Vol. 8 No. 2. September 2023

DENTINO

Jurnal Kedokteran Gigi

Terbit Setiap Maret dan September

TERAKREDITASI : No. 85/M/KPT/2020
DIREKTUR JENDERAL PENGUATAN RISET DAN PENGEMBANGAN
KEMENTERIAN RISET, TEKNOLOGI, DAN PENDIDIKAN TINGGI

Dentino. Vol. 8 No. 2. September 2023. P-ISSN 2337-5310. E-ISSN 2527-4937
Indexed Directory of Open Access Journals (DOAJ),
Sosialisasi Science and Technologi Index (SINTA)

Vol VIII. No 2. September 2023

P-ISSN : 2337-5310
E-ISSN : 2527-4937

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DAFTAR ISI

DENTINO
JURNAL KEDOKTERAN GIGI
Vol VIII. No 2. September 2023

THE INHIBITION TEST OF RED POMEGRANATE (*Punica granatum* L.) PEEL EXTRACT AGAINST *Staphylococcus aureus*

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ABSTRACT
Background: Staphylococcus aureus is a bacteria that commonly infects the oral cavity and linked to angular cheilitis. The most often used therapy for angular cheilitis is fusidic acid 2% cream, however it can cause urticaria, skin rashes, and irritation, thus an alternative is required to solve this issue. Pomegranate peel contains a variety of bioactive chemicals, including phenolics, which have antimicrobial activity. **Purpose:** To analyze the inhibition zone of red pomegranate peel extract at concentrations of 20%, 40%, 60%, and 80%, and determine the concentrations that have the greatest inhibition on the growth of Staphylococcus aureus. **Method:** The total phenolic content was measured using UV-Vis spectrophotometry method. The inhibition test was carried out by well-diffusion method with 6 sample groups, including positive control (2% fusidic acid), negative control (aquadest), and red pomegranate peel extract concentrations of 20%, 40%, 60%, and 80%. **Results:** Red pomegranate peel extract inhibited the growth of S. aureus at concentrations of 20%, 40%, 60%, and 80% with the inhibition zone diameter respectively 11.63 ± 0.88 mm, 13.84 ± 0.39 mm, 16.85 ± 0.58 mm, and 19.19 ± 0.43 mm **Conclusion:** Based on the results, red pomegranate peel extract at all concentrations have the capacity to inhibit the growth of S. aureus, with the highest concentration extract (80%) having the greatest inhibition.

Keywords : Inhibition Test, Phenolic, Red Pomegranate Peel Extract, *Staphylococcus aureus*,
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INTRODUCTION

Staphylococcus aureus is a bacteria that frequently infects the oral cavity; microbiological studies reveal that *S. aureus* and *Candida albicans* are the major etiology of Angular cheilitis. According to studies, 60% of angular cheilitis are caused by a combination of *S. aureus* and *C. albicans* infections.[1] *Angular cheilitis* is a common condition in children, with a prevalence of 0.2-15.1%.[2] The development of this disease is very fast and therapy must be administered immediately. Angular cheilitis is severe enough can cause pain when opening the mouth makes eating difficult and worsens malnutrition in individuals.[3]

The use of fusidic acid 2% cream is a common treatment for angular cheilitis. The usage of fusidic acid may result in urticaria, skin rashes, and irritation. Long-term fusidic acid usage also raises the chance of resistance.[4] Treatment with plant substances may be used as an option to overcome this issue. Natural antibiotics will be safer if utilised for an extended period of time.[5]

Pomegranate (*Punica granatum* L.) is a plant that is often used as a traditional medicine in treating gastrointestinal, cardiovascular and endocrine diseases.[6] Pomegranate cultivars in Indonesia are classified into three types based on colour differences: red, black, and white pomegranate cultivars. Pomegranate with red skin has a sweeter taste and contains more bioactive flavonoids than pomegranate with white skin. Flavonoids and polyphenols are found in red pomegranate which are effectively used as antibacterial.[4]

