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# Effect of Active Compounds of *Eruca Sativa* Plant Extract on *Staphylococcus aureus* Bacteria

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## Abstract

***Background:*** Medicinal plants play an important role in agricultural production due to their therapeutic significance, particularly in the treatment of various pathological conditions.

***Methods:*** Active compounds in Eruca sativa were identified using High-Performance Liquid Chromatography (HPLC). Fifty isolates of *Staphylococcus aureus* (*S. aureus*) bacteria were obtained. The effect of Eruca sativa plant extract on biofilm formation of bacterial isolates was tested using the standard plate method.

***Results:*** The highest percentage of S. aureus was found in wound samples, with 22 isolates (44%). The isolates showed variability in their ability to form biofilms. The efficacy test revealed that plants treated with different concentrations of brassinolide (0, 1.5, 2.5, 3.5 mg/L) and Eruca sativa showed inhibition of S. aureus growth in isolates (S2, S7, S10, S15, S16, S31, S42, S48, S50) with alcohol concentrations (5, 25, 75 mg/ml). The effect of Eruca sativa plant extract was dependent on the concentration of brassinolide applied. The highest inhibition was observed with brassinolide concentration of 3.5 mg/L-1 and alcohol extract concentrations of 50 and 75 mg/ml.

***Conclusion:*** The alcoholic extract from the leaves of Eruca sativa, combined with brassinolide, proved effective in inhibiting the growth and biofilm formation of *S. aureus* bacteria.

**Keywords:** Biofilm, Eruca Sativa, HPLC, *Staphylococcus aureus.*

## Introduction

Staphylococcus bacteria are among the most common bacterial pathogens and are some of the most virulent and widespread bacteria in nature (1). They are found in the air, soil, skin, sebaceous glands, and mucous membranes of mammals and birds (2). *Staphylococcus aureus* (*S. aureus*) is the most significant pathogenic type of Staphylococcus that infects humans (3, 4). It has the potential to cause various opportunistic infections, including gastroenteritis, scalded skin syndrome, impetigo, pneumonia, meningitis, osteomyelitis, septic arthritis, cellulitis, otitis media, and bacteremia (5).

In addition to forming biofilms, extracellular polymeric substances produced by bacterial cells that bind them in clusters, these materials contain sugars, proteins, and nucleic acids. The biofilm provides a protective environment for bacteria, shielding them from antibodies and phagocytes, and creating high-level resistance against antibiotics. The biofilm is one of the virulence factors that help the pathogen evade host defense systems (6, 7).

Continuous antibiotic administration has led to the emergence of antibiotic-resistant strains of S. aureus bacteria characterized by multiple resistances, making infections difficult to treat despite the production of new

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generations of antibiotics (8, 9). Medicinal plants currently occupy an important place in agricultural production due to their therapeutic importance. They are also safe to use and easy to apply without the need for special skills or experience in their preparation, and they are available in most countries, making them accessible at cheap prices (10, 11) and a valuable source of alternative medicine (12). Plant materials can be processed through extraction, fractionation, purification, and concentration to produce pharmaceutical products for pharmacological treatments (13). *Eruca sativa* is a medicinal plant that belongs to the Brassicaceae family, classified among Angiosperms and dicotyledons in the order Brassicales. *Eruca sativa* is one of the vegetable crops with significant nutritional, medical, and economic importance worldwide (14). It is widespread in the Mediterranean area and traditionally known as jarjeer or rocket salad (15). Studies have shown that *Eruca sativa* is important in the prevention of colds and chest diseases, and *Eruca sativa* oil is significant in eliminating fats from the blood, leading to a noticeable and significant reduction in both total fat and cholesterol levels in the body. It also plays a role in improving the effectiveness of sex hormones progesterone and estrogen, as well as sexual glands, increasing sperm fertility, and improving liver function. Additionally, the leaves of the *Eruca sativa* plant are used as stimulants, help relieve poor digestion, promote hair growth, and are used in ointments for wounds and burns (16).

