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Screening of *Punica granatum* extract for antimicrobial activity against oral micro organisms

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ABSTRACT

Background and Objectives: A lot of research work in both dental and medical fields support the curative properties of pomegranate. Accordingly, it was decided to prepare a pomegranate mouthwash and evaluate it among Dental patients diagnosed with Chronic Gingivitis. The objective of the present invitro study is to assess the Minimum Inhibitory Concentration (MIC) of the commercially available pomegranate extract powder against few oral pathogenic microorganisms. **Methodology:** Serial dilution method using thioglycolate broth medium was used for anerobes like Streptococcus mutans, Fusobacterium nucleatum, Aggregatibacter actinomycetomcomitans, Prevotella intermedia and Mueller hinton agar mediated growth was used for aerobe like Staphylococcus aureus. Following which microdilution assay was performed and accordingly evaluated the MIC. Based on this report, the test rinse was prepared and further evaluated using the same methodology for both aerobes and anerobes. **Results and Inference:** It was observed from the MIC report for both aerobes and anerobes that at a concentration of 0.2% the formulated mouth rinse was effective against all the chosen organisms. The results of the study infer that products like mouthwash, dental gels etc made from this concentration could be possibly used for the control of dental infections.

Keywords: *Punica granatum*; Minimum Inhibitory Concentration, Microdilution Assay, anti-bacterial effect.

INTRODUCTION

Pomegranate or Punica granatum, is a plant native to India and other tropical and sub-tropical countries. It is one of the oldest and most consistently grown fruits in India. Much research work is in progress evaluating the beneficial effects of pomegranate having realized its antibacterial [1, 2] and antioxidant properties. Pomegranate has proven benefits in the management of various systemic conditions including Diabetes mellitus and Cardiovascular diseases [3, 4]. Pomegranate has shown great potential to be useful in dentistry as well as gathered from initial reports [5, 6]. Methanol extracts of pomegranate are high in hydrolyzable tannins (punicalins and punicalgins), ellagic acid, a component of ellagi-tannins, and gallic acid, a component of gallotannins. These molecules could also be the most potent antibacterial compounds in pomegranate apart from anthocyanins (pelargonidin-3-galactose and cyanidin-3-glucose) and flavonols (quercetin and myricetin) [7].

A lot of research work in both dental and medical fields support the curative properties of pomegranate. Accordingly, it was decided to prepare a pomegranate mouthwash and evaluate it among Dental patients diagnosed with Chronic Gingivitis. The mouthwash is prepared from a commercially available pomegranate extract. It is very essential to formulate the drug at an effective concentration which can bring out all the desired actions of the drug when used appropriately. To evaluate that particular concentration, it is required to assess the Minimum Inhibitory Concentration of the Pomegranate extract.

Hence the aim of the present study is to screen the Pomegranate extract for its Minimum Inhibitory Concentration against selected oral micro-organisms.

MATERIALS AND METHODS

The study is approved by the institutional ethical committee of Manipal university, KMC, Manipal. The pomegranate extract was procured from the Verdure Sciences laboratories, available under the trade name PomElla® from United States with the product Code: POM030EPPH. This is a proprietary complex standardized to 30% punicalgins. It was evaluated for the minimum inhibitory concentration against gram positive and gram negative aerobic and anerobic bacteria like Staphylococcus aureus, Aggregatibacter

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actinomycetomcomitans (A.a), Streptococcus mutans (S.m), Porphyromonas gingivalis (P.g), Prevotella intermedia (P.i), Fusobacterium nucleatum (F.n) and Streptococcus mutans, at the Maratha Mandal Research Institute, Belgaum.

Minimum Inhibitory Concentration (MIC) procedure

The lowest concentration of a drug which will inhibit the visible growth of an organism after overnight incubation is defined as the Minimum Inhibitory Concentration (MIC). This period is extended for organisms like anaerobes, which require prolonged incubation for growth [8]. (Andrews J.M, 2001)

MIC procedure (For Anaerobes)

Serial dilution method [9] was adopted for testing the MIC of the anerobes. 9 dilutions of the drug were through with Thioglycollate broth for MIC. Within the initial tube 20microlitre of drug was added into the 380 microlitre of Thioglycollate broth. For dilutions 200microlitre of Thioglycollate broth was added into subsequent 9 tubes separately. Then from the initial tube 200microlitre was transferred to the primary tube containing 200microlitre of Thioglycollate broth. This was considered as 10-1 dilution. From 10-1 diluted tube, 200microlitre was transferred to second tube to form 10-2 dilution. The serial dilution was repeated up to 10-9 dilution for every drug. From the maintained stock cultures of required organisms, 5microlitre was taken and added into 2ml of Thioglycollate broth. In each serially diluted tube 200microlitre of above culture suspension was added. The tubes were incubated for 48-72 hours in anaerobic jar at 37^0C and observed for turbidity.

