suspension solution has been treated by the ultra-sound (40kHz) for 30min, autoclaved at 121^{o}C for 20min and cooled down after that to the temperature of the room.

Preparation of Lime juice:

According to the methods listed by (7). Lime Juice extract was prepared by cutting the Lemon fruits that collected from the local market into halves using sterile knife, squeezed out to obtain the liquid from it individually in sterile container. Care was taken for avoiding the contaminations of lemon juice throughout squeezing. Yield extract has been marked as 100% concentrated extract of the juice; a 50% concentration has been produced through the dilution of 100% extract with the correct volume of the sterilized D.W.

Antibiofilm study of lime juice:

The antibiofilm activity of lime juice was studied by following the same procedure used for biofilm producing isolates detection, as it mentioned earlier, with the different of adding lime juice concentrations in volume 100 with the same volume for bacterial suspension in wells as triplicate for every one of the concentrations, which have been incubated at a temperature of

37°C for 24hr, then, all the wells have been washed 3 times with DW, stained with 0.10% crystal violet, and read OD by the ELIZA reader at 490nm.

Detecting anti-biofilm activities of LiO_2 against bacterial isolates:

The mentioned procedure was followed but with various concentration levels of the LiO_2 NPs which have been added with the bacterial suspension to the wells, as triplicate for every value of the concentration, then the plate was incubated for 24hr at 37°C, after the period of incubation, and have been washed, stained, and read OD at 490nm.

Detecting synergetic anti-biofilm activity of LiO2 and Lime juice against bacterial isolates:

The antibiofilm activity of LiO_2 NPs in combination with Lime juice extract was studied by using TCP assay also. Individual wells have been filled with 100.0µl of bacterial suspension, then, a mixture of LiO_2 NPs and lime extract has been added (50µl from every one of them). The plat was incubated for 24h at a temperature of 37°C, after incubation, the wells have been washed 3 times with D.W, stained with crystal violet for 15min. The stain has been rinsed off, resolubilized with ethanol and optical density has been measured at 490nm. The control has been considered to be representing 100% of the bio-film formation isolates and achieved from the first result of biofilm formation.

Results and Discussion:

Isolation and identification:

The results of present study showed contamination with different bacterial species, especially; Coliform, *Salmonella* and *Staphylococcus*. Coliform bacteria were isolated from all of 30 specimens of fresh white cheese that collected from the district bazaars and markets of different regions of Baghdad city. Principally *E. coli* isolates were isolated in percentage of (91%), the result is agree with (1) and approach to (16) that reported contamination percentage with E. coli was (98.70%). Cattle can harbor *E.coli*, with no ill effect, shredding them in their feces from which they get the entrance to raw milk. Which is why, this has given a reason why the raw milk that has been obtained from a variety of the producers had tested positive for *E.coli*. None-the-less, in the case where the pasteurization has been applied to milk, then bacteria would be destroyed, thereby, resulting in production of the white cheese with milk that is *E.coli* free

(17). The insufficient quality of the raw milk that has been utilized in local cheese producing due to the low hygiene level throughout the process of milking and cleanliness of containers that are utilized to store and transport milk, in addition to unsanitary conditions during processing, inefficient heat treatment and the improper ways of handling and marketing of cheese can be risk factors that are likely to cause microbial contamination (18,19); Pathogenic bacteria like the *Salmonella*, *L. monocytogenes* and enteropathogenic *E. coli* (EPEC) were classified as organisms of high risk to cheese industry. In addition to that, the outbreaks of the foodborne diseases as a result of a variety of the different chees types from a number of the countries were reported as well. The earlier researches have indicated that the *E. coli* O157:H7 could survive throughout the manufacturing and ripening of the cheeses. Several aspects, like the starter culture, competitive flora, pH level, heat, a_W value, salt, method of cheese production, inoculation level, and conditions of storage impact the growth of *E. coli* in the cheese. In general, the number of micro-organisms continuously decrease throughout the storage (20)

Biofilm assay

The gold-standard method for this study was TCP method, in compare with data from TM and CRA methods (21).The results of this study indicate that all tested isolates have the ability of producing biofilm in varying degrees of intensity; tested isolates of *E. coli* formed biofilm in the following percentage, 5 isolates gave strong biofilm (25%), 13 isolates produced moderate biofilm (60%), while only three isolates showed weak biofilm (15%) (figure 1). *Salmonella* spp. has confirmed the ability to biofilms producing on a number of the surfaces, which include various stainless steel types (AISI 304 or AISI 316), polystyrene, polyethylene, glass, acrylic, and metal inert gas (MIG) and tungsten inert gas (TIG) melts (22).