To address the danger posed by *Staphylococcus aureus* bacteria, which have many virulence factors as well as side effects caused by antibiotics, the aim of this study is to investigate the effect of *Eruca sativa* leaf extract on the growth of *S. aureus* isolated from various clinical conditions.

## Materials and Methods

### *Plant specimens*

The seeds of the watercress (*Eruca sativa*) plant were obtained from markets in Baghdad, Iraq. After being classified by the plant herbarium at the College of Education for Pure Sciences, Ibn Al-Haitham, University of Baghdad, the seeds were cultivated and treated with brassinolide at different concentrations. The leaves of the plant were then collected after the plant reached maturity.

### *Preparation of leaf extract of Eruca sativa plant.*

One hundred grams of dried *Eruca sativa* leaf vegetative powder is placed in the thimble of the Soxhlet extractor. The alcoholic solvent is prepared at a concentration of 80% (80 ml of methanol alcohol with 20 ml of distilled water) and placed in a 500 ml round-bottom flask of the Soxhlet extractor. The apparatus is left in a water bath at 80 °C for 8 hours. The plant extract is then concentrated using a rotary evaporator under reduced pressure, and the solvent is removed at 45 °C. The concentrated extract is dried in an electric oven at 45 °C until completely dry, weighed, and stored at 4 °C until further use (17). The diagnosis of active compounds was performed using High-Performance Liquid Chromatography (HPLC) (18).

### *Diagnosis of Culture isolates*

Bacterial isolates were collected from different hospitals in Baghdad, Iraq, and cultured on the following culture media: Mannitol Salt Agar and Blood Agar for diagnosing *S. aureus* (19).

The VITEK-Compact System Device was used to confirm the diagnosis of *S. aureus*.

The susceptibility of bacterial isolates to biofilm formation was investigated using the microtiter plate method (MTP) with 96-well plates (20).

### *Effect of Eruca sativa plant extract on biofilm formation of bacterial isolates by standard plate method*

The standard plate method was used to study the effect of the extract and alcohol of the *Eruca sativa* leaf plant on biofilm formation by *S. aureus* bacteria. Ten isolates were selected for *S. aureus* that formed the most biofilm, as follows, according to the method of (21): 200 µl of brain-heart infusion medium containing 1% glucose, different concentrations of *Eruca sativa* extract, and 10 µl of bacterial suspension,



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with three replicates for each concentration and bacterial isolate. The last three wells in the last column were filled with 200 µl of brain-heart infusion broth and 1% glucose as a negative control. The plate was covered, and parafilm tape was used to prevent evaporation of the contents of the wells. It was incubated at 37 °C for 24 hours.

After incubation, the contents of the wells were gently emptied, and the plate was washed three times with saline phosphate buffer and left to dry for 15 minutes. Each well was then filled with 200 µl of crystal violet stain and left for 20 minutes. Afterward, the plate was washed three times with saline phosphate buffer and left to dry for 15 minutes. Finally, 200 µl of 33% glacial acetic acid were added to each well, and the absorbance was measured using an ELISA reader at a wavelength of 630 nm to estimate the ability of the extract to inhibit biofilm formation, according to the following equation:

$$\text{Percentage of biofilm inhibition} = \frac{\text{OD control light density} - \text{OD in the presence of the extract}}{\text{Optical density OD of the control}} \times 100.$$

***Statistical analysis***

The data were tabulated in a datasheet using IBM SPSS version 25.0, which was utilized for statistical analysis. Significant differences were tested using analysis of variance (ANOVA), followed by the least significant difference (LSD) test. Statistical significance was defined as a probability value ($P \leq 0.05$) and ($P \leq 0.001$).

## Results

***Effect of brassinolide on some active compounds in watercress plant.***

The results in Table 1 showed that the watercress plant contains three active compounds that are affected by different concentrations of brassinolide, namely: progoitrin, glucoalyssin, and gluconapin. The concentration of 2.5 mg/L presented the highest values of 15.87%, 12.42%, and 4.94%, compared to no spraying with brassinolide. Plants sprayed with concentrations above 2.5 mg/L increased the concentration of these active ingredients.

