

The *In Vitro* Inhibitory Effects of Red Pomegranate (*Punica granatum* Linn) Extract on *Fusobacterium Nucleatum's* and *Porphyromonas Gingivalis's* Growth

Sri Hernawati^{1*}, Pratiwi Soesilawati²

¹Department of Oral Medicine, Faculty of Dentistry, Jember University, Jember, Indonesia

²Department of Oral Biology, Faculty of Dental Medicine, Universitas Airlangga, Surabaya Indonesia

*Correspondence: Sri Hernawati, Department of Oral Medicine, Faculty of Dentistry, Jember University, Jl. Kalimantan No 37, Kampus Tegal Boto, Jember 68121, Jawa Timur, Indonesia
Telp.+62331-333536, Fax.+62331-331991, E-mail: srihera.fkg@unej.ac.id

ABSTRACT

Gram-negative anaerobic bacteria such as *Fusobacterium nucleatum* and *Porphyromonas gingivalis* are associated with halitosis since it can release the aroma of ammonia. Previous research has posited that flavonoids can inhibit bacterial growth. Red Pomegranate fruit is known to contain very effective flavonoids. Red pomegranate fruit is even known to contain polyphenols flavonoid and its derivatives and alkaloids. Based on this fact, Red pomegranate fruit is expected to be used as a mouthwash with anti-bacterial effect on *Fusobacterium nucleatum* and *Porphyromonas gingivalis* to control halitosis. The aim of this research was to reveal the inhibitory activity of red pomegranate extract on the growth of *Fusobacterium nucleatum* and *Porphyromonas gingivalis* by obtaining the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). The red pomegranate extract was produced via ethanol extract to obtain 100% concentration of red pomegranate. Dilution was conducted in order to obtain extract in 100%, 50%, 25%, 12.5% and 6.25% concentration. Minimum Inhibitory Concentration (MIC) tests and Minimum Bactericidal Concentration (MBC) of pomegranate extracts against *Fusobacterium nucleatum* and *Porphyromonas gingivalis* bacteria was conducted to determine the in vitro inhibitory effect. Red pomegranate extract with a concentration of 50% is determined as a minimum inhibitory level and a minimum bacterial concentration against the growth of *Fusobacterium nucleatum* and Red pomegranate extracts with a concentration of 12.5% are determined as minimal inhibitory levels and Minimum Bactericidal Concentration against bacterial growth of *Porphyromonas gingivalis*. Red pomegranate extract had the potential to become an antibacterial agent toward the growth of *Fusobacterium nucleatum* and *Porphyromonas gingivalis*.

Keywords: MIC, MBC, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, Red pomegranate extract

Correspondance:

Sri Hernawati,
Department of Oral Medicine, Faculty of Dentistry, Jember University,
Jl. Kalimantan No 37, Kampus Tegal Boto, Jember 68121, Jawa Timur,
Indonesia
Telp.+62331-333536, Fax.+62331-331991, E-mail: srihera.fkg@unej.ac.id

INTRODUCTION

Fusobacterium nucleatum is an anaerobic gram negative bacterium associated with halitosis since it releases gas containing sulphur, cysteine and methionine.¹ This collection of gases produces odors that resembles ammonia, hydrogen sulphide and methyl mercaptan. Several subspecies of *Fusobacterium nucleatum*, including polymorphum, are found in gingival sulcus and subspecies nucleatum in the periodontal pocket.² *Fusobacterium nucleatum* is largely associated with other bacteria that can trigger early and end-of-plaque colonies.³

Similarly, *Porphyromonas gingivalis* is an anaerobic gram negative bacterium normally present in plaque which plays a role in the development of halitosis because it breaks the protein substrate down into peptide chains and sulphur-containing amino acids such as methionine, cysteine, cysteine cytolism and methionine, as well as volatile sulphur

compounds.⁴ Therefore, mouthwash can be considered an essential therapy in inhibiting both *Fusobacterium nucleatum* and *Phorphyromonas gingivalis* bacteria since it readily makes contact with the entire surface of the oral cavity including areas that cannot be mechanically cleaned by brushing.^{5,6} Chlorhexidine, considered to be the most effective anti-bacterial mouthwash currently available, unfortunately, induces several side-effects including: tooth staining and restoration, calcium formation, allergic reactions, as well as discomfort immediately after rinsing. In cases of long-term use, it may also lead to desquamation and exfoliation of the oral mucosa. Consequently, an alternative ingredient is required to improve mouthwash, but one which must be economical and have a significant anti-bacterial impact with minimal side-effects.⁷