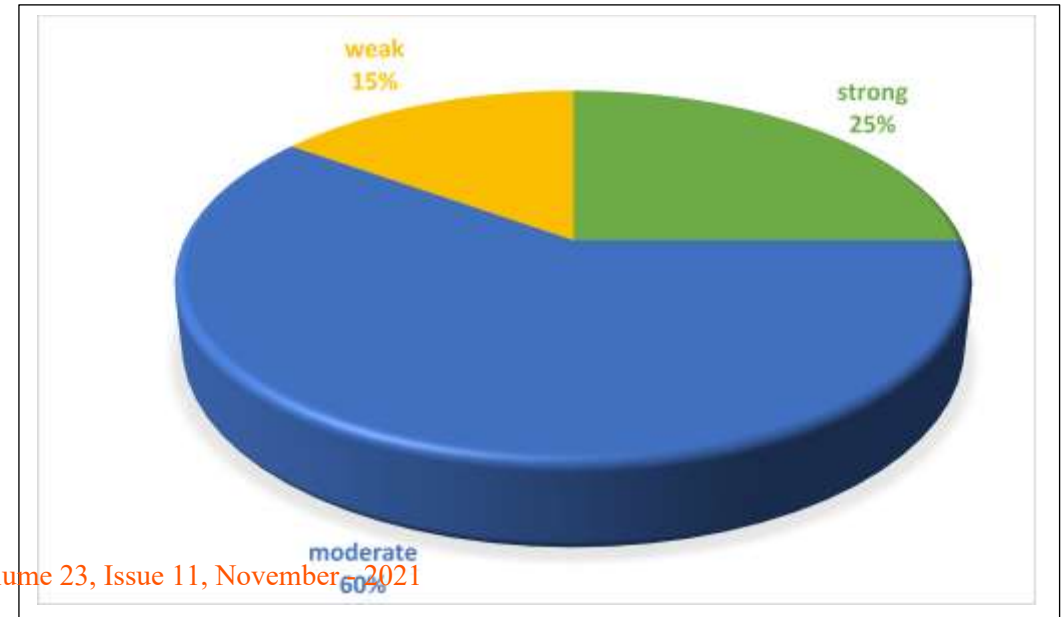

Figure (1) : Biofilm producing by *E. coli*

The formation of the bio-film by the *E. coli* plays a role in occurrence of a variety of the infections and complicates its eradication. Some factors, such as the variety of the extra-cellular appendages contributing in the colonization of the E. *coli* surface and their finely controlled expression and activity result in the formation of the mature bio-films (5).

E. coli has been found as the most common one of the urinary tract infection causes; biofilm formation is a significant factor of virulence in a variety of the pathogenic bacteria that cause the human UTIs based on a study that has been carried out by (23).

UTI have been found to be amongst the most common bacterial of the diseases all over the world, involving infection of approximately 250 million people in the developing countries each year. The uropathogenic *E. coli* on its own is responsible for 70-90% of UTIs and their patterns of susceptibility towards a variety of the antibiotics differ in a variety of the geographical areas. The bacterial biofilms are most of the time related to the long-term persistence of the bacteria in a variety of the environmental conditions. Bacteria in the bio-film exhibited considerably increased resistance to the antibiotics (24).

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 (25).

The results of this study have shown that all of the concentrations of lime juice effect on biofilm *E.coli* producer, the higher antibiofilm effect against *E.coli* 17 and 19 was achieved by concentration 25% (figure2 and 3). Previous studies have shown lemon extract have significant anti-microbial activity towards the *S. aureus, P. aeruginosa, Klebsiella pneumoniae* and *E. coli* (26). Other study had shown that Lemon juice isn't merely an astringent, it is also one of the good anti-microbial agents (27). The fresh crude lemon juice displayed the maximum anti-microbial activity against *Salmonella* para typhi B and *Shigella sonnei* that is followed by the *E.coli* (28).

