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Antimicrobial Activity of Pomegranate (*Punica Granatum*) Pericarp Extract against *Streptococcus Mutans*- A Source For Natural Mouth Rinse: An *In-vitro* and *In-vivo* Study

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Plant materials were known as source of new antimicrobial agents. Many efforts have been made to develop alternative mouth rinses from natural products which are safe, easily available and substitute thestandard pharmaceutical remedies. Thus, considering the therapeutic value of pomegranate fruit, the presentstudywas designed to compare the antimicrobial efficacy of aqueous extract of Pomegranate pericarpand commercially availableChlorhexidinemouth wash against caries causative microorganisms in both *in-vitro* and *in-vivo*. To evaluate the antimicrobial efficacy of pomegranate pericarp extract (PPE) against *Streptococcus mutans* (S.*mutans*) andto determine its usefulness as anti caries mouth rinse. In-vitro antimicrobial efficacy was evaluated by disc inhibition zone method and broth dilution assay considering minimum inhibitory concentration of PPE. In-vivo evaluation was done as a randomised controlled trial which included thirty children aged between 6-12years. They were divided randomly into 3 groups of ten each and subjected to different mouth rinses - Group I: PPE mouth rinse, Group II: 0.2% Chlorhexidine mouth rinse and Group III: distilled water (control). The salivary samples whichwere collected before and after (5 minutes) mouth rinsing were inoculated on Mutans Sanguis agar and the bacterial count was calculated. Statistically significant decrease in salivary S.*mutans* count was observed in group I and II compared to group III after mouth rinsing. However, there was no statistically significant difference between groups I and II. PPE mouthwash was effective in reducing the salivary S.*mutans* count and was comparable to chlorhexidine mouth rinse. Hence PPE mouth rinse may be considered as a potential anti caries mouth rinse.

Keywords: Chlorhexidine, Minimum Inhibitory Concentration, Mouthwash, Pomegranate pericarp extract, *Streptococcus mutans*.

Dental caries is one of the most common chronic infectious diseases in the world whichis influenced by multiple factors such as diet, host characteristics and cariogenic microorganisms, of which *Streptococcus mutans* (S.*mutans*)is a significant contributor to tooth decay.[1]

The prime mode of preventing the development and progression of dental caries is through mechanical plaquecontrol(regular tooth brushing). Mouth rinses are medicated solutions which are recommended as an antimicrobial, topical anti-inflammatory solutions so as to decrease halitosis and deliver fluoride for caries prevention in general. They arebeneficial especially to mentally and physically challenged patients who lack manual dexterity. A multitude of products have arisen, during the past few decades which contain different active chemical ingredients like chlorhexidine, triclosan, fluoride mouth rinses etc.[2]

Chlorhexidineis considered as a gold standard anti-plaque agent because of its broad spectrum antimicrobial activity.[3] However, its long term usage can cause tooth staining, unpleasant taste, increased calculus formation and mucosal erosion at higher concentrations.[4] These short comings has led to the need for further research and introduction of new antibacterial agents which are derived naturally with minimal / no side effects on the oral tissues especially in children.

Nature has enormous plant source which have good medicinal value and work against pathogenic microorganisms. Pomegranate(*Punica granatum*)is one such natural source that is currently finding important applications in the field of dental health.[5] The healing property of pomegranate was discussed in one of the oldest medical texts, the Eber's Papyrus from ancient Egypt (1500 BC).[3] In Ayurvedic medicine, pomegranate is considered "a pharmacy unto itself" and as a remedy for diabetes in Unani medicine. Various components of this plant such as the leaves, flowers, roots, bark and fruit extracts have been used for a variety of ailments. [6]

Even though there is ample evidence regarding the antimicrobial efficacy of PPE in various *In Vitro*studies, its clinical evidenceis very minimal. Considering this fact, the present study is an attempt to evaluate the clinical applicability of naturally available PPEas mouth rinse in children.

MATERIALS AND METHODS

Following the approval from the institutional ethical committee, the presentmicrobiological study was conducted in the department of Pedodontics and Preventive dentistry in collaboration with department of Microbiology and Pharmacology.

