Time-kill assay of pomegranate (*punica granatum* L) seed ethanolic extract against streptococcus sanguis; the cause of recurrent aphthous stomatitis

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Abstract

Objective: To examine the antimicrobial activity of pomegranate seed using a time-kill-assay by measuring the number of bacteria left at a various time points after exposure to the substance.

Material and Methods: Pomegranate seeds were extracted by a maceration method using 70% ethanol as the solvent. The extract was evaluated for minimum inhibitory concentration and minimum bactericidal concentration against streptococcus sanguis ATCC 10556, using a microdilution method. The concentration used in time-kill-assay were 2 to 4 times MBC value and 48 hours incubation time as the initial value. Correlation of the concentration and time duration

using semi-log graph, i.e. the x-axis as time duration with variation interval of 30, 60 and 90-seconds. Y-axis is the logarithmic value of the bacterial colonies number.

Results: Bacteriostatic effect was observed at 4000 ppm concentration at 30, 60 and 90-seconds time intervals. The bactericidal effect was observed at 8000 ppm of concentration at the 90-second time interval, whereas 30 and 60-seconds remained bacteriostatic.

Conclusion: The pomegranate seeds ethanolic extract is considered as antibacterial with a concentration-dependent category.

Keywords: Pomegranate seeds, Streptococcus sanguis, Time-kill assay

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Introduction

The pomegranate (*punica granatum* L.) is a versatile fruit that has many benefits such as improving health and is used as a traditional medicine.¹ It contains high concentrations of phytonutrients and phytochemicals and it is rich in antioxidants and tannins. The main antioxidant in pomegranate is polyphenols, which contain flavonoids, tannins and vitamin C. Besides as antioxidant, flavonoids are antibacterial, antiviral, and anti-inflammatory too, while tannins have hemostatic, antibacterial and anti-inflammatory properties.²⁻⁴ The antibacterial flavonoid and tannin content in pomegranate is suspected to cope with streptococcus sanguis, also known as the bacteria that causes Recurrent Aphthous Stomatitis (RAS).

RAS, known as oral lesion, can occur at any part of the oral cavity accompanied with pain and may occur recurrently. It is a mild disease that is not life-threatening and can self-heal within 10-14 days without treatment but may interfere with eating and talking that can decrease the quality of life.⁵⁻⁷ The aetiology of RAS is still unknown, but there are some predisposing factors which were thought to play an important role. Those factors are local factors, allergy, bacteria, immune status, haematinic, hormonal and psychological stress.⁵⁻⁹

The number of RAS incidence is about 10-25% in a population. It is quite a large number, and therefore it is relevant to many researchers to find the best treatment for RAS.⁸ One of the bacteria suspected to cause RAS is streptococcus sanguis and currently, an antiseptic mouthwash can be used for the treatment of this condition.

Mouthwash will contact with the oral mucosa for 30-60-seconds whereas the in vitro evaluation of the MBC against bacteria streptococcus sanguis done in 48 hours. Therefore, this requires a time-kill assay to optimize the contact time of the test extracts and bacteria. Time-kill assays are also known as the "suspension tests or suspension time-kill analysis" and are used to determine the time required by the antimicrobial agent to kill the microorganism. It is used in microbiology to assess a test object's in vitro antimicrobial activity in relation to time.¹⁰⁻¹⁹

Material and Methods

This experimental research was carried out in the Laboratory of Chemistry Padjadjaran University. The pomegranate fruits were collected from the region of Cisarua Lembang, dried under the sun, mashed it into powder and extracted by maceration method using 70% ethanol as the solvent. The extract was evaluated for Minimum Inhibitory Concentration (MIC) and Minimum Bacterial