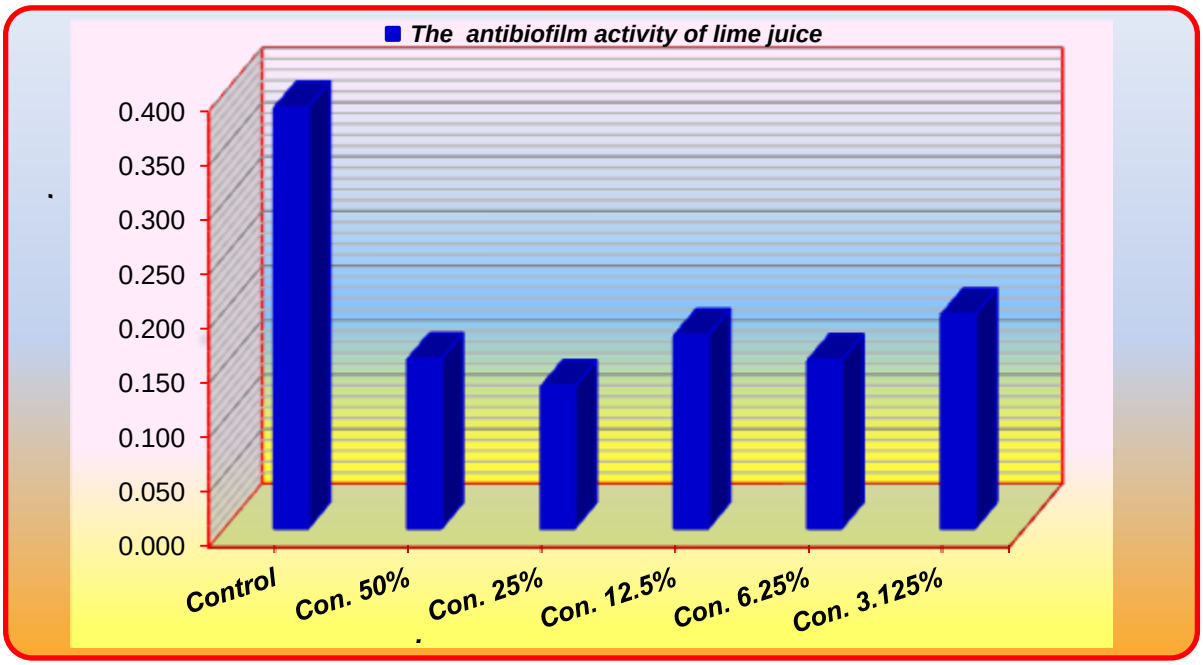

Figure 2: Antibiofilm study of lime juice against *E.coli 17*

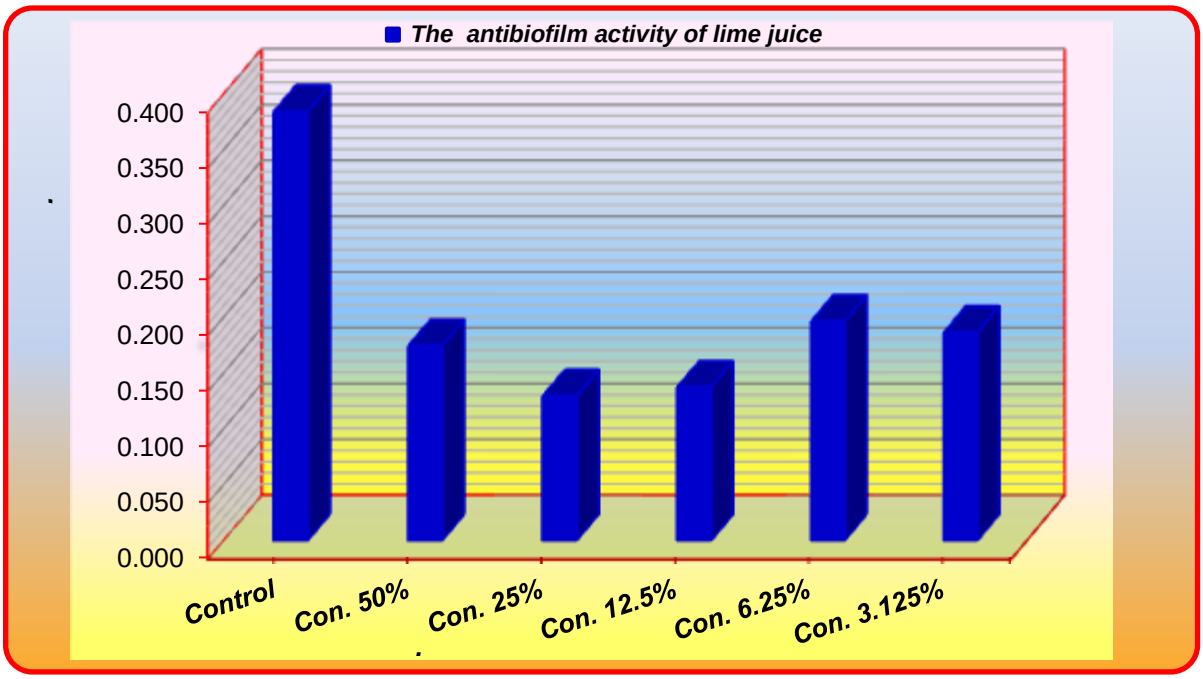

Figure (3): Antibiofilm study of lime juice against *E.coli* 19

The figures 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).

