

Investigating the antimicrobial and anti-adhesion properties of the alcoholic extract of *Verbascum sinuatum* plant on the biofilm formation of three intestinal bacteria

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Abstract

Introduction: Intestinal bacteria enter the body through contaminated food and water or contact with infected people and animals. These bacteria cause blood, urine, bone or nervous system infections in humans. The most important bacteria that cause these diseases include *Salmonella typhimurium*, *Klebsiella pneumoniae* and *Shigella sonnei*. The increasing drug resistance of bacteria created the necessity of investigating the antibacterial effects of *Verbascum sinuatum* plant extract on the planktonic form and biofilm of all three bacteria in question.

Methods: After preparing and cultivating standard bacteria and preparing biofilm from three intestinal bacteria, the effect of alcoholic extract of *V.sinuatum* on planktonic bacteria and the biofilm caused by them was investigated by well plate method. The value of minimum inhibitory concentration (MIC) and minimum bacteriocidal concentration (MBC) was determined on planktonic form and biofilm of bacteria.

Results: results showed that the alcoholic extract of *V.sinuatum* has antimicrobial effect on *Salmonella typhimurium* and *Klebsiella pneumoniae*. *V.sinuatum* also has inhibitory effect and has antimicrobial effect on the biofilm of *Salmonella typhimurium* and *Klebsiella pneumoniae*.

Conclusion: The formation of biofilm is an important factor in stability and as a result, it is the cause of infections that are difficult to deal with. *Klebsiella pneumoniae* and *Salmonella typhimurium* also have the ability to form biofilm and it is considered an important cause of many infections, so it is important to fight this infectious factor using the extract of *V.sinuatum* plant.

Keywords: *Salmonella Typhimurium*, *Klebsiella Pneumoniae*, *Shigella sonneii*, Biofilm, *V.sinuatum* extract

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Introduction

Salmonella is a Gram-negative and motile bacillus. It is one of the common causes of food poisoning. Most *salmonellae* are pathogenic to humans, animals, or both.¹ Salmonellosis is a disease that is transmitted through food and has an infectious or toxic effect. A number of domestic animals, including cats, dogs, and reptiles, can also spread the infection. In humans, *Salmonella* strains sometimes cause infection in the urine, blood, bones, joints, or nervous system (fluid spinal cord and brain) and can cause severe disease.² If a *Salmonella* infection enters a person's bloodstream (bacteremia), it can infect tissues throughout the person's body such as the tissues around

the brain and spinal cord (meningitis), the lining of the heart or its valves (endocarditis), bone or bone marrow (osteomyelitis) and blood vessels.³

Shigella are Gram-negative, anaerobic, non-motile, spore-free bacilli that are closely related to *Escherichia coli* and *Salmonella*.¹ Shigellosis is a bacterial infection that affects the digestive system. This disease is caused by a group of bacteria called *Shigella*. *Shigella* spreads through contaminated water and food or through contact with contaminated feces. The bacterium releases toxins that irritate the intestines and cause the initial symptoms of diarrhea.⁴ The high-risk group includes very young,

elderly and immunocompromised people. The global prevalence of shigellosis is about 180 million cases per year, and about 1 million cases lead to death.⁵

Klebsiella belongs to the Enterobacteriaceae family and is a part of Gram-negative, non-motile, oxidase-negative and cylindrical bacteria. They have a polysaccharide capsule. The most famous member of this genus is *Klebsiella pneumoniae*. These bacteria form the second type of Enterobacteriaceae in the normal flora of human feces. *Klebsiella* are motionless and usually have a capsule. *Klebsiella* can grow on previous lesions such as wounds, trauma, degenerative tumors, congenital injuries and other infections such as gall bladder and genital lesions.⁶

