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The effect of Pomegranate Peels Aqueous Extract against Streptococcus Mutans and the Adherence to tooth surface in Comparison to Chlorhexidine Gluconate (in Vitro Study)

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Abstract
For centuries, nature has been an enormous source of agents of medical importance and this study was conducted to evaluate the antibacterial activity of aqueous extract of pomegranate peels against Streptococcus mutans by using agar diffusion technique and the adherence to tooth surface in comparison to chlorhexidine gluconate in vitro was also studied.
This study revealed that pomegranate peels exhibit good antibacterial activity and this activity was found to be increased as the concentration of extract increased and the result also showed that the pomegranate peels aqueous extract was effective in inhibiting the adherence of Streptococcus mutans to tooth surface
Keywords: pomegranate peels, streptococcus mutans, adherence

1. Introduction

Dental caries is one of the most common chronic infectious diseases in the world (Anusavice 2002).

Dental caries (holes in teeth) occur when the hard tissues of the tooth and dentine are softened by demineralization caused by bacteria on foods especially sugars, producing an acid that demineralizes the hard tooth(Brook MD *et al.* 2007).

Streptococcus mutans is a gram positive cocci facultative anaerobic bacterium commonly found in the human oral cavity and it is a significant contributor to tooth decay (Barry 2004).

These streptococci can attach to the proteins covering the tooth enamel, where they then convert sucrose into extra cellular polysaccharides (mutan, dextran, levan) (Cleaton-Jones *et al.* 1989). These sticky substances, in which the original bacterial layers along with secondary bacterial colonizers are embedded, form dental plaque. The final metabolites of the numerous plaque bacteria are organic acids that breach the enamel, allowing the different caries bacteria to begin destroying the dentin(Franco *et al.* 1996).

For centuries; nature has been an enormous source of agents of medical importance and the beneficial health effects of many plants have been claimed for not only preventing food deterioration but also acting as antimicrobials against pathogenic microorganisms (Iwu *et al*. 1999). Scientists are searching for natural products and these products Natural compounds have been recently investigated as promising agents for the prevention of dental caries.

Punica granatum Linn. (Pomegranate) is a member of family *Punicaceae* which is a deciduous spreading shrub or small tree and has thorns with it. This plant is found all over India. Pomegranate peel is an inedible part obtained during processing of Pomegranate juice. Pomegranate peel is a rich source of tannins, flavonoids, polyphenols and some anthocyanins as Delphinidins, Cyanidins (Li *et al.* 2006). Antioxidant and antibacterial properties of pomegranate peel in in-vitro model systems have been reported (Reddy *et* al. 2007). All the compounds of pomegranate peels are reported to have therapeutic properties. Extracts of peels of pomegranate show antibacterial property against bacterial strains (Alzoreky 2009) The aim of the present study was to decipher the antibacterial properties of so that they could be used as efficient antimicrobials in near future.

2. MATERIALS AND METHODS

2.1. sample collection

Stimulated saliva samples were collected under standard conditions to obtain five microbial samples. Dental students aged 18-22 years old and with no medical history were selected to participate in the study. Each individual was instructed to chew a piece of Arabic chewing gum (0.4-0.5gm) for five minutes to stimulate salivary flow as much as possible (Al-Bazaz 2010). Methods of isolation and identification of Streptococcus mutans were according to those described by Holbrook & Beighton (1986) and Finegold & Baron (1986). Saliva was collected in sterilized screw capped bottles.

2.2. Bacterial Isolation

The collected saliva was homogenized by vortex mixer for two minutes. Ten-fold serial dilutions were prepared using sterile normal saline. Two dilutions were selected and inoculated on Mitis-Salivarius Bacitracin Agar (MSB Agar), the selective media for Streptococcus mutans, which was prepared according to the manufacturer's instructions. 0.1ml was withdrawn from dilutions of 10^{-1} and 10^{-2} using adjustable micropipette with disposable tips and then spread in duplicate by using sterile microbiological glass spreader on the plates of MSB agar. The plates were then incubated anaerobically by using a gas pack supplied in an anaerobic jar for 48 hours at 37°C followed by aerobic incubation for 24 hours at 37°C. A single colony from Streptococcus mutans was transferred to 10 ml sterile Brain Heart Infusion Broth (BHI-B) and then incubated aerobically for 24 hours at 37°C to activate the inoculums. The purity of the isolates was checked by inoculation of 0.1 ml of the isolates from BHI-B suspensions on media by spreader as mentioned before, and then a selective colony was transferred to 10 ml of sterile BHI-B and incubated aerobically for 24 hours at 37°C.

2.3. Identification

Identification of Streptococcus mutans was carried out by 4 stages:- a) Colony morphology. b) Morphological test of bacterial cell. c) Biochemical test. d) Identification system for Streptococcus mutans of Analytic Profile Index (API) 20 strep.

2.4. Antimicrobial activity

The antimicrobial activity of the pomegranate peels aqueous extract was assessed in this study using the agar diffusion test. Four different concentrations were prepared from the stock of pomegranate peels aqueous extract. The concentrations prepared were: 15mg/ml, 25mg/ml, 50mg/ml, and 75mg/ml. Bacterial isolates of Streptococcus mutans were spread on Brain Heart Infusion Agar (BHI-A). Wells of equal sizes and depths were prepared in the agar using Kork porer for the evaluation of the antimicrobial effect of the different concentrations of the pomegranate peels aqueous extract and 0.2% chlorhexidine gluconate. Each well was filled with 50µl of a concentration that was prepared from the stocks of the extract. Plates were left for 15 minutes at room temperature and then incubated aerobically for 24 hours at 37°C. diameter of Inhibition zones were measured. The resistance of the isolates to the tested agents was indicated when there were no zones of inhibition.

2.5. The minimum bactericidal concentration determination (MBC)

To determine The minimum bactericidal concentration (MBC) of the pomegranate peels aqueous extract, all the concentrations of the pomegranate peels aqueous extract that revealed inhibition zones were mixed with BHI-A to get 25ml of agar and extract then poured into Petri dishes and allowed to harden and inoculated with 0.1ml from the activated isolates of Streptococcus mutans. All these Petri dishes were incubated for 24 hours at 37°C including the control plates (negative control which contained BHI-A with microbial inoculums without the addition of the extract and the positive control plates which contained BHI-A and different concentrations of the aqueous extract without microbial inoculums). Each Petri dish was checked and examined for microbial growth. The minimum bactericidal concentration (MBC) was determined as the lowest concentration of the extract that killed the microorganisms.

2.6. Adherence

To study the effect of the pomegranate peels aqueous extract on the adherence of Streptococcus mutans to tooth surface, three concentrations were used: the minimum bactericidal concentration (MBC) and two concentrations just lower than the MBC. These three concentrations were compared with 0.2% chlorhexidine gluconate, control positive (broth and bacteria without agent), and control negative (broth and agent without bacteria). A stainless steel wire was threaded from one end in the root of a previously cleaned, polished and sterilized first premolar. The teeth were then immersed in 10 ml of the agent for 2 minutes except for control positive. The wires and the teeth were then washed with sterilized deionized water and dried, immersed in 10ml Brain Heart Infusion Broth containing 5% sucrose (pH=7). The study and control tubes were incubated with 2% of bacterial isolates and incubated aerobically at 370C for seven days. A positive score was given to the microbial growth on wire, teeth and bottle indicating a non-effective treatment and vice versa. This method was described by Al Bazaz 2010 .

3. RESULTS

Table (1) demonstrate the diameters of the inhibition zones of the different concentrations of the pomegranate peels aqueous extract against Streptococcus mutans these diameters increased when the concentration of the extract increased. the sensitivity of Streptococcus mutans was higher to pomegranate peels aqueous extract at concentrations (50mg/ml, 75mg/ml) than chlorhexidine this was also illustrated in figure (1) .

T-test was used to compare each concentration used of pomegranate peels aqueous extract and

chlorhexidine, statistically highly significant difference (p 0.001) was shown and this was demonstrated in table (2)

Table 1: Mean values of the inhibition zones (in mm) produced by the different concentrations of the pomegranate peels aqueous extract against Streptococcus mutans.

Extract	Concentration	Mean diameter of inhibition zone (mm)± SD
Pomegranate peels aqueous extract	15(mg/ml)	12.98 0.85
	25(mg/ml)	15.33 0.25
	50(mg/ml)	18 0.8
	75(mg/ml)	21.05 0.81
chlorhexidin	0.2%	16 0.34

F value = 426.18, P value = 0.000 (highly significant), df =4

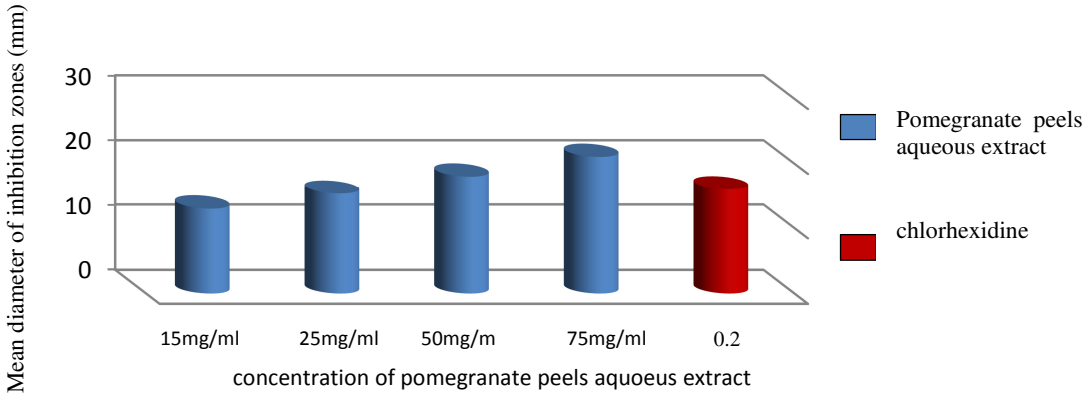

Figure 1: Bar Chart Graph showing the mean diameter of inhibition zones of the different concentrations of the pomegranate peels aqueous extract and chlorhexidine gluconate against streptococcus mutans.

Table 2: t-test between each concentration of black seed oil extract and chlorhexidine (CHX)

Concentration of pomegranate peels aqueous extract	CHX	t-test
15(mg/ml)	0.2%	11.88*
25(mg/ml)	0.2%	11.5*
50(mg/ml)	0.2%	-9.81*
75(mg/ml)	0.2%	-25.22*

*P<0.001 (highly significant)

The results of this study showed that the MBC of the pomegranate peels aqueous extract for Streptococcus mutans was 25mg/ml. This concentration showed no bacterial growth when mixed with the agar and inoculated with the Streptococcus mutans, while the concentrations of 15mg/ml still showed bacterial growth when mixed with the agar and inoculated with the Streptococcus mutans.this concentration could be regarded as inhibitory but not bactericidal. This extract was also effective in the prevention of adherence of Streptococcus mutans to tooth surface at the concentration of 25mg/ml as shown in table (3)

Table 3: The effect of the different concentrations of the pomegranate peels aqueous extract, chlorhexidine gluconate, on the adherence of Streptococcus mutans.

Agents	Adherence
pomegranate peels aqueous extract 25mg/ml (MBC)	-ve
pomegranate peels aqueous extract 15mg/ml	+ve
pomegranate peels aqueous extract 10mg/ml	+ve
Control positive	+ve
Control negative	-ve
Chlorhexidine 0.2%	-ve

4. DISCUSSION

There is a recent interest in the use of natural products and their effect on oral health. The potential antibacterial effect could be of use in the prevention and treatment of oral diseases (Edgar 1998).

Dental caries continues to be the most common oral disease in infants and children, and its prevalence continues to rise in developing countries (Pai *et al.* 2010).

The pomegranate fruit has been consumed for centuries without adverse effects or toxicity.

(Bhadbhade *et al.* 2011) The antimicrobial activity of Punicagranatum Linn has been widely investigated. The rind or peel, juice and seeds of the fruit have been studied. (AbdollahzadehSh *et al.* 2011) Human trials examining the antibacterial properties of pomegranate extracts have primarily focused on oral bacteria (Nascimento *et al.* 2000; Menezes *et al.* 2006)

In this study pomegranate peel aqueous extract was used because it is rich in tannin, ellagitannins and phenolic acids

The results of this study confirmed that Pomegranate peels aqueous extract have strong antibacterial activity against streptococcus mutans and these results were in agreement with the results of other researchers (Vijayanand & Hemapriya 2011). whom reported that punica granatum peels extracts have strong antibacterial activity against gram positive and gram negative bacteria.

The antibacterial activity of pomegranate peels aqueous extract may be explained by the presents of various phytochemicals such as phenolic punicalagins, gallic acids, catechin, quercetin and retina.

the findings of this study were in accordance with results of DiSilvestro *et al* (2009) who reported that the use of mouth rinses that contain extracts of pomegranate have demonstrated reduction in plaque microflora. This may be explained by the changes in salivary measures relevant to oral health. The changes were reduced total protein, reduced activities of aspartate aminotransferase (an indicator of cell injury), reduced alpha-glucosidase activity (a sucrose degrading enzyme), increased activities of the antioxidant enzyme ceruloplasmin (which could give better protection against oral oxidant stress) and increased radical scavenging (DiSilvestro *et al.* 2009)

several studies found that Topical applications of pomegranate preparations have been found to be particularly effective in controlling oral inflammation, as well as bacterial and fungal counts in periodontal disease and Candida associated denture-stomatitis. (Vasconcelos *et al.* 2003) & (Sastravaha *et al.* 2005). The ellagitannin, punicalagin, was thought to be responsible for pomegranate's antibacterial activity (Menezes *et al.* 2006)

In this study the adherence of streptococcus mutans to tooth surface was found to be prevented at a concentration of 25mg/ml of pomegranate peels aqueous extract. This finding suggests that the pomegranate peels aqueous extract could be used as a antibacterial agent in a concentration of 25mg/ml to prevent bacterial colonization on the tooth surface as 0.2% chlorhexidine gluconate

This was in agreement with other Studies that have shown the specific antimicrobial action of Punicagranatum Linn on dental biofilm bacteria, i.e., disturbance of polyglycan synthesis, and thus acting on the adherence mechanisms of these organisms to the dental surface (Kakiuchi *et al.* 1986). other researcher also confirmed the finding of this study by their suggestion that pomegranate might be used in the control of adherence of different microorganisms in the oral cavity (Vasconcelos *et al.* 2006). There is a growing interest in the use of tannins in the prevention of dental caries. The effect of tannins on microbial metabolism can be measured by their action on membranes. They can cross the cell wall that is composed of several proteins and polysaccharides, and bind to its surface. They can precipitate proteins and may also suppress many enzymes such as glucosyl transferase. The antibacterial agents present in pomegranate- the hydrolysable tannins- form complexes of high molecular weight with soluble proteins, increase bacterial lysis and interfere with bacterial adherence (Machado *et al.* 2003).

Pomegranate is a potent antioxidant. Ellagic acid has been detected in human plasma following consumption of pomegranate juice (Seeram *et al.* 2004). No toxic effects of the antioxidant polyphenol punicalagin, abundant in pomegranate juice, have been reported (Cerdá *et al.* 2003).

5. CONCLUSION

After further purification and characterization of the active metabolites present in *Punica granatum* followed by a detailed study of toxicity and pharmacological effects of the compound, the peel extracts of pomegranate may be used as a mouthwash against oral bacteria causing dental caries. However, the potential use of pomegranate as a convenient alternative to antimicrobial products, especially for children should be considered.

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