Red pomegranate fruit (*Punica granatum* Linn) is known to contain active ingredients that can be used as a substitute for

chlorhexidine in mouthwash. The red pomegranate fruit contains flavonoid polyphenols, anthocyanins and tannins including: ellagitannins, ellagic acid and punicalgin all of which have anti-bacteria functions.⁸⁻¹⁰ This research, therefore, aimed to investigate the inhibitory activity of red pomegranate extract in relation to the growth of *Fusobacterium nucleatum* and *Porphyromonas gingivalis*.

MATERIALS AND METHODS

Samples and Ethical clearance

The research reported here constituted a laboratory experiment featuring post-test only control group design. 42 samples were divided into seven groups, each consisting of six samples. This study was approved by Ethic Committee approval number 531/UN25.8/KEPK/DL/2019 Faculty of Dentistry, Jember University.

Red pomegranate extract preparation

Red pomegranate extract was obtained by means of a maceration method involving the use of ethanol. The results of maceration were evaporated until a thick extract of 100% concentrate was obtained. The extract was then diluted to produce its concentrations of 50%, 25%, 12.5% and 6.25%, for *Fusobacterium nucleatum* and *Porphyromonas gingivalis*

examinations. The extract was produced at the Bioscience Laboratory, Dentistry Faculty, Jember University. In addition to the extract, 0.2% chlorhexidine was also prepared for the positive control group, while sterile distilled water was produced for the negative control group.

Preparation of *Fusobacterium nucleatum* and *Porphyromonas gingivalis*

A disc-diffusion method (Bauer-Kirby) was subsequently employed to determine the antibacterial properties of the research groups. Whatmann paper no. 42 was cut using a paper punch, then sterilized for five minutes in an oven, before being immersed in all seven groups. After soaking for five minutes, they were placed in inoculated BHI-A media and placed on petridishes that had contained suspension of *Fusobacterium nucleatum* and *Porphyromonas gingivalis* at a concentration of 0.5 Mc Farland. The petridishes were inserted into a desiccator and incubator at a temperature of 37°C for 24 hours. The clear zone around the disc (Figure 1) was subsequently measured with digital calipers. The research was primarily conducted at the Biomedical Laboratory, Dentistry Faculty, Jember University.