Over the last few decades, there has been a significant increase in resistance to a wide range of antibiotics by strains derived from "classical" *K. pneumoniae*.⁷ As a consequence of this antibiotic resistance, simple infections such as urinary tract infections (UTIs) have become recalcitrant to treatment, and more serious infections such as pneumonias and bacteremias have become increasingly life-threatening.^{8, 9} Two mechanisms of antibiotic resistance have been commonly observed in *K. pneumoniae*. One mechanism involves the expression of extended-spectrum β -lactamases (ESBLs), which render bacteria resistant to cephalosporins and monobactams. The other mechanism of resistance is the expression of carbapenemases by *K. pneumoniae*, which renders bacteria resistant to almost all available β -lactams, including the carbapenems.¹⁰ Healthy people are not usually infected with *Klebsiella*. The only way to contract *Klebsiella* is if a person is exposed to this bacterium, for example, if *Klebsiella* enters the respiratory system, it will lead to pneumonia, and if it enters the blood, it will lead to a blood infection.¹¹ In medical environments, *Klebsiella* is transmitted from one patient to another through the contaminated hands of staff or other people.¹²

Materials and methods

Extracting *V.sinuatum*

About 200g of dried *V. sinuatum* was powdered and mixed with 80% ethanol in a ratio of 1:4 and placed on a shaker for 48h at room temperature. After 48h, the solution was passed through filter paper and the obtained liquid was poured into glass plates and placed in a 40°C until it was completely concentrated. Then the dried extract was kept in the refrigerator until use.

Inoculation of *V.sinuatum* extract by diffusion method in the well on the planktonic form of *Salmonella Typhimurium*

0.5 McFarland's microbial suspension prepared from *Salmonella Typhimurium* bacteria was cultured in Mueller

Hinton Agar culture medium using the Lawn method in three directions. On the cultures, wells were created using a test tube with a diameter of 10mm, and the bottom of the wells was cultured with Mueller Hinton agar. After closing the bottom of the wells, the alcoholic extract of *V.sinuatum* with a concentration of 900g/ml and 1000g/ml was poured into the wells in the amount of 180 and 200 μ l. One well was considered as a control. Then the plates were incubated in 40°C for 24h. After 24h, the diameter of the non-growth halo formed in the plates was measured. This experiment was repeated 3 times.

Inoculation of *V.sinuatum* extract by diffusion method in the well on the planktonic form of *Klebsiella pneumoniae*

0.5 McFarland's microbial suspension prepared from *Klebsiella pneumoniae* was cultured in Mueller Hinton agar culture medium by the lawn culture method in three directions. On the culture medium, wells were created with a diameter of 10mm, and the bottom of the wells was cultured with Mueller Hinton agar, after closing the bottom of the wells, the alcoholic extract of *V.sinuatum* with a concentration of 700g/ml and 800g/ml was added. One well was considered as a control. Then the plates were placed in an incubator for 24h. After 24h, the diameter of the non-growth halo formed in the plates was measured with a caliper and this experiment was repeated 3 times.

Inoculation of *V.sinuatum* extract by diffusion method in the well on the planktonic form of *Shigella sonnei*

0.5 McFarland's microbial suspension prepared from *Shigella sonnei* was cultured in Mueller Hinton agar culture medium by lawn culture method in three directions. On the cultures, wells were created using a test tube with a diameter of 10mm, and the bottoms of the wells were cultured with Mueller Hinton agar, after closing the wells, the alcoholic extract of *V.sinuatum* with concentrations of 700, 800, 900, and 1000g/ml was added. One well was considered as a control. Then the plates were placed in an incubator for 24h. After 24h, the diameter of the non-growth halo formed in the plates was measured with a caliper and this experiment was repeated 3 times.

Minimum inhibitory concentration (MIC) method of *V.sinuatum* extract on the planktonic form of *Salmonella typhimurium*

Determining the minimum inhibitory concentration of MIC was done by tube method. In this method, 10 sterile test tubes containing Mullerhinton Broth medium were used for the planktonic form of bacteria formed. At first, to dilute the amount, 1000 μ l of *V.sinuatum* alcoholic extract with a concentration of 900g/ml was added to tube

number 1 and mixed. After tube number 1, 1000µl of solution (Müller Hinton Broth medium + *V.sinuatum* extract) was removed and added to tube number 2, and in the same way until tube number 10. After performing these steps, 100µl of 0.5 McFarland's microbial suspension was added to each of the tubes. The MIC result was checked based on the turbidity of the culture medium, which indicates bacterial growth. The MIC of *V.sinuatum* extract on the planktonic form of *Salmonella typhimurium* with a concentration of 1000g/ml was also determined by the same method.