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Figure 1 A. Colonies of *S. sanguis* (1.3×10^6) after 30-seconds treatment with ethanol as the solvent, B. Colonies of *S. sanguis* (13×10^5) after 30-seconds treatment with pomegranate seeds ethanolic extract at the concentration of 4000 ppm (2x MBC), C. Colonies of *S. sanguis* (10×10^4) after 30-seconds treatment with pomegranate seeds ethanolic extract at the concentration of 4000 ppm (2x MBC), D. Colonies of *S. Sanguis* (3×10^4) after 60-seconds treatment with pomegranate seeds ethanolic extract at the concentration of 4000 ppm (2x MBC), E. colonies of *S. sanguis* (2×10^5) after 60-seconds treatment with pomegranate seeds ethanolic extract at the concentration of 4000 ppm (2x MBC), F. Colonies of *S. sanguis* (6×10^4) after 90-seconds treatment with pomegranate seeds ethanolic extract at the concentration of 4000 ppm (2x MBC), G. Colonies of *S. sanguis* (6×10^5) after 90-seconds treatment with pomegranate seeds ethanolic extract at the concentration of 4000 ppm (2x MBC), H. Colonies of *S. sanguis* (11×10^5) after 30-seconds treatment with ethanol as the solvent, I. Colonies *S. Sanguis* (1×10^5) after 30-seconds treatment with pomegranate seeds ethanolic extract at the concentration of 8000 ppm (4x MBC), J. Colonies *S. sanguis* (1×10^4) after 60-seconds treatment with pomegranate seeds ethanolic extract at the concentration of 8000 ppm (4x MBC), K. Colonies *S. sanguis* (1×10^4) after 90-seconds treatment with pomegranate seeds ethanolic extract at the concentration of 8000 ppm (4x MBC)

concentration (MBC) against streptococcus sanguis ATCC 10556, using a microdilution method through a 96-well microplate. Dilution was carried out in stages starting from 8000 ppm concentration. The obtained MBC value was the benchmark concentration for the time-kill-assay.

The extract was then prepared in concentrations 2 to 4 times the MBC value for the time-kill-assay. Samples were incubated at 37°C for 48 hours in an anaerobic state. This procedure was carried out for 30 sec, 60 sec and 90 sec, and then colonies of the growth bacteria were counted.

The intention is for the substance to be used as a mouthwash, therefore the duration of action for the test subject was planned accordingly. It was required to utilize a specific method that will represent the closest simulation on various exposure times.

To find out the correlation between concentration and time duration, semi-log graphs were used, i.e. the x-axis as time duration, variation interval of 30, 60 and 90-seconds. Y-axis is logarithmic value of the number of bacterial colonies. The results were the number of bacterial colonies present after an exposure at each concentration and each time interval.

Results

MIC of ethanolic extract pomegranate seeds against *S. sanguis*; pomegranate seeds ethanolic extract have bacteriostatic activity against bacteria streptococcus sanguis ATCC 10556 with a MIC of 2000 ppm (at the 5th dilution). The MBC value of pomegranate seeds ethanolic extract against bacteria streptococcus sanguis was 2000 ppm, it showed that no occurrence of bacterial growth in the media at the minimum concentration.

Time-kill assays of 4000 ppm and 8000 ppm ethanolic extract pomegranate seeds; to determine the duration of action for the test subject that relevant with the intention for the substance to be used as a mouthwash, then it was required to utilize a specific method that will represent the closest simulation of the various exposure times.

Figure 1 shows the colony count of *S. sanguis* on blood agar plates. Plate A and H reveal the ethanolic solvent as solvent control colony of *S. sanguis* with the result was 10^6 and similar result reveals by the negative control as well. Plates B and C show the bacterial colony count after the 30-second intervention of time-kill assay for concentration of the sample 4000 ppm (2xMBC) was 10^5 . Plates of D and E show the 60-second intervention of 4000 ppm with a result $10^{4.5}$ and plates F and G show the 90-second intervention at the same concentration with $10^{4.5}$ *S. sanguis* colony as a result.

The higher concentration, which is 8000 ppm as 4xMBC was tested on plate I, J and K figure 1, in

Figure 2 Linear regression comparison of the *S. sanguis* colonies count (CFU/mL) after 30s, 60s and 90s exposure time of the pomegranate seeds ethanol extract with 4000 ppm, 8000 ppm concentration and the negative control

Table 1 The colony count of the *S. sanguis* on the blood agar medium for 4000 ppm and 8000 ppm based on the time of exposure interval 30s, 60s and 90s

Time	Control (-)	Solvent Control	4000 ppm	8000 ppm
0	6.0	6.0	6.0	6.0
30	6.0	6.0	5.0	5.0
60	6.0	6.0	4.5	5.0
90	6.0	6.0	4.5	4.0

sequence 30, 60 and 90-second. The results show 10^5 , 10^4 , 10^4 *S. sanguis* colony count in sequence. Based on the colonies count of the *S. sanguis* on the blood agar medium before, the data recapitulation is summarized in table 1.