**Table 1.** Effect of brassinolide concentrations on the watercress plant active compounds.

| Brassinolide (mg $L^{-1}$) | Progoitrin (%) | Glucoalyssin (%) | Gluconapin (%) |
|---|---|---|---|
| **0** | 7.41 | 5.28 | 3.25 |
| **1.5** | 12.83 | 11.79 | 4.56 |
| **2.5** | 15.87 | 12.42 | 4.94 |
| **3.5** | 10.83 | 11.08 | 3.92 |
| **LSD α 0.05*** | 3.76 | 2.59 | 0.71 |

*Significant differences at $P \leq 0.05$.

***The ability of S. aureus bacteria biofilm formation.***

The ability of all *S. aureus* isolates to form biofilms using the microtiter plate method was tested based on the optical density reading from the ELISA device. The isolates showed variation in their ability to produce biofilms. The results recorded a biofilm formation percentage of 98%, with the intensity of the formation ranging from weak (60%), moderate (30%), to strong (6%) (Fig. 1).

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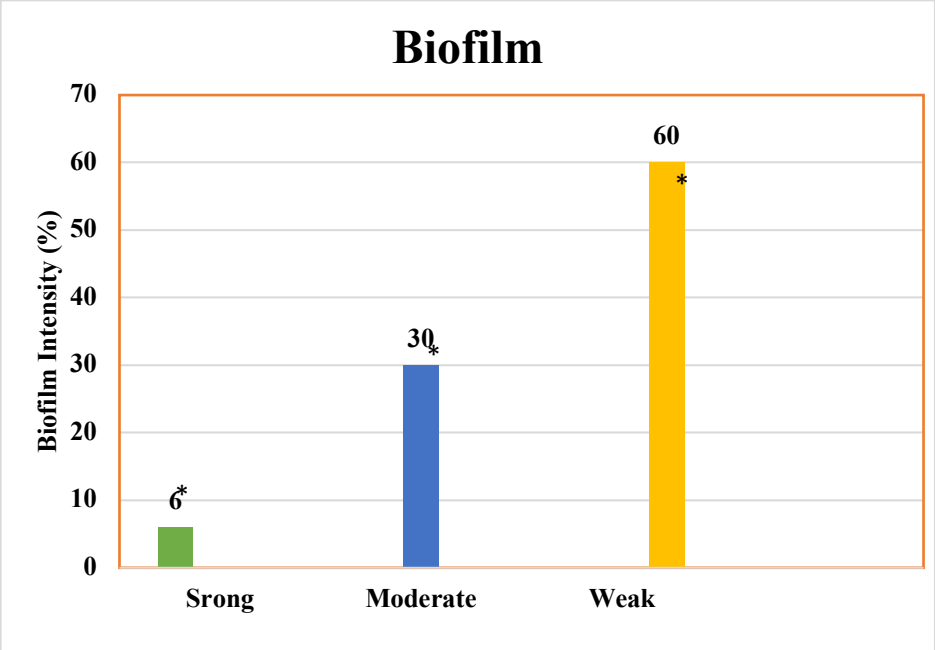


**Fig. 1.** Biofilm intensity for *S. aureus* isolates. *Significant differences at probability P≤ 0.001.

***Effect of Eruca sativa plant extract on biofilm formation of bacterial isolates by standard plate method***

This test was conducted to evaluate the effectiveness of the brassinolide-treated *Eruca sativa* plant against *S. aureus*. The susceptibility of the alcoholic extract of the *Eruca sativa* leaves, with the effect of different concentrations of brassinolide (0, 1.5, 2.5, 3.5 mg/L), was tested for inhibiting bacterial growth in the isolates under study (S2, S7, S10, S15, S16, S31, S42, S43, S48, S50) with diluted alcohol concentrations (5, 25, 50, 75 mg/ml). *Eruca sativa* extract at a concentration of 0 mg/L (no spraying with brassinolide) had no effect on the growth of bacterial isolates under study.

