Inhibition growth of pomegranate seeds extract against streptococcus sanguis: the cause of recurrent aphthous stomatitis

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Abstract

Objective: Pomegranate (*punica granatum* L.) seeds contain high of phytonutrients and phytochemicals, rich in polyphenol antioxidants namely tannins and flavonoids which also have antibacterial activity. *Streptococcus sanguis* is a bacterium known as one of the factors causing Recurrent Aphthous Stomatitis (RAS). To examine the potential antibacterial of pomegranate seeds against *S. sanguis*.

Material and Methods: In vitro study of pomegranate seed were extracted with maceration method using 70% ethanol as the solvent to obtain stable extract, continued with phytochemical screening against phenolic, flavonoids, alkaloids, steroids, triterpenoid, saponins and tannins. The extract was evaluated for Minimum Inhibitory

Concentration (MIC) and Minimum Bactericide Concentration (MBC) against *S. sanguis* ATCC 10556, using microdiluted method through 96 wells microplate.

Results: Chlorhexidine was used as positive control while 70% ethanol was used as solvent as well as negative control. Phytochemical screening gave positive results for phenolics, flavonoids, steroids, saponins and tannins. Microdilution test showed the concentration of 500 ppm as MIC and MBC value at 2000 ppm.

Conclusion: Pomegranate seeds extract have a growth inhibitory against *S. sanguis* with MIC value of 500 ppm and 2000 ppm as MBC.

Keywords: Pomegranate seeds, *Streptococcus sanguis*, Recurrent aphthous stomatitis

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Introduction

Recently people start to choose herbs as medicine to cure various diseases because it is affordable and also quite efficacious. Pomegranate (*punica granatum* L.) is a long-living tree native from the Middle east, cultivated in the subtropical to the tropic regions.¹ It has many benefits for health and usefulness as traditional medicine, used in several systems of medicine for a variety of ailments. In Indonesia as one of the country in the tropic region, Pomegranate is easy to grow in the home yard as well as an ornamental plant at the lowland to below 1000 m dpi region.

Pomegranate is a very versatile fruit which can be directly eaten fresh, or made as juice. It contains of high phytonutrient and phytochemical as well as rich in tannin antioxidant. Antioxidant is very beneficial for maintaining health and treating diseases. The main antioxidant in pomegranate is polyphenol which contains flavonoids, tannins and vitamin C. Besides as antioxidant, flavonoids, tannins also have antibacterial action. Jurenka¹ stated the therapeutically beneficial pomegranate constituents are ellagic acid ellagitannins (including punicalagins), puniceic acid, flavonoids, anthocyanidins, anthocyanins and estrogen

flavonols and flavones.¹ In Ayurvedic medicine the pomegranate is considered as "a pharmacy unto itself" and is used as an antiparasitic agent, a "blood tonic" and to heal aphthae, diarrhea and ulcers.¹ The MIC of adherence of pomegranate against *S. mitis*, *S. mutans*, *S. sanguis* and *C. albicans*.²

In daily life there are certain people who often have lesions in their mouth and people call it as aphthae. Medically this lesion known as Recurrent Aphthous Stomatitis (RAS), can occur at any part of the oral cavity accompanied with pain and may occur recurrently. It is a mild disease and not life threatening, self healing within 10-14 days without treatment but may interfere with eating and talking that can decrease the quality of life.³⁻⁵ Scully⁶ stated that the number of RAS incidence of about 10-25 % in a population. It is a quite large number, so it is relevant if many researchers tried to find the best treatment for RAS.⁶

The etiology of RAS is still unknown, but there are some predisposing factors which were thought played an important role. Those factors are local factors, allergy, bacteria, immune status, haematinic, hormonal and psychological stress.

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One of the bacteria suspected as the cause RAS is *S. sanguis*.^{6,7}

Until now only a few studies about pomegranate were reported, especially the study of the seeds as a drug that can inhibit the growth of bacteria *S. sanguis* in RAS. The purpose of this study was to find out the MIC and MBC of pomegranate seeds extract against bacteria in RAS *S. sanguis*.

