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A comparative study of the antibacterial efficiency of some Phyto flavonoids, Antibiotics and the synergistic combinations between them against Staphylococcus aureus

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Abstract

Eight synergistic combinations were prepared between the crude flavonoid extracts of the two studied plants (prepared by three different extraction methods) and the antibiotics (Amoxylin and Doxycyclin), in which their antibacterial effect was higher than the effect of the extracts alone، for all studied treatments.The best combination was (fenugreek and Amoxylin) in which it recorded the biggest inhibition zone reached (24mm).The minimum inhibition concentration (MIC) inhibiting against Staphylococcus aureus was the crude flavonoid extract of Trigonella foenum-graecum L. seeds and Ocimum basilicum seeds at 25 mg/ml with inhibition zone diameter of 6 mm and 4 mm for respectively.The mechanism of anti-bacterial action of the studied extracts were determined by their ability to change the cellular PH of the *Staphylococcus aureus* in addition to causing some morphological changes in the bacterial cells.

Keywords: Phytoflavonoids, synergistic combinations, antibacterial, *Staphylococcus aureus* , Antibiotic.

Introduction

Plant extracts and herbal products are complex substances that contain a variety of primary and secondary metabolite products, their biological activity is a result of the synergy of the different chemical compounds that they contain. In addition, these products may demonstrate different mechanisms of biological and pharmacological activity (Gupta and Birdi, 2017).

Flavonoids are a large group of different biologically active compounds with a low toxic effect includes flavonols, flavones, flavanones and other compounds which are commonly found in various medicinal plants (Gutiérrez-Grijalva et al., 2018). They an anti-oxidant and anti-inflammatory effects (Gonçalves *et al.,* 2017). In addition, they play an important role in preventing chronic diseases, protecting nerves, as well as an anti-cancer effect. (Kumar Singh et al., 2019).

A large group of studies demonstrated that crude plant extracts containing Flavonoids possess inhibitory activity against different microorganisms especially Staphylococcus aureus (Chattopadhyay et al., 2001). Staphylococcus aureus, is a Gram-positive bacterium belonging to the order Bacillales, family Staphylococcaceae, genus Staphylococcus (Masalha *et al.,* 2001). it can be observed as large, non-motile colonies on blood agar and appear, On a microscopic scale, round, golden yellow cells as a bunch of grapes (Gulzar and Zehra, 2018).

S. aureus bacteria is characterized by its ability to transmit and infect different groups of species, in which it can transmit from human to animal through contamination of skin wounds by direct contact with infected animals (Gulzar and Zehra, 2018). This bacterium is considered as the main cause of a group of diseases, including skin infections, gastric poisoning, and other diseases like Bacteremia, pneumonia and Endocarditis (Shaw *et al.,* 2004).

Antibiotic resistance genes and exotoxins production has led to the formation of an antibiotic resistance strain, that was first identified when a S. aureus strain developed resistance against penicillin by producing Penicillinase (hydrolysis enzyme) during 1940 (Bitrus *et al.,* 2018).

Phytoflavinoids was confirmed to induce harnessable antibacterial activities, in which it can synergist with antibiotics and suppress bacterial virulence (Cushnie and Lamb, 2011).

The study that was reached by (Resende *et al.,* 2015), which aims to verify that the hydroxylation group in flavonoid compounds has an effective role in antibacterial effects, and that the compound Kaempferol, is the most active flavonoid against *Staphylococcus aureus.*

The study conducted by (Farhadi, Khameneh *et al.* 2019) on *Staphylococcus aureus* and Escherichia coli bacteria found that the effect of flavonoids in inhibiting the growth of bacterial species was significant.

Also, (Alcaraz, Blanco *et al.* 2000) concluded from his experiments that the effect of flavonoids in inhibiting the growth activity of *Staphylococcus aureus* bacteria.

And (Wu, Yang *et al.* 2019) when studying extracts of licorice plant, Glabrol and Licochalcone, two flavonoid compounds, showed high efficiency against Staphylococcus aureus bacteria. In addition, it showed low cellular toxicity when examining the therapeutic safety assessment.

