Antimicrobial effect of ethanolic extract of orange peel against bacteria isolated from burn infection

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ABSTRACT

Objective: A burn is a type of damage to the skin. In this skin damage, the first defense barrier of the body is destroyed, and the patient's wound becomes the best environment for the growth of bacteria that cause dangerous infections. Nowadays, due to the problems of side effects and resistance of bacteria to antibiotics, medicinal plants are used to deal with bacteria. This research amied to investigate the effect of ethanolic extract of orange peel on bacteria isolated from the wounds of burn patients in Jiroft hospitals and compare them with selected antibiotics.
Methods: For this purpose. an experiment was conducted with three replications in 2022 .after collecting the orange peel of Thomson variety, its extract was performed by percolation method and after isolation, the bacteria causing the infection were identified using morphological and biochemical tests. Then, the effect of ethanolic extract of orange peel and common antibiotics against bacteria was investigated.
Results: The results showed that *Pseudomonas aeruginosa* (52%), *Staphylococcus aureus* (37%) and *Escherichia coli* (11%) frequency had the most role in burn wound infections. And the orange peel extract had a better antibacterial effect against bacteria compared to cefixime and azithromycin antibiotics, so that the diameter of the inhibition zone of orange peel extract in *S. aureus* at 300.12 mg/ml was 31.22 mm and the antibiotic cefixime and azithromycin were 17.23 and 27.52 mm, respectively.
Conclusions: Therefore we can use orange peel extract instead of antibiotics and chemical drugs to fight the burn infection bacteria.

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1- Introduction

A burn is an injury to flesh or skin caused by heat, electricity, chemicals, friction, or radiation. Burns are of great interest due to their severe physical and psychological complications and high mortality rate (Holems and Heimbach, 2005; Park *et al*., 2013).As a result of burns, the body's first defense barrier is destroyed, and as a result, patients are exposed to numerous bacterial infections (Askarian *et al*. 2004). Wound infection control is one of the main problems in the burn ward, and every year a large number of patients die or become disabled due to this complication (Mohamed 2016). According to the Iranian Society of Microbiology, 80% of the deaths in the burn ward are related to burn infections and a high percentage of different organs of the body (Cakir and Yegen, 2004). In many cases, the use of antibiotics saves the lives of many patients Schult, z *et al*., 2013. But since 2010, due to concerns about antibiotic resistance and the high risk of fungal infection, their general use do not recommend. (Behzadnia *et al*., 2014).

The use of plants for the treatment of diseases has been common in our traditional medicine culture for a long time due to the absence of side effects (Rezaeizadeh *et al*., 2009). Smith Papyrus in 1500 BC in Egypt described the treatment of burns using honey and pine gum poultice (Avni *et al*., 2010). The use of tea leaves by the Chinese in 600 BC has been used over time (Wasiak *et al*., 2013; Kumar *et al*., 2007). Even today, research has shown that some plants have strong antibacterial effects on microbes. Therefore, the use of herbal medicines is preferred instead of antibiotics to fight infections (Osman *et al*., 2010). In traditional and modern medicine, the use of plant extracts is widely used due to the medicinal properties in their compounds (Zareian *et al*., 2007). Also, due to the increase in microbial resistance and the side effects of antibiotics, medicinal plants are a suitable alternative to deal with bacterial infections (Salavatifar *et al*., 2023).Therefore, the researchers investigated the active compounds extracted from different plant species for use as antibacterial drugs (Gachkar *et al*., 2013).

Orange is a tree in the family Rutaceae. This plant grows well in the northern and southern parts of Iran. Orange increases the body's natural resistance. It has a diuretic, laxative and blood-thinning effect. It is effective in anemia and the treatment of indigestion. It has an effect on relieving inflammation of the gum and mouth mucosa (Zargari, 1997). Pharmacologically oranges are used to treat colds, fevers, liver disorders, gastrointestinal disorders, and skin spots (Paul, 1995).

