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ZOUSH Ointment with the Properties of Antibacterial Moreover, Burn Wound Healing

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Abstract
Pseudomonas aeruginosa is a third hospital infection agent, and it is the second essential factor in wound infections. Despite many scientific advances in the treatment of burns, burn is one of the significant public health problems worldwide, particularly in developing countries. In this study, natural ointment ZOUSH was formulated with alcoholic extracts of *Satureja khuzestaniea, Zataria multiflora* and *Mentha Mozaffariani Jamzad* with Honey, and Polyurethane. A comparison between ZOUSH ointment and antibiotic was made by the disc diffusion method. PCR techniques carried out to confirm the genes exoS, lasA, and lasB, the housekeeping genes gyrA and fabD were used as a control. The burn wound was created by Ian Allen Holder method. The treatment was performed in 20 days, and the size of the burn was measured. Silver sulfadiazine ointment was considered as a positive control. The results indicated that 10, 13, 17 and 20 µg concentration of ZOUSH ointment have 12, 14, 15, 18 mm inhibition zone. Also, ZOUSH ointment can decrease the size of the wound and has wound healing properties. Due to the effectiveness of ZOUSH ointment on the size of burn wound and its antibacterial properties on *P. aeruginosa*, this ointment is recommended for the treatment of burn wounds.

Keywords *Pseudomonas aeruginosa* · Burn · *Satureja khuzestaniea* · *Zataria multiflora* · *Mentha Mozaffariani Jamzad*

Background

Pseudomonas aeruginosa is an opportunistic gram-negative bacterium. When the bacterium was entered into the host cell, it uses several virulence factors such as cell-associated factors and external factors. Factors associated with the cell include fimbria, flagellum, lipopolysaccharide (LPS), and Pyocyanin, which is contributing to adhesion of the bacterium to the host cells. LPS have endotoxin properties, and Pyocyanin catalyzes the production of toxic oxygen species such as superoxide and hydrogen peroxide (Cobb et al. 2004).

The most critical extracellular factors of Pseudomonas include exotoxin A, type III secretion proteins, exotoxin U, exotoxin S, exotoxin T, exotoxin Y, elastase, alkaline protease, and protease IV (Cobb et al. 2004). Biofilm formation is under the influence of the quorum sensing system (QS) (Karatuna and Yagci 2010).

Other proteases of this bacterium can be the enzyme serum protease and the metalloprotease enzyme. Proteases destructed physical defense systems by interfering with host defense mechanisms, and cause bacterial replication in tissues by eliminating elastin (Kessler et al. 1998, 2004).

On the other hand, exotoxin S is a two-function protein, the N-terminal end which is activated the effect of GTPase which destroys cytoskeletal actin and prevents from entering of the bacterium to the epithelial and macrophage cells, and the carboxylic terminus has a property of ADP ribosyltransferase (Chakotiya et al. 2016). This activity leads to the

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rearrangement of cellular actin proteins and ultimately leads to cell death. Exotoxin S is involved in phagocytosis modulation by phagocytes and induces invasions of non-phagocytic cells and programmed death of epithelial cells, lymphocytes, and fibroblasts, and it is the most crucial factor in the spread of infection in Burns (Aiello et al. 2010).

Despite the many scientific advances in burn treatment, burns are still a significant public health problem worldwide, especially in developing countries (Alaghehbandan et al. 2001; Darvishpour et al. 2007).

Pseudomonas aeruginosa is the most common cause of human diseases in the genus Pseudomonas. This bacterium is the most common cause of hospital infections and burns (Delden and Iglewski 1998), which is responsible for more than 75% of post-burn deaths (Sadikot et al. 2005). The location of deep and superficial wounds is a protein-rich medium containing necrotic tissue which provides a special place for colonization of microbes and their proliferation. *P. aeruginosa* can form biofilms, and also it can spread burn infections. Because of the presence of drug resistance genes and several virulence agents, it is difficult to treat this bacterium and threatens the risk of death for these patients (Church et al. 2006).

Pseudomonas aeruginosa is resistance to a variety of new antibiotics, which has led researchers to seek new methods, such as herbal medicine and safe drugs for the treatment and prevention of infections caused by *P. aeruginosa*. (Holder 2004).