This study aims at evaluating the the antibacterial activity of some flavonoids extracted from two medicinal herbs include basil (*Ocimum basilicum* L.) and fenugreek (Trigonella sp.), and comparing their activity with some Antibiotics and synergistic combinations (flavonoid extracts and antibiotics) against *Staphylococcus aureus*.

Materials and Methods

Preparation of Plant material

Seeds of basil (Ocimum basilicum L.) and fenugreek (Trigonella sp.) were obtained from the local market of Al-Diwaniyah as a Pharmaceutical package traded for commercial purposes. in which basil seeds were packed by killi company of an Indian origin. While the fenugreek seeds were packed by herbal hills company of an Indian origin too. Samples were washed with distilled water

and dried for about 48h at 45c̊ temperature, then grounded and placed in sterile containers until use.

Preparation of bacterial strains

The bacterial isolates were obtained from the microbiology laboratory at biology Department , college of Science, Al-Qadisiyah University. These isolates were cultured several times sequentially on Macconkey agar and blood agar for the purpose of obtaining pure colonies , purified colonies was activated by using nutrient broth before starting each test.

Preparation of flavonoid extracts

50 grams of fenugreek and basil seeds were weighed separately, after grinding them with an electric grinder, and adding 100 ml of methanol alcohol at a concentration of 80%. Then it was placed in a closed flask, and the beaker was placed on a hot plate at a temperature of 45 ° C with continuous stirring with a magnetic stir bar. Magnetic bar for one hour, and after completion, the solution was filtered, the saliva was taken and placed in plastic test tubes, then placed in a centrifuge at a speed of 300 r/min for 5 minutes, then the saliva was taken and left in the shade, so that the alcohol evaporated and the solution concentrated to 10 ml.

For the purpose of determining the optimal method of extraction, to obtain the greatest concentration of flavonoids, the following method was used:

1: extraction by changing the concentration of methanol alcohol with constant time and temperature

In this method, a constant temperature (45 °C) was used, and the time was fixed (one hour) depending on the general method with the use of increasing concentrations of methanol alcohol (40%, 50%, 60%, 70%, and 80%).

2: Extraction by changing the temperature with constant time and methanol alcohol concentration

In this method, the extraction time was constant (one hour), as was the concentration of methanol alcohol (80%), depending on the general method, and the temperature was increasing (35°C, 45°C, 55°C, 65°C, 75°C).

3: extraction by changing time with constant temperature and methanol alcohol concentration

In this method, the time required for extraction was changed (15 minutes, 30 minutes, 45 minutes, 60 minutes, 75 minutes) with a constant concentration of methanol alcohol (80%) and temperature (45 °C) as mentioned in the general method.

As a result, 15 extracts of 10 ml were obtained and placed in the refrigerator.

Evaluation of Antibacterial Activity of Flavonoid extracts

The agar diffusion method described by (Mahmoud, Jawad *et al.* 1989) was followed to test the effectiveness of the prepared extracts in inhibiting microorganisms as follows:

1. Inoculating the nutrient media with a sterile swab using a sterile swab using the method of spreading, then taking 0.1 ml of bacteria cultures containing (1.5 x 108) cells / ml by comparing it with a Macfarland standard turbidity solution. Then the dishes were left to dry at room temperature.

2. A drill with a diameter of 5 mm was made in the center of the pellets fed by the cork borer, 0.1 ml of each extract was added to each hole, and a control drill was made by adding 0.1 ml of the extraction solution only and the dishes were left in the refrigerator for half an hour; For the purpose of spreading the extract in the agar.

3. The plates were incubated at 37 ° C for 24 hours, after which the inhibitory activity was determined for each extract by measuring the diameter of the inhibition zone around each pit.

Determination of Minimum inhibitory concentration (MIC)

The Minimum inhibitory concentration (MIC) of prepared flavonoid extracts against S.aureus strain was determined in Muller-Hinton agar(MHA) using the agar diffusion method as described above. in which the extracts were diluted in to several concentrations included (25,50,75,100,150,200) g/ml and tested for their antibacterial efficiency (Collins et al., 1995).