Staphylococcus aureus, gram-positive cocci and facultative anaerobes, is one of the five most common causes of hospital infections, especially post-surgical wound infections (Ogston, 1984). This bacterium creates yellow colonies due to the production of staphyloxanthin pigment. This pigment plays a role in pathogenicity because it acts as an antioxidant and protects bacteria from oxygen free radicals (Clauditz *et al*., 2006). This bacterium causes a wide range of simple skin infections to threatening diseases. When the skin barrier is removed, the bacteria invade the tissue. At first, penicillin was used to treat staphylococcal infections, but due to the production of penicillinase by bacteria that breaks down penicillin; Therefore, newer antibiotics (oxacillin and methicillin) were used (Chambers, 2001). Unfortunately, this bacterium has also become resistant to these antibiotics over time. These antibiotics are used along with gentamicin to treat serious infections such as endocarditis. Aminoglucoside antibiotics such as gentamicin, streptomycin, and kanamycin used to work well against staphylococci, but staphylococci have become resistant to these antibiotics over time. These antibiotics lead to bacterial death by binding to the ribosomal 30S subunit (Carter *et al*., 2000).

Pseudomonas aeruginosa is a gram-negative bacterium and opportunistic pathogen. This bacterium causes urinary tract infections, respiratory system, inflammation and edema, soft tissue infections, bacteremia, bone and joint infections, stomach and intestinal infections, and various systemic infections, especially in patients with severe burns, patients with cancer and AIDS (Aljeboury, 2013. *P. aeruginosa* is mostly resistant to common antibiotics, but some strains respond to gentamicin, tobramycin, colicitin, norfoxacin, ciprofloxacin, and amicasein. Gentamycin and carbinicillin are mostly used to treat severe infections.

Escherichia coli is a gram-negative bacillus of the Enterobacteriaceae family (George *et al*., 2009). *it* is one of the most diverse bacterial species, with 20% of its genome shared between different strains (Lukjancenko *et al*., 2010).

In 2016, Razmjoo investigated the antimicrobial effects of methanolic extract of orange peel and its effect on the shelf life and microbiological and chemical properties of flavored milk during 7 days of storage at different temperatures. The results showed that methanolic extract of orange peel had the highest effect on Candida and the least effect on *S. aureus* and *E. coli* (Razmjoo, 2016). And, In 2009, Nakhaei Moghaddam performed an antimicrobial effect of methanolic extract of orange peel against 37 isolates of clinical bacteria of *Helicobacter pylori* in comparison with amoxicillin and metronidazole *in vitro*.The results indicated that all isolates were sensitive to 2 mg of the extract. The mean diameter of the inhibition zone was 13.28 mm. Also, the antibacterial activity of the extract was maintained after heating at autoclave temperature for 20 minutes (Nakhaei Moghaddam, 2009). The antibacterial effect of ethanolic extract of orange peel on five pathogenic bacteria (*E. coli*, *S. aureus*, Bacillus, Salmonella and Klebsiella) showed that the effect of this extract on gram-positive microorganisms is more than gram-negative microorganisms (Ashook Kumar *et al*., 2011).

Due to the fact that the orange plant is very compatible with the climate of southern Iran and is grown on a large scale, its antibacterial effect has not been fully investigated until today. In this study, we investigated the antibacterial effect of ethanolic extract of orange peel on the bacteria causing infection in burn patients hospitalized in

Jiroft's hospitals (Imam Khomeini and Qaim) and compared them with common antibiotics so that if the tests are positive, this plant can be used instead of antibiotics to fight the bacteria causing the infection in order to prevent the bacteria from becoming resistant to antibiotics and their complications on the body of burned patients should be prevented. In this way, they can be of great help to these patients and the health of the society.

Materials and Methods:

1-1-Isolation of bacteria causing infection from burn wounds of patients

Isolation of bacteria causing infection from burn patients referred to hospitals in Jiroft city (Qaim and Imam Khomeini) was done in 2022. First, after opening the burn wound dressing and cleaning the wound from extra substances (ointment, etc.) and disinfecting it using a sterile syringe or swab, samples were taken from the wound and under standard conditions. The samples were sent to the laboratory of Azad University, Jiroft branch. In order to grow bacteria, the samples were cultured on blood agar, chocolate agar, Macconkey agar and eosin methylene blue agar (EMB) made by Merck Company of Germany. And after incubation at 37 °C for one day and night, they were evaluated in terms of growth and colony formation.