The results of the current study also showed that the *Eruca sativa* extract at a concentration of 1.5 mg/L brassinolide had an effect at a concentration of 75 mg/ml in inhibiting bacterial growth for the following isolates: (S7, S10, S16, S31, S42, S43, S48), with inhibition diameters of 12, 12, 10, 15, 15, 13, 15 mm, respectively. It had no effect on bacterial isolates (S2, S15, S50). The results at concentrations of 25 mg/ml and 50 mg/ml showed an effect on isolates (S31, S42), with inhibition diameters of 12 mm and 15 mm, respectively, and had no effect on the rest of the isolates under study. At a concentration of 5 mg/ml, it did not affect bacterial growth.

*Eruca sativa* extract with a concentration of 2.5 mg/L brassinolide had an effect at a concentration of 75 mg/ml in inhibiting bacterial growth for all selected isolates in the current study (S2, S10, S16, S31, S42, S48, S50) with inhibition diameters of 11, 14, 14, 15, 13, 17, 11, 10, 10 mm, respectively, except for isolate S43, which showed no effect. The results also showed that the other concentrations (5, 25, 50 mg/ml) had no effect on the bacterial growth of the selected isolates.

The results of the current study also found that *Eruca sativa* extract at a concentration of 3.5 mg/L brassinolide had an effect at a concentration of 75 mg/ml in inhibiting bacterial growth for all selected bacterial isolates (S2, S7, S10, S15, S16, S31, S42, S43, S48, S50) with inhibition diameters of 14, 13, 16, 16, 15, 19, 11, 16, 14, 12 mm, respectively.

The results of the current study also showed that at a concentration of 50 mg/ml, there was an effect on eight bacterial isolates (S2, S7, S10, S15, S31, S42, S43, S48), with inhibition diameters of 12, 1, 1, 1, 12, 0.7, 1, 12 mm, respectively, and no effect on the rest of the isolates (S16, S50). At a concentration of 25



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mg/ml, the extract had an effect on three bacterial isolates (S7, S31, S48) with inhibition diameters of 0.7, 0.9, 0.8 mm, respectively, and had no effect on the rest of the bacterial isolates. At a concentration of 5 mg/ml, the extract affected only one isolate, S31, with an inhibition diameter of 0.9 mm, and had no effect on the rest of the isolates under study.

It was noted from the results that the effect of *Eruca sativa* extract depended on the concentration of brassinolide applied to the plant. The highest rate of inhibition of bacterial growth in the isolates under study was observed at the highest concentration of brassinolide (3.5 mg/L), compared with *Eruca sativa* extract at concentrations of 1.5 mg/L and 2.5 mg/L. At a concentration of 0 mg/L (no spraying with brassinolide), no inhibition of bacterial growth was observed. This suggests that the higher the concentration of brassinolide in the *Eruca sativa* plant, the greater the inhibition of bacterial growth. The inhibition diameters increased with the increasing concentration of brassinolide (Table 2 and Figs. 2 & 3).

**Table 2:** Effect of brassinolide and *Eruca sativa* plant active compounds on *S. aureus* bacteria.

| Bacteria | Brassinolide concentration (mg/L$^{-1}$) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1.5 | | | 2.5 | 3.5 | | | |
| | 25 | 50 | 75 | 75 | 5 | 25 | 50 | 75 |
| **S2** | --- | --- | --- | 11 | --- | | 12 | 14 |
| **S7** | --- | --- | 12 | 14 | --- | 1 | 1 | 13 |
| **S10** | --- | --- | 12 | 14 | --- | --- | 1 | 16 |
| **S15** | --- | --- | --- | 15 | --- | --- | 1 | 16 |
| **S16** | --- | --- | 10 | 13 | --- | --- | --- | 15 |
| **S31** | 12 | 12 | 15 | 17 | 1 | 1 | 12 | 19 |
| **S42** | 15 | 15 | 15 | 11 | --- | --- | 1 | 11 |
| **S43** | --- | --- | 13 | --- | --- | --- | 1 | 16 |
| **S48** | --- | --- | 15 | 10 | --- | 1 | 1 | 14 |
| **S50** | --- | --- | --- | 10 | --- | --- | --- | 12 |

*Significant differences (P≤ 0.05).