Minimum Bactericidal Concentration (MBC) method on the planktonic form of *Salmonella typhimurium*

After MIC test, the tubes were cultured on sterile nutrient agar culture medium and these plates were incubated at 37°C for 24h. In this case, the lack of bacterial growth indicates the MBC point.

MIC method on the planktonic form of *Klebsiella pneumoniae*

Determining the minimum inhibitory concentration of MIC was done by tube method. In this method, 10 sterile test tubes containing Mueller Hinton Broth medium were used for the planktonic form of the bacteria. At first, to dilute the quantity, 1000µl of the alcoholic extract of *V.sinuatum* with a concentration of 700g/ml was added to tube number 1 and mixed. After tube number 1, 1000µl of solution (Muller Hinton Broth medium + *V.sinuatum* extract) was removed and added to tube number 2, and in the same way until tube number 10. After performing these steps, 100µl of 0.5 McFarland's microbial suspension was added to each of the tubes. The MIC result was checked based on the turbidity of the culture medium. The MIC of *V.sinuatum* extract on the planktonic form of *Klebsiella pneumoniae* with a concentration of 800g/ml was also determined by the same method.

MBC method on the planktonic form of *Klebsiella pneumoniae*

After the MIC was done, the tubes were cultured on sterile nutrient agar culture medium by swapping, and these plates were placed in an incubator at 37°C for 24h. In this case, the lack of bacterial growth indicates the MBC point. High well tests were also performed for *Shigella sonnei* with a concentration of 700g/ml and 800gr/ml, but because the results were not significant and no growth halos were created due to the effect of the extract on the bacteria, as a result, MIC and MBC tests were not performed for this bacterium.

Biofilm preparation method

Day 1: To perform this test, a small amount of *Salmonella Typhimurium* grown in Mueller Hinton Agar medium was removed by a sterile needle and cultured on glucose TSA medium and placed in an incubator for 24h.

Day 2: It was removed from the bacteria grown in TSA medium and prepared from it by a 0.5McFarland spectrophotometer. 1ml was taken from this prepared 0.5McFarland and poured into a 96-well microplate containing sterile glucose-containing TSB, then the extract was added into a 96-well microplate containing medium, and incubated at 37°C for 18-28h.

Day 3: Antimicrobial effect was checked.²⁰

Investigating the antimicrobial effect of the alcoholic extract of *V.sinuatum* on the biofilm formed by *Salmonella typhimurium*

After the biofilm process, a sterile swab was drawn on the biofilm formed in the microplate to be stained with *Salmonella Typhimurium*. Then, on Mueller Hinton Agar medium, lawn culture method was done in three directions. Then, 3 wells were created with a test tube whose diameter is 10ml, and the bottom of the wells was filled with Mueller Hinton Agar medium. After 5 to 10min, 180µl and 200µl of the alcoholic extract of *V.sinuatum* added to the well. 180µl and 200µl for the first well 1 and 2 respectively. The third well were considered as control and no substance was poured into it. Then the prepared plates were placed in the incubator for 24h to check the results the next day.

Investigating the antimicrobial effect of the alcoholic extract of *V.sinuatum* on the biofilm formed by *Klebsiella pneumoniae*

After the biofilm process, a sterile swab was drawn on the biofilm formed in the microplate to be stained with *Klebsiella pneumoniae*. Then, on Mueller Hinton Agar medium, lawn culture method was done in three directions. Then, 3 wells were created with a test tube whose diameter is 10 ml, and the bottom of the wells was filled with Mueller Hinton Agar medium. After 5 to 10min, 180µl and 200µl of the alcoholic extract of *V.sinuatum* added to the well. 180µl and 200µl for the first well 1 and 2 respectively. The third well were considered as control and no substance was poured into it. Then the prepared plates were placed in the incubator for 24h to check the results the next day.