Examination of the mechanism of action of flavonoid extracts

In order to determine the mechanism of action of plant extracts in inhibiting bacterial growth, the effect of the extracts on the cytoplasmic pH and its effect on causing morphological abnormalities was studied.

A: the changes in Cytoplasmic pH

The changes in the pH of the bacterial cells due to plant extracts are considered an antimicrobial mechanism for plant extracts. The changes in Cytoplasmic pH of bacterial cells were examined under incubation with plant extract and was determined according to the method described by (Sánchez, García et al. 2010).

B: morphological changes

plant extracts that exhibit antimicrobial action are known to induce the morphological changes in the bacterial cells due to incubation with these extracts and this is considered an antimicrobial mechanism for plant extracts too. The lytic effect of the plant extracts on bacterial cells was studied by Microscopic examination following the method described by (Abbas 2018).

Results and discusion

Study of Antibacteria activity of Crud flavonoids extraction of *Trigonella foenum-graecum L.* and *Ocimum basilicum L.* against *Staphylococcus aureus* growth

The attractive activity of plant extract and basil at a concentration of 200 mg/ml against the bacterial isolate *Staphylococcus aureus* and Corona was studied under the influence of two radiation (Amoxicillin and Doxycycline). The pristine flavonoid extract of its seeds contributed to

the growth of the studied bacteria. The diameter of the stem of the extract reached 18 mm using the Hot Plate and Ultrasonic actuators. The seeds of the basil plant also had a clear effect on bacterial isolation, as the diameter of the stem reached 14 and 19 mm using the Hot Plate and Ultrasonic actuator. The results of the study also showed that there was no effect of the root flavonoid extract of seeds of different plants and basil prepared using the device. Vortex was compared to the studied extracts of the effect generating trains from Safi Nessim studied, where the filtering diameter of the antibiotic Amoxylin was 23 mm, while the filtering diameter of the antibiotic Doxycycline was 20 mm. The results showed that the Brazilian extract of the seeds of the Madyan and basil plants possessed 78.2% and 82.6% of the effectiveness of eliminating Amoxicillin and 90% and 95% of the effectiveness of Doxycycline is shown (Panel 1). The reason for this is that the basil and fenugreek plant groups contain a high number of flavonoids that are created by the bacteria Staphylococcus aureus, as they work to disrupt cell membranes through selection with the external members of the cell wall (Maeeny *et al*., 2007). It reached the conclusions of Wali *et al*. (2015) who emphasized their different effect in achieving the growth of *S.auerus* bacteria at a concentration of 200 mg/ml, as it gave a sputum diameter of 7 to 12 mm compared to the gentamicin chemistry, which recorded a diameter of up to 18 ml. Researcher Al-Tamimi (2019) showed its effect on the concentration of agents for *S.aureus* bacteria at a concentration of 250 mg/ml. The study by researcher Sharma *et al*. (2013) noted that the methanolic extract of anti-basil seeds had antibacterial activity that inhibited 9% of 146 bacterial strains tested, including *S.aureus*.