Material and Methods

Pomegranate (*punica granatum* L.) fruits used in this study were obtained from the farmers' gardens in Cisarua Lembang, Indonesia. Standard *S. sanguis* (ATCC 10556) strains were used. The study was conducted at The Chemical Laboratory, Padjadjaran University. The process was started with preparing the material and plant determination i.e dried the pomegranate seeds under the sun, mashed it into powder and subjected to extraction with 70% ethanol. The powder were soaked in 70% ethanol as the solvent for 24 hours, the macerat were screened then evaporated with the rotary evaporator to obtained a stable extract. Then it was continued with phytochemical screening against phenolic, flavonoids, alkaloids, steroids,

triterpenoid, saponins and tannins. The next step was to determine the MIC and MBC of pomegranate seeds extract against *S. sanguis* ATCC 10556 through several stages i.e bacteria rejuvenation procedures, made the Mueller Hinton broth, determined the Optical Density (OD), antibacterial test with Disk Diffusion Test. The determination of MIC was carried out using microdiluted method through 96 wells microplate. Every two rows of wells duplodata was used namely media and samples in series 1 and 2. The media and the solvent on a series 3 and 4 media, sample and bacteria *S. sanguis* in series 5, 6 and on series 7, 8 were filled with media, solvents and the bacteria *S. sanguis*. Chlorhexidine was used as positive control while 70% ethanol as the solvent as well as negative control.

Results

The weight of pomegranate seeds powder sample was 364 gram, after three steps of maceration process with 70% ethanol, 37.14 gram of extract were obtained. The depreciation value was 10.2 %.

Phytochemical tests was performed after Pomegranate seed extract were obtained. It was proposed to identify the content of secondary metabolites which was presented in the sample. The phytochemical screening showed positive result for phenolic, flavonoids, steroids, saponnins and tannins [table 1](#).

The next step was assessing the extract for the MIC and MBC against *S. sanguis*. The result of inhibitory test showed that there was antibacterial activity marked by the formation of inhibition zone against *S. sanguis* [table 2](#) and [figure 1](#).

According to the above table and figure, there were inhibition zones on pomegranate seed extract sample at the concentration of 20.000, 10.000 and 5000 ppm. 70% ethanol as the solvent was also act as negative control while chlorhexidine as positive control showed the inhibition zone at the concentration of 2000 ppm.

The results showed that there was inhibition activity against *S. sanguis* of each concentration. That was the reason why it was necessary to do MIC test as the next step of anti-bacterial test. The MIC test was conducted using microdilution method through 96 well microplates. Every two rows of the wells using duplodata, which was media and sample in rows 1 and 2. The Media and solvent were in rows 3 and 4. The media, sample and *S. sanguis* bacteria were in rows 5 and 6. The media, solvent and *S. sanguis* bacteria were in rows

Table 1 Screening of secondary metabolites

No.	Secondary metabolites	Test method	The results
1.	Phenolic	FeCl ₃ 5% Reagent	+
2.	Flavonoids	HCl conctr + Mg Reagent	+
		H ₂ SO ₄ 2N Reagent	+
		NaOH 10% Reagent	-
3.	Alkaloids	Dragendorf Reagent	-
4.	Steroids	Lieberman-Burchard Reagent	+
5.	Triterpenoids		-
6.	Saponnins	HCl + H ₂ O Reagent	+
7.	Tannins	FeCl ₃ 1% Reagent	+

Figure 1 Antibacterial test analysis of Pomegranate seeds extract against *S. sanguis*

Table 2 Antibacterial test analysis of pomegranate seeds extract against *S. sanguis*

No	Sample & Concentration (ppm)	Inhibitory Diameter, (d/mm)	D Average (mm)		Note
1	Punica granatum L. Seed Extract (20.000)	8.40	8.15	8.28	Active
2	Punica granatum L. Seed Extract (10.000)	8.10	8.70	8.40	Active
3	Punica granatum L. Seed Extract (5.000)	8.10	7.80	7.95	Active
4	Control Negative: Ethanol solventPositive	-	-	-	Not Active
5	Control: Chlorhexidine (2.000)	11.30	11.40	11.35	Susceptible

Table 3 MIC analysis of pomegranate seeds extract against *S. sanguis*

Well	Concentration (ppm)											
	8.000	4.000	2.000	1.000	500	250	125	62,5	31,25	15,63	3,81	3,91
Media + Sample	0.712	1.703	1.178	0.849	0.559	0.324	0.183	0.107	0.072	0.056	0.051	0.045
	0.574	1.237	1.013	0.601	0.368	0.218	0.134	0.084	0.061	0.051	0.045	0.043
Media + Solvent	0.042	0.039	0.038	0.041	0.040	0.042	0.040	0.040	0.040	0.043	0.041	0.041
	0.043	0.038	0.038	0.041	0.040	0.041	0.041	0.043	0.043	0.043	0.041	0.040
Media + Sample + Bacteria	0.623	1.045	0.890	0.596	0.367	0.216	0.134	0.100	0.095	0.092	0.083	0.079
	0.656	0.737	0.890	0.658	0.410	0.250	0.151	0.112	0.106	0.094	0.086	0.074
Media + Solvent + Bacteria	0.043	0.042	0.042	0.043	0.047	0.087	0.115	0.111	0.105	0.108	0.110	0.109
	0.045	0.044	0.044	0.046	0.047	0.084	0.095	0.105	0.101	0.106	0.110	0.107

Figure 2 MBC value of pomegranate seeds extract against *S. sanguis*

7 and 8. Dilution carried out in stages, starting from the concentration of 32.000 ppm.