MIC procedure for Staphylococcus aureus (Aerobe)

Media used - Mueller Hinton agar

Bacterial strain used: Gram positive - Staphylococcus aureus (NCIM 2079)

Preparation of Inoculum

For the preparation of inoculum, growth from the agar slant was scrapped by adding 3ml of sterile saline. This saline cell suspension was then spread evenly on large sterile Petri plates containing solidified agar employing a sterile glass spreader. These plates were incubated in bacteriological incubator at 370C for twenty-four hours. After profuse growth of the organism within the Petri dish, it had been scrapped using sterile spatula and adding small portion of sterile saline. This suspension was transferred to a sterile 100ml conical flask. the ultimate volume of the suspension was made up to 50ml with sterile saline (Clinical and Laboratory Standards Institute, 2011) [10].

Standardization of Inoculum

For determination of MIC, the inoculum density was adjusted to contain 5 x 106 CFU/ml which have turbidity adequate to 0.5 McFarland standard. For this, 0.5McFarland standard was prepared by adding 0.05ml of 0.048M BaCl2 (1.17% w/v BaCl2.2H2O) to 9.95ml of 0.18M H2SO4 (1% w/v) with constant stirring. the quality was transferred to a glass screw capped bottle. Absorbance of the McFarland standard was checked at 625nm (absorbance at 625nm should range between 0.08-0.13).

Microdilution Assay

Microdilution assay was performed in 96 well plate to estimate reciprocal inhibition of the expansion of the organism by the drug. Double strength and single strength Mueller Hinton medium were prepared and sterilized. In first row, alternatively 100µL of double strength medium was added and in remaining wells, 100µL of single strength medium was added. Concentrated solution of drug (1000µg/mL) was added in first row in triplicate and further diluted serially till fourth well of column. Then wells were inoculated with 10??L of ordinary inoculum of test organism. Similarly solvent controls were also tested in triplicate for every test organism. In remaining columns positive and negative control were prepared in triplicate. After 24 hours, plate was read by the ELISA plate reader at 590nm. Optical density of growth in each well was calculated by reducing the absorbance of sample blank. The Inhibitory concentration for 50% of microorganisms (IC50) for the copper nanoparticles was calculated as compared to positive control of every test organisms.

Percentage inhibition of growth = (control – test /control) × 100

RESULTS

To consider a compound as antimicrobial, its Minimum Inhibitory Concentration range should be less than 1mg/mL. This is a standard acceptable limit for antimicrobial activity. If a compound is having Minimum Inhibitory Concentration more than 1mg/ml then it will not be considered as a good antimicrobial agent. So, the MIC was assessed for the stock solution of 2mg/mL, which has given 1mg/mL starting concentration (which is under the acceptable limit). The results of the MIC of the extract against anerobes and aerobes are shown in table 1 and 2 respectively. The results from table 2 can be inferred that 2mg/ml concentration was effective even when it is diluted > 5times, which means the extract was effective against Staphylococcus aureus even at a concentration of <31.25ug/ml.

Having obtained the MIC report, mouthwash formulations were prepared taking into consideration the effectiveness at a dilution till 0.8% where all the chosen anerobes were sensitive. The 0.8% formulated mouthwash was again evaluated for the Minimum Inhibitory Concentration for all the chosen organisms. The results of the MIC of the 0.8% formulation against anerobes is shown in table 3.

It was interpreted from the MIC report of the 0.8% formulation that is effective even when diluted till 6.25 i.e. 5 times the original dilution. So, it was decided to prepare further diluted concentrations of 0.4 and 0.2% taking into consideration of the MIC report.

The prepared 0.4% and 0.2% formulations were also evaluated microbiologically against aerobes as well as anerobes. The results of MIC of the 0.2% & 0.4% formulations against anerobes is shown in table 4 and against aerobes in table 5 & 6 respectively.

From the Table 5 & 6, it can be inferred that the 0.4% mouth wash was effective against S. aureus even when diluted >6 times and 0.2% mouth wash was effective against S. aureus even when diluted further 4 times. Having known that both the concentrations were effective, the lower concentration was opted for formulation. Hence, 0.2% test formulation was finalized for the clinical trial planned.