1-2-Identification of disease-causing bacteria

Identification of bacteria in burn samples was done by suitable morphological and biochemical tests (lactose fermentation, gram staining, catalase, coagulase, lipase and phosphatase tests, etc.). All samples were identified by biochemical tests.

1-3-Preparation of orange peel extract

Preparation of extract by percolation method for orange peel of Thomson cultivar collected from the research farm of Jiroft Azad University and their accurate identification was approved by Kerman University of Medical Sciences. To produce the ethanolic extract of orange peel extract of Thomson cultivar, the desired orange was prepared from the citrus orchards of the university's research farm, and the peel was thoroughly washed with water and dried in the shade away from the sun, and the powder obtained from the plant was mixed with a certain volume of ethanol solvent in a ratio of 1 to 5 and at 60 °C for four hours. At the end, the obtained extract was centrifuged at a high speed and the extract was extracted from the upper phase of the filtered solution after 72 hours (Ranade *et al*., 2012).

1-4-The effect of ethanolic extracts on bacteria isolated from burn wound infection

The effect of extracts on bacteria isolated from burn wound infection of patients was investigated by the Kirby-Bauer method (Wikler *et al*., 2006). For this purpose, first, from the 24-hour colonies of the identified strains on the blood agar medium, bacterial suspension with a concentration of half McFarland was prepared using sterile physiological serum. Then, microbial suspension was cultured as a table on Müller-Hinton agar medium. Then the blank disc soaked in ethanolic extract of orange peel with a concentration of 1.1, 2.25, 4.50, 9.01, 18.02, 36.37, 73.27, 150.81 and 300.12 mg/ml was placed separately on the culture medium at equal distances from each other. The plates were placed at 37 °C for 24 hours. After the incubation period, the diameter of the inhibition zone was measured in mm

1-5-The Effect of common antibiotics on bacteria isolated from burn wound infections

To investigate the effect of common antibiotics on burn wound bacteria of patients, three selected antibiotics (azithromycin, gentamicin and cefixime) and as well as the effect of the extract, the Kirby-Bauer method was used and the inhibitory power of the selected antibiotics against the three studied bacteria was measured by measuring the diameter of the inhibition zone. Blank disks impregnated with antibiotics were placed on the Müller-Hinton agar medium which was cultured by microbial suspension with concentration of half McFarland. Then the plates were placed at 37 °C for 24 hours. After the incubation period, the diameter of the bacterial no-growth zone was measured in mm.

1-6-Data Analysis

For each of the different concentrations of orange peel extract and antibiotics on each bacterium, the experiments were repeated three times and the diameter of the non-growth of bacteria was measured by caliper and the mean comparison of the data was processed using SAS software and the tables and the graphs were performed using Excel software.

Results

2-1-Isolation and identification of bacteria causing infection from burn wounds

After taking samples from burn patients in Jiroft hospitals and culturing the samples in blood agar, chocolate agar, Macconkey agar and eosin methylene blue agar (EMB) were isolated from a total of 54 samples, and the analysis of the samples showed that 59.8% of the cultures were positive and 40.2% of the cultures were negative. After identification using morphological and biochemical tests, three bacteria namely *Escherichia coli, Staphylococcus aureus* and *Pseudomonas aeruginosa were* confirmed (Table 1). *P. aeruginosa* with a frequency of 52% had the most role in burn infections, followed by *S. aureus* with 37% and *E. coli* with 11% frequency were the three most common bacteria in burn wound infections (Fig.1). Gram staining shows the bacteria as gram-positive clustered cocci. The special saline mannitol medium (7-9% salt) is used for the growth of *S. aureus*. This bacterium tolerates salt and produces yellow colored colonies after growing on the latter medium, that are formed due to the fermentation of manthiol and the decrease in the pH of the culture medium. All Staphylococcus species are positive for catalase test. *S. aureus* is positive for the coagulase test. This bacterium produces the enzyme DNAse. It is also positive for lipase and phosphatase tests. *P. aeruginosa* is a gram-negative bacterium that grows well on blood agar and EMB culture mediums. Identification of this

bacterium is based on gram morphology, inability to ferment lactose, fruit odor (with grape flavor) and the ability to grow at 42 °C.

