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Inhibition Test of Pomegranate Peel Extract (*Punica granatum*) Against the Growth of *Staphylococcus aureus* with In Vitro

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Abstract:
Background*: Staphylococcus aureus is a bacterium that contributes to the development of oral abscesses. If this oral abscess is not treated right away, odontogenic infection will develop. Antimicrobial drugs can be used to prevent oral abscesses. Antimicrobial substances made from natural materials are increasingly being used because they can make chemical drugs less effective due to side effects and drug resistance. Pomegranates are among the natural elements that may help prevent oral abscesses. Pomegranate peels contains active compounds that can be utilized by the community as a medicine for healing abscesses. It is widely known that pomegranate peel extract contains tannins, saponins, and flavonoids. The study's purpose was to determine inhibitory ability of pomegranate peel extract on the growth of Staphylococcus aureus.*
Materials and Methods*: This kind of study is a laboratory experiment with a post-test only control group design. The pomegranate peel extract was evaluated at concentrations of 3,125%, 6,25%, 12,5%, 25%, and 50% with DMSO as a control with four repetitions. Kirby-Bauer Disc Diffusion is the technique used to test the antibacterial efficacy.*
Results*: The Kruskal Wallis test findings revealed a p value of 0.001 on inhibition, indicating that pomegranate peel extract had the ability to stop Staphylococcus aureus bacteria from growing. The average value of the inhibition zone that resulted was 9.03 mm at 3.125% concentration, 9.63 mm at 6.25% concentration, 10.98 mm at 12.5% concentration, 15.03 mm at 25% concentration, and 18.3 mm at 50% concentration.*
Conclusion:*According to the study's findings, pomegranate peel extract can inhibit the growth of Staphylococcus aureus.*
Key Word*:Pomegranate peels, Staphylococcus aureus, Antibacterial.*

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I. Introduction

Staphylococcus aureus is a cocci-shaped and gram-positive bacterium that is extensively distributed in nature and found in the axillae, inguinal and perineal regions, and the anterior nostrils of humans. 25-30% of people carry *Staphylococcus aureus* in their nasal passages and on their skin. *Staphylococcus aureus* is disease-causing or pathogenic in humans[1].

According to the results of Riset Kesehatan Dasar in 2018 (Riskesdas), 45.3% of dental problems in Indonesia involve damaged teeth, cavities, and pain. Meanwhile, the majority of oral health problems experienced by Indonesians are swollen gums and abscesses of 14%[2]. Abscess is a localized infection that causes a chronic inflammatory disease. The collection of pus in the cavity is created by granulation tissue in response to a bacterial infection. Abscesses that have been developed but are not treated promptly will worsen and cause the patient to experience excruciating pain. *Staphylococcus aureus* is a type of bacteria that is frequently found in the oral cavity and is known to be a cause of abscesses. *S. aureus* is a Gram-positive bacterium that plays a crucial part in the invasion of the causative agent of oral abscesses, and it is a member of the Staphylococcus genus, which is the most prevalent pathogen[3].

Red pomegranate, scientifically known as *Punica granatum Linn*, is one of the plants that can be used as an alternative to reduce the usage of chemicals. Red pomegranate is the most well-known and an accessible kind. It is believed that the *polyphenols (flavonoids, saponins, and tannins*) and *alkaloids* found in red pomegranate have antibacterial properties. It is believed that this plant originated in Iran; nonetheless, it has been cultivated throughout the Mediterranean region for a very long time. Also commonly cultivated in South China and Southeast Asia[4].

Practically every part of the pomegranate plant is healthy, including the leaves, blossoms, fruit, root bark, etc. Pomegranate juice contains a high concentration of flavonoids, which are rich in anti-carcinogenic properties, specifically antioxidant compounds that can prevent the formation of free radicals in the body while repairing damaged cells. Capable of preventing cardiovascular disease, skin cancer, and prostate cancer. The leaves are useful as a laxative for women's menstruation, alleviating gas and pain. The fruits of pomegranate can heal gingivitis and bronchitis[4,5].