MIC method of alcoholic extract of *V.sinuatum* on biofilm formed by *Salmonella Typhimurium*

10 test tubes containing Muller Hinton Broth medium were used for the formed biofilm. To dilute the amount, 1000µl of the alcoholic extract of *V.sinuatum* with a concentration of 900g/ml was added to tube number 1 and

mixed. After tube number 1, 1000µl of solution (Müller Hinton Broth medium + *V.sinuatum* extract) was removed and added to tube number 2, and in the same way until tube number 10. The formed biofilm was added to 10 tubes. The MIC result was checked based on the turbidity of the culture medium, which indicates bacterial growth. The MIC of the alcoholic extract of *V.sinuatum* on the biofilm of *Salmonella typhimurium* with a concentration of 1000g/ml was also performed with the same method.

MBC method of alcoholic extract of *V.sinuatum* on biofilm formed by *Salmonella Typhimurium*

After the MIC test, the tubes were cultured on sterile nutrient agar culture medium by swapping, and incubated at 37°C for 24h. In this case, the lack of bacterial growth indicates the MBC point.

MIC method of alcoholic extract of *V.sinuatum* on biofilm formed by *Klebsiella pneumoniae*

10 test tubes containing Muller Hinton Broth medium were used for the formed biofilm. To dilute the amount, 1000µl of the alcoholic extract of *V.sinuatum* with a concentration of 700g/ml was added to tube number 1 and mixed. After tube number 1, 1000µl of solution (Müller Hinton Broth medium + *V.sinuatum* extract) was removed and added to tube number 2, and in the same way until

tube number 10. The formed biofilm was added to 10 tubes. The MIC result was checked based on the turbidity of the culture medium, which indicates bacterial growth. The MIC of the alcoholic extract of *V.sinuatum* on the biofilm of *Klebsiella pneumoniae* with a concentration of 800g/ml was also performed with the same method.

MBC method of alcoholic extract of *V.sinuatum* on biofilm formed by *Klebsiella pneumoniae*

After the MIC test, the tubes were cultured on sterile nutrient agar culture medium by swapping and incubated at 37°C for 24h. In this case, the lack of bacterial growth indicates the MBC point.

Results

The results of MIC and MBC of alcoholic extract of *V.sinuatum* on the planktonic form and biofilm of *Salmonella Typhimurium*

At this stage, it was observed that in the planktonic form of *Salmonella Typhimurium*, test tubes No. 1 and 2 with the extract of *V.sinuatum* in both concentrations (900g/ml and 1000gr/ml) were transparent and they were MIC. The alcoholic extract of *V.sinuatum* in these two concentrations has an inhibitory effect on the planktonic form and a lethal effect on the biofilm form of *Salmonella Typhimurium*(Figurer 1 and 2).