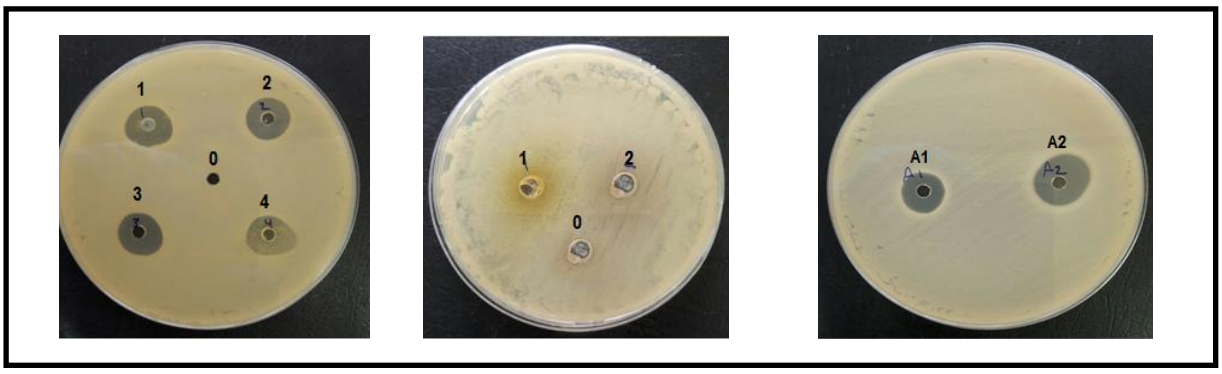


Figure 1: Antibacterial Activity of Raw Flavonoid Extracts from Fenugreek and Basil Seeds Against Staphylococcus aureus.

A: Inhibition diameters for extracts (1. Fenugreek extract with Hot Plate, 2. Basil extract with Ultrasonic Device, 3. Basil extract with Hot Plate, 4. Fenugreek extract with Ultrasonic Device, 0. Solvent: Distilled Water)

B: Inhibition diameters for extracts with Vortex (1. Fenugreek extract, 2. Basil extract, 0. Solvent: Distilled Water)

C: Inhibition diameters for antibiotics (Amoxicillin A1, Doxycycline A2)

Study of the Synergistic effect between Crud flavonoids extraction of Trigonella foenum-graecum L. and Ocimum basilicum L. against Staphylococcus aureus growth

Synergistic combinations were prepared between the raw flavonoid extracts of the fenugreek and basil plants with the two antibiotics Amoxylin and Doxycyclin, which included (1- fenugreek extract using a Hot Plate + Amoxylin device, 2- basil extract using an Ultrasonic device + Amoxylin, 3- basil extract using a Hot Plate + Amoxylin device, 4- Fenugreek extract using the Ultrasonic + Amoxylin device, 5- Fenugreek extract using the Hot Plate +Doxycyclin device, 6- Basil extract using the Ultrasonic +Doxycyclin device, 7- Basil extract using the Hot Plate +Doxycyclin device, 8- Fenugreek extract using the Ultrasonic +Doxycyclin device) The effectiveness of this was studied Synergistic combinations prepared against the growth of *S.aureus* bacteria. shows the antibacterial effectiveness of these synergistic combinations, as it is clear that combination 2 was the most efficient combination in inhibiting the growth of the studied bacterial isolate, as it gave an inhibition diameter of 24 mm, followed by combinations 4, 6, and 8 in diameter. Inhibition of 23 mm, then combination 3 and 5 with an inhibition diameter of 22 mm, then combination 7 with an inhibition diameter of 21 mm, while the minimum inhibition diameter of 20 mm was when using combination 1.

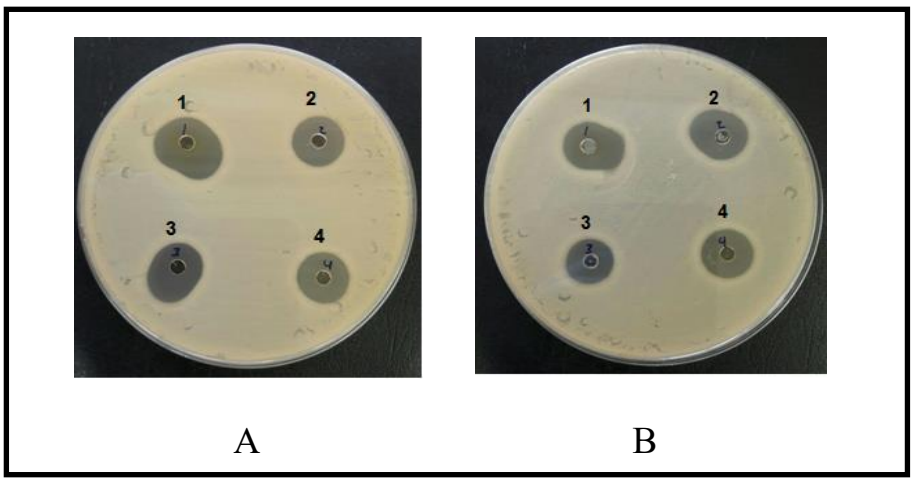


Figure 2: Antibacterial Activity of Synergistic Combinations of Raw Flavonoid Extracts from Fenugreek and Basil Seeds with Antibiotics Against Staphylococcus aureus.