Thyme and Satureja are not only used in traditional medicine but also due to phenolic compounds such as thymol and carvacrol which has antimicrobial activity on some fungal isolates has been proven (Soltani et al. 1998; Mozingo and Pruitt 1994; Abbasi et al. 2014). In Meskini et al. research, the inhibitory effect of *Satureja khuzestanica* against *P. aeruginosa* was reported (Meskini et al. 2015). Another study by Jalalvand et al. confirmed the impact of *Satureja khuzestanica* against the mexR and mexA genes in *P. aeruginosa*, which reduced the expression of these genes due to the presence of carvacrol compounds that is effective in reduction resistance *P. aeruginosa* by decreasing the expression of mexA (Jalalvandi et al. 2015). Carvacrol acts similarly to other phenolic compounds, which increases bacterial membrane permeability to H+, K+ and ATP reduction, and the effect on DNA and RNA leads to bacterial death (Manohar et al. 2001; El Gendy et al. 2015). Naturally, Thymol is the main phenolic component in Thyme and Carvacrol (Leung 1980). Antibacterial activity of *Zataria multiflora* is related to Thymol and Carvacrol too (Behravan et al. 2007).

The anti-bacterial and antioxidant activity of the *Mentha Mozaffariani Jamzad* has been proven due to phenolic compounds such as Carvacrol and Thymol (Lange and Croteau 1999; Moreno et al. 2002). The high antimicrobial activity of *Mentha* essential oil against a wide range of bacteria, including various species of *Staphylococcus, Pseudomonas, Bacillus*, and *Escherichia*, as well as some fungal species such as *Aspergillus, Fusarium*, and *Penicillium* are proved (Gulluce et al. 2007). In recent years, due to accelerating the process of wound healing by plant extracts and anti-microbial properties (Rahman and Hossain 2010), anti-inflammatory, analgesic and tissue regeneration of high-level polymeric methylene diisocyanate substrates due to increased surface-to-volume ratio, it is possible to release the extract over the course of burn treatment. Polyurethane was used to development of cell linkage which is destroyed by burnt (Vasita and Katti 2006). The aim of this study was formulation a new effective ointment can inhibit wound infection which is the creation by *P. aeruginosa*, and this ointment can heal wound which is a creation by burning.

Methods

Preparation of Herbal Extracts

Satureja khuzestanica is collected from Ahvaz, Khuzestan, Iran, Mentha Mozaffariani Jamzad is collected from Kermanshah, Iran, and Zataria multiflora is collected from Shiraz, Iran. The alcoholic extraction was prepared as a powder by Barij Essence, Kashan Iran.

Preparation of ZOUSH Ointment

The method for preparing ZOUSH ointment is described in another article (Meskini and Esmaeili 2018).

Disc Diffusion Method

Disc diffusion method was used to determine the inhibition zone of the ZOUSH ointment to compare it with Gentamycine 30 µg and Polymyxin B. For this purpose, and the following steps were taken: Muller Hinton Agar culture medium was prepared with sterile plates. An overnight culture of *P. aeruginosa* strain PAO1 was prepared in Pseudomonas agar medium. From overnight culture, Stoke McFarland (based on CLSI 2016) was made. Insert a sterile swab into a bacterium (Stoke of 0.5 McFarland) and press it to the wall of the tube to take its backup solution, then we will cultivate the swab into a Muller Hinton Agar medium. Different concentration of ZOUSH ointment stained on blank discs, and put on the culture media with the help of pence and observing the required distance. The plates incubated for 24–18 h at 37 °C. Then, measure the zone inhibition of grow's discs.