Polyphenols (flavonoids, ellagic acid, tannins, and ellagitanins) and alkaloids are the active components in red pomegranate that are hypothesized to serve as antibacterials and are obtained through the maceration extraction method with 70% ethanol. The active components in pomegranate have their own mechanism for suppressing bacterial growth, such as alkaloids reacting with amino acid molecules that compose bacterial cell walls and bacterial DNA[6].

The purpose of this research was to determine whether or not pomegranate peel extract (*Punica granatum*) inhibits the development of *Staphylococcus aureus* bacteria in vitro.

II. Material And Methods

The type of research conducted was laboratory experimental research with a post-test only control group research design. The purpose of this study is to analyze the effect of pomegranate peel extract concentration on the development of *Staphylococcus aureus* using the Kirby-Bauer Disc Diffusion method.The research phase marked approximately three months, from December 2022 to February 2023.

Study Design: Post-Test Only Control Group Design

Study Location: The study was carried out at the Laboratory of Microbiology in the Faculty of Pharmacy of University of North Sumatra

Study Duration: December 2022-February 2023

Sample size: Pomegranate peel extract with different concentration which is 50%, 25%, 12.5%, 6.25% and 3.125%*and DMSO (dimethyl sulfoxide) as control group*. Pomegranate were collected from Sei Mati, Medan Maimun, Kota Meda, Indonesia

Sample size calculation: The Federer formula was used to calculate the size of the experimental research sample.

Subjects & selection method: In this investigation, the test microorganism was a *Staphylococcus aureus* with ATCC25923 which culture grown on blood agar at the Laboratory of Microbiology in Faculty of Pharmacy, University of North Sumatra. Subjects which is *Staphylococcus aureus* must be pure, free from contamination and standardized with the ATCC code.

Inclusion criteria:

1. Pomegranate fruit that meets ripeness criteria (± 5-7 months after the blossom appears)
2. Fresh
3. Pests-free

Exclusion criteria:

1. Pomegranate that has pests
2. Pomegranate that has rotted

Procedure methodology

The cold extraction procedure known as maceration is used to prepare pomegranate peel extract. 80 grams of pomegranate peel simplicial were weighed, then 250 ml of 70% ethanol were added. Stir for the first six hours. Let at room temperature for 18 hours, stirring occasionally. The substance is then filtered using cotton, filter paper, and a filtering container.

Using 125 ml of 70% ethanol, repeat the extraction process on the dregs to yield macerate II. Combine the macerates together. To achieve a thick extract, evaporate the macerate at a temperature of 40°C using a Rotavapor.

Using a Bunsen flame, sterilize the inoculating loop and allow it to cool for some time. Then, introduce a sterile inoculating loop or syringe into the bacterial culture and transfer four to five isolated colonies of *Staphylococcus aureus* to a test tube. The test tube is put into the vortex mixer for one minute to ensure that the mixture is homogeny.

The heated and sanitized Nutrient Agar (NA) is then chilled to a temperature between 400 and 450°C. Thereafter, 0.1 mL of inoculum was placed in a sterile petri dish, followed by the pouring of 15 mL of liquid nutrient agar (NA) at 400-450°C. In addition, the cup was shaken on the surface of the table until the media and bacterial suspension were thoroughly combined, and the media was then allowed to settle.

Inhibition Test

1. Pomegranate peel extract and dimethyl sulfoxide solution are dripped onto sterile paper discs (control).
2. With tweezers, carefully remove the disc paper that has dripped.
3. Place it firmly on top of the nutrient agar that has been inoculated with bacteria, ensuring that the entire surface of the disc is in contact with the agar.
4. 5 discs containing the extract concentration were placed symmetrically on top of the agar in each petri dish.
5. Disc paper impregnated with control, namely dimethyl sulfoxide, is placed on each bacterium, and the petri dish is then labeled with label paper and markers.
6. The petri dish is then placed in the incubator and incubated at 37°C for 24 hours.