Almost every part of the pomegranate plant has antibacterial abilities, including the pulp, skin, and

seeds.[7] However, when compared to seed extracts, fruit juice, and pomegranate fruit, peel extract had the most antimicrobial activity.[8] Studies that have been carried out by many experts show that pomegranate skin is source of bioactive compounds such as phenolic compounds. Phenol in plants causes damage to the cytoplasmic membrane of bacteria and cause inhibition of growth and death of bacteria.[9]

Pomegranate peels have been shown in previous research to suppress the growth of *S. aureus*. The results indicated that at a very low concentration, 3%, the diameter of the inhibition zone created was 14.67 mm, placing it in the strong category.[10] Different results were obtained in other studies, it was concluded that at a greater concentration, 15%, the diameter of the inhibition zone was 7.5 mm, classified as the medium category.[11] Based on this background, researchers are interested in conducting further research to test the inhibitory ability of red pomegranate peel extract (*Punica granatum* L.) on the growth of *Staphylococcus aureus*.

MATERIAL AND METHODS

The research was carried out in an in vitro laboratory setting using a post test only group experimental design. The number of research samples used was 24 samples which were grouped into 6 treatment groups consisting of positive control (2% fusidic acid), negative control (aquadest), and Red Pomegranate Peel Extract concentrations of 20%, 40%, 60%, and 80 %. Each treatment is repeated 4 times based on the calculations of *Resource Equation Approach* formula.

Red pomegranate fruit was obtained from Pomegranate Plantation Mangaran, Situbondo while *Staphylococcus aureus* isolate obtained from Microbiology Laboratory, Faculty of Dentistry, University of Jember. The preparation of Red Pomegranate Fruit Extract (RPFE) was carried out at the Bioscience Laboratory of the RSGM University of Jember. The total phenolic content of RPFE was conducted at the Chemical Analysis Laboratory, Faculty of Pharmacy, University of Jember. The inhibition power of RPPE was conducted at Microbiology Laboratory, Faculty of Mathematics and Natural Sciences, University of Jember.

Ethical Approval

This research has been conducted through an ethical feasibility test by the Ethical Committee of Medical Research Faculty of Dentistry University of Jember with an ethical sertificate No.1791/UN25.8/KEPK/DL/2022.

Determination Test

Pomegranate identification tests were carried out at the Plant Laboratory, Politeknik Negeri Jember, while *S. aureus* bacteria were identified at the Microbiology Laboratory, Faculty of Dentistry, University of Jember.

The Making of RPPE

Fresh pomegranate fruits are washed and then separated from the skin of the fruit. Pomegranate skin is cut into small pieces and dried using an oven for 72 hours at 40°C. Dried pomegranate peels were blended and then filtered using a 60mesh sieve. The simplicia powder was then macerated using 70% ethanol at a ratio of 1:10. The filtrate was then filtered with Whatman paper and evaporated with a rotary evaporator.[12]

Total Phenolic Content Test

The total phenolic content of the extract was measured by UV-Vis spectrophotometry using standard gallic acid and Folin Ciocalteu reagent. The absorbance of gallic acid was measured and then a linear curve equation was made. About 30 mg of ethanol extract of pomegranate peel is diluted with 10 ml of 70% ethanol. As much as 0.5 ml of the sample was redissolved using 70% ethanol to 10 ml. 0.1 ml of this solution, added by 0.5 ml of Folin-Ciocaltaeau reagent then homogenized and left for 6 minutes. Then 0.4 ml of Na_2CO_3 solution was added and homogenized.[13] The solution was measured for its absorbance value with 5 times repetition. The phenolic content is expressed in units of mg Gallic Acid Equivalent/g extract (mg GAE/g extract).

Dilution of RPPE

The concentrated extract obtained from the rotary evaporator is a 100% concentration of red pomegranate skin extract. The extract was then diluted using distilled water to obtain concentrations of 20%, 40%, 60% and 80%.

