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HERBAL OINTMENT BLEND AND ANTIBACTERIAL ACTIVITY

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Abstract

Background and Aim*: Bacteria are the most important cause of infection worldwide. Patients with weakened immune systems, infants and the elderly are more prone to infection. Staphylococcus aureus and Pseudomonas aeruginosa are important causes of nosocomial infections and multidrug resistance. Due to drug resistance, the treatment of infections caused by them is facing serious problems. Wound infection is major problem in the all over the world and considering to fund new therapeutic especially herbal is increase, therefore the focus of this study was to evaluate the Antibacterial activity of herbal ointment blend on Staphylococcus aureus and Pseudomonas aeruginosa in vitro study.*

Materials and Methods*: In this study, extracts were first prepared from Ricinus Communis, Achillea millefolium, Calendula officinalis, Onosma dichroanthum Boiss. Then, to determine the amount of Minimum Inhibitory Concentration and Minimum Bactericidal Concentration, microdilution broth method was used. Then to investigate the synergistic effects of extracts by dilution in broth for each extract separately. After data collection, The Kappa coefficient test was used for data analysis, using SPSS 21 software, and the results were reported at the significance level of 0.05.*

Results*: Achillea millefolium have strange antimicrobial effect in Staphylococcus aureus and Calendula officinalis have great antimicrobial effect in Pseudomonas aeruginosa and Ricinus communis do not antimicrobial effect reported.*

Conclusion*: The results of the study indicate that Extracts of aqueous and ethanolic plants extracted from the plants of this study caused better antibacterial activity and can be use with compound of commercial drug to best therapeutic result.*

Keywords: *herbal ointment, ricinus communis, achillea millefolium, calendula officinalis, onosma dichroanthum boiss, antibacterial activity.*

Introduction

The skin is the body's first layer of protection and plays a major role in the body's protection against microorganisms, maintenance of homeostasis, and prevention of incursion. Traditional materials for wound healing process such as gauze and cotton bandages are so absorbent, protect wounds against hemorrhages and contact with the outside environment. But, changing bandages may cause hemorrhage, poor transpiration, and damages to the newly formed tissues. Wound drainage from these dressings may also lead to bacterial infections [*Harris-Tryon & Grice, 2022*]. An ideal dressing should be non-toxic, non-adherent, and no allergenic and should be

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produced from available biomaterial with antimicrobial attributes, which can improve the skin's wound healing like dressing which made from natural bio polymers [*Hadisi et al., 2020*] One of the most frequent and major complications in patients with wound injuries is the infection and is the main cause for prolonged in-hospital stay the colonization and infection of these wounds are a dual clinical problem [*Li et al., 2021*].

P. aeruginosa able to tolerate a variety of physical conditions and survive on minimal nutritional requirements. *P. aeruginosa* is one of the important pathogens that caused nosocomial infection, especially in immune suppressed patients like severe wound [*Mahmoudi et al., 2019*]. Selective antibiotic pressure led to emerging of acquired multidrug resistance in several countries in the past; and some multidrug resistant *P. aeruginosa* infections have been untreatable [*Pourmbarak, Mahmoudi, 2020*]. The most common human pathogen is *P. aeruginosa*; it is severe and is mainly the cause of hospital infections. Also, *S. aureus* has become one of the most important health problems in the world due to its potential pathogenicity and increasing resistance to antimicrobial drugs[*Zarei et al., 2018*]. Therefore, it is necessary to prevent the occurrence of infections caused by these bacteria and find the root of its spread in hospitals [*Mahmoudi et al., 2019*]. Mass consumption of antibiotics such as third-generation cephalosporins, macrolides and fluoroquinolones are factors that stimulate and produce antibiotic-resistant strains [*Azizi et al., 2022*]. Due to the increasing resistance of bacteria to some types of antibiotics, efforts have been made to obtain more information about the effective use of compounds in plants and their use in the treatment of various diseases [*Yaghootdoos et al., 2022*]. Among the different nations of the world, the ancient Egyptians should be considered the first nation that used medicinal plants in an amazing way. The oldest medical book of China, which contains a description of more than a hundred species of plants, is attributed to one of the emperors of that country named Shino, who lived about 2800 BC [*Metwaly et al., 2021*]. In the Middle Ages, the knowledge of medicinal plants spread from the monastery gardens to the urban communities, and in fact, Western countries started herbal medicine from the beginning of the Middle Ages. Among the old scientists of Iran who were pioneers in the field of herbal medicine, we can mention *Mohammad bin Zakariya Razi, Abu Ali Sina* [*Ghaffari et al., 2022*]. The reasons for extracting effective plant substances and purifying them and using them in pharmaceutical formulations can be summarized in the following cases