Statistical analysis

Data was analyzed using SPSS. Student's *Kruskal Wallis Test* was used to ascertain the significance of differences between mean values of two continuous variables and confirmed by nonparametric Mann-Whitney test. In addition, Kruskal Wallis Test shows the inhibition ability of each concentration while Mann-Whitney test was carried out to compare the concentration of 2 different group.

III. Result

Results revealed that pomegranate peel extract at doses of 3.125%, 6.25 %, 12.5%, 25%, and 50% inhibited the growth of *Staphylococcus aureus* with varying diameters of inhibition. In contrast, in the control group,which is DMSO did not discover the clear zone, indicating that it does not have antibacterial action against *Staphylococcus aureus*.

Table no 1 shows the diameter value of inhibition of pomegranate peel extract (*Punica granatum*) withconcentrations 3.125%, 6.25 %, 12.5%, 25%, and 50% and control group (DMSO) in suppressing the growth of *Staphylococcus aureus*.

Table no 1 : Diameter Value of Inhibition of Pomegranate Peel Extract (*Punica granatum*) Concentrations 3.125%, 6.25 %, 12.5%, 25%, and 50% and Control (DMSO) in Suppressing the Growth of *Staphylococcus aureus*.

Sample Group		Replication				Diameter Mean (mm)	SD
		1	2	3	4		
Pomegranate Peel Extract (*Punica granatum*)	3.125%	9.7	9.9	8.1	8.4	9.03	0.91
	6.25%	10.1	10.2	9.0	9.2	9.63	0.61
	12.5%	10.8	11.2	10.9	11.0	10.98	0.17
	25%	14.8	15.1	15.4	14.8	15.03	0.29
	50%	17.8	17.3	20.6	19.6	18.23	1.54
Control Group (DMSO)	0	0	0	0	0	0,00	0,00

Concentrations of 3.125%, 6.25 %, 12.5%, 25%, and 50% of pomegranate peel extract (*Punica granatum*) inhibited the development of *Staphylococcus aureus* bacteria as shown by a clear zone around the disc paper in nutrient agar. However, the result for each replication were not homogenic, indicating that statistic analysis will be carried out using the non-parametric test, Kruskal Wallis test and followed by Mann-Whitney test.

Table no2: Kruskal Wallis Test Results Inhibitory Ability of Pomegranate Peel Extract (*Punica granatum*) at Concentrations of 3.125%, 6.25 %, 12.5%, 25%, and 50%, and Control Group (DMSO)

Sample Group	N	Mean Rank	P value
3.125%	4	7.50	0.001
6.25%	4	9.50	
12.5%	4	14.50	
25%	4	18.50	
50%	4	22.50	
Control Group (DMSO)	4	2.50	

Table no3 : Mann-Whitney Test result of Pomegranate Peel Extract (*Punica granatum*) with Concentration 3.125%, 6.25%, 12.5%, 25%, 50% and DMSO

Comparison Between Samples		Mean Difference	Sig.
Concentration 3.125%	Concentration 6.25%	-2.00	0.248
	Concentration 12.5%	-4.00	0.021
	Concentration 25%	-4.00	0.020
	Concentration 50%	-4.00	0.021
	DMSO	4.00	0.014
Concentration 6.25%	Concentration 3.125%	-2.00	0.248

	Concentration 12.5%	-4.00	0.021
	Concentration 25%	-4.00	0.020
	Concentration 50%	-4.00	0.021
	DMSO	4.00	0.014
Concentration 12.5%	Concentration 3.125%	-4.00	0.021
	Concentration 6.25%	-4.00	0.021
	Concentration 25%	-4.00	0.020
	Concentration 50%	-4.00	0.021
	DMSO	4.00	0.014
Concentration 25%	Concentration 3.125%	-4.00	0.020
	Concentration 6.25%	-4.00	0.020
	Concentration 12.5%	-4.00	0.020
	Concentration 50%	-4.00	0.020
	DMSO	4.00	0.013
Concentration 50%	Concentration 3.125%	-4.00	0.021
	Concentration 6.25%	-4.00	0.021
	Concentration 25%	-4.00	0.021
	Concentration 50%	-4.00	0.020
	DMSO	4.00	0.014
DMSO	Concentration 3.125%	4.00	0.014
	Concentration 6.25%	4.00	0.014
	Concentration 25%	4.00	0.014
	Concentration 50%	4.00	0.013
	DMSO	4.00	0.014