The characteristic of fluorescence under ultraviolet light is also effective in the immediate detection of *P. aeruginosa* colonies and helps in detecting its presence in wounds. *E. coli* produces purple colonies on the McConkey agar medium because the bacterium is lactose positive and ferments sugar and produces acid. The same thing happens in the EMB medium and forms dark purple colonies with a metallic green sheen. The bacteria in TSI medium is acid/acid and negative with gas and H2S production. In terms of the IMViC test, it is indole positive, MR positive, VP negative and citrate negative (Paton *et al*., 1998).

Table 1: Biochemical Tests for Identification of *E. coli, S. aureus and P. aeruginosa*

Bacteria	Lactose fermentation	Indole	Gas production from glucose	Move	Citrate
E. coli	+	+	+	+	-
Staphylococcus aureus	+	+	+	+	-
P. aeruginosa	-	+	-	-	+

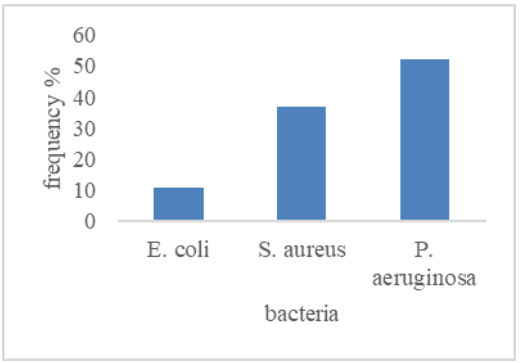

Figure 1: Frequency of bacteria in burn samples of patients referred to hospitals in Jiroft (Qaim and Imam Khomeini) in 2022.

2-2-Preparation of orange peel extract

Ethanolic extract was prepared for orange peel .

2-3-Effect of ethanolic extract of orange peel on infection-causing bacteria in burn wounds

The results of ethanolic extract of orange peel at concentrations of 1.1, 2.25, 4.50, 9.01, 18.02, 36.37, 73.27, 150.81 and 300.12 mg/ml on three bacteria isolated from burn wounds by three replications and measuring of the diameter of the inhibition zone, it showed that *S. aureus* showed resistance to the ethanolic extract of orange at a concentration of 1.1 and at a concentration of 2.25 it formed a halo with a diameter of 12.44 mm. In this bacterium, a halo with a diameter of 31.22 mm was formed at a concentration of 300.12.

P. aeruginosa showed resistance up to 9.01 concentration, and at 300.12 concentration, the inhibition zone of the extract was 27.54 mm. The most resistant bacterium to ethanolic extract of orange peel is *E. coli*, which showed resistance up to a concentration of 18.02 mg/ml, and at the last concentration, the halo diameter was 25.88 mm compared to other bacteria. It was lower (Figure 2). We performed experiments from a concentration range of 1.1 mg/ml to 300.12 mg/ml. Also, results showed that with increasing the concentration of orange peel extract, its antibacterial effect increased.