Molecular Methods

DNA Extraction Method

DNA extraction according to Promega kit steps was done as follows (Miller et al. 1988; Beutler et al. 1990; Safety and Administration 1991): Add 1 ml of overnight culture to a 1.5 ml microcentrifuge tube. Centrifuge at 13,000–16,000×*g* for 2 min to pellet the cells. Add 600 µl of Nuclei Lysis Solution. Gently pipet until the cells are resuspended. Incubate at 80 °C for 5 min to lyse the cells; then cool to room temperature. Add 3 µl of RNase Solution to the cell lysate. Invert the tube two to five times to mix. Incubate at 37 °C for 15–60 min. Cool the sample to room temperature. Add 200 µl of Protein Precipitation Solution to the RNase-treated cell lysate. Vortex vigorously at high speed for 20 s to mix the Protein Precipitation Solution with the cell lysate. Incubate the sample on ice for 5 min. Centrifuge at 13,000–16,000×*g* for 3 min. Transfer the supernatant containing the DNA to a clean 1.5 ml microcentrifuge tube containing 600 µl of room temperature isopropanol. Gently mix by inversion until the thread-like strands of DNA from a visible mass. Centrifuge at 13,000–16,000×*g* for 2 min. Carefully pour off the supernatant and drain the tube on clean absorbent paper. Add 600 µl of room temperature 70% ethanol and gently invert the tube several times to wash the DNA pellet. Centrifuge at 13,000–16,000×*g* for 2 min. Carefully aspirate the ethanol. Drain the tube on clean absorbent paper and allow the pellet to air-dry for 10–15 min. Add 100 µl of DNA Rehydration Solution to the tube and rehydrate the DNA by incubating at 65 °C for 1 h. Periodically mix the solution by gently tapping the tube. Alternatively, rehydrate the DNA by incubating the solution overnight at room temperature or 4 °C. Store the DNA at 2–8 °C.

PCR

In this step, the PCR molecular technique was used to confirm the *P. aeruginosa* strain PAO1 for presence exoS, lasA, and lasB genes.

Selection and Design the Primer

After reviewing the different sources of the primers, the primers were designed with Primer 3 plus and Genscript Taq Man software and blast performed in NCBI Blast software. To select and design specific primers for the diagnosis of the agent, consider some issues. Because of the diversity of primer sequences, to design the best primer, the sequence of identified genes in the genome databases is collected and then analyzed. The desired primers are compared by a set of analytical programs and hypothesized analogy with sequences recorded in the gene bank. After studying various papers, the design of the Forward and Reverse primers for the three exoS, lasA, lasB and Housekeeping DNA gyrA and fabD genes were performed.

After bioinformatics studies, the exoS gene primer with 144 nucleotides, the lasA gene primer with 67 nucleotides, the lasB gene primer with 78 nucleotides, the gyrA gene primer with 121 nucleotides and the fabD gene primer with 158 nucleotides were designed (Table 1).

PCR Reactions

According to the Pishgam protocol, PCR reaction was prepared in 25 µl total volume as follow: 12.5 µl Master Mix (1×), 1 µl (10 µm) F Primer (0/1–1 µm), 1 µl (10 µm) R primer (0/1–1 µm), 1 µl (20 pg) Template DNA, and 9.5 µl Sterile Deionized Water. The PCR reaction in the American Corbett Thermocycler was carried out in 35 cycles according to following steps: Initial Denaturation 95 °C for 5 min, Denaturation 95 °C for the 30 s, Annealing

Table 1 Oligonucleotide primers of exoS, lasA, lasB, gyrA and fabD

Name	Sequence	Strand	Position	Tm °C	Purification method
exoS:query_L1	GACCAAGCGCCATCACTTCG	Forward	552	59.11	HPLC
exoS:query_R1	CGGTAGAGAGCGAGGTCAGC	Reverse	676	59.17	HPLC
lasA:query_L1	CTCGCCGTTCCTCTTCGTCT	Forward	39	59.03	HPLC
lasA:query_R1	GCCATCGTCATGGGCATTGG	Reverse	86	59.16	HPLC
lasB:query_L1	AGTTTGGACACGTCGATCAG	Forward	264	58.4	HPLC
lasB:query_R1	GCTTGACCTGTTGTTCGTTG	Reverse	267	58.4	HPLC
gyrA:query_L1	GGTCTGGGCATAGAGGTTGT	Forward	220	56.8	HPLC
gyrA:query_R1	GAAGATCGAGGGTATTTCCG	Reverse	236	54.7	HPLC
fabD:query_L1	GCTCTTCAGGACCATTCTGG	Forward	774	59.8	HPLC
fabD:query_R1	ATCCCTCGCATTCGTCTTC	Reverse	931	60.2	HPLC

60 °C for 45 s, Extension 72 °C for 40 s, Final Extension 72 °C for 5 min. Electrophoresis performed after preparing the electrophoresis gel. For this purpose, 5 µl of each PCR product plus Loading buffer is poured into the wells and thrown electrophoresed with a voltage of 80 V about 30 min.