Table no4 Shows that there is a significance difference between each group except group with concentration 3.125% and 6.25% ($p>0.05$). This indicate that both concentrations have effects and the ability of inhibiting the growth of *Staphylococcus aureus* but there is no better option when both groups are compared.

IV. Discussion

The purpose of this study was to determine the effectiveness of pomegranate peel extract in inhibiting the development of *Staphylococcus aureus* for 24 hours by measuring the diameter of the inhibition zone using a paper disc. This study was conducted in vitro utilizing the Disk Diffusion technique (Kirby-Bauer test) with nutrient agar. The Disc Diffusion method (Kirby-Bauer test) is conducted by placing a disk containing an antimicrobial agent on the test microbe-inoculated media. The clear area indicates the presence of antimicrobial chemicals on the surface of the agar media that inhibit the development of microorganisms. Based on the inhibition area, antibacterial strength is classified as follows: an inhibition area of 5mm or less indicates weak antibacterial strength, an inhibition area of 5-10mm indicates medium antibacterial strength, an inhibition area of 10-20mm indicates strong antibacterial strength, and an inhibition area of 20mm or more indicates very strong antibacterial strength.

This research implemented 6 treatment groups: group one with a concentration of 3.125 percent, group two with a concentration of 6.25 percent, group three with a concentration of 12.5 percent, group four with a concentration of 25 percent, group five with a concentration of 50 percent, and group six with DMSO as the control. This study tested each concentration of pomegranate peel extract and DMSO four times.

According to the findings of previous research, pomegranate peel extract can prevent the growth of *Staphylococcus aureus*. This is demonstrated by pomegranate peel extract concentrations of 3.125 percent, 6.25 percent, 12.25 percent, 25 percent, and 50 percent, but DMSO as a control did not exhibit any distinct zones on the blank disc. The clear zone indicates that antimicrobial chemicals on the surface of the agar media inhibited the growth of bacteria, specifically *Staphylococcus aureus.*

Based on the result, the first three concentration of pomegranate peel extract which is 3.125%, 6.25% and 12.5% were categorized as concentration with medium inhibition ability while extract with 25% and 50% were classified as a concentration with strong ability of inhibiting.According to these findings, pomegranate peel extract (*Punica granatum*) has an antibacterial property that can prevent the growth of *Staphylococcus aureus*.

According to earlier research conducted by Kholisa et al in 2018, pomegranate extract (*Punica granatum*) includes active components with antibacterial, antioxidant, and anti-inflammatory activities, including tannins, saponins, and flavonoids[6.]

The toxicity of tannins can destroy the bacterial cell membrane. Tannins are believed to exert their effect by contracting the cell wall or cell membrane, impeding the permeability of the cell. Hence, the cell cannot carry out its functions[7].In addition, saponin chemicals can limit protein production because they accumulate and cause harm to bacterial cell constituents[8].

Flavonoids serve as antibacterial by building complex compounds with extracellular proteins and dissolved proteins, damaging the bacterial cell membrane and triggering the release of intracellular chemicals. These flavonoid compounds are lipophilic, meaning they are toxic to cell membranes[9].

V. Conclusion

In vitro studies have shown that *Staphylococcus aureus* growth can be inhibited by pomegranate peel extract (*Punica granatum)* at concentrations of 3.125%, 6.25%, 12.5%, 25%, and 50%. The diameter of the inhibitory zone that forms around the disc paper increases according to the concentration of pomegranate peel extract. It may be concluded that pomegranate peel extract is capable of inhibiting the growth of *Staphylococcus aurues* bacteria due to the presence of antibacterial components in the form of tannins, saponins, and flavonoids..

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