Treatment of wounds is one of the most fundamental issues that mankind has faced since the beginning of creation. Many medicines and ointments are used to repair open wounds, each of which has several shortcomings, limitations, and side effects [*Giannenas et al., 2020*]. Iran is considered one of the richest regions in the world due to the wide distribution of medicinal plants, as well as in terms of weather conditions, geographical location and the field of growth of these plants. Investigations on these plants in terms of their antibacterial properties provide a good basis that their results can be used to replace drugs of natural origin to control and treat bacterial infections and this can reduce the use of drugs [*Giannenas et al., 2020*].

Recently many studies considering herbal Potential to treatment wound and herbal ointment is suitable because easy to produce and cheap and user-friendly. Therefore, we aimed in this study to test the effect of *Ricinus communis, Achillea millefolium, Calendula officinalis, Onosma dichroanthum boiss* herbal ointment blend antibacterial activity.

Materials and Methods

Preparation and formulation of herbal ointment: For Preparation of herbal ointment, all plant samples were purchased and authenticated at the herbal medicines research center of Jahad Daneshgahi Ardabil, Iran. 100 g of powdered *ricinus communis, achillea millefolium, calendula officinalis, onosma dichroanthum boiss* was packed into a thimble and transferred into Soxhlet extractor with 0.5 L of 80% ethanol for about 72 h until the end point of extraction. The extract was harvested and concentrated in a rotary evaporator (50 °C) by separating the ethanol from the extract. The ethanolic extract was collected in the plastic container. To inhibit enzymatic degradation, air oxidation, and loss of active ingredients, the extract was stored at 10 °C in the laboratory. After extraction, the process of making the ointment continued by

preparing the ointment bases. The ointment bases containing *ricinus communis* (purchased from Jahad Daneshgahi Ardabil, Iran) and Bee Honey Wax (purchased from beekeepers in Ardabil, Iran) as the oily and waxy materials respectively were prepared by fusion method. Then, according to our traditional medicine practices, 20 g of Honey Wax were melted on water bath at 40°C. After that, 20 g of yellow, clear, and odorless *ricinus communis* was gradually added to the melted oily mixture with continuous stirring. They were melted together with constant stirring until congealing Honey wax and oil are homogenized. Then, 4% (v/v) of each *achillea millefolium, calendula officinalis, onosma dichroanthum boiss* extract was added to the homogeneous mixture and stirred. Finally, the whole homogenous ointment was prepared and stored in the sterilized container in cold condition until consumption.

Bacterial strains: Bacterial strains were obtained from a standard laboratory, Iranian Research Organization for Science and Technology (Tehran. Iran). The antibacterial activity of the ointments was investigated using the most common pathogens including *Pseudomonas aeruginosa* (PTCC 1310), *Staphylococcus aureus* (PTCC 1112). The type cultures of the above bacterial species were sub-cultured on Nutrient agar (Oxoid) and stored at 4°C until required for further studies. The antibacterial activity of the ointment was carried out using in-vitro susceptibility tests including agar well diffusion assay and resazurin microtitre-plate assay with determination of Minimum Inhibitory Concentration and d Minimum Bactericidal Concentration.

Agar well diffusion assay: The antibacterial activity of the herbal ointment at different concentrations against the growth of selected Gram-negative (*P. aeruginosa*) and Gram -positive *(S. aureus*) bacterial strains was carried out using agar well diffusion assay. Muller-Hinton agar (Merck, Germany) plates were directly inoculated with the bacterial suspension containing 1.5×10^8 CFU/mL (equivalent to 0.5 McFarland standard) from the bacterial stock cultures grown in Muller Hinton broth (Merck, Germany) medium at 37°C for 24 h. Five cylindrical wells of 6 mm in both depth and diameter were made on the Muller-Hinton agar plates. In this study, nine concentrations of the herbal ointment were prepared as follows 540, 270, 135, 67.5, 33.75, 16.87, 8.43, 4.21, and 2.1 *mg/mL* in such a way that a series of nine tubes make serial doubling dilutions of the ointment ranged from 100% to 0.39% (v/v). To prepare these concentrations, 1 ml of the stock solution made from the herbal ointment (54% w/v) was distributed into the tube 1 and 2. Then, 1 ml of sterile 10% dimethyl sulfoxide (DMSO; BDH, UK) was distributed in the tube 2 _9. After that, 1 *ml* of the contents of the second tube was sequentially mixed the 10% DMSO, achieving two-fold serial dilutions. The remaining 1 ml from the last dilution mix was discarded. All the contents of the tubes were sterilized by passing 0.45 mm pore size filters. The 25 _30 *ml* of each different concentration was then dispensed into the wells, allowed to stand for 30 min at room temperature, and then incubated at 37 °C for 18 _24 h. Afterward, the diameter of growth inhibition zone around each well was measured in millimeter (*mm*) using a metric ruler.