RT-PCR

For Sample Preparation and Cell Lysis we Pelleted 1×10^7 bacterial cells. Then discarded the supernatant and add 500 µl of Lysis Buffer (2-ME added). After that, we lysed the sample by repetitive pipetting or vortexing for 10 s. In column activation steps we placed a spin column into a 2 ml collection tube. Then added 100 µl activation Buffer into the Spin Column. Centrifuged at 10,000×*g* for 30 s. After that discard the flow-through. Immediately proceeded to next step. 3 Column Loading: we added 300 µl (or 0.6 × volume of the cell lysate) Isopropanol to the prepared lysate and vortex. Then, we transferred the mixture directly into the spin column. Centrifuge at 10,000×*g* for 30 s. Discard the flow-through.

Due to first Column Washing, we applied 700 µl of first washing buffer (ethanol added) to the Spin Column. Centrifuged at 10,000×*g* for 30 s. then discarded the flow-through. Due to second Column Washing: we applied 700 µl of second washing buffer (ethanol added) to the Spin Column. Centrifuged at 10,000×*g* for 30 s. then discarded the flow-through. Centrifuge again at 10,000×*g* for 2 min to remove residual ethanol.

Due to elution of RNA: we placed the Spin Column into a DNase/RNase-free microcentrifuge tube. Then, added 40–50 µl elution buffer to the center of the column membrane. Incubated at room temperature for 1 min. Centrifuged at 10,000×*g* for 1 min to elute the RNA and Stored RNA at − 20 or − 80 °C.

Due to elimination of remaining DNA: Remaining genomic DNA may be particularly a problem in subsequent RT-PCR or quantification of low-copy transcripts. For complete removal of gDNA from RNA preparations we used Jena Bioscience gDNA Removal Kit (Cat.-No. PP-219) (Stumpf et al. 2016). After RNA extraction, we used One step RT-PCR kit (2× HyperScript, GeneAll) which was used 20 µl reaction mixture under the following condition 10 µl of one step-RT PCR master mix, 1 µl (10 µm) of Primer Forward, 1.5 µl (10 µm) of Primer Reverse, 2 µl (20Pg) of total RNA,and 5.5 µl of D.D.W. The RT step conditions for all primer sets were 30–60 min at 42–60 °C, 94 °C for 2–5 min, 94 °C for 30 s, 60 °C for 30–60 s, 72 °C for 1 min, 72 °C and for 2–5 min (McCarthy et al. 2015).

Wound Burn and Treatment

The burn wound was created in the back of mice by Ian Allen Holder method. The grouping was previously described (Meskini and Esmaeili 2018). The procedure was performed in 20 days, at two times in the morning and evening for burned mice. Every 5 days, four mice were exited for testing. The wound size of all mice was measured and reported as average. In this step, silver sulfadiazine ointment was considered as a positive control.

Results

Disc Diffusion Method

Obtained results showed component of ZOUSH ointment agents (*Satureja khuzestaniea, Zataria multiflora, Mentha Mozaffariani Jamzad*, Honey, and Polyurethane) in different concentrations have the bigger zone of inhibition in contrast with a single, dual, and triple agent. The best zone of inhibition (the biggest) was measured for ZOUSH ointment with 20 µg dose. The other dose of the different component was reported in Chart 1. Statistical analysis showed that there was different significant between the the zone of inhibition of ZOUSH ointment in different concentration with the other groups.

PCR

The results of PCR shown that Amplicon is multiplied according to the Amplicon model designed with the software. The PCR results for exoS, lasA, lasB, gyrA, and fabD genes are shown in Fig. 1.

RT-PCR

The results of RT-PCR has shown the level expression of gyrA and fabD (House Keeping genes) didn't have any qualifications and semi-quantify difference before and after treatment with ZOUSH ointment, but the level expression of exoS, lasA, and lasB genes has been reduced (Fig. 2; p value < 0.05).