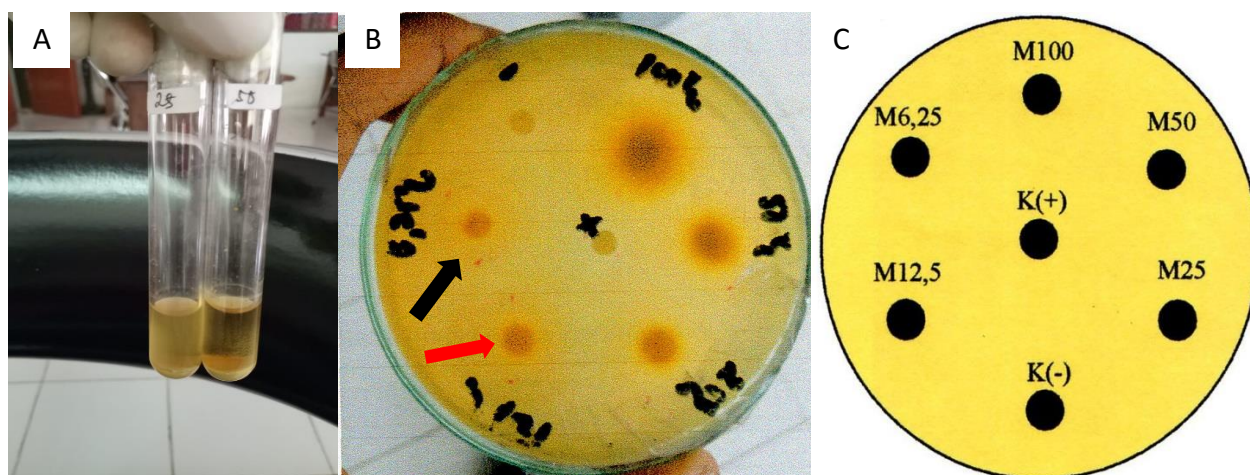


Figure 1: Observation of turbidity with dilution method (A) and Measurement of inhibition zone around the disc.

The area indicated by red arrow did not show growth of bacterial colony, area indicated by black arrow showed growth of bacterial colonies (B). Tube Number 1-7 consecutively with 100%, 50%, 25%, 12.5%, 6.25%, positive control and negative control (C).

Minimum Inhibitory Concentration (MIC) tests of pomegranate extracts against *Porphyromonas gingivalis* and *Fusobacterium nucleatum* bacteria

The MIC test was conducted referring to the previous study series concentration of pomegranate extracts. *Porphyromonas gingivalis* bacteria and *Fusobacterium nucleatum* bacteria were diluted with 1% CMC solvent prepared in advance.¹¹⁻¹² Five of the 7 sterile test tubes containing 8.8 mL of sterile Nutrient Broth were added 1 mL of *Porphyromonas gingivalis* extract (group 1). *Fusobacterium nucleatum* (group 2). 200 μ L of bacterial culture, respectively. 1 and group 2 with concentrations of 6.25%, 12.5%, 25%, 50%, 100% and 200 μ L of bacterial culture. The negative control was given aquades 1 mL and 200 μ L of bacterial culture. Positive control was given 1 mL chlorhexidine and given 200 μ L of bacterial culture. All tubes were vortexed so that they were homogeneous then 2 mL were taken to measure the *Optical Density* (OD) value of the

bacteria using a spectrophotometer (λ 630 nm).

The seven tubes above were incubated for 18 hours at 37°C. The post incubation OD value was measured again by taking 2 mL to measure the OD value using a spectrophotometer (λ 630 nm), if the difference in OD value with the lowest concentration was negative, then it was set as the Minimum Inhibitory Concentration (MIC).

Minimum Bactericidal Concentration (MBC) tests of pomegranate extracts against *Porphyromonas gingivalis* and *Fusobacterium nucleatum* bacteria

The MBC test uses the pour plate method which refers to the previous study by melting the Nutrient Agar in advance and preparing seven sterile petri dishes for 1 group of bacterial extracts and the concentration series and also the controls were put into a tube containing Nutrient Broth. The tube is added to a bacterial suspension and then homogeneous and

then poured into a sterile petri dish and wait until the media solidifies. After that it is incubated at 37°C, incubation results can be seen with no growth in the BHIA media. Total colony counted by colony counter. MBC Test procedure uses pour plate method can be seen in the following steps. First, prepare a bacterial suspension which is equivalent to turbidity with a standard Mcfarland 0.5 solution. The suspension is diluted to 106 CFU / mL. Then make a series of extract concentrations diluted with Aquadest. MIC test series held on seven tubes, each with 1 mL of extract dilution plus 8.8 mL of BHIB and 200 micron suspension. Negative control is filled with the media and suspension only. While positive control with chlorhexidine. All tubes are vortexed to be homogeneous then 2 mL was taken to measure the optical OD of bacteria using spectrophotometry. All tubes were incubated for 18 hours at 37°C. The post incubation OD values were remeasured. If the difference in OD values with the lowest concentrations was negative, then it was determined as MIC. MBC test was carried out on all tubes, by inoculating bacteria into the BHIA media, incubated 24 hours at 37°C, the results of incubation

can be seen with no growth in bhia media. Total colony was calculated by colony counter.