A: Synergistic effect with the antibiotic Amoxicillin (1. Fenugreek extract with Hot Plate, 2. Basil extract with Ultrasonic Device, 3. Basil extract with Hot Plate, 4. Fenugreek extract with Ultrasonic Device)

B: Synergistic effect with the antibiotic Doxycycline (1. Fenugreek extract with Hot Plate, 2. Basil extract with Ultrasonic Device, 3. Basil extract with Hot Plate, 4. Fenugreek extract with Ultrasonic Device)

The results of the study confirmed the existence of a synergistic effect between the two antibiotics studied and the flavonoids present in the seed extracts of the fenugreek and basil plants, which made the combination more efficient in penetrating the outer membrane of the *S.aureus* bacteria by tearing the cell wall and increasing the permeability of the membrane. Omoya and Ajayi (2016). These results agreed with the findings of Aparna and Gayathri (2018), who found a synergistic effect between the antibiotic Amoxycillin and the methanolic extract of fenugreek seeds for S.aureus bacteria with an inhibition diameter of 16 mm. Cui *et al*. (2021) also found a

synergistic effect between basil seed extract and the antibiotics Amoxilin and Tetracyclin (from the same Dixocyclin family) against S.aureus bacteria. The diameter of inhibition reached 13 mm for the two combinations. The results of the study by Omoya and Ajayi (2016) gave evidence of the synergistic action between the alcoholic extract of fenugreek seeds and the antibiotic Amoxilin against *S.aureus* bacteria with an inhibition diameter of 12.8 mm, while the antibiotic Tetracyclin with the same extract recorded an inhibition diameter of 65 mm. These results agreed with the results of Aguilar-Urquizo *et al*. (2020) that the synergistic action of the aqueous basil seed extract with the antibiotic Doxycycline recorded a diameter of inhibition against *S.aureus* bacteria that reached 9.6 mm, while the alcoholic extract with the same antibiotic gave a diameter of inhibition of 16.4 mm.

Determination of Minimum Inhibition concentration (MIC)

A set of serial concentrations of StockSolution with a concentration of 200 mg/ml were prepared for the crude flavonoid extracts of the seeds of Arabidopsis plants and basil (200, 100, 50, 25) ml to determine the low concentration of the inhibitor to the growth of Staphylococcus aureus bacteria in a manner that begins with the etching described previously. Among the results shown in Table (4-4) is that the lowest concentration inhibitory to the growth of the studied bacteria was 25 mg/ml per plant, and upon completion of identification, the diameter of the release reached 6 mm, compared to the other concentrations and the two aspiration targets studied. As for the basil plant, the concentration was recorded at 25 mg/ml on ultrasound examinations, the lowest concentration, as the diameter of the inflorescence reached 4 mm, compared to the other concentrations studied.

Table 1. Minimum Inhibitory Concentration of Raw Flavonoid Extracts from Fenugreek and Basil Seeds Against Staphylococcus aureus.

Extraction Method	Plant Studied	200 mg/mL	100 mg/mL	50 mg/mL	25 mg/mL
Traditional Extraction	Fenugreek	18 mm	16 mm	11 mm	6 mm
	Basil	18 mm	14 mm	12 mm	-
Ultrasonic Extraction	Fenugreek	19 mm	15 mm	9 mm	-
	Basil	14 mm	12 mm	10 mm	4 mm