Preparation of *Staphylococcus aureus* Suspension

The suspension was prepared by taking a *S. aureus* colony from the end of the loop wire and then dissolving it in 5 ml of NaCl solution before vortexing it. The turbidity of the suspension was then visually adjusted using the Mc Farland 0.5 standard.[14]

Preparation of Mueller Hinton Agar (MHA) Media and Bacterial Inoculation

MHA powder in the amount of 3.8 grams was dissolved using 100 ml of distilled water. The solution was stirred and heated to boiling and then sterilized in an autoclave.[14] 1 ml of bacterial suspension was inoculated into 15 ml of MHA media then poured into a petridish and waited for it to become solid.

The Inhibition Test of RPPE

The inhibition test was carried out with well diffusion methods. The solid media was then perforated using a 5.5 mm diameter cork borer. Each media has 6 holes. The wells that have been made are dripped with the test material, red pomegranate peels of various concentrations, positive control and negative control each of 20µL. The Petridish was closed again and incubated for 24 hours using an incubator at 37°C.

After 24 hours of incubation, the formation of an inhibition zone around the well was observed which was indicated by the presence of a clear area around the well. Calculation of the diameter of the inhibition zone

is carried out by measuring the vertical and horizontal diameters around the hole minus by the diameter of the well then summed and averaged.[15]

Data analysis was carried out with SPSS software by first carrying out the Shapiro Wilk normality test, then the Levene Test homogeneity test, then the non-parametric Kruskal-Wallis Test was carried out, followed by the Mann-Whitney Test.

RESULT

The total phenolic content of RPPE was tested using Folin-Ciocalteu reagent and standard gallic acid, measured using UV-Vis spectrophotometry. Table 1. shows the results of determining the total phenolic content of red pomegranate peel extract.

Tabel 1. Total Phenolic Contents of RPPE

Total Phenolic Contents	Percentage	Standard Deviation (SD)
402,2 mg GAE/g	40,2%	7,762

In determining the total phenolic content, five repetitions were carried out for the sake of data accuracy. Based on the table, the total phenolic content of red pomegranate peel extract was 402.2 ± 7.76 mgGAE/g, which means that in every gram of red pomegranate peel contains of phenolic content equivalent to 402.2 milligrams of gallic acid.

The extract inhibition test was carried out by observing the formation of a clear zone around the wells that had been dripped with the test substance. The measurement results in table 2 display the average diameter of the inhibition zone for each group.

Tabel 2. Average Diameter Inhibition Zone of RPPE

No	Groups	N	Average Diameter of the Inhibition Zone (mm) and Standard Deviation (SD)
1.	K (+)	4	21,33 ± 1,09
2.	K (-)	4	0
3.	KP1 (20%)	4	11,63 ± 0,88
4.	KP2 (40%)	4	13,84 ± 0,39
5.	KP3 (60%)	4	16,85 ± 0,58
6.	KP4 (80%)	4	19,19 ± 0,43

Table 2. shows the higher the concentration of extract resulted in the formation of a larger diameter of inhibition zone. The largest inhibition zone was found in the K(+) group, followed by the pomegranate peel extract group with concentrations of 80%, 60%, 40%, and 20%. The antibacterial content at high concentrations will cause the formation of a larger diameter of the inhibition zone. The formation of an inhibition zone was not found at K(-) because there was no clear area found around the well.

The data obtained was then tested with the Shapiro Wilk normality test and a significance value of >0.05 was obtained, which means that the research sample data was normally distributed. Then a homogeneity test was carried out with the Levene Test and a significance value of <0.05 was obtained, which means that the research data was not homogeneous, so the non-parametric Kruskal-Wallis Test was carried out, followed by the Mann Whitney Test.

The results of the Kruskal-Wallis test obtained an *Asymp Sig* value of < 0.05, which means that there was a significant difference in the average diameter of the inhibition zone for all treatment groups. After the Mann Whitney Test was carried out, the *p value* in each group was <0.05, which meant that there was a significant difference between all treatment groups.