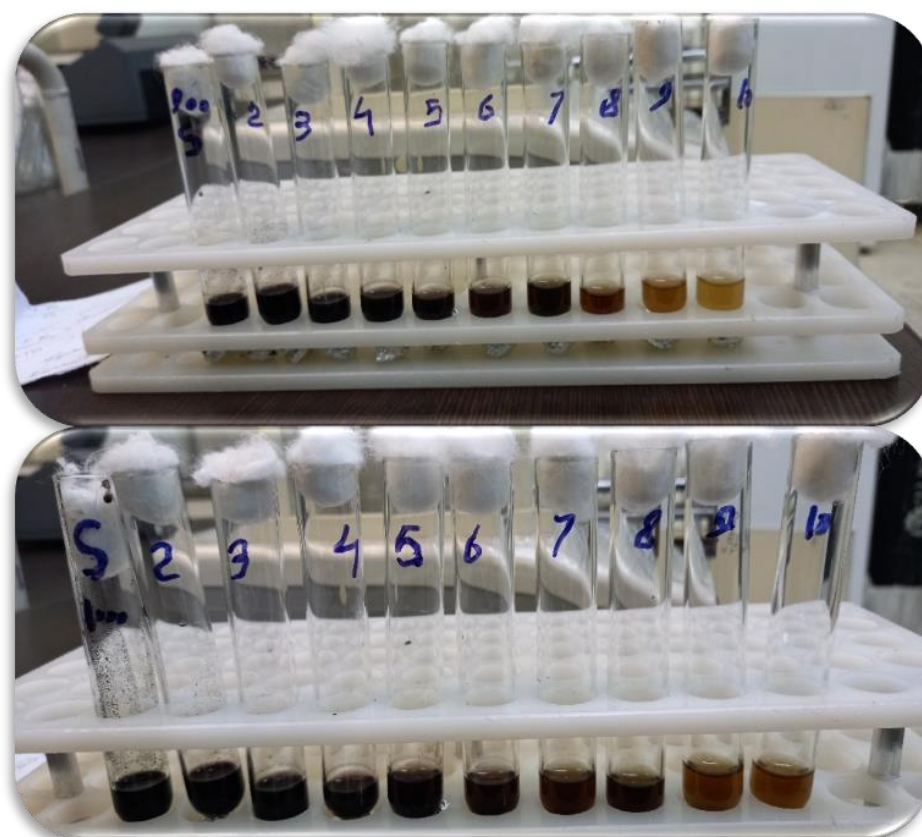


Figure 1. MIC of the alcoholic extract of *V.sinuatum* on the planktonic form and biofilm of *Salmonella Typhimurium*.



Figure 2. MBC of alcoholic extract of *V.sinuatum* on the planktonic form and biofilm of *Salmonella Typhimurium*

The results of MIC and MBC of alcoholic extract of *V.sinuatum* on the planktonic form and biofilm of *Klebsiella pneumoniae*

At this stage, it was observed that in the planktonic form of *Klebsiella pneumoniae*, the test tube number 1 with the alcoholic extract of *V.sinuatum* in both concentrations

(700g/ml and 800gr/ml) was transparent and considered as MIC point. Two concentrations the alcoholic extract of *V.sinuatum*, have inhibitory effect on the planktonic form and a lethal effect on the biofilm form of *Klebsiella pneumoniae* (Figurer 3 and 4).