Detection the antibiofilm activity of LiO_2 NPs against bacterial isolates:

The antibiofilm activity of LiO_2 was carried by microtiter plate; also LiO2 NPs were prepared in different concentrations and used as antimicrobial agents against biofilm activity of *E. coli*. The results of this study revealed that all prepared concentration of LiO2NPs had the antibiofilm activity against *E. coli* 17 and 19 but higher activity was achieved by 7.8 µg / ml concentration (figure 4 and 5). Studies about the LiO_2 activity on biofilm are limited but local study by Al-Salmany,(29) reported that Nano-particles (Li O_2 and Ag) had effects towards the adherent cells and mature bio-film which had been formed by the *C. albicans* .The mechanisms causal the antimicrobial activities of the nano-particles are not totally understood and differ from formations of the oxidative and/or free radical formation stressors to the damages of the DNA (30).

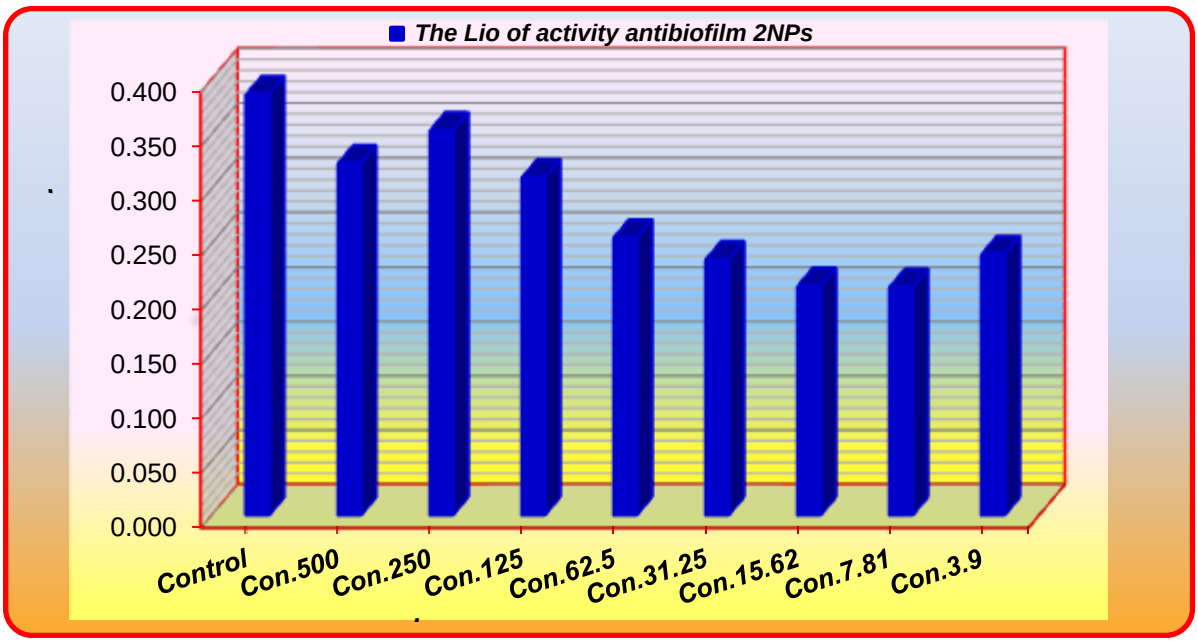

Figure (4): Antibiofilm activity of LiO_2 against *E.coli* 17

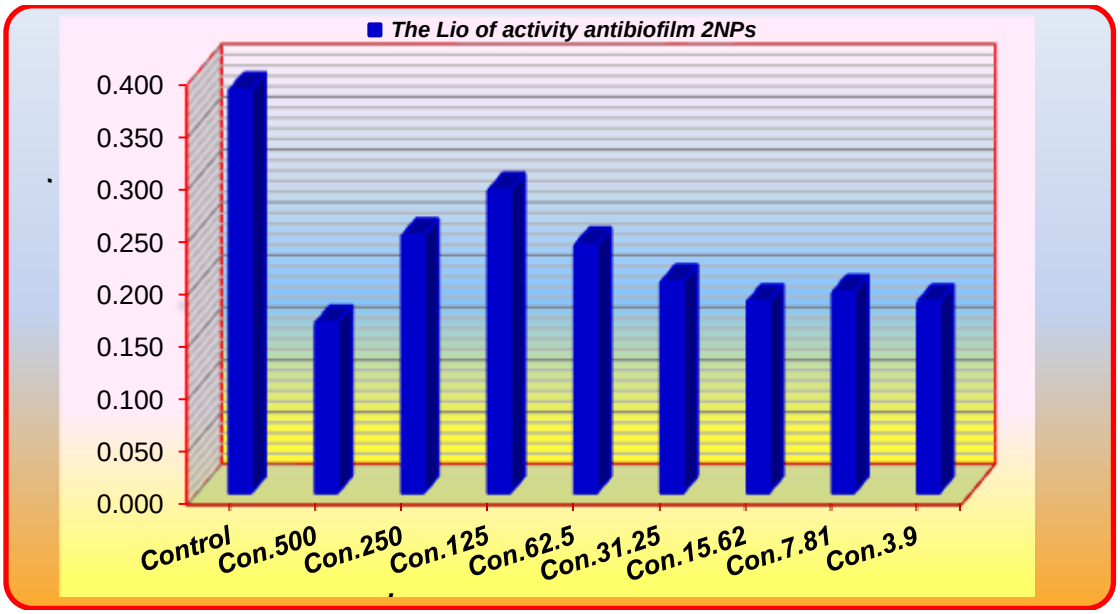

Figure (5):Antibiofilm activity of LiO_2 against *E. coli* 19

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