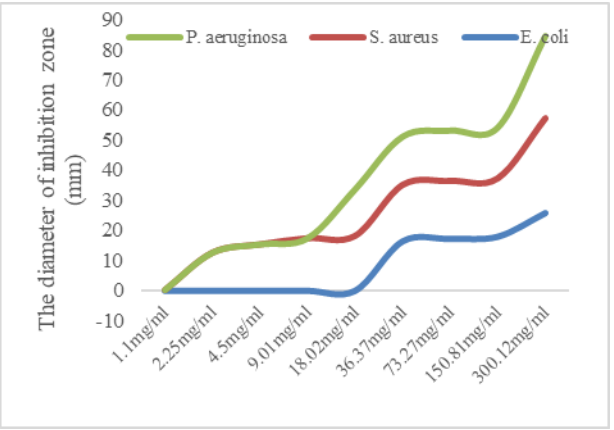

Figure 2: The diameter of the inhibition zone of bacteria *S. aureus*, *P. aeruginosa* and *E. coli* due to different concentrations of orange peel extract

2-4-The Effect of selected antibiotics and orange peel extract on burn bacteria

Three antibiotics azithromycin (E-15), gentamicin (GM-10) and cefixime (CFM-5) were used for the experiments of this research. Tests showed that *E. coli* showed resistance to two antibiotics, azithromycin and cefixime (Fig. 2). However, this bacterium has shown a halo with a diameter of 8.18 mm against the concentration of 36.37 mg/ml of orange peel. Gentamicin was the strongest antibiotic among the selected antibiotics, so that the diameter of the halo of non-growth by *S. aureus* (30.02 mm), *P. aeruginosa* (28.72 mm) and *E. coli* (25.03 mm), which showed resistance to two other antibiotics and cefixime is the weakest antibiotic among the selected antibiotics. So that *E. coli*, *P. aeruginosa* and *S. aureus* created a halo with diameters of 11.72, 4.26 and 17.23 mm, respectively. And the orange peel extract had a better antibacterial effect compared to cefixime and azithromycin antibiotics, so that the diameter of the inhibition zone of orange peel extract at 300.12 mg/ml on *S. aureus* was 31.22 mm and the antibiotic

cefixime and azithromycin were 17.23 and 27.52 mm, respectively (Fig. 2).

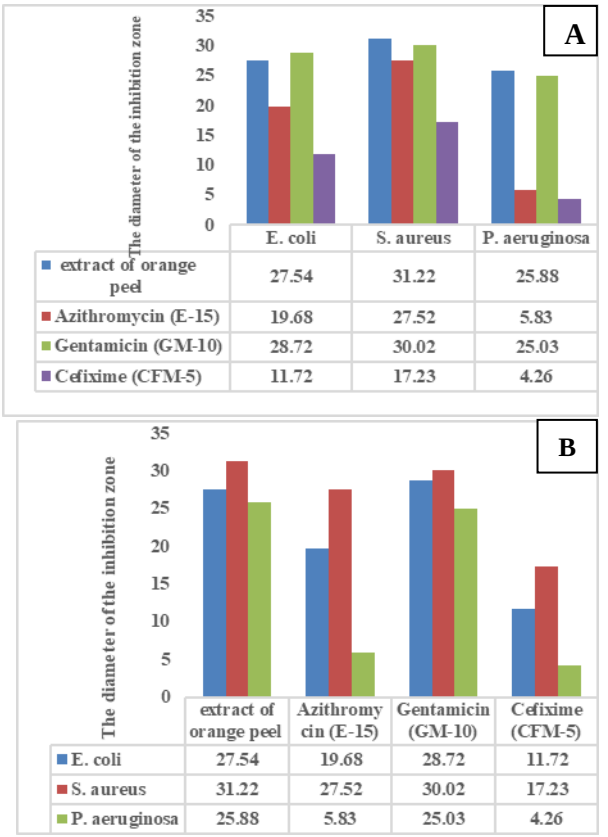

Figure 2: Comparison of the effect of selected antibiotics and ethanolic extract of orange peel at a concentration of 300.12 mg/ml on *S. aureus*, *P. aeruginosa* and *E. coli*(A) and (B).

Discussion and Conclusion

The use of antibiotics to fight pathogenic bacteria is increasing. These pharmaceutical antibiotics, in addition to having very adverse effects on different parts of the body such as kidneys, liver, heart, stomach, etc. Bacteria have become resistant to them over time and cannot have a beneficial effect on bacteria. For the reason of the resistance of bacteria to antibiotics, the number of new antibiotics to fight the bacteria is increasing sharply. But today, the use of medicinal plants to fight bacteria instead of antibiotics and chemical drugs has become common due to the lack of dangerous side effects. These medicinal plants can show good antibacterial effects and some of them have better effects than many antibiotics. The results of this study confirm the accuracy of this issue.