Microtitre-plate assay with determination of Minimum Inhibitory Concentration and d Minimum Bactericidal Concentration: The broth microdilution method was used to determine Minimum Inhibitory Concentration and Minimum Bactericidal Concentration. All tests were performed in Muller Hinton broth supplemented with Tween 80 (Sigma-Aldrich, USA). Briefly, serial doubling dilutions made from the herbal ointment ranged from 100% to 0.39% (v/v) were prepared in a 96-well microtiter plate. To prepare these concentrations, 100 *ml* of sterile Muller Hinton broth was distributed in the well 2 _12. Then, 100 ml of the stock solution made from the herbal ointment (54% w/v) was distributed into the well 1 and 2 and only 50 *ml* was added into the well 12. After that, 100 *ml* of the contents of the second well was sequentially mixed the Muller Hinton broth until the well 9, achieving two-fold serial dilutions. The remaining 100 *ml* from the contents of the well 9 was discarded. Diluted solution of the ointment was not added to the well 10 (control 1: cultured along with the bacterium without the ointment) and 11. Then, 10 ml of bacterial suspension containing 1.5×10^8 *CFU/mL* (equivalent to 0.5 McFarland standard) from the bacterial stock cultures grown in Muller Hinton broth medium at 37 °C for 24 h was added to all wells except the well 11 (control 2: cultured

without the bacterium and ointment) and 12 (control 3: cultured along with 50 *ml* of the ointment without the bacterium). Then, the samples were placed in an incubator (37°C, 15 h). After that, 30 ml of indicator solution (Resazurin 0.1%) was added to each well and followed by 4 h of incubation at 37 °C. The Minimum Inhibitory Concentration is defined as the lowest concentration of the stock solution made from the herbal ointment at which has no growth of the bacteria. Inhibition of bacterial growth was indicated by the color change which visually assessed. The Minimum Bactericidal Concentration of the herbal ointment against the selected bacterial strains was determined using the wells which had no visible growth of the bacteria when determining the Minimum Inhibitory Concentration [*Sheikhbagheri et al., 2022*]. From the wells with no growth of the bacteria, 10 *ml* was transferred to Brain Heart Infusion (BHI) agar plates. Then, the plates were placed in an incubator at 37 °C. After 24 h, the lowest concentration of the herbal ointment that showed not any colony growth on the BHI agar plates was determined as the d Minimum Bactericidal Concentration for the target bacterium [*Sheikhbagheri et al., 2022*].

Statistical analysis: Statistical analysis was performed using SPSS software. One-way ANOVA with Tukey post hoc test was used to comparing wound area and the percentage of wound contraction among the groups which expressed as mean ±SEM. Differences between the groups was considered significant at $P < 0.05$.

Results

Minimum Inhibitory Concentration and Minimum Bactericidal Concentration results

After the dilutions of Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of *Ricinus communis, Achillea millefolium, Calendula officinalis, Onosma dichroanthum boiss* extracts against *Staphylococcus aureus, Pseudomonas aeruginosa* were determined, then the Minimum Inhibitory Concentration and d Minimum Bactericidal Concentration values were converted to mg/ml with the dry weight obtained from the extracts. The value to inhibit the growth of bacteria, Minimum Inhibitory Concentration, in most of the mentioned groups is about half of the minimum Bactericidal concentration of bacteria. *Ricinus communis* No antimicrobial effect against *Staphylococcus aureus, Pseudomonas aeruginosa, but Achillea millefolium, Calendula officinalis, Onosma dichroanthum boiss* have effective antimicrobial activity. The result was shown in table1.