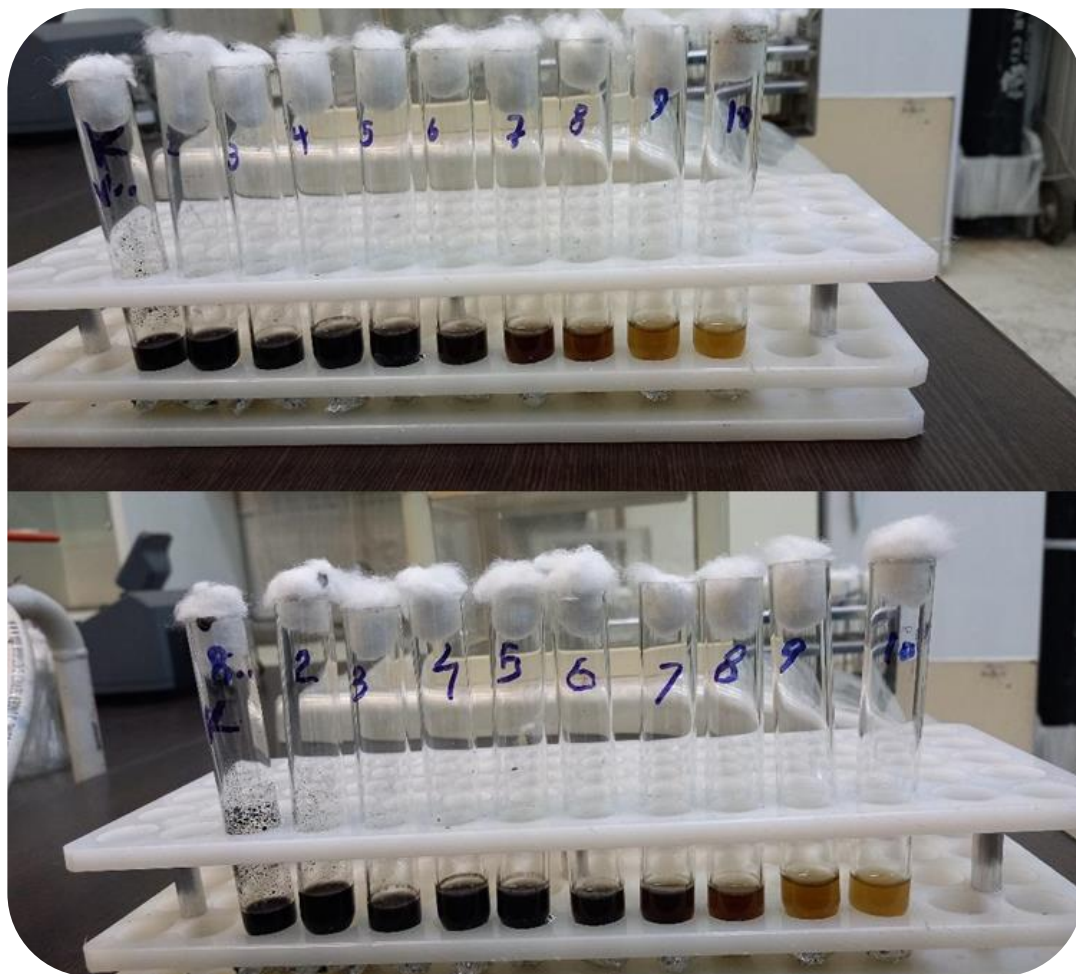


Figure 3. Forms related to the MIC results of the alcoholic extract of *V.sinuatum* on the planktonic form and biofilm of *Klebsiella pneumoniae*.



Figure 4. Forms related to MBC, the results of the alcoholic extract of *V.sinuatum* on the planktonic form and biofilm of *Klebsiella pneumoniae*

Discussion

According to the results obtained from the graph comparing the results of the average aura of non-growth of bacteria and biofilm in relation to different volumes of antimicrobial compounds, it shows that the well with the volume of 200 μ l of the antimicrobial substance has the greatest effect, and then the well with the volume of 180 μ l shows this, respectively. The greater the volume of the antimicrobial agent, the greater its effect, and according to the results obtained from the change graph, the average diameter of the halo of non-growth in the biofilm is smaller than the planktonic form of bacteria. In this research, SPSS version 22 software was used for data analysis and graphs were drawn using Excel software. According to the statistical analysis, the sensitivity of the samples was determined according to the diameter of the halo of non-growth created in the well in bacteria and biofilm to the alcoholic extract of *V.sinuatum* plant. The obtained results are as follows: the antimicrobial substances of the alcoholic extract of *V.sinuatum* have a significant antimicrobial effect on bacteria and its biofilm, and its effect is significant.

In the MBC and MIC method, which was observed on the planktonic form, in *Salmonella typhimurium*, test tubes number 1 and 2 in the alcoholic extract of *V.sinuatum* with a concentration of 900g/ml and 1000g/ml are transparent tubes and are considered as the MIC point. That is, the alcoholic extract of *V.sinuatum* in these two concentrations has an inhibitory effect on the planktonic form and a lethal effect on the biofilm form of *Salmonella Typhimurium*. In the bacterium *Klebsiella pneumoniae*, tube number 1 in the alcoholic extract of *V.sinuatum* with a concentration of 700g/ml and 800g/ml

are transparent tubes and are considered as the MIC point, that is, the alcoholic extract of *V.sinuatum* at a concentration of 700g/ml and 800g/ml has an inhibitory effect on the planktonic form and a lethal effect on the biofilm form of *Klebsiella pneumoniae*. In the current research, the antimicrobial effects of the alcoholic extract of *V.sinuatum* on *Salmonella typhimurium* and *Klebsiella pneumoniae* and its biofilm were investigated. *Salmonella typhimurium* and *Klebsiella pneumoniae* showed sensitivity to the alcoholic extract of *V.sinuatum* and halos were formed. Also, the created biofilm showed sensitivity to the alcoholic extract of *V.sinuatum* and halos were observed.