Fresh ripen pomegranate fruits were procured from local market and the pericarps wereseparated manually, shade dried for 7 days, powdered and stored under freezing condition until its use[Figure I]. This powder was mixed in different concentrations (250, 500, 750 and 1000 mg) with 10 ml of distilled water in Jiffy's centrifuge tubes. These four concentrations of extracts were immersed in thermostatic water bath at a temperature of 60^{o}C for 20 minutes following which they were left to cool and subjected to centrifugation at 2500 rpm for 10 minutes and the resultant supernatants were used to analyse the antimicrobial efficacy.[7]

The antimicrobial activity of PPE was assessed using disc inhibition zone method. S.mutans was first isolated from saliva by inoculation on Mitis Salivarius Bacitracin agar and PPE wasloaded on the sterile filter paper discs at a concentration of 25, 50, 75 and 100mg/ml, respectively. Filter paper disc dipped in 0.2% chlorhexidine was taken as positive control and distilled water as negative control. S.mutans streaked agar plates impregnated with discs were incubated in anaerobic jar at 37^{0}C for about 24 hrs. The zone of inhibition was assessed by measuring the diameter of inhibited growth[Figure II]. Broth dilution method was adopted to determine minimum inhibitory concentration (MIC) of the active extract. The lowest concentration of extract resulting in bacterial density lower than 300 colonies per plate was determined as MIC. As there was no bacterial colony growth at all the three concentrations (50, 75, 100 mg/ml) ofPPE mouthwash, the lowest concentration i.e., 50mg/ml was taken as MIC andwas used to prepare the mouth rinse without adding any sweeteners.

A total of two hundred children between the age group of 6-12 years who were following aroutine oral hygiene practice withDMFT score e" 4 were screenedwithout sex predilection. Subjects with draining abscess, sinus, cellulitis or any other conditions that require emergency dental treatment and patients with history of recent antibiotic usage (atleast for past 1 month) were excluded from the study.After explaining the test procedure for the forty two children who have fulfilled the inclusioncriteria, only thirty parentshave gave

their consent to participate in the study voluntarily. Further these children were randomly divided into three groups depending upon the mouth rinse used - group I: PPE mouth rinse (experimental); group II: 0.2% chlorhexidine mouth rinse (positive control) and group III: Distilled water (negative control) with ten subjects in each group.

Salivary samples were collected in the morning in order to eliminate any bias in the concentration of saliva due to circadian rhythm. Following an initial swallow, about 1 ml of unstimulated salivawas collected in a sterile vialby instructing the children to drool for 2 minutes. Each child in their respective group was given 5ml of mouth rinse and asked to squish for about one minute. The same procedure was followed for salivary samplecollection after 5 minutes following mouth rinsing. The collected samples were transported in an icebox within 2 hours to maintain the viability of microorganisms.

The collected saliva was inoculated on Mutans Sanguis agar and the plates were incubated in an anaerobic jar for 48 hours followed by bacterial count using conventional plate count method [Figure III].

The whole procedure was conducted by a single investigator and the scores were recorded. To avoid bias in the results, a second investigator who was unaware of the prior results randomly evaluated the agar plates. As the inter examiner variability was not significant(P value < 0.5), the scores given by the first investigator were only considered. The values thus obtained were tabulated and subjected to statistical analysisusing Wilcoxon signed rank testand Mann-Whitney U test.

RESULTS

Intra group comparison in S.*mutans* count before and after mouth rinsing revealedsignificant decrease in number of S.*mutans* colony count in groups I and II (p=0.001). Nevertheless, this reduction was not statistically significant ingroupIII (p=1) [Table I].

Intergroup comparison of salivary S.mutans count showed significant decrease in colony count between groups I and III; II and III (p=0.001). However, no statistical significant difference was observed when groups I and II were compared (p=0.48) [Table II].

DISCUSSION

Epidemiologicalstudiesshowed that the prevention of dental caries was done by inhibiting plaque biofilm formation or removing plaque from the teeth that enhances oral hygiene. Commonpreventive strategies of dental caries aremechanical cleansing techniques such as regular brushing and flossing; use of systemic and topical fluorides; dietary modifications include altering frequency of sugar intake, use of sugar substitutes, fissure sealants, antimicrobial agents in mouth washes and probiotics.[8] Among these, use of topical antimicrobial agents suchas mouth rinses minimize caries risk by reducing the number of S.mutans in the mouth there by altering the oral environment.[9]

A variety of synthetic mouth washes containing Chlorhexidine, Triclosanand Cetylpyridinium chloride are available in the market. Over a period of last 40 years,chlorhexidinehas been thoroughly investigated and successfully