Chart 1 Inhibition zone of different concentration dose of mixed extracts as single, dual, triple and five components of ZOUSH ointment agents

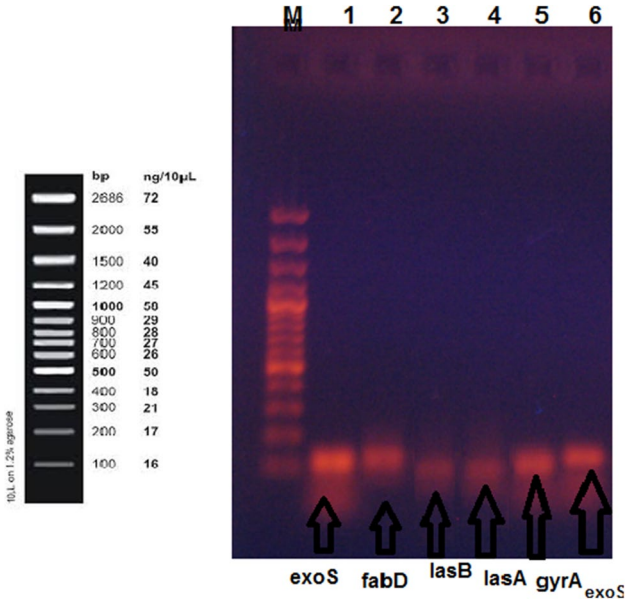

Fig. 1 The results of PCR of exoS, lasA, lasB, gyrA and fabD genes: well 1: exoS, well 2: fabD, well 3: lasB, well 4: lasA, well 5: gyrA and well 6: exoS. The results of PCR shown that Amplicon is multiplied according to the Amplicon model designed with the software. Positive control and negative control samples were used to control the PCR process

Wound Burn and Treatment

The average size of burn wound after treatment with Silver Sulfadiazine ointment and ZOUSH ointment was measured in 5, 10, 15, and 20 days. Obtained results showed the size of the wound is reduce in the 1st group which is treated with ZOUSH ointment, the size of the wound was 1 cm × 1 cm in the 1st day and reduce to 0 cm × 0 cm in 20th day, this means that the wound was an improvement in this group. The detail of wound size in the different day was reported in mm and showed in Table 2. Statistical analysis showed that there was significant difference between ZOUSH ointment and Silver sulfadiazine ointment wound healing in 5th, 10th, 15th, and 20th day of treatment (p value < 0.05).

Discussion

Prevention of *P. aeruginosa* in the hospital is difficult due to the inherent and acquired resistance of this bacterium to many antibiotics. Given the fact that the primary way of controlling infection is the use of antibiotics, today the emergence of MDR and XDR strains is a worldwide problem (Chakotiya et al. 2016). The incidence of these strains is increasing due to the selective inappropriate antibiotics pressure and increased dosage.

In this study, our results in disc diffusion method showed ZOUSH ointment agent with different concentration have more prominent zone inhibition than *Satureja khuzestaniea, Zataria multiflora, and Mentha Mozaffariani Jamzad as single, dual and triple*. The best zone of inhibition which is 18 mm (the biggest) was measured for ZOUSH ointment with 20 µg dose.

This zone inhibition was similar zone inhibition of Polymyxin B. Because the herbal extracts of this study have a high level of Carvacrol and Thymol which have the property of bactericidal effect (Meskini et al. 2015; Jalalvandi et al. 2015).

In this study, the Silver Sulfadiazine ointment was used as a positive control. According to the results obtained in the mean score of the wound burn size, as it is clear that the Silver Sulfadiazine ointment has a lower power in the mean reduction in the size of the wound burn relative to the ZOUSH ointment, So that in the treatment group with