Statistical analysis

The data collected was tabulated and statistically analyzed with a Normality test (Kolmogorov-Smirnov) being performed whose results confirmed a p value of more than 0.05. A Levene's test of homogeneity was subsequently conducted, the results of which confirmed a p value of less than 0.05. Since both of the tests found the data to be normal and non-homogeneous distributed, a non parametric Kruskal-Wallis statistic test was carried out. The results of the Kruskal-Wallis test showed a p value of less than 0.05. Therefore, a Mann-Whitney test was performed which indicated a p value of less than 0.05.

RESULTS

The results of the research on the inhibitory effects of red pomegranate extract on the growth of *Fusobacterium nucleatum* are illustrated in Table 1.

Table 1: The mean and standard deviation of inhibitory zone diameters of red pomegranate extract in relation to *Fusobacterium Nucleatum* bacteria

Treatment Group	N	Ø	SD
M 100	6	16.46	1.71
M 50	6	13.55	1.68
M 25	6	12.09	0.50
M 12.5	6	10.89	1.44
M 6.25	6	8.84	1.47
K+	6	8.85	1.58
K-	6	0.00	0.00

- N : Number of samples
- Ø : The mean value of the inhibitory zone diameter
- SD : Standard deviation of the inhibitory zone
- M100 : Red pomegranate extract at a concentration of 100%
- M50 : Red pomegranate extract at a concentration of 50%
- M25 : Red pomegranate extract at a concentration of 25%
- M12.5 : Red pomegranate extract at a concentration of 12.5%
- M6.25 : Red pomegranate extract at a concentration of 6.25%
- K + : Positive control (0.2% clorhexidine)
- K- : Negative control (sterile distilled water)

Table 2: The mean and standard deviation of inhibitory zone diameters of red pomegranate extract in relation to porphyromonas gongivalis bacteria

Treatment Group	N	Ø	SD
M 100	6	19.10	1.05
M 50	6	14.28	0.57
M 25	6	13.16	0.41
M 12.5	6	11.00	1.02
M 6.25	6	8.87	1.07
K+	6	5.18	1.44
K-	6	0.00	0.00

- N : Number of samples
- Ø : The mean value of the inhibitory zone diameter
- SD : Standard deviation of the inhibitory zone
- M100 : Red pomegranate extract at a concentration of 100%
- M50 : Red pomegranate extract at a concentration of 50%
- M25 : Red pomegranate extract at a concentration of 25%
- M12.5 : Red pomegranate extract at a concentration of 12.5%

M6.25 : Red pomegranate extract at a concentration of 6.25%
 K + : Positive control (0.2% chlorhexidine)
 K- : Negative control (sterile distilled water)

The results of the Kruskal-Wallis test showed a significant difference in all research groups with a significant value (p) of less than 0.05 (0.000). The group of red pomegranate extract is also known to have an inhibitory effect vis-a-vis the growth of *Fusobacterium nucleatum*. The results of the Mann-Whitney test indicated, moreover, that there were significant differences between the groups with a significance value (p) of less than 0.05 in each group, except between Group M6.25 (red pomegranate extract at a concentration of 6.25%) and the positive control (0.2% chlorhexidine). The red pomegranate extract at concentrations of 100%, 50%, 25% and 12.5% is also known to have larger inhibitory zone diameters than 0.2% chlorhexidine (p <0.05). In other words, there was a meaningful difference between the M100, M50, M25, M12.5 and M6.25 groups with the K + and K- groups. This shows that the red pomegranate extract at concentrations of 100%, 50%, 25%, 12.5% and 6.25% has a greater ability to inhibit the growth of *Porphyromonas gingivalis* than does 0.2% chlorhexidine. The discs in the groups M100, M50, M25, M12.5 and M6.25 also indicate that red pomegranate extract contains active antibacterial substances.