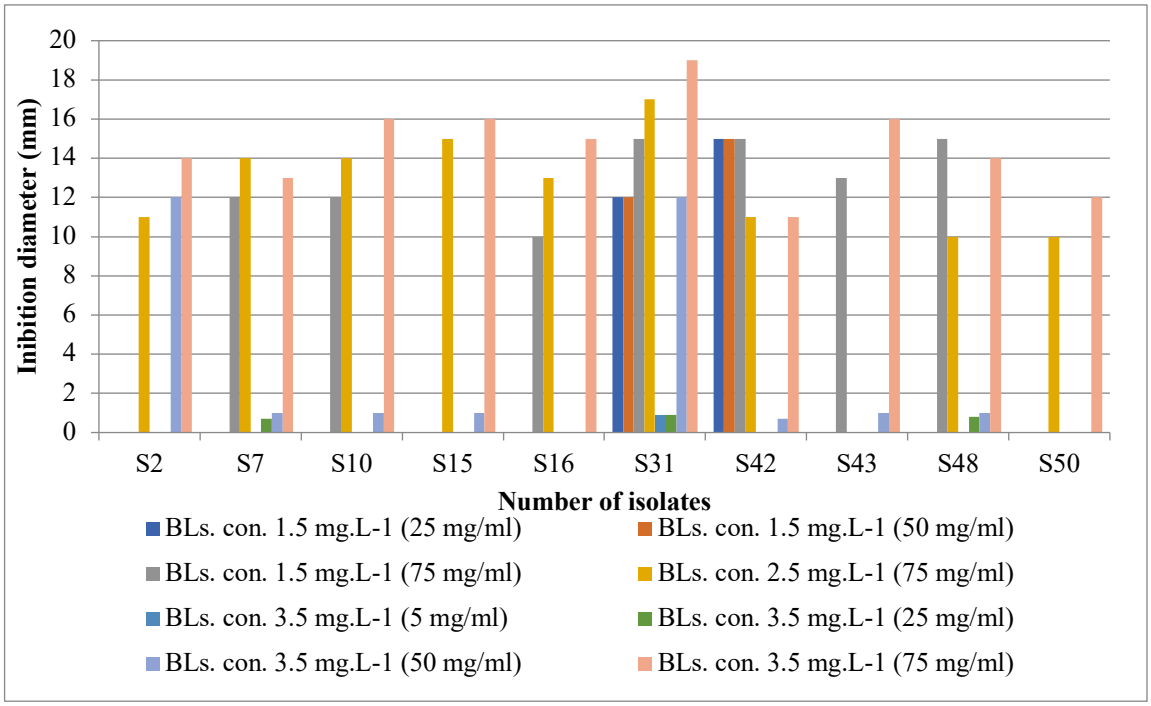


**Fig. 2.** The different effect of *Eruca sativa* extract on the growth of *S. aureus* bacteria. *Significant differences at probability P≤ 0.001.



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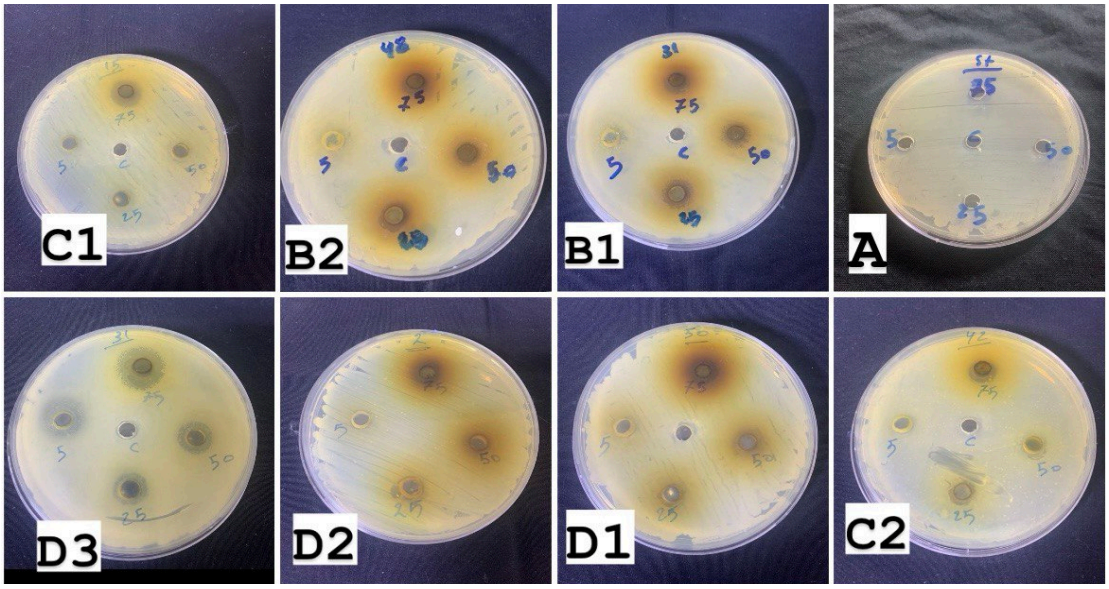