In [table 3](#), along with the decrease in extract's concentration by the multiple of $\frac{1}{2}$, there was a growing in the number of bacteria at the concentration of 250 ppm for wells consisting of media and sample. This condition provides that the value of MIC was before the concentration of 250 ppm, i.e. at concentration of 500 ppm. Wells consisting of *S. sanguis* in media and solvent showed that there was *S. sanguis* growth at the concentration of 1000 ppm,

it gave information that solvent had influence in *S. sanguis* growth inhibition by MIC 2000 ppm.

[Figure 2](#) the MBC determination test of Pomegranate seeds extract against *S. sanguis* showed there was still a growing of bacterial colonies at the concentration of 250, 500 and 1000 ppm, whilst in the subsequent concentration (2000 ppm) found no bacterial growth. It could be determined that MBC for pomegranate seeds extract against *S. sanguis* was on the value of 2000 ppm [table 2](#).

Discussion

The important phytochemical test was conducted after the maceration process with 70% ethanol and pomegranate seeds extract were obtained. It is necessary to identify the content of the extract. The phytochemical screening results proved that pomegranate seeds extract contains of phenolics, flavonoids, steroids, saponins and tannins. The phenolics and flavonoids group have been known of its antibacterial activity. The former study stated that antimicrobial activity from 6 variety Pomegranate were correlated with the respond of phytonutrient substance, such as total phenolics and anthocyanins compound.^{8,9}

Phytochemical screening of pomegranate peel and leaf indicated alkaloids, tannins, sterol, volatile oil, carbohydrate, flavonoids, glycosid, resin, balsams, terpenes and Free-Reducing Sugar but

saponins was undetectable. The content of these metabolites substances showed great potential as medicinal plant.^{10,11}

The result of phytochemical test pomegranate seeds extract as a guide to do the next test i.e the test for *S. sanguis* inhibition. The phenolics and flavonoids group have been known to have antibacterial activity, that is why the result of phytochemical test could support if there were antibacterial properties during inhibition test (disk diffusion), MIC and MBC.^{12,13} MIC of an antimicrobial extract was determined using broth serial dilution technique as was done in this study, antimicrobial substance was diluted several times using tube test contained of nutrient compound and then reacted with the pathogenic bacteria. The tube test then incubated, the growth of pathogenic bacteria was detected using spectrophotometer 600 nm. Concentration on the tube test which showed the bacterial growth increases dramatically expressed as MIC.¹⁴

The result of this study showed that MIC of pomegranate seeds extract was 500 ppm while MIC for media and solvent was 2000 ppm. It indicated that MIC value of pomegranate seeds extract was better and if the evaporation stage in maceration process had successfully vaporized the whole 70% ethanol as a solvent, the only remaining substance was active compound or thick extract. Even though if there was still little substance was left and carried out, the effect was not expected to change the MIC value which was obtained significantly.

Vasconcelos et al.¹⁵ stated that pomegranate peel extract can be used to control adherence of different microorganisms in the oral cavity. MIC of adherence of pomegranate gel against *S. mutans* and *S. sanguis* were 1:16, 1:128 for *S. mitis* and 1:64 for *C. albicans*.¹⁵ MIC adherence of pomegranate gel against *S. mitis* (1:512), *S. mutans* (1:256), *S. sanguis* (1:128) and *C. albicans* (1:4).¹⁶

There were differences between the MIC against *S. sanguis* then MIC was 500 ppm the MIC was 1:16 (62.500 ppm) and 1:128 (7800 ppm) respectively. These differences occur because the pomegranate extract used in this study was derived from the seeds. The result of this study indicated that MIC of pomegranate seeds extract was better than peel's extract.

The value of the lowest concentration of pomegranate seeds extract was known, could inhibit the growth of *S. sanguis* bacteria MIC. The next step was determining the MBC value. MBC is the lowest concentration of an antimicrobial that will prevent the growth of organisms in a culture broth.¹⁶ The determination test for MBC value of pomegranate seeds extract indicated that at the concentration of 250, 500 and 1000 ppm there were still bacterial colonies growth, while at the subsequent concentration of 2000 ppm no growth of colony bacteria, thus MBC value for pomegranate seeds extract sample against *S. sanguis* was at the value of 2000 ppm.

Conclusion

Pomegranate seeds extract has proven containing phenolics, flavonoids, steroids, saponins and tannins at phytochemical test and has bacteriostatic activity against *S. sanguis* with 500 ppm as MIC value and bactericidal MBC at the concentration of 2000 ppm.

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Conflict of Interest

The authors report no conflict of interest.

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