In this research, after taking samples from burn patients in the hospitals of Jiroft city, a total of 54 samples of bacteria causing infection were isolated, and the examination of the samples showed that 59.8% of the cultures were positive and 40.2% of the cultures were negative. After identification using morphological and biochemical tests, three bacteria namely *E. coli, S. aureus* and *P. aeruginosa were* confirmed. *P. aeruginosa* with a frequency of 52% had the highest role in burn infections, followed by *S. aureus* with 37% and *E. coli* with 11% frequency were the three most common bacteria with a high frequency in burn wound infections. In a research conducted by Faqri on burn patients hospitalized in Isfahan burn accident hospital. Out of the 106 examined patients**,** 91 of them had an infection, of which 85.5% had a burn wound infection, and *P. aeruginosa* was the most common organism among the infectious agents of burn wounds with 54.4% (Faqri, 2005). In 2009, THi Akhi in a descriptive study of 126 samples of infectious wounds resulting from burns of selected patients admitted to the burn department of Sina Educational and Medical Center in 1999-2000 achieved these results. Out of a total of 126 samples, 73 bacterial strains were isolated, among which (64.38%) *P. aeruginosa* and (35.62%) were other bacterial species. *Staphylococcus aureus* (13.69%) was the second most important bacteria isolated from infected burn wounds. 91% of patients who died had positive cultures. Despite the sensitivity of *P. aeruginosa* to third-generation cephalosporins such as cefoprazone (79.9%), ceftazidine (71%) and cefoctoxime (59.3%), the increase of its resistance to some antibiotics was significant (Taghi Akhi, 2009). In a study conducted by Vindenes, it was found that *P. aeruginosa* was the most common bacterium infecting burn wounds with 31.7% and the most important cause of burn wound infection (Vindenes *et al*., 1995).

This research was conducted on the topic of comparing the antimicrobial effect of the ethanolic extract of orange peel with selected antibiotics against bacteria isolated from the infection caused by the burn wounds of Jiroft hospital patients and showed that native plants can be used to fight bacteria. The results of the experiments of this study showed that the ethanolic extract of the peel of this plant had a significant antibacterial effect on three bacteria, *P. aeruginosa, S .aureus* and *E. coli,* which are the causative agents of burn wound infection in burn patients, and it had the greatest effect on *S. aureus*, which is consistent with the results of Razmjoo *et al*. (2016). The results of Razmjo *et al*. (2016), in the study of the antimicrobial effects of methanolic extract of orange peel and its effect on the shelf life of flavored milk determined that the methanolic extract of orange peel had a high inhibitory effect on the growth of *S. aureus*. Also, orange peel extract had the least effect on *E. coli*, which could be due to the lower effect of the extract on Gram-negative bacteria compared to Gram-positive bacteria. The results of Shamlou *et al*.'s research (2016) also showed that gram-positive bacteria are more sensitive to ethanolic extract of *Aloe vera* than gram-negative bacteria. It is believed that this phenomenon is due to the inherent tolerance of Gram-negative bacteria, due to the special structure of the cell wall and the strength of this wall compared to Gram-positive bacteria, as well as the nature and effective plant compounds (Ashook kumar *et al*., 2011).

The Kirby-Bauer method or disk diffusion test is used to identify an organism that is resistant or sensitive to a

specific antibiotic. Even this test in a very specific way can be used to determine how sensitive an organism is to a specific antibiotic. In clinical environments, the Kirby-Bauer test is a completely standardized and defined method. In order to obtain high concentrations of the effective substances in the extract, the extract must be evaporated at a suitable temperature that does not cause the decomposition of the active pharmaceutical ingredients and under low pressure and concentrated. Experience has shown that one kilogram of solvent is suitable for 200 grams of dry plants, that is, in a ratio of 1:5. Extracts whose solvent content is less than this ratio should be used with caution (Samsam Shariat, 1992).