According to the results of our study in this research and the results of other mentioned studies, it can be said that the formation of biofilm is an important factor in stability and as a result, it is the cause of infections that are difficult to deal with. Among the bacteria, *Klebsiella pneumoniae* and *Salmonella typhimurium* also have the ability to form biofilm and are considered to be an important cause of many infections, so it is important to fight this infectious agent using plant extracts.

In the study of Ghorbani et al., the flower extract of *V.sinuatum* region of Bojnord region, the maximum halo of non-growth in the well disk method on *Staphylococcus aureus* was 33.12 and 66.14 mm respectively, and for *Bacillus cereus* it was 33.11mm respectively. 66.13 mm was obtained and the minimum inhibitory and lethal concentration of *V.sinuatum* flower extract from Bojnord region on bacteria (*Staphylococcus aureus*) was observed as 5.12 mg/ml as the most sensitive bacteria. In this research, it was found that among the different

ecotypes of North Khorasan, the methanolic extract of *V.sinuatum* flower of Bojnord region had the most antibacterial effect on bacteria (*Bacillus cereus* and *Staphylococcus aureus*), but it was not effective on *Escherichia coli*¹³.

Hamid Kalalian Moghadam et al., in investigating the antibacterial and anti-adhesion activity of the ethanolic extract of *Verbascum Thapsus* l large mullein on biofilm formation in laboratory conditions of three oral *streptococci*, stated that the ethanolic VT extract has inhibitory effects on the biofilm formation of oral VT *streptococci* as a reduction in bacterial growth and a reduction in the ability are biofilm formation¹⁴. During their research, Mihailovic et al concluded that the extracts of aerial parts, especially their flowers, have optimal medicinal, anti-inflammatory, antioxidant, and antibacterial functions due to the abundance of phenolics and flavonoids¹⁵.

The results of Senatore et al showed that the methanolic extract of *V.sinuatum* inhibited all the bacterial strains tested in this project. Gram-positive bacteria were more sensitive to the extract. *S.epidermidis* showed the lowest MIC (15.5µg/ml).¹⁶

Selseleh et al investigated the antimicrobial and antioxidant activity of *Verbascum* and their results showed that *Verbascum* had an inhibitory effect on all the bacteria they studied and *Verbascum* showed the highest antibacterial activity (MIC 0.12mg/mL) against *Staphylococcus aureus*.¹⁷

Bao et al. performed the antimicrobial activity of *V.sinuatum* by disk diffusion method on *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Candida albicans* and found that *V.sinuatum* had strong antimicrobial activity against gram-positive bacteria and yeast cultures¹⁸. The result of this experiment was similar to our research on the bacterium *Klebsiella pneumoniae*, against which the *V.sinuatum* showed antimicrobial activity.¹⁸

Sener et al isolated *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Candida albicans* from the wards of patients with office infections and evaluated the inhibitory effect of *V.sinuatum* on these microorganisms. The results showed that the extracts Strong antimicrobial activity against *Enterococcus faecalis*, *Proteus mirabilis* and *Candida albicans* has a greater inhibitory effect and has a moderate inhibitory effect on other studied microorganisms.¹⁹