Table 1. Intragroup comparison of salivary S.mutans count before and after mouth rinsing (in 10^3 CFU/ml)

Group	Before IQR (in 10^3CFU/ml)	After IQR (in 10^3CFU/ml)	P-value
I	383	218	0.005*
II	350	203	0.005*
III	350	375	1

Wilcoxon signed rank test
* Statistically highly significant if Pd"0.01, IQR: Interquartile range

Table 2. Intergroup comparison of difference in salivary S.mutans count before and after mouth rinsing (in 10^3 CFU/ml)

Inter group comparison of difference (after – before count) in IQR	P-value
I(285) Vs II(308)	0.48
I(285) Vs III(105)	0.001*
II(308) Vs III(105)	0.001*

Mann-Whitney U test
*Statistically highly significant if Pd"0.01, IQR: Interquartile range

used as antiplaque agent in dental practice. [10] It is a synthetic bisbiguanide which is positively charged showing high affinity for negative ions found in the cell membrane ofthemicroorganisms. It indirectly affects the enzymatic function of dehydrogenase and adenosine triphosphatase present in the cell wall of bacteria resulting in disruption of cell membrane leading to cell death. Proposed mechanism of caries inhibition is by interfering with the metabolic activity of S.*mutans,*particularly inhibition of phosphonyl pyruvate enzyme.[11]

Chlorhexidine has high substantivity of 12 hours which is attributed to its controlled release system regulated by beta cyclodextrine. Greater the amount of beta cyclodextrine, the more progressive release of chlorhexidine.[4] In the present study chlorhexidine was taken as positive control as it was considered to be gold standard anti plaque mouth rinse due to its prolonged broad spectrum antimicrobial activity.However certain local side effects were reported with its long term usage.[12]

To overcome these side effects, researchers are shifting their attention to herbal remedies to fight against microbial infections. Since plant extracts were known to be a good source of new antimicrobial agents, efforts have been made for development of alternate mouth wash from natural products which were anticipated to be safer, easily available and substitute standard pharmaceutical remedies.

Pomegranate fruit is currently finding important applications in the field of dental health due to its consumption in ancient cultures for its medicinal purposes without adverse effects or toxicity. There are several *In Vitro* studies determining the antimicrobial activity of Pomegranate extract against S.*mutans* but very few *In Vivo*studies were conducted to prove its efficacy against dental caries. Thus PPE was selected in the present study to determine its clinical usefulness as anti caries mouth rinse.

Pomegranate pericarp contains different bioactive compounds like phenolics, flavonoids, proanthocyanidine compounds, minerals such as potassium, nitrogen, sodium and complex polysaccharides.Consuming pomegranate pericarp was considered beneficial for treatment of colic, colitis, menorrhagia, oxyuriasis, headache, diuretic, acne, piles, allergic dermatitis and treatment of oral diseases.[2]

Pomegranate fruit has many properties which include antimicrobial, anti-oxidant, anti-inflammatory, anti-mutagenic,anti-carcinogenic and inhibitory effect on invasion/motility, cell cycle arrest and apoptosis.[13]

The main compounds responsible for most of the purposeful properties of Pomegranate pericarp are phenolic compounds like ellagitannins and flavonoids. Chemically phenolic acids are defined as substances that possess an aromatic ring bound to one or more hydrogenated substituent.

Eating Pomegranate as a food could place antibacterial and antioxidant agents into the mouth and gum areas. On the other hand, better oral exposure to these agents could come from more

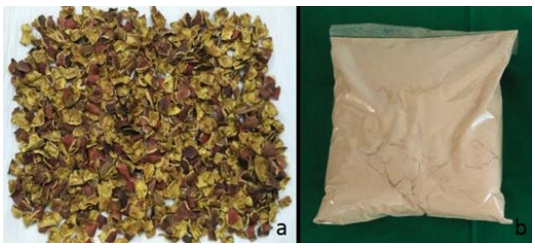

Fig. 1a. Dried pomegranate pericarp;**b**: Pomegranate pericarp powder."

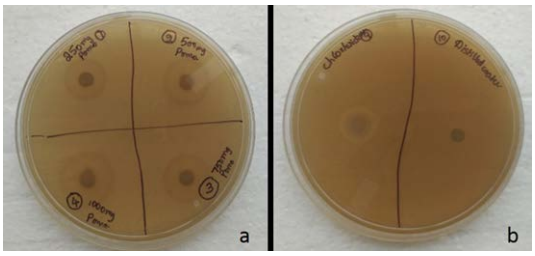

Fig. 2a. Zone of inhibition for PPE at different concentrations;**b**:Zone of inhibition for chlorhexidine and distilled water."