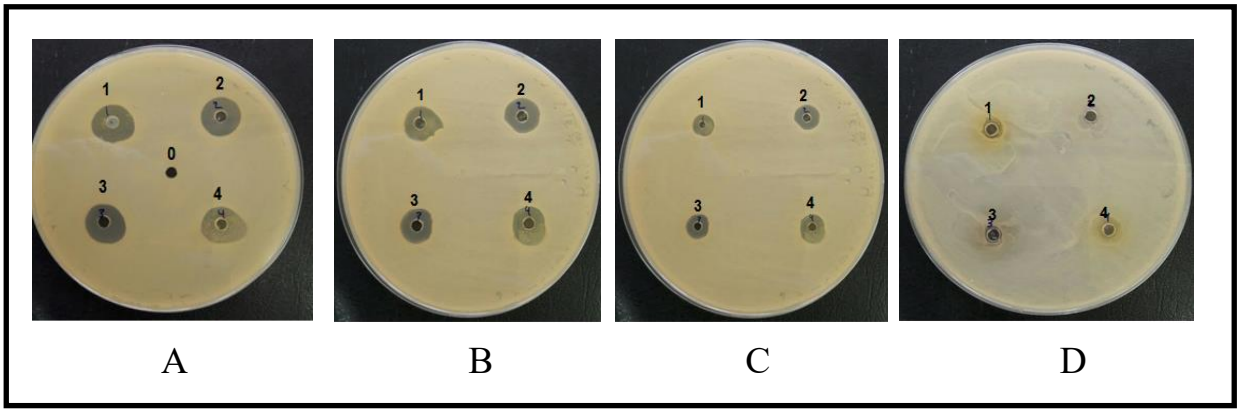


Figure 3: Minimum Inhibitory Concentration of Raw Flavonoid Extracts from Fenugreek and Basil Seeds Against Staphylococcus aureus.

A: Inhibition diameters for extracts at 200 mg/mL (1. Fenugreek extract with Hot Plate, 2. Basil extract with Ultrasonic Device, 3. Basil extract with Hot Plate, 4. Fenugreek extract with Ultrasonic Device)

B: Inhibition diameters for extracts at 100 mg/mL (1. Fenugreek extract with Hot Plate, 2. Basil extract with Ultrasonic Device, 3. Basil extract with Hot Plate, 4. Fenugreek extract with Ultrasonic Device)

C: Inhibition diameters for extracts at 50 mg/mL (1. Fenugreek extract with Hot Plate, 2. Basil extract with Ultrasonic Device, 3. Basil extract with Hot Plate, 4. Fenugreek extract with Ultrasonic Device)

D: Inhibition diameters for extracts at 25 mg/mL (1. Fenugreek extract with Hot Plate, 2. Basil extract with Ultrasonic Device, 3. Basil extract with Hot Plate, 4. Fenugreek extract with Ultrasonic Device)

Determining the Effect of Extracts on Cytoplasmic pH Changes in S. aureus Bacteria

The results of the study indicated the effect of the different treatments on the pH of the *S.aureus* bacteria cells studied using spectrophotometry, the pH value decreased in the studied treatments to which the extracts were added. When adding the fenugreek seed extract prepared in the traditional way using a Hotplate device to the bacterial cells, the absorbance reached 0.18 and decreased to 0.021 when using the fenugreek extract prepared using ultrasound. As for the basil seed extract, adding it to bacterial cells led to a decrease in the pH value to reach 0.124 when prepared in the traditional way. When it was prepared using the ultrasound method, the pH value decreased to 0.097. It is clear that the most efficient extract in reducing the pH value is the fenugreek seed extract prepared using the ultrasound method

Effect of Flavonoid Extracts on the Morphology of Staphylococcus aureus Cells

The results of our study showed the effect of the flavonoid extract of fenugreek seeds on the morphology and shape of the cells of Staphylococcus aureus bacteria. Image (4-1) shows the bacteria studied under the microscope without treating them with any extract. It is evident that the bacterial cells are colored dark purple as a result of the use of gram stain. It is also noted that the solution surrounding the cells is clear and transparent. It is also observed that cells naturally gather together as a result of the presence of what is called a biofilm. This biofilm is a thin membrane consisting of the accumulation of living cells on nonliving surfaces, such as metals and the bodies of dead organisms, or on living surfaces, such as plants and animals. These cells may be homogeneous or heterogeneous, embedded in a matrix of extracellular polymeric materials consisting mainly of polysaccharides, as well as proteins, fats, and nucleic acids. (Tremblay *et al.,* 2013). Biofilms are one of the virulence factors and pose a risk from a pathogenic standpoint. They are responsible for causing various disease infections, in addition to their presence on the surfaces of medical equipment such as catheters, laparoscopic instruments, and others. It facilitates the process of transferring genes between bacterial cells, which leads to an increase in the number of harmful strains on the one hand, and on the other hand these cells themselves are able to avoid the host's defenses in addition to their resistance to antibiotics, as the filling made of extracellular polymeric materials works to impede the spread of antibiotics through the biofilm. Thin biofilms