Table 1: Results of MIC of the extract powder for anerobes

	100	50	25	12.5	6.25	3.12	1.6	0.8	0.4	0.2
A.a	S	S	S	S	S	S	S	S	S	S
S.mutans	S	S	S	S	S	S	S	S	R	R
P.g	S	S	S	S	S	S	S	S	S	S
F.n	S	S	S	S	S	S	S	S	S	S
P.i	S	S	S	S	S	S	S	S	R	R

S - Sensitive; R- Resistant, A.a – Aggregatibacter actinomycetomcomitans,
S.mutans – Streptococcus mutans, P.g - Porphyromonas gingivalis, F.n – Fusobacterium nucleatum
P.i – Prevotella intermedia

Table 2: MIC for aerobe (Staphylococcus aureus)

Date of EXP: 01-09-2013 to 2-09-2013
Cell line: Staphylococcus aureus
Cell density: 10,000 cells/well
Absorbance at: 590 nm
Treatment: 24 h
Assay: Microdilution assay for MIC

Drug Name	**Concentration** (ug/ml)	**Absorbance at 540 nm**			**% inhibition of growth**			**Average**	**IC 50 value** (ug/ml)	**MIC value** (ug/ml)
2mg/ml powder	1000	0.149	0.129	0.143	76.97063	80.06182	77.89799	78.31015	<31.25	<31.25
	500	0.207	0.118	0.198	68.00618	81.76198	69.39722	73.05513		
	250	0.203	0.199	0.193	68.62442	69.24266	70.17002	69.3457		
	125	0.194	0.203	0.19	70.01546	68.62442	70.63369	69.75786		
	62.5	0.186	0.2	0.21	71.25193	69.0881	67.5425	69.29418		
	31.25	0.187	0.186	0.191	71.09737	71.25193	70.47913	70.94281		

Table 3: Results of MIC of the 0.8% formulation against anerobes

S. No.	**Mouthwash Concentration**	**100**	**50**	**25**	**12.5**	**6.25**	**3.12**	**1.6**	**0.8**	**0.4**	**0.2**
1	**Aa**										
	0.8%	S	S	S	S	S	S	S	S	S	R
2	Fn										
	0.8%	S	S	S	S	S	S	S	R	R	R
3	Pg										
	0.8%	S	S	S	S	S	S	S	S	S	S
4	Pi										
	0.8%	S	S	S	S	S	S	S	S	S	S
5	Sm										
	0.8%	S	S	S	S	S	R	R	R	R	R

A.a – Aggregatibacter actinomycetomomitans, F.n – Fusobacterium nucleatum
P.g – Porphyromonas gingivalis, P.i – Prevotella intermedia, S.mutans – Streptococcus mutans

Table 4: MIC of 0.4% and 0.2% formulations against anerobes

S.No	**Samples**	**100 ug/ml**	**50 ug/ml**	**25 ug/ml**	**12.5 ug/ml**	**6.25 ug/ml**	**3.125 ug/ml**	**1.6 ug/ml**	**0.8 ug/ml**	**0.4 ug/ml**	**0.2 ug/ml**
	Aa										
1.	0.2%	S	S	S	S	S	S	S	S	S	S
2.	0.4%	S	S	S	S	S	S	S	S	S	S

S.No	Samples	100 ug/ml	50 ug/ml	25 ug/ml	12.5 ug/ml	6.25 ug/ml	3.125 ug/ml	1.6 ug/ml	0.8 ug/ml	0.4 ug/ml	0.2 ug/ml
	Pg										
1.	0.2%	S	S	S	S	S	S	S	S	S	S
2.	0.4%	S	S	S	S	S	S	S	S	S	S
	Pi										
1.	0.2%	S	S	S	S	S	S	S	S	S	S
2.	0.4%	S	S	S	S	S	S	S	S	S	S
	Fn										
1.	0.2%	S	S	S	S	S	S	S	S	S	S
2.	0.4%	S	S	S	S	S	S	S	S	S	S
	Sm										
1.	0.2%	S	S	S	S	S	R	R	R	R	R
2.	0.4%	S	S	S	S	S	R	R	R	R	R

S – Sensitive, R – Resistant,

A.a – Aggregatibacter actinomycetomcomitans, P.g – Porphyromonas gingivalis, P.i – Prevotella intermedia, F.n – Fusobacterium nucleatum, S.m – Streptococcus mutans

Table 5: MIC of 0.4% formulation against aerobes

Drug Name	Concentration (ug/ml)	Absorbance at 540 nm			% inhibition of growth			Average	IC 50 value (ug/ml)	MIC value (ug/ml)
0.4%	50	0.201	0.233	0.178	68.93354	63.98764	72.48841	68.46986	<1.56	<1.56
	25	0.288	0.276	0.228	55.48686	57.34158	64.76043	59.19629		
	12.50	0.345	0.312	0.287	46.67697	51.77743	55.64142	51.36528		
	6.25	0.29	0.272	0.296	55.17774	57.95981	54.25039	55.79598		
	3.13	0.255	0.245	0.238	60.58733	62.13292	63.21484	61.97836		
	1.56	0.222	0.242	0.246	65.68779	62.5966	61.97836	63.42092		