**Fig. 3.** Shows the effect of *Eruca sativa* extract on the growth of *S. aureus* bacteria. (A) Extract at 0 concentration of non-spraying of Brassinolide (B1 and B2) extracted at a concentration of (1.5) mg/L$^{-1}$ of Brassinolide (C1 and C2) extracted at a concentration of (2.5) mg/L$^{-1}$ of Brassinolide (D1, D2 and D3) extracted at a concentration of (3.5) mg/L$^{-1}$ of Brassinolide.

***Effect of Eruca sativa leaf extract on the biofilm of study isolates***

The 96-well standard plate method was used to study the susceptibility of the bacterial isolates under study, based on their production of biofilms, as mentioned earlier. The effect of *Eruca sativa* leaf extract activated with brassinolide was then examined on the selected bacterial isolates (S10, S15, S20, S28, S30, S33, S34, S38, S39, S40). These isolates were selected because they are high biofilm producers, as shown in Table 3.

The inhibitory effect of the *Eruca sativa* leaf extract, activated by the hormone brassinolide at a concentration of 3.5 mg/L, on the production of biofilms of the selected isolates was observed, while the rest of the concentrations did not show any results in inhibiting biofilm production.

It was noted from the results that the effect of *Eruca sativa* leaf extract depended on the concentration of brassinolide applied to the plant. The highest percentage of inhibition of biofilm production in the selected isolates was recorded at the highest concentration of brassinolide (3.5 mg/L), compared to the other concentrations of *Eruca sativa* leaf extract (0, 1.5, 2.5 mg/L) that did not show any effect or inhibit biofilm production in the selected isolates.

**Table 3.** Effect of diluted concentrations of Brassinolide on isolated of *S. aureus* bacteria.

| Number of isolates | Diluted concentrations of Brassinolide 3.5 mg/L$^{-1}$ | |
|---|---|---|
| | 50 | 75 |
| S10 | 0.3 | 1.13 |
| S15 | 0.1 | 1.05 |
| S20 | 0.1 | 0.03 |
| S28 | 0 | 0 |
| S30 | 0 | 0 |
| S33 | 0 | 0 |
| S34 | 0 | 0 |
| S38 | 0 | 0 |
| S39 | 0 | 0 |
| S40 | 0 | 0 |

*Significant differences at probability P≤ 0.001.



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## Discussion

The plants sprayed with brassinolide showed an increase in the concentration of active ingredients. This is attributed to the role of brassinolide in enhancing the vegetative growth of the plant, which results in primary and secondary metabolic by-products, including medically active compounds in volatile oils. This may be due to the role of the hormone, which is primarily a derivative of secondary metabolism in medicinal plants and participates as a product of oil compounds from the metabolism of sterols (22).

The hormone also plays a role in enhancing both vegetative and reproductive growth by increasing cell division and promoting the synthesis of nucleic acids and cyclins, which are phosphorylated proteins involved in increasing cell division. Cyclins have high activity in this process, particularly in activating the enzyme Cyclin-Dependent Kinase (CDK) (23).