DISCUSSION

The total phenolic content in the ethanol extract of red pomegranate peel obtained in the study was 402.2 ± 7.76 mg GAE/g extract. Previous research on Egyptian pomegranate showed that the total phenolic content of the aqueous extract of pomegranate peel ranged from 179.30 ± 4.82 mg GAE/g to 246.37 ± 4.61 mg GAE/g, whereas in methanol extract it ranged from 148.13 ± 2.99 mg GAE/g to 214.91 ± 3.29 mg GAE /g.[16] The difference in results in this study compared to previous studies could be due to differences in the solvents used during extraction. The polarity of the solvent used affects the total phenolic content of the extract. The polarity level of the ethanol solvent used in this study is likely to be close to the polarity of the phenolic compounds, allowing for greater extraction of phenolic compounds. Furthermore, semipolar plant cell walls are more readily disrupted by ethanol extracts, allowing bioactive phenolic chemicals to escape the cells more quickly.[17] Differences in geographical location, climate, also have a major influence on the bioactive compounds contained in pomegranates.[18]

Research was done proves that red pomegranate skin has the ability to inhibit the growth of *S. aureus* at concentrations of 20% to 80%. The results of the various tests across groups revealed that there was a significant difference at all concentrations. Concentration that has the greatest capacity to inhibit the growth of *S. aureus* is at 80%. Extracts with high concentrations will contain more antibacterial compounds causing the ability to inhibit bacteria to be more optimal.[19]

This study used a positive control of fusidic acid because fusidic acid is a narrow spectrum antibiotic that is often used in topical preparations for infections caused by *S. aureus*.[20] Fusidic acid 2% cream had a higher ability to inhibit *S. aureus* than the other treatment groups. This might be because fusidic acid is an antibiotic that has been proven to have antibacterial ability against *S. aureus* and is one of the antibiotics with high antibacterial activity. The bacteriostatic action of fusidic acid antibiotics is achieved by suppressing bacterial protein synthesis.[4]

After 4 times of replication and calculation of the average diameter of the inhibition zone in the study, the

ability of the antibacterial agent to inhibit *S. aureus* in red pomegranate peel extract with a concentration of 20% (11.63 ± 0.88 mm), 40% (13 .84 ± 0.39 mm), 60% (16.85 ± 0.58 mm), and 80% (19.19 ± 0.43 mm) classified as a strong category for inhibiting the growth of *S. aureus* because of the range of clear zone diameters formed is in the range of 10-20 mm.

The research results obtained showed differences with previous studies which concluded that concentrations of 15% to 60% the ability of the antibacterial agent of RPFE were classified as the medium category.[11] Differences in the inhibitory test methodologies utilised may have resulted in different research outcomes. In earlier research, the inhibition test technique was disc diffusion, however in this study, the inhibition test method was well diffusion. The well approach produced a larger inhibition zone than the disc diffusion method when testing the antibacterial agents capabilities. This is due to the osmosis process in the antibacterial agent that is put into the well, which happens more thoroughly due to direct contact with the agar media. [21]

This study findings are consistent with earlier investigations, which show that pomegranate peel extract has a strong antibacterial effect.[22] Research using imported Indian pomegranate samples proved that at a concentration of 21% the formation of an inhibition zone was 21.33 mm, while this study using Pomegranate grown in Indonesia showed that at a concentration of 20% the diameter of the inhibition zone was 11.63 mm. Different research results can be caused by the origin of the pomegranate skin preparations used. Indian pomegranate skin methanol extract has a phenolic content of 485 mg/g,[22] whereas in this study the phenolic content of pomegranate peel extract was 402 mg GAE/g extract. The greater the phenolic content will result in stronger antibacterial activity.[23]

Based on the results of the study it can be concluded that the diameter of the inhibition zone on the growth of *Staphylococcus aureus* in red pomegranate peel extract (*Punica granatum* L.) 20% concentration was 11.63 ± 0.88 mm, 40% concentration was 13.84 ± 0.39 mm, 60% concentration of 16.85 ± 0.58 mm, and 80% concentration of 19.19 ± 0.43 mm. Red pomegranate peel extract concentration of 80% has the greatest inhibition against the growth of *S. aureus*.

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