Table 6: MIC of 0.2% against aerobes (S.aureus)

Drug Name	Concentration (ug/ml)	Absorbance at 540 nm			% inhibition of growth			Average	IC 50 value (ug/ml)	MIC value (ug/ml)
0.2% Sample	50	0.268	0.251	0.257	58.57805	61.20556	60.27821	60.02061	<6.25	<6.25
	25	0.384	0.33	0.327	40.64915	48.99536	49.45904	46.36785		
	12.50	0.303	0.278	0.293	53.16847	57.03246	54.71406	54.97166		
	6.25	0.445	0.502	0.548	31.22102	22.41113	63.21484	51.90417		
	3.13	0.238	0.36	0.452	15.30139	44.35858	30.1391	22.97785		
	1.56	0.328	0.431	0.392	49.30448	33.38485	39.41267	40.70067		

DISCUSSION

The in vitro aspect of the study included assessing the minimum inhibitory concentration (MIC) of the extract and accordingly formulating the test rinse. The targeted patients in the clinical trial planned were those diagnosed with Chronic marginal plaque induced Gingivitis. The MIC was performed against various organisms like Staphylococcus aureus, Streptococcus mutans, Aggregatibacter actinomycetomcomitans, Porphyromonas gingivalis, Prevotella intermedia & Fusobacterium nucleatum in order to widen the scope of anti-bacterial activity. These organisms were chosen so as to see the effect of the formulation on aerobes and anerobes, both gram positive as well as gram negative. Also, to have an effect on the primary colonizers, bridging species as well as the secondary colonizers, respective organisms were evaluated to ensure the possible antiplaque action.

Initially the extract powder(2mg/ml) was evaluated for the MIC against both aerobes and anerobes and found that the organisms were effective up to 8 times dilution and few organisms were resistant after that. Accordingly, 0.8% concentrated mouthwash was formulated and again tested for the MIC against the same microorganisms as the antibacterial efficacy might be altered in the process of preparation of the mouthrinse. It was observed that the 0.8% was effective even after further diluting it 5 times. Hence 0.4% and 0.2% were formulated and evaluated for the MIC and it was found that both the concentrations were effective against aerobes and anerobes even under further dilutions. It was decided to choose the lowest possible concentration for

the preparation of the mouthwash. Also, the 0.4% formulated rinse had a strong bitter taste and a dark yellowish-brown color. Thus, 0.2% was opted for the final formulation.

The concentration used in the present study is similar to the one evaluated by Di Silvestro R(2009) [11], where he used 100mg of pomegranate extract dissolved in 35ml of deionized water i.e 0.286% W/V of extract for thrice daily rinsing.

A randomized controlled clinical trial was done by Bhadbhade SJ *et al*, (2011) [12] to evaluate the antibacterial effect of pomegranate-containing mouth rinse prepared from Pomella extract on plaque. The pomella extract was dissolved in distilled water to make up the effective concentration at 4g% unlike the present study where a 0.2% concentration was also found effective. This difference could be attributed to the method of performing MIC, where the MIC of the extract alone was considered in their work unlike in the present study where the MIC of the formulations were also considered and cross-checked for aerobes and anerobes. They noted that the pomegranate extract showed an inhibition of A.a, P.g, P.i strains in vitro at various concentrations. This study highlighted the anti-plaque effect of the pomegranate extract-based mouth rinse and was able to demonstrate certain prophylactic benefits.

Pomegranate flavonoids have shown antibacterial action in vitro against gingivitis causing microbes [13]. Streptococcus sanguis (S. sanguis) was sensitive to pomegranate extract and the inhibitory action was similar to chlorhexidine as observed by Pereira J.V(2001) [14]. S. sanguis is known to be one of the initial colonizers in dental plaque formation. The possible reasons for this antibacterial effect are the tannins which increase bacteriolysis and also interfere with bacterial adherence mechanisms onto the tooth surface. Pomegranate extracts were also found out to inhibit the growth of Staphylococcus aureus, Streptococcus pyogenes, Diplococcus pneumoniae, Escherichia coli O157:H7, and Candida albicans [15, 16].

CONCLUSION

The Minimum Inhibitory Concentration of a pomegranate extract powder as well as the formulation were evaluated successfully. The findings of the present study support the possible use of this particular extract for the preparation of mouthwash, gels, chips etc. for the control of dental infections especially Gingivitis and Periodontitis.

Conflict of interest

The authors do not claim any conflict of interest

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