The synergistic effect of LiO_2 NPs and Lime juice was also detected, it was found that when the combination between them was done the same antibiofilm activity was achieved but the higher effect against *E.coli* 17 observed by the concentration 250 µg/ml from Lime juice and 25 µg/ml from LiO_2 NPs. The biofilm of *E.coli* 19 gave highly effect by concentration 62.5µg/ml from lime juice and 6.25µg/ml from LiO_2 NPs.

Many studies were indicated that utilizing a combination of antimicrobial agents has been more effective in comparison with the use of any agents alone. The combination of LiO_2 NPs and Lime juice had a significant activity towards *Salmonella* more the activity of each material alone (25), in addition to other studies like Monteiro *etal.* (31) who reported the anti-fungal effect of Ag NPs in combination with chlorhexidine digluconate and nystatin against *Candida albicans* and *C. glabrata* bio-films. Local result achieved by (36) had shown that NPs (LiO_2 and Ag) combination with the anti-fungal (i.e. Itraconazole) had obtained significant inhibition in the mature bio-film in comparison to adherent cells, considerable inhibition had occurred at the majority of the nanoparticle combinations (such as LiO_2 and Ag) with anti-fungal (Itraconazole)

that had been utilized. Which is why, those finding coincided with the present results on effects of lime extract and LiO_2NPs against biofilm.