Based on the results of the Kruskal-Wallis test, there was a significant difference in the inhibitory activities of red pomegranate extract in relation to *Porphyromonas gingivalis* in all research groups (p value = 0.000). Furthermore, the results of the Wann-Whitney test indicated significant differences between the groups in the inhibitory activities of red pomegranate extract vis-a-vis *Porphyromonas gingivalis*. The red pomegranate extract with concentrations of 100%, 50%, 25%, 12.5% and 6.25% had larger inhibitory zone diameters than 0.2% chlorhexidine (p <0.05). In other words,

there was a significant difference between the M100, M25, M25, M12.5 and M6.25 groups and the K + group. This indicates that red pomegranate extract at concentrations of 100%, 50%, 25%, 12.5% and 6.25% has greater capacity to inhibit the growth of *Porphyromonas gingivalis* than does 0.2% chlorhexidine.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) tests of pomegranate extracts against *Porphyromonas gingivalis* bacteria

The results of measurements of OD values on the Minimum Inhibitory Concentration Test (MIC) using a spectrophotometer (¥ 630 nm) showed a definite inhibition of the growth of *Porphyromonas gingivalis* bacteria by observing a decrease in OD values in incubation and post incubation presented in table 3. The Minimum Inhibitory Concentration (MIC) value can be determined by measuring the difference between the pre incubation and post incubation OD values. A negative ΔOD value indicates a decrease in absorbance value indicating a decrease in the number of *Porphyromonas gingivalis* bacteria that have been incubated for 18 hours, showed negative values at pomegranate extract concentrations of 12.5%, 25%, 50%, 100% and positive control (chlorhexidine 0.2%), at a concentration of 6.25% and negative control (aquadest steril) did not decrease in value. Post incubation OD is marked by a positive ΔOD difference, meaning that there is still an increase in the growth of *Porphyromonas gingivalis* bacteria. Based on the above results, pomegranate extracts with a concentration of 12.5% are determined as Minimal Inhibitory Concentration (MIC) or minimal inhibitory levels and Minimal Bactericidal Concentration (MBC) of pomegranate extract against *Porphyromonas gingivalis*.

Table 3: Results of measurements of OD values in MIC and MBC tests of pomegranate extracts against *Porphyromonas gingivalis* bacteria

Pomegranate extracts concentration	Pre-incubation	Post incubation	ΔOD	MBC
6.25%	0.62	0.95	0.33	54
12.5%	0.68	0.60	-0.08	0
25%	1.03	0.95	-0.08	0
50%	1.05	0.95	-0.1	0
100%	1.4	0.95	-0.45	0
K +	0.85	0.8	-0.05	0
K-	0.05	0.14	+0.09	3.1x10 ⁵

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) tests of pomegranate extract against *Fusobacterium nucleatum*

The results of measurements of OD values in the Minimum Inhibitory Concentration Test (MIC) using a spectrophotometer (¥ 630 nm) showed a definite inhibition of the growth of *Fusobacterium nucleatum* bacteria by observing a decrease in OD values in incubation and post incubation presented in table 2. The Minimum Inhibitory Concentration (MIC) value can be determined by measuring the difference between the pre incubation and post incubation OD values. A

negative ΔOD value indicates a decrease in absorbance value indicating a decrease in the number of *Fusobacterium nucleatum* bacteria that have been incubated for 18 hours. A positive ΔOD value indicates no decrease in OD values, which means an increase in the number of *Fusobacterium nucleatum* bacteria after incubation. Table 4 showed negative values at the extract concentration of pomegranate 50%, 100% and positive control (chlorhexidine 0.2%), at concentrations of 6.25%, 12.5%, 25% and negative control (aquadest steril) did not decrease in value Δ Post incubation OD is marked by the positive ΔOD difference which means that there is still an increase in the

growth of *Fusobacterium nucleatum* bacteria. Based on the above results, the pomegranate extract with a concentration of 50% is determined as a minimum inhibitory concentration (MIC) or a minimum inhibitory level and a minimum

bacterial bacterial concentration (MBC), or a minimum kill rate of pomegranate extract against the growth of *Fusobacterium nucleatum*.