perform many functions, including protection from antibiotics, disinfectants, and changing dynamic environments, communication between cells within the thin biofilm, which allows rapid time adaptation, as channels for transport are formed between cells gathered within the biofilm, and the ability to survive in conditions of lack of food, where living cells can Consume Matrix filling materials as food when needed. (Bogino *et al.,* 2013; Silva *et al.,* 2014; Costa-Orlandoi *et al.,* 2017; Raghupathi *et al.,* 2017). When S.aureus cells were treated with the studied extracts, many phenotypic variations were observed in the prepared slices, and as shown in Plate 4, dissolution of the dye, swelling of the cells and their separation from each other, and turbidity of the solution surrounding the cells were observed for all of the studied extracts. The dissolution of the dye is due to the extracts containing active substances that affect the bacterial cell wall, as it was found that flavonoids form complexes with the external proteins of the cell walls of the bacterial cell and lead to its rupture (Maeeny *et al*., 2007). The rupture of the wall led to the osmosis of some components of the cytoplasm into the solution surrounding the cells, making it cloudy and unclear. The ability of flavonoids to form complexes with the external proteins of the cell wall of bacteria, which are often considered components of the biofilm, led to damage to the biofilm responsible for grouping cells together and giving the usual clustered shape to the studied bacteria. All the factors that affected the morphological shape of the bacterial cell weakened it and reduced its resistance against the active substances present in the extracts, which explains the mechanism of action of these extracts against the *S.aureus* bacteria.

The results of our study agreed with the findings of Farhan (2018), who confirmed the effectiveness of the alcoholic extract of fenugreek against some bacterial species. He also confirmed the ability of these extracts to degrade the bacterial cell wall and dissolve the proteins surrounding it. It also agreed with the findings of Ngobein et al. (2020), who observed the appearance of morphological changes in Bacillus cereus bacteria as a result of their treatment with Buxus macowanii plant extract. These phenotypic changes included cell swelling, cell division, and cell wall rupture when examined under a scanning electron microscope.

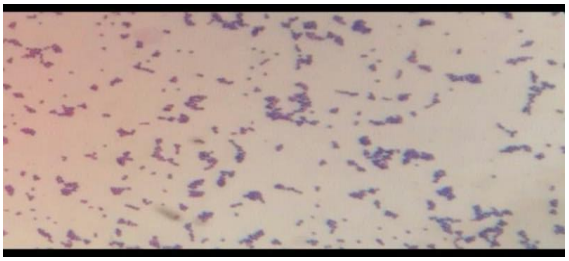
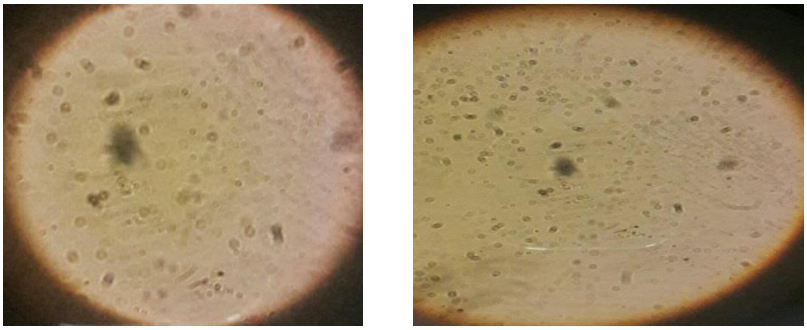


Figure 4. Staphylococcus aureus bacteria under an electron microscope.



A

B

Figure 5. Effect of Fenugreek Seed Flavonoid Extracts on Staphylococcus aureus Cells: A) Treatment with Hot Plate, B) Treatment with Ultrasonic Device.

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