The table above shows duration of time contact between the *S. sanguis* and samples in sequence 30, 60 and 90-seconds. Then it was illustrated as a linear regression graph to clearly depict the decrease of the colonies count from each concentration and time of exposure as seen in figure 2.

Figure 2 shows linear regression as a way to illustrate a comparison of the *S. sanguis* colonies count (CFU/ml) after 30s, 60s and 90s exposure time of the pomegranate seeds ethanol extract with 4000 ppm, 8000 ppm, and the negative control. Based on figure 2, the negative and solvent controls are shown as a straight line at the same level, which depict the same results. However, the 4000 ppm (2xMBC) with a green line and 8000 ppm (4xMBC) with a purple line, show different regression. The 90-second contact duration time with 8000 ppm (4xMBC) shown the lowest *S. sanguis* colony count, which is 10^4 as a result.

Discussion

A time-kill assay at 4000 ppm and 8000 ppm ethanolic extract pomegranate seeds was used to determine the duration of action for the test subject

with the intention for the substance to be used as a mouthwash. This required a specific method that will represent the closest simulation of various exposure times.

The time-kill assay uses concentrations of 2 to 4 times of incubation time MBC 48 hours as the initial value. To find out the correlation between concentration and time duration, a semi-log graph was used, i.e. the x-axis as time duration, variation interval of 30, 60 and 90-seconds. Y-axis is logarithmic value the number of bacterial colonies.

The X value is the number of colonies of *S. sanguis* (CFU/mL), with the value of the initial colonists prior to treatment (the interval of 0-seconds) is in log 10^6 . Through a series of time intervals of 30, 60 and 90-seconds, decrease in the number of results retrieved the colony to 4000 ppm concentration (2xMBC) i.e. the decline rate of $1.5 \times \text{Log}_{10}$ to interval duration of 30, 60 and 90-seconds, whereas in concentration 8000 ppm (4xMBC) obtained a decrease of up to 2 levels of Log_{10} in the interval duration of 90-seconds.

The pharmacodynamic aspects of antibacterial drugs include the nature of bacteriostatic or bactericide and also time-dependent or concentration-dependent. When a decrease of the number of bacterial colonies is $\geq 99.9\%$ (two levels decrease of Log_{10}) then the antibacterial effect can be categorized into the bactericidal, but if it is less, than these values are categorized as bacteriostatic. Time dependency is a category where the antibacterial effect is not affected even though the value of concentration continues to be raised. Only the duration of working time can affect the antibacterial effects. Concentration-dependency is a category for antibacterial activity that is unaffected by the duration of working time, which acts thus only based on the increase in concentration.

Based on the results of the time-kill assay obtained through linear regression graphs, the antibacterial test results clearly illustrated of its efficacy. At 4000 ppm concentration, it is considered as bacteriostatic on the duration interval of 30s, 60s and 90s. Hence, for the 8000 ppm concentration, it was obtained that the existence of the effect of bactericide starts from the duration interval of 90-seconds, whereas at 30 and 60-seconds fixed categories include bacteriostatic. These finding suggest that the ethanol extract of pomegranate seeds was determined in the category of antibacterial concentration-dependent.

Conclusion

The present study was set out to examine the antimicrobial activity of pomegranate seed using a time kill assay, by measuring the number of bacteria

left at a various time points that were relevant to the exposure time using by topical drug administration in the oral region. This study has identified that the ethanol extract of pomegranate seeds are a concentration-dependent antibacterial and proven for its potential. The implication of this finding suggests for further research for its potential efficacy of the substance on recurrent aphthous stomatitis therapy.

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Conflict of Interest

All authors confirm that there is no conflict of interest associated with this publication.

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