Table 4: Results of OD values measured in MIC and MBC tests of pomegranate extract against *Fusobacterium nucleatum* pomegranate extracts

concentration	Pre-incubation	Post incubation	Δ OD	MBC
6.25%	0.62	0.86	0.24	115
12.5%	0.68	0.9	0.22	77
25%	1.03	1.15	0.14	65
50%	1.05	0.85	-0.2	0
100%	1.4	0.68	-0.72	0
K +	0.85	0.8	-0.05	0
K -	0.05	0.24	0.19	1.36x10 ⁶

DISCUSSION

The results showed that red pomegranate extract at a concentration of 100% demonstrates the greatest ability to inhibit the growth of *Fusobacterium nucleatum* and *Porphyromonas gingivalis* compared with concentrations of 50%, 25%, 12.5% and 6.25%, as well as with 0.2% chlorhexidine in the negative control group. The term 'concentration' refers to the amount of solute contained in a specific amount of solution. The stronger the concentration of red pomegranate extract, the longer the diameter of the inhibitory zone will be since the higher the concentration, the more solute is contained. In other words, a lower concentration signifies the presence of less soluble substances and more solvent in the solution. The formation of inhibitory zones around the discs of M100, M50, M25, M12.5 and M6.25 indicates that red pomegranate extract contains a bioactive material with antibacterial properties.¹³

Red pomegranate extract, furthermore, possesses the ability to inhibit the growth of mutant streptococcus significantly in *in vivo* studies. Antibacterial substances contained in red pomegranate include polyphenols (Flavonoid and its derivatives, such as Ellagic acid, Tannin and Ellagitannins) and alkaloid.^{1,14-16} Unfortunately, the antibacterial mechanism of polyphenols is still unknown since various reactions such as interference with the membrane causing cell leakage, disruption of the active transport of metabolic enzymes and energy loss, occur simultaneously.¹⁷ Other mechanisms then inhibit the DNA and RNA synthesis processes. Benzene rings (B ring) of flavonoids are believed to interact in the intercalation or bonding of hydrogen derived from RNA and DNA.¹⁸ Quercetin inhibits DNA gyrase of certain bacteria, while catechin destroys the lipid layer of the bacterial cell membrane. This damage causes membrane leakage, with the result that barrier function becomes impaired since the membrane is not only a selective barrier to movement in and out of the active substance into the cell, but also an enzyme biosynthesis site. The leakage of cell membranes can also cause cell lysis. Polyphenols act as anti-bacterials by denaturing enzymes and attaching themselves to certain substrates such as minerals, vitamins and carbohydrates with the result that those substrates cannot be used for bacterial metabolism. Polyphenols can also be absorbed into the cell wall, but not without causing disruption of bacterial cell membrane structure and function.^{19,20}

In addition, alkaloids constitute other compounds that are extracted by ethanol. Alkaloids have an antibacterial effect by inhibiting DNA synthesis²¹ and also react with the acid compound presented in the DNA as the main constituent of the cell nucleus. With the disruption of DNA synthesis, the bacteria will experience difficulty in multiplying, leading to a decrease in their number. The disruption of DNA will then inhibit protein synthesis, triggering an interference with bacterial metabolism.^{16,22}

Flavonoids can decrease the activities of aspartate aminotransferase and alpha glucosidase enzymes, while increasing the activities of the antioxidant enzyme, namely ceruloplasmin^{17,23} and inhibiting bacterial growth.²⁴ For instance, quercetin (a flavonoid contained in pomegranate) can inhibit bacterial DNA gyrase leading to the death of the bacteria. This is caused by increasing the permeability of bacterial membranes, damaging their capability inhibiting the production of bacterial ATPs, as well as disrupting membrane transport and bacterial movement.¹⁹

Flavonoids also possess the ability to form extracellular complexes and dissolve layers of lipids and proteins in bacterial cell membranes. Damage to these then induce changes in cell membrane permeability through which important intracellular components such as nucleic acids and nucleotides can exit, resulting in the inhibiting of the function of the cytoplasmic membrane. This, in turn, will cause bacterial metabolism disruption, thereby impeding the growth of bacteria or even resulting in their death.^{25,26}

CONCLUSION

Based on the research results, it can be concluded that red pomegranate extract possesses the ability to inhibit the growth of *Fusobacterium nucleatum* and *Porphyromonas gingivalis*. It is also evident that the strongest inhibitory activity is found in red pomegranate extract at a concentration of 100%.

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