Dulger et al evaluated the ability of *Verbascum* species to inhibit 35 hospital isolates of methicillin-resistant *Staphylococcus aureus* (MRSA). The antibacterial activity of the extracts was tested by disk diffusion and modified agar dilution method. Ethanolic extracts of the plants were most effective with MICs for MRSA isolates of 0.2–0.4, 0.1–0.4 and 0.4–1.6 mg/mL, respectively, and for *S.aureus* ATCC 25923 of 1.6, 0.2 and 1.6 mg/mL respectively. MBCs for MRSA isolates were 0.8–1.6, 0.4–1.6 and 6.3–12.5 mg/mL, respectively and for *S.aureus* ATCC 25923 were 12.5, 1.6 and 25 mg/mL, respectively.²⁰

Dulger et al. conducted their studies to investigate the anti-staphylococcal activity of the ethanolic extract obtained from the leaves of *V.sinuatum* against *Staphylococcus aureus* by agar-well diffusion method and microdilution method. Ethanol extract showed antibacterial activity against staphylococcal strains. Therefore, the extract obtained from *V.sinuatum* can be used as an anti-staphylococcal agent.²¹

Amirnia et al investigated the antibacterial activity of aqueous and alcoholic extracts of *Verbascum speciosum*. They investigated the antibacterial effect of *Verbascum speciosum* in laboratory conditions on three strains of *Bacillus subtilis*, *Bacillus cereus* and *Escherichia coli*. These microorganisms were treated using disk diffusion method and plant extracts were used in three concentrations (12.5, 25 and 50µg/ml). The results showed that these natural substances had an inhibitory effect on the growth of the above substances in all doses. Moreover, in both cases, the diameter of the inhibitory zones increases with the increase of the extract concentration. On the other hand, the results show that the ethanolic extract was more effective than water in all the doses emphasized on the three microorganisms.

In the case of both aqueous and ethanol extracts, maximum antibacterial activity was shown against *Bacillus cereus* followed by *B. subtilis* and *E. coli* was the most resistant strain.²²

Karamian et al. investigated the antibacterial effects of methanolic extracts of three *Verbascum* species on Gram-positive and Gram-negative bacteria by disc diffusion method. The results showed that the methanolic extract of *V.sinuatum* has the highest amount of phenolic compounds and *V.speciosum* has the highest amount of flavonoids. In the β-carotene/linoleic acid system, linoleic acid oxidation was effectively inhibited by *V.speciosum* extract (58.4±18.1 mg/g), followed by *V.sinuatum*

(51.41±2.28 mg/g). In addition, methanol extracts of three *Verbascum* species showed strong antibacterial activity against all tested bacteria.²³

Considering the results of our study in this research and the results of other studies mentioned, it can be said that the formation of biofilm is an important factor in stability and as a result it is the cause of infections that are difficult to deal with. Among the bacteria, *Klebsiella pneumoniae* and *Salmonella typhimurium* also have the ability to form biofilms and are considered to be the main cause of many infections, so it is important to combat this infectious agent using plant extracts.

The results of the research showed that the alcoholic extract of *V.sinuatum* has an antimicrobial effect on *Salmonella typhimurium* and *Klebsiella pneumoniae* and has an inhibitory effect, and it also has an antimicrobial effect on the biofilm of *Salmonella typhimurium* and *Klebsiella pneumoniae*.

Highlights

What Is Already Known?

The most important bacteria that cause these diseases include *Salmonella typhimurium*, *Klebsiella pneumoniae* and *Shigella sonnei*. The increasing drug resistance of bacteria created the necessity of investigating the antibacterial effects of plant extract

What Does This Study Add?

The results of the research showed that the alcoholic extract of *V.sinuatum* has an antimicrobial effect on *Salmonella typhimurium* and *Klebsiella pneumoniae* and has an inhibitory effect, and it also has an antimicrobial effect on the biofilm of *Salmonella typhimurium* and *Klebsiella pneumoniae*.

Authors' Contributions

FSS conceived and designed the study, conducted research, and collected and organized data. ZHB wrote the initial and final draft of the article and provided logistic support. All authors have critically reviewed and approved the final draft.

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Conflicts of Interest Disclosures

The authors declare that they have no conflict of interest.

Consent For Publication

All authors declare their consent to publish this manuscript.

Ethics approval

not applicable

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