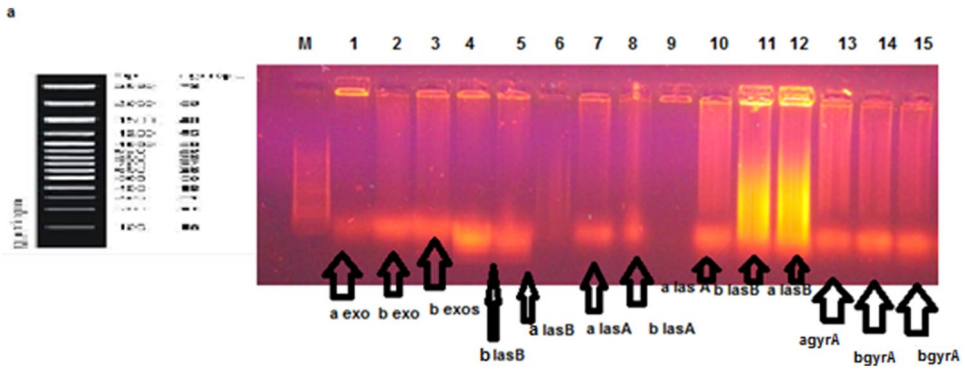

Fig. 2 The results of RT-PCR of exoS, lasA, lasB, gyrA and fabD genes: well 1: exoS, well 2: exoS, well 3: exoS, well 4: lasB, well 5: lasB, well 6: negtaive control (without any template), well 7: lasA, well 8: lasA, well 9: negtaive control (without any template), well 10: lasA, well 11: lasB, well 12: lasB, well 13: gyrA, well 14: gyrB, and well 15: gyrB. The results of RT-PCR has shown the level of expression of gyrA and fabD (House Keeping genes) didn't have any qualify and semi-quantify difference before and after treatment with ZOUSH ointment, but the level of expression of exoS, lasA, and lasB genes has been reduced

Table 2 Average size of burn wound after treatment with Silver Sulfadiazine ointment and ZOUSH ointment (Reported in mm)

Day	Silver Sulfadiazine ointment (Reported in mm)	ZOUSH ointment (Reported in mm)
0	1×1	1×1
5	0.6×0.6	0.55×0.6
10	0.6×0.8	0.3×0.4
15	0.3×0.5	0.25×0.4
20	0.2×0.2	0.0×0.0

a ZOUSH ointment after 20 days, the size of the wound has reached zero, which is meaning that the treatment is successful and the wound has completely recovered. In Nímia et al. studies emphasized that new dressings with and without silver show better results than Sulfadiazine for wound healing, this result is consistent with our research because the ZOUSH ointment has better effects on the treatment of burn wounds compared to Silver Sulfadiazine ointment (Nímia et al. 2018). An analysis that was done by Chacko et al. revealed that the wheatgrass extract is cost-effective and faster-wound healing and pain relief than silver sulfadiazine in subjects with 1–5% burns. This study, as well as the results of our research, showed to control the wound infection and to reduce the size of the wound and improve it, other ointments such as ZOUSH ointment can be used (Chacko et al. 2018). In the other research which is done by Aziz et al. the result revealed that wound could be depressed by Honey and these dressings promote better wound healing than Silver Sulfadiazine for burn wounds. In our study, Honey was also used as a wound healing agent and antimicrobial properties. The research also confirmed the results of our research and found that one of the substances that has a better effect on the treatment of burn wound compared to Silver Sulfadiazine is Honey (Aziz and Hassan 2017).

Conclusion

The disc diffusion results showed that *Satureja khuzestaniea, Zataria multiflora, Mentha Mozaffariani Jamzad*, Honey, and Polyurethane have positive effects on growth inhibition of *P. aeruginosa*. The antibacterial effects ZOUSH ointment compared with Gentamycine 30 µg, and Polymyxin B 300 µ. The In vitro results indicated that improvement in wound size and wound burn healing in the treatment group compared to control treatment groups (Silver Sulfadiazine ointment). Subsequently, ZOUSH ointment can be used as a candidate for the treatment of wound burns.

Abbreviations

ZOUSH Ointment: Herbal ointment ZOUSH prepared with alcoholic extracts of plants *Satureja khuzestaniea, Zataria multiflora, Mentha Mozaffariani Jamzad*, Honey, and Polyurethane were formulated.

exoS: Exotoxin S is virulence factor *P. aeruginosa* that contribute to spreading of the bacterium.

lasA and lasB: This code elastase that contributes to inhibition of the immune system.

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Data Availability All data and documents are available.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no competing interests.

Ethics Approval All experiments were conducted based on the License Number (BMSU1352308) on May 30, 2016, in the Baqiyatallah University of Medical Sciences Ethics Committee.

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