Fig. 3. Bacterial colonies before and after PPE mouth rinsing."

direct and chronic exposure with active agents, such as mouth rinses.This is thought to occur due to the fact that the oral tissue would directly be exposed to polyphenols, which would subsequently get activated by enzymes, thereby destroying pathogenic bacteria.[2] Hence an approach has been tried out in the present study to use PPE as mouth rinse.

In the current study S.*mutans* was isolated using Mitis Salivarius Bacitracin (MSB) agar as suggested by Gold *et al*. (1973)[14] who stated that MSB agar can be used as selective medium for isolating S.*mutans* from saliva.

Broth dilution assay, Agar dilution method, Disc diffusion method, Cup plate method and Ditch plate methodare used to assess the antimicrobial activity of any natural or synthetic agent. In the present study disc diffusion method and broth dilution assay was followed as it was considered as standard and reliable.[15] Furthermore this method involves direct contact of the tested substances with the microbial cultures, which is important for the evaluation of mouth rinses.

In a study conducted by Aldhaher *et al* (2015)[1] the MIC of PPE was 15mg/ml. However, in this study it was 50mg/ml. This difference in the results may be attributed to the difference in the type of extract used and method of extract preparation. In the current study, aqueous extract was prepared rather than alcoholic extracts because of its easy availability and highest extraction capacity with water followed by methanol and ethanol.This is due tothe relative polar nature of polyphenols in pomegranate and they are strongly soluble in polar solvents like water rather than non-polar solvents such as alcohol. [16]

The aqueous extract used in this contemporary study was prepared at 60^{o}c temperature for 20 minutes. This procedure was followed according to the findings by Wissam *et al* (2012)[17] who stated that, there is effective extraction of polyphenols and PA at 60^{o}c temperature using water as a solvent; however temperature above 70^{o} c and time longer than 30 minutes may lead to possible polymerization of flavonoids leading to loss of phenolic compounds.

Results of the present study showed significant reduction in salivary S.*mutans* count with PPE compared to distilled water, whereas no significant difference was noticed withchlorhexidine group. This implies that pomegranate mouth rinse is equally efficacious with chlorhexidine mouth rinse.The possible reason for this isdue to the presence of tannins, which crosses bacterial cell wall and precipitate proteins through complex formation, increase bacterial lysis and impede bacterial adhesion by suppression of enzymes like glucosyl transferase which plays an important role in adhesion of S.*mutans* to tooth surface. [6] According to Machado *et al*. (2002) [18] ellagitannin-punicalgin is thought to be the primary constituent involved in the antimicrobial effect of pomegranate pericarp.

Similar results were noted by Smruti *et al*. (2011)[19] who compared antiplaque efficacy of pomegranate mouth rinse against chlorhexidine. This study also concluded that pomegranate mouth rinse could be explored as a long-term anti-plaque rinse with prophylactic benefits.

The data obtained with the mouth wash of Punica granatum on S.*mutans* are consistent with the results shown in a clinical studyconducted by Umar *et al*.(2016) [20] who stated that pomegranate mouth rinse may be used as an adjunct to prevent dental caries and maintain good oral hygiene.

There are certain limitations in the use of plant extracts as mouth rinse when compared to synthetic mouth rinses as they are time consuming, need of elaborate apparatus to isolate and characterise active molecules and shelf life. The isolation of active components faces many other challenges like inconsistency of source material, obscurity in isolating active components and cost of extraction.

However to consider the clinical applicability of this study certain issues have to be addressed, which include - appropriate concentration of mouth wash to be used, cost effectiveness, addition of preservatives for better shelf life, addition of colouring agents to improve acceptability by children, addition of flavouring agents for better palatability and its long term effectiveness as anti caries mouth rinse on large sample group.

CONCLUSION

The results of thisstudy gives an inference that both PPE and chlorhexidine mouth rinse possess remarkable antimicrobial activity against

*S.*mutans. Hence PPE mouth rinse may be used as an alternative to chlorhexidine and also as an adjunct to conventional tooth brushing for prevention of dental caries and maintenance of oral hygiene in children.

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