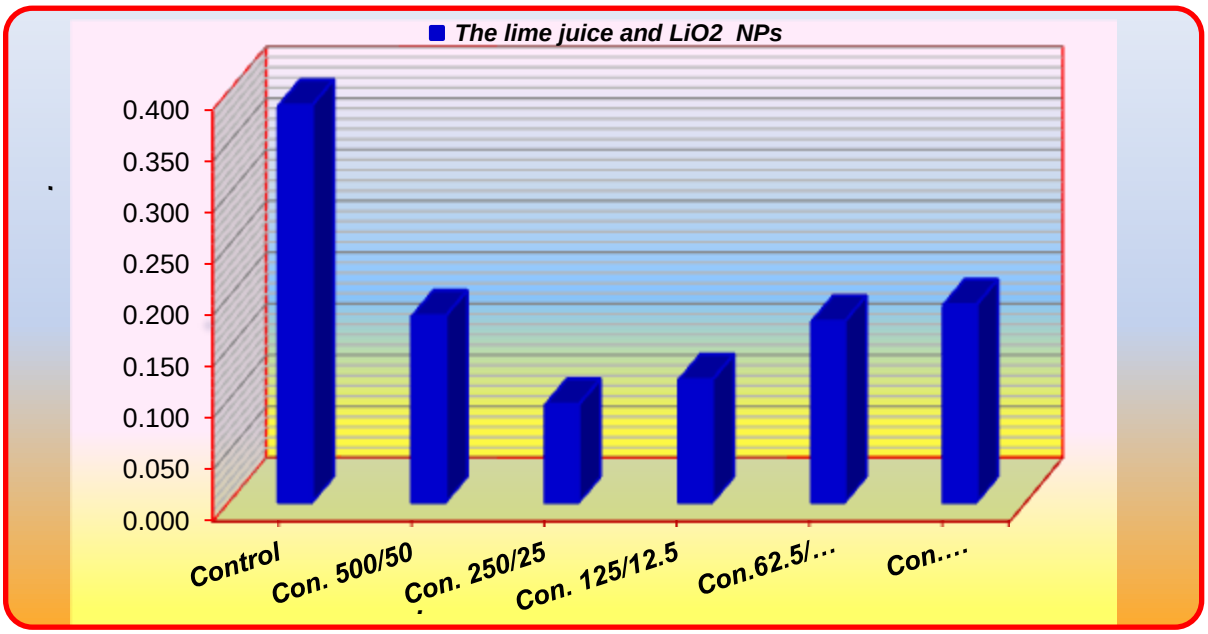

Figure (6): The synergetic antibiofilm activity of LiO_2 and Lime juice against *E.coli 17*

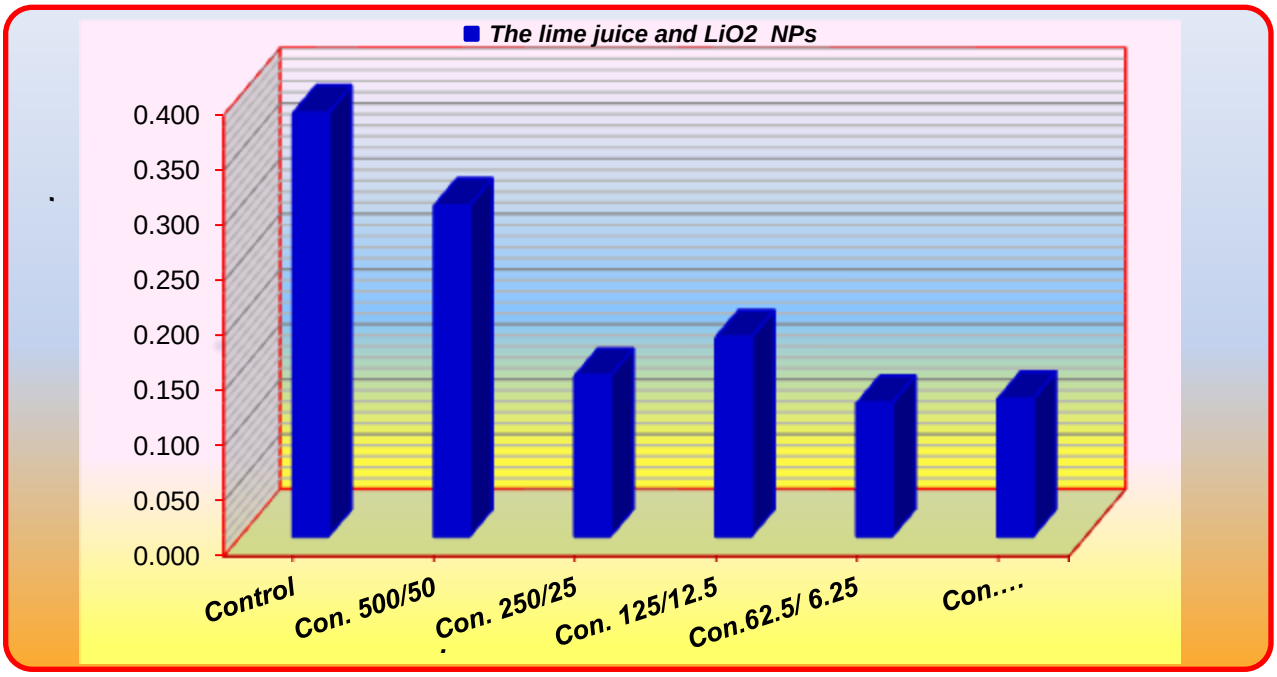

Figure (7): The synergetic antibiofilm activity of LiO_2 and Lime juice against *E.coli 19*

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