Upon diagnosing these samples after culture and microscopic examinations, it was found that there were 50 isolates of *Staphylococcus aureus* bacteria, including 22 isolates from wounds, 16 isolates from burns, and 12 isolates from urine. In contrast, a study in which samples of *S. aureus* bacteria were collected from various sources in many hospitals in Baghdad (24) reported 23.1% from burns and 23.6% from wounds, which is much lower than the findings of the current study, where 32% of isolates were from burns and 44% from wounds.

The findings of the current study are inconsistent with those of (25) and (26), which found all Staphylococcus spp. isolated from vaginal infections. The widespread prevalence of *S. aureus* may be attributed to contamination in hospitals, needle contamination during injections and sampling, or bloodstream infections caused by wound infections, burns, or other skin infections (27).

Regarding biofilm formation, the results of the current study were very similar to those of (28), which showed that the percentage of biofilm formation by *S. aureus* bacteria was 96.35%. In contrast, the study by (29) found the biofilm formation percentage to be 100%, which is also a result close to the findings of the current study. (30) reported that the percentage of biofilm formation by clinical bacterial isolates of *S. aureus* was 62.5% at a strong degree, which is a high rate compared to the current study's result of 6%. The study by (31) showed that the biofilm formation by clinical bacterial isolates of *S. aureus* was 40.6% at a weak degree, which does not correspond to the current study's result of 60%.

Recently, there has been a strong interest in the use of plant extracts and bioactive compounds found in medicinal plants. These plants are an inexhaustible source of many natural products with inhibitory effects on the growth of various microorganisms, including *S. aureus* (32).

The results also indicated that the effect of *Eruca sativa* extract depended on the concentration of Brassinolide applied to the plant. This is because the active compounds in *Eruca sativa* increase when treated with the growth regulator Brassinolide, which has a high ability to enhance the plant's efficiency in terms of morphological and chemical traits. Additionally, it increases the active and secondary metabolic compounds of medicinal and aromatic plants, even at very low concentrations, as confirmed by the current study's results in Table 2 regarding the effect of Brassinolide on all studied traits.

Brassinolide is one of the growth regulators that plays a role in encouraging the absorption of mineral and nutrient ions and works to increase the primary and byproducts of metabolism, represented by medically active compounds. This, in turn, increases the efficiency of plants treated with Brassinolide, which leads to an increase in the efficiency of the plant extract in inhibiting bacterial growth. These results were consistent with the findings of (19), which showed that the alcoholic extract of the leaves of the *Eruca sativa* plant has a high inhibitory ability against gram-positive bacteria, including *S. aureus*.

While the results of a study conducted by (33) in Baghdad indicated the ability of the aqueous extract of the leaves of *Eruca sativa* to inhibit the growth of gram-positive bacteria



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more effectively than gram-negative bacteria, it was also found to be an inhibitor of the growth of some yeasts and molds. The results of (34) showed that the extract of the leaves of *Eruca sativa* has a high inhibitory ability against gram-positive bacteria compared to gram-negative bacteria.

The effect of *Eruca sativa* extract on both gram-positive and gram-negative bacteria is due to its content of medically effective compounds. The leaves of this plant contain glycosides, sulfur glycosides, alkaloids (such as euric acid, jacoline, dehydrojacoline, and erucifolin) (35), tannins, saponins, resins, and terpenes. The mechanism of action of sulfur glycosides involves interaction with groups (-SH) in cell proteins, where they interact with the amino acid cysteine and form a disulfide bond (S-S). Since the sulfur group (-SH) acts as a special catalyst for cell replication, sulfur compounds break down this group, thereby inhibiting bioactivity and replication in the cell (36).

As for alkaloids, their mechanism of action is to stop the synthesis of nucleic acids in the bacterial cell by inhibiting the enzyme DNA gyrase and simultaneously affecting the coenzyme aids produced by bacterial cells (37). Many medicinal plants possess effective compounds similar to those found in *Eruca sativa*, including the anise (*Pimpinella anisum*) plant, which contains saponins, steroids, and resins. *Artemisia herba alba* also contains many active substances and compounds like those of *Eruca sativa*, including glycosides, saponins, tannins, and large amounts of terpenes. The alcoholic extracts of these plants also have an inhibitory effect against different types of gram-positive and gram-negative bacteria. The results of (21) showed that the alcoholic extract of *anise* and *Artemisia herba alba* has a high ability to inhibit bacterial growth and can be described as a natural antimicrobial agent. These results were consistent with the current findings on the *Eruca sativa* plant.