According to the results, the sensitivity of gram-positive bacteria to the ethanolic extract of orang peel is more than gram-negative bacteria, which is thought to be due to the inherent tolerance of gram-negative bacteria or the special structure of the cell wall in gram-negative bacteria and the strength of this wall compared to gram-positive bacteria. effective plant compounds (Brooks *et al*., 2002).

E. coli is a gram-negative, motile, facultatively anaerobic bacillus without spores. In anaerobic conditions, this bacterium produces a mixture of acids such as lactate, succinate, ethanol, acetate and carbon dioxide (Madigan *et al*., 2006). The optimal growth of bacteria is at 37°C, but they can tolerate up to 49°C and continue to grow (Fotadar *et al*., 2005). *E. coli* can grow in aerobic and anaerobic conditions (Darnton, 2007).In the study of the antibacterial effect of ethanolic extract of orange peel on three bacteria, *P. aeruginosa, S .aureus* and *E. coli,* which are the causative agents of burn wound infection, it was shown that the effect of this extract on gram-positive microorganisms was more than gram-negative microorganisms. We performed experiments from a concentration range of 1.1 mg/ml to 300.12 mg/ml. Also, results showed that with increasing the concentration of orange peel extract, its antibacterial effect increased. Similar to Shamlou *et al*.'s research (2016), with increasing the concentration of orange peel extract, the droplet of bacterial growth or its antibacterial effect increased. The results of other studies show that the antibacterial properties of medicinal plants are greater at higher concentrations (Narimani and Moghaddam 2016). And, the results of the research on the antibacterial properties of the extract and essential oil of the medicinal plant *Echinophorasibthorpiana* on *E. coli* and *S. aureus* bacteria by Abbasi *et al*., 2016, showed that gram-positive bacteria are more sensitive than gram-negative bacteria to the essential oil of this medicinal plant. Also, the chemical compounds that make up the essential oil and extract of a specific plant in different regional, climatic, geographical and age conditions can affect the antibacterial properties of medicinal plants (Bagamboula *et al*., 2004). In general, the higher the levels of phenolic substances in the essential oil, the more their antibacterial properties will be against pathogenic bacteria.

Also, three antibiotics azithromycin (E-15), gentamicin (GM-10) and cefixime (CFM-5) were used for the experiments of this research. Tests showed that *E. coli* showed resistance to two antibiotics, azithromycin and cefixime. However, this bacterium has shown a halo with a diameter of 8.18 mm at the concentration of 36.37 mg/ml of orange peel. Gentamicin was the strongest antibiotic among the selected antibiotics, so that the diameter of the halo of non-growth by *S. aureus* (30.02 mm), *P. aeruginosa* (28.72 mm) and *E. coli* (25.03 mm), which showed resistance to two other antibiotics and cefixime is the weakest antibiotic among the selected antibiotics. So that *E. coli*, *P. aeruginosa* and *S. aureus* created a halo with diameters of 11.72, 4.26 and 17.23 mm, respectively. And the orange peel extract had a better antibacterial effect compared to cefixime and azithromycin antibiotics, so that the diameter of it extract at 300.12 mg/ml on *S. aureus* was 31.22 mm and the antibiotic cefixime and azithromycin were 17.23 and 27.52 mm, respectively.

According to the above, and the results of orange peel tests, it was found that we can use plant extracts instead of antibiotics and chemical drugs to fight dangerous bacteria. However, this research showed that in some cases, herbal medicines have a better effect than antibiotics. Not having severe side effects on patients' bodies and the low cost of providing them to patients can be acceptable reasons for using herbal medicines instead of antibiotics.

Suggestions

In the end, it is suggested to conduct research on the antibacterial properties of other plants on burn bacteria and to investigate the effect of this plant on other human pathogenic bacteria. In addition, in order to more confidently investigate the effect of these extracts on the body of living laboratory organisms, it is better to investigate these extracts.

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