Ointment quantities: First, the dry weight of the extracts was measured, then ointments were made for all antibacterial substances. Ointments were prepared from extracts of *Ricinus communis, Achillea millefolium, Calendula officinalis, Onosma dichroanthum boiss* and silver sulfadiazidine antibiotic containing their Minimum Bactericidal Concentration per gram (*g*). In preparing the ointment for the treatment groups, the desired extracts with the final Minimum Bactericidal Concentration per 1 gram of silver sulfadiazidin ointment was used.

Discussion

Antibacterial and healing effect of the ointment prepared from the extracts of *Ricinus Communis, Achillea millefolium, Calendula officinalis, Onosma dichroanthum Boiss* along with the antibiotic silversulfadiazidin on the infection caused by *Staphylococcus aureus* and *Pseudomonas aerogenes*. The extracts of plant can have effects on *Staphylococcus aureus* and *Pseudomonas aerogenase*, and according to the results obtained, the Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of the mentioned substances for *Staphylococcus aureus* and *Pseudomonas aerogenase* are different. In this experiment,

Table 1:

Minimum Inhibitory Concentration and Minimum Bactericidal Concentration values for extracts (*mg/ml*)

Extracts	Minimum Bactericidal Concentration		Minimum Inhibitory Concentration	
	S aureus	P aeruginosa	S aureus	P aeruginosa
Ricinus Communis (*mg/ml*)	It doesn't	It doesn't	It doesn't	It doesn't
Achillea millefolium (*mg/ml*)	46.84	46.84	23.42	23.42
Calendula officinalis (*mg/ml*)	20.9	83.58	10.45	41.79
Onosma dichroanthum Boiss (*mg/ml*)	44.00	44.00	22.00	22.00

standard strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa* were used.

The antibacterial effect of mangrove leaf extract on *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* was investigated by Taj *Bakhsh et al.* (*2015), Sattari et al. (2006),* based on the results of the research they conducted on the antibacterial effect of eucalyptus extract, concluded that the crude alcoholic and aqueous extracts of eucalyptus can effectively prevent the growth of *Pseudomonas aeruginosa* and the reduction of *Pseudomonas aeruginosa* growth was seen in the experiments [*Sattari et al., 2006*].

In the present study, result show the antimicrobial activity of the mentioned substances and their increasing effects against *Staphylococcus aureus* as well as *Pseudomonas aeruginosa*. It is noteworthy that the bacteria used in this research were all hospital isolates and were resistant to most of the antibiotics used, and the prepared ointment was able to prevent the growth of these bacteria. Based on the results of the research in order to investigate the antimicrobial effects of herbal essential oils and antibiotics that we conducted, it was consistent with the results obtained in the present research and showed its effect on the tested bacteria. *Nayak et al.,* reported that 13% of *staphylococci* isolated from clinical sources with Minimum Inhibitory Concentration limits between 1-3 µg/ml were resistant to pine oil [*Nayak et al.,2015*].

Rutala et al., measured the d Minimum Bactericidal Concentration of 94 strains of *Staphylococcus aureus* against *pine oil* biocide. According to their tests, it was found that all the studied samples had an d Minimum Bactericidal Concentration of less than 0.05% (W/V) compared to the biocidal substance. The results obtained from Rotella's studies are somewhat consistent with the results obtained from this study [*Rutala et al., 2000*].

Trivedi *et al.*, showed that eucalyptus essential oil is very effective against resistant *Staphylococcus aureus* bacteria [*Trivedi & Hotchandani, 2004*].

In recent years, a lot of research has been done to evaluate the antimicrobial effects of essential oils and extracts, which indicates the power and ability of these compounds in preventing the growth of a wide range of microorganisms that cause diseases. Since these compounds are natural and somehow in many cases they contain other healthy compounds, therefore they are highly emphasized in order to maintain human health. Medicinal plants, having active medicinal and nutritional compounds, have always been of interest in botanical terms [*Swamy et al., 2016*].

It is important to point out that the traditional methods of using medicinal plants can show new ways to discover new and biologically active compounds along with medicinal plants with antibiotics.

CONCLUSION

Results indicated that topical application of the herbal ointment as a safe, inexpensive and easy to produce herbal agent has a rapid wound healing effect. Based on the results of this study, it can be concluded that this herbal ointment could be more effective therapeutic medicine as it is leaded to shorter treatment compared to the commercial topical ointments.

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