As the concentration of the hormone brassinolide increases in the *Eruca sativa* plant, the inhibition of biofilms of the selected isolates also increases. This is due to the higher concentration of active compounds in the *Eruca sativa* plant treated with the growth regulator brassinolide, which has a high ability to increase the plant's efficiency in terms of morphological and chemical traits. Additionally, brassinolide promotes an increase in the active and secondary metabolic compounds of medicinal and aromatic plants, as confirmed by the results of the current study in Table (2) on the effect of brassinolide in all studied traits. Brassinolide is one of the growth regulators that plays a role in encouraging the absorption of mineral and nutrient ions. It works to increase the primary and secondary products of metabolism, represented by medically active compounds, thereby increasing the efficiency of plants treated with brassinolide. This leads to a cycle that increases the efficiency of the plant extract at a concentration of (3.5) mg/L-1 in inhibiting biofilms.

The extract of *Eruca sativa* leaves activated with brassinolide at a concentration of (3.5) mg/L-1 proved its high inhibitory ability against the biofilms of the selected bacterial isolates. The effect of the extract can be attributed to its content of medically effective compounds, including glycosides, sulfur glycosides, alkaloids, tannins, saponins, resins, and terpenes. (38) showed that the mechanism of action of saponins can be summarized as removing microbiological membranes and disrupting living cells in them, thus inhibiting biofilm formation. (39) stated that tannins act to inhibit enzymes and carrier proteins in the cell membrane.

Brassinolide increases the concentration of amino acids in the plant because it enhances vegetative growth and increases protein synthesis in the *Eruca sativa* plant. This, in turn, activates and increases the primary and secondary products and proteins within the plant, leading to an increase in amino acids. (40) explained that brassinolide can activate and increase the effectiveness of DNA and RNA polymerases, facilitating the synthesis of DNA, RNA, and proteins. Additionally, brassinolide stimulates the activity of the enzyme ATPase, which in turn stimulates the enzyme carboxylase, responsible for increasing soluble protein and, consequently, amino acids. The



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results of the study by (41) showed that the rate of inhibition of the biofilm of *S. aureus* bacteria increases with increasing concentrations of amino acids in the medium. This prevents biofilm formation in a positive, dose-dependent relationship. Tyrosine, one of the amino acids found in *Eruca sativa*, has a high inhibitory ability toward biofilm formation. Many bacteria, including *S. aureus*, produce amino acids in the stationary phase (42).

This is consistent with the results of the study by (43), which showed that amino acids are effective against *B. subtilis* bacteria. The use of amino acids led to the separation and prevention of biofilm formation. The breakdown of the biofilm of *S. aureus* bacteria by amino acids is due to their interference with poly-N-acetyl glucosamine in the bacterial cell wall, which causes the separation and decomposition of the biofilm (44).

Fifty isolates of *S. aureus* were obtained from 200 samples collected from clinical sources after diagnosing *S. aureus*. The results showed that the highest percentage of these bacteria came from wound samples, followed by burns, and then urine. The alcoholic extract of *Eruca sativa* leaves proved effective in inhibiting the growth of *S. aureus* bacteria at the highest concentration of brassinolide (3.5 mg/$L^{-1}$) compared to *Eruca sativa* extract at concentrations of (1.5 and 2.5 mg/$L^{-1}$). At a concentration of (0 mg/$L^{-1}$), no spraying with brassinolide showed no effect on bacterial growth. The alcoholic extract of the *Eruca sativa* leaves also proved efficient in inhibiting the biofilm of *S. aureus* bacteria at high concentrations. The leaves of medicinal plants, including *Eruca sativa*, widespread in Iraq, play a major role in inhibiting various types of microorganisms, including staphylococcal bacteria.

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## Conflict of interest

The